

Visualizing and Controlling Redox Processes at the Single Molecule/particle Level

Robert Godin

Department of Chemistry

McGill University

Montréal, Québec, Canada

July, 2015

A Thesis submitted to McGill University in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

© Robert Godin, 2015. All rights reserved.

Dédicace

Je dédie ma thèse à mes parents, Gilles et Annette

Vous m'avez donné tout ce dont j'avais besoin pour pouvoir m'épanouir

Tous mes succès sont grâce à vous, et j'en suis éternellement reconnaissant

Merci

Acknowledgements

It's natural for me to start by thanking Gonzalo for his guidance, support, and patience throughout my studies. You have played a large role in shaping me and my future self, both professionally and personally. My career as a researcher is still in its early stages and every positive experience and lessons learned, directly or indirectly through you, will help me succeed at the next level.

I also thank Dr. Hsiao-Wei Liu for guiding me through my initial exposure to single molecule spectroscopy. I am confident in my abilities as a single molecule experimentalist because of your training.

Special thanks to the support staff in the department of chemistry for always being helpful: Claude and Mario from Stores, Robert for computer help, Rick and Weihua from the electronics shop, Jean-Philippe from the machine shop, Mohini and Petr from CSACS, Chantal for administrative help, everyone in the departmental offices, and others that I'm sure I missed.

I also extend my gratitude to the entire Cosa group where the whole is greater than the sum of its parts. I mean this strictly as a compliment!! I've been lucky to have such a great group of labmates and friends that can make any outing a blast! There's been many of us who have called the PP302 office and the corridor home: An, Pierre, Wayne, Robert, Katerina, Amani, Ryan, Laura, Lana, Christina, Viktorija, Ricky, Mathieu, Andreas, Yasser, and Mei. A special thanks to those of you I had the chance to work alongside of, as you bear with me as start rambling on and on and on. I also thank our neighbours from the Mittermaier group, for letting me infiltrate your cluster when we first moved in the new office and for being part of the larger family. I'd especially like to thank Jason, literally my best right hand man, for keeping me up to date with all the important

internet news, and for great lunches over the years. I couldn't have asked for a better friend sitting next to me.

Thanks to all my friends, within the department and outside of chemistry. I would not have been able to thoroughly enjoy my time in Montreal without all of you. I will miss the people more than the city as our lives take us all over the world.

Merci également à toute ma famille pour leur support. J'ai été choyé dans la vie d'avoir toujours été encouragé par vous de poursuivre mes rêves.

My studies have taking me along a wonderful path. I feel like all the pieces have fallen in the perfect place. Along the way, I met the love of my life, Denise. Merci Denise pour tout ton amour qui me rend si heureux, tout ton soutien quand les temps sont sombres. Je te remercie aussi pour tout l'effort que tu as mis pour m'aider dans mes études et dans mes écritures. I outspread my mostfully felt heartwarmest gratitude in relation to your constantly supporting neighborliness in slaying the fiendish adversary that is my impertinent incessant inglorious hypocubage (<http://smbc-comics.com/index.php?db=comics&id=1957#comic>).

Contribution of Authors

This thesis consists of eight Chapters. Chapter 2 has been published in *Chemical Science*. Chapter 3 has been published in *Langmuir*. Chapter 4 has been published in *The Journal of Physical Chemistry C*. Chapter 5 has been published in *The Journal of Physical Chemistry Letters*. Chapter 6 is based on a manuscript that is currently in preparation. Chapter 7 includes final remarks and an outlook. All papers presented in chapters 2 to 6 were co-authored with the research advisor Prof. Gonzalo Cosa (Department of Chemistry, McGill University), who participated in project design as well as manuscript writing and editing.

- ❖ Chapter 2: Robert Godin, Hsiao-Wei Liu and Gonzalo Cosa, Ambient Condition Oxidation in Individual Liposomes Observed at the Single Molecule Level, *Chemical Science*, **2014**, 5, 2525 – 2529.

Robert Godin contributed to project design, and performed all single molecule and ensemble experiments. Contributions also include analysis and interpretation of data, in addition to manuscript writing and editing.

Hsiao-Wei Liu contributed to project design and manuscript editing.

- ❖ Chapter 3: Robert Godin, Hsiao-Wei Liu, Laura Smith and Gonzalo Cosa, Dye Lipophilicity and Retention in Lipid Membranes: Implications for Single-Molecule Spectroscopy, *Langmuir*, **2014**, 30, 11138–11146

Robert Godin contributed to project design, acquisition of single molecule and ensemble experiments data. Contributions also include analysis and interpretation of data, in addition to manuscript writing and editing.

Hsiao-Wei Liu contributed to project design and manuscript editing.

Laura Smith contributed to liposome preparation and acquisition of ensemble fluorescence quenching experiments.

- ❖ Chapter 4: Robert Godin, Rodrigo Palacios and Gonzalo Cosa, Heterogeneous Charge Mobility in Individual Conjugated Polyelectrolyte Nanoparticles Revealed by Two-Color Single Particle Spectroelectrochemistry Studies, *Journal of Physical Chemistry C*, **2015**, *119*, 12875–12886

Robert Godin contributed to project design, and performed all single molecule and ensemble experiments. Contributions also include analysis and interpretation of data, in addition to manuscript writing and editing.

Rodrigo Palacios contributed to project design, interpretation of data, and manuscript editing.

- ❖ Chapter 5: Robert Godin, Benjamin D. Sherman, Jesse J. Bergkamp, Carlos A. Chesta, Ana L. Moore, Thomas A. Moore, Rodrigo Palacios, and Gonzalo Cosa, Fluorescence-Mediated Visualization of Charge Transfer Dynamics in Dye-Sensitized SnO₂ Electrodes under Applied Biases, *Journal of Physical Chemistry Letters*, **2015**, *6*, 2688–2693

Robert Godin contributed to project design, and performed all single molecule and ensemble experiments. Contributions also include analysis and interpretation of data, in addition to manuscript writing and editing.

Other authors were part of the Inter-American Collaboration in Materials Research (CIAM) collaboration and contributed to project design, data analysis and manuscript editing.

- ❖ Chapter 6: Robert Godin and Gonzalo Cosa, Counting Single Redox Turnovers: Fluorogenic Antioxidant Conversion and Mass Transport Visualization via Single Molecule Spectroelectrochemistry, *Manuscript in preparation*

Robert Godin designed the experiments, and performed all single molecule and ensemble experiments. Contributions also include analysis and interpretation of data, in addition to manuscript writing and editing.

Contributions to Knowledge

The main contributions to knowledge stemming from each chapter are as follows.

In Chapter 2, we devised a strategy to monitor the extent of oxidation that takes place in immobilized liposomes. This strategy allowed us to observe for the first time individual oxidation processes occurring in lipid membranes. We also concluded that oxidation takes place during standard liposome preparations. The previously unknown blinking behaviour of H₂B-PMHC was also reported.

In Chapter 3, a novel technique to quickly determine the fraction of dye that is associated with a lipid membrane under dilute conditions was developed. The technique based on Fluorescence Correlation Spectroscopy (FCS) allowed us to quantify the impact parallel reaction pathways may have on the lipid partitioning of chemical probes. By comparing the results obtained at various lipid concentration, we experimentally demonstrated that the lipid concentration greatly influences the lipid partitioning behaviour of dyes. We highlight the importance of this concept in Single Molecule studies where the lipid concentration is vanishingly small.

In Chapter 4, the novel technique of dual-color Single Molecule Spectroelectrochemistry (SMS-EC) was applied to the study of conjugated polyelectrolyte nanoparticles. The spectral distinction of non-aggregated and aggregated sites was key to measure differential oxidation potentials of these distinct sites within individual nanoparticles. The measured fluorescence response to applied bias gave us novel insight on the charge and energy transfer characteristics and led us to propose physical models to explain the observed behaviour. We believe that there is a particle-to-particle heterogeneity in the polymer chain packing that results in heterogeneous external ion motion through the nanoparticles.

In Chapter 5, we applied SMS-EC to dye-sensitized SnO₂ films to explore interfacial charge transfer (ICT) dynamics that are relevant to dye-sensitized solar cells. The sample geometry importantly excluded the contribution of dyes that may desorb from the semiconductor surface when an external potential is applied. We were thus able to assign all changes in photophysical properties to changes in ICT. We observed similar responses to applied potential with two different organic dyes, characterized by different excited state redox potentials, and thus concluded that the changes in occupancy of the electronic states of the semiconductor is the main factor influencing the ICT in this system. This unprecedented dataset also demonstrated that a dye photoreduction pathway was active. Dye photoreduction has been largely ignored so far in the design of solar cells, but may lead to a decrease in the power conversion efficiency.

In Chapter 6, we use SMS-EC to monitor individual electrochemically-generated fluorescent products. This was performed for the first time in organic solvents, greatly expanding the scope of electrochemical reactions that may be studied by SMS-EC. The products of electrochemical reactions could be observed one at a time, improving the limit of detection of spectroelectrochemical techniques down to the single molecule level. This allowed us to experimentally measure the spatiotemporal concentration profile of electrochemical products at a very low (0.5 nM) analyte concentration.

Table of Contents

Acknowledgements	iii
Contribution of Authors.....	v
Contributions to Knowledge	viii
Table of Contents	x
List of Schemes	xv
List of Tables	xvi
List of Figures.....	xvii
List of Abbreviations and Symbols	xxvii
Abrégé	xxxii
Abstract.....	xxxiv
1. Introduction.....	1
1.1. Single Molecule Spectroscopy (SMS).....	2
1.2. Single Molecule Chemistry	4
1.3. Fluorescence	10
1.3.1. Excited State Processes	11
1.3.2. Fluorescence Quenching	14
1.3.2.1. Fluorescence Quantum Yield and Fluorescence Lifetime	14
1.3.2.2. Fluorescence Quenching by O ₂	17
1.3.3. Fluorescence Quenching by Photoinduced Electron Transfer (PeT)	20
1.3.3.1. Marcus Theory	24
1.3.4. Interfacial Charge Transfer	28
1.3.4.1. Effect of Density of States	28
1.3.4.2. ICT in Dye-Sensitized Solar Cells	30
1.4. Oxidation at the Single Molecule Level	33
1.4.1. Oxidation-sensitive Fluorescent Probes	35
1.4.2. Lipid Peroxidation.....	40
1.4.2.1. Initiation	41
1.4.2.2. Propagation.....	44
1.4.2.3. Termination	44
1.4.2.4. Inhibition	44
1.5. Single Molecule Spectroelectrochemistry	47
1.5.1. Other combined SM and EC System.....	51
1.6. Single Molecule Experimental Setup	54
1.6.1. Confocal Microscopy	58

1.6.2.	Total Internal Reflection Fluorescence Microscopy	60
1.6.3.	Single Molecule Spectroelectrochemistry (SMS-EC).....	63
1.7.	Research Goals	65
1.8.	References	67
2.	Ambient Condition Oxidation in Individual Liposomes Observed at the Single Molecule Level.....	79
2.1.	Preface	80
2.2.	Abstract.....	82
2.3.	Introduction	82
2.4.	Results and Discussion	83
2.5.	Conclusions	92
2.6.	Notes.....	92
2.7.	Supporting Information	93
2.7.1.	<i>Materials</i>	93
2.7.2.	<i>Liposome Preparation</i>	94
2.7.3.	<i>Coverslip Preparation</i>	95
2.7.4.	<i>Scanning Confocal Microscopy Imaging of Single Liposomes</i>	96
2.7.5.	<i>ABAP Oxidation Experiment</i>	96
2.7.6.	<i>Ambient Oxidation Experiments</i>	97
2.7.7.	<i>H₂B-PMHC and DiD Spectral Discrimination</i>	98
2.7.8.	<i>H₂B-PMHC Fluorescence Enhancement over Time under Oxidative Conditions</i>	98
2.7.9.	<i>Comparison of Linear and Gaussian Fitting of Individual Liposome Data</i>	100
2.7.10.	<i>Gaussian Fitting of Fluorescence Intensity Ratios Recorded Under Oxidation Conditions Induced by ABAP</i>	102
2.7.11.	<i>Gaussian Fitting of Fluorescence Intensity Ratios Recorded Under Ambient Conditions and Different Antioxidant Loads</i>	102
2.7.12.	<i>Confocal Fluorescence Intensity Images Recorded Under Ambient Conditions and Different Antioxidant Loads</i>	103
2.7.13.	<i>Effect of PMHC on H₂B-PMHCox: Preoxidized H₂B-PMHC Is Not Reduced By PMHC</i>	104
2.7.14.	<i>Confocal Imaging of Regions That Have Been Pre-Irradiated with 633 nm Laser Light</i>	105
2.7.15.	<i>Ensemble Irradiation of DiD Under Maximum Power</i>	107
2.7.16.	<i>Number of Fluorescent Bursts in Individual Liposomes</i>	107
2.7.17.	<i>Summary of Antioxidant Additive Effects</i>	109
2.8.	References	110
3.	Dye Lipophilicity and Retention in Lipid Membranes: Implications for Single-Molecule Spectroscopy	112
3.1.	Preface	113
3.2.	Abstract.....	115
3.3.	Introduction	115

3.4.	Experimental Section.....	119
3.4.1.	Materials.....	119
3.4.2.	Liposome Preparation	120
3.4.3.	Activation of Fluorogenic BODIPY Probes.....	120
3.4.4.	Fluorescence Quenching Experiments	121
3.4.5.	Dye Retention during Dialysis	122
3.4.6.	Single-Particle Confocal Fluorescence Imaging.....	122
3.4.7.	FCS Measurements	124
3.5.	Results and Discussion	125
3.5.1.	Ensemble Fluorescence Quenching	125
3.5.2.	Ensemble Dye Retention.....	128
3.5.3.	Single-Particle Confocal Fluorescence Imaging.....	129
3.5.4.	FCS.....	133
3.5.5.	Summary	137
3.6.	Conclusion.....	139
3.7.	Acknowledgments	140
3.8.	Supporting Information	140
3.8.1.	<i>Single Particle Confocal Microscopy Imaging of Individual Liposomes ..</i>	140
3.8.2.	<i>Fluorescence Correlation Spectroscopy (FCS) Experiments</i>	141
3.8.3.	<i>FCS Theory and Analysis.....</i>	142
3.8.4.	<i>Synthesis of 8-(Decanoyloxy)-methyl-2,6-diethyl-1,3,5,7-tetramethyl Pyrromethene Fluoroborate (Et₂B-CO₂C₉)</i>	148
3.8.5.	<i>BODIPY and DiD Spectral Discrimination</i>	149
3.8.6.	<i>Ensemble Quenching Results</i>	150
3.8.7.	<i>Calculation of % Retention in Lipid Membrane from Confocal Single Particle Imaging.....</i>	153
3.8.8.	<i>FCS Autocorrelation Traces</i>	154
3.9.	References	154
4.	Heterogeneous Charge Mobility in Individual Conjugated Polyelectrolyte Nanoparticles Revealed by Two-Color Single Particle Spectroelectrochemistry Studies.....	157
4.1.	Preface	158
4.2.	Abstract.....	160
4.3.	Introduction	160
4.4.	Experimental Section.....	162
4.4.1.	Preparation of MPS-PPV solution in H ₂ O/organic solvent for ensemble measurements.....	162
4.4.2.	Ensemble spectroscopy	163
4.4.3.	SEM imaging.....	163
4.4.4.	Single molecule spectroelectrochemistry.....	164
4.5.	Results and Discussion	165

4.5.1.	Effect of water content in H ₂ O/THF and H ₂ O/MeCN mixtures	165
4.5.2.	Single Molecule Spectroelectrochemistry of MPS-PPV particles	170
4.5.2.1.	Results for + 1.03 V $\geq$ E _{app} $\geq$ +0.03 V	171
4.5.2.2.	Results for + 1.03 V $\geq$ E _{app} $\geq$ - 0.97 V	181
4.6.	Conclusions	186
4.7.	Acknowledgements	187
4.8.	Supporting Information	187
4.8.1.	<i>Particle Size Distribution from SEM Micrograph</i>	188
4.8.2.	<i>Ensemble Spectroscopy of MPS-PPV in either H₂O/THF or H₂O/MeCN Mixtures</i>	189
4.8.3.	<i>Examples of Non-normalized SMS-EC Fluorescence Intensity Trajectories...</i>	192
4.8.4.	<i>Full Two Potential Cycles of SMS-EC Experiments</i>	193
4.8.5.	<i>Comparing the Effect of External Bias for the Different NP Subsets</i>	195
4.8.6.	<i>Comparing NP Subsets for Applied Potentials from + 0.03 to + 1.03 V...</i>	196
4.8.7.	<i>Mechanism of Fluorescence Enhancement under Negative Applied Biases....</i>	196
4.9.	References	197
5.	Fluorescence-Mediated Visualization of Charge Transfer Dynamics in Dye-Sensitized SnO₂ Electrodes under Applied Biases	202
5.1.	Preface	203
5.2.	Abstract.....	205
5.3.	Introduction	205
5.4.	Results and Discussion	207
5.5.	Conclusions	220
5.6.	Experimental Methods.....	220
5.6.1.	Materials.....	220
5.6.2.	Sample Preparation	221
5.6.3.	Single molecule/particle spectroelectrochemistry.....	222
5.7.	Acknowledgements	223
5.8.	Supporting Information	223
5.8.1.	<i>Surface Characterization of the SnO₂ Films</i>	223
5.8.2.	<i>Control of Dyes Bound to ITO Surface</i>	224
5.8.3.	<i>Photobleaching of Perylene and SQ2 Dyes Bound to Inert SiO₂ Film</i>	224
5.8.4.	<i>Additional Representations of Perylene Photobleaching Trajectories</i>	225
5.8.5.	<i>Electrochemical Characterization of Thick SnO₂ Films</i>	225
5.9.	References	226
6.	Counting Single Redox Turnovers: Fluorogenic Antioxidant Conversion and Mass Transport Visualization via Single Molecule Spectroelectrochemistry	230
6.1.	Preface	231
6.2.	Abstract.....	232

6.3.	Introduction	232
6.4.	Results and Discussion	234
6.5.	Conclusions	242
6.6.	Materials and Methods	243
6.6.1.	SMS-EC setup.....	243
6.7.	Supporting Information	244
6.7.1.	<i>Fluorescence Enhancement of Chemically Oxidized H₂B-PMHC</i>	245
6.7.2.	<i>Electrochemical Characterization of H₂B-PMHC</i>	245
6.7.3.	<i>Effect of O₂ under Electroreductive Conditions</i>	246
6.8.	References	246
7.	Concluding Remarks and Outlook.....	249
7.1.	Concluding Remarks	249
7.2.	Outlook	252
7.3.	References	255

List of Schemes

Scheme 1.1. Radiative and non-radiative singlet excited state deactivation pathways.	16
Scheme 1.2. Photoinduced electron transfers (PeTs) where the photoexcited molecule is the electron acceptor (Case I) or the electron donor (Case II).	23
Scheme 1.3. Mechanism of electrochemical and chemical oxidation of α -tocopherol (α -TOC). Redox potentials indicated are referenced to the ferrocene/ferrocenium couple. Reprinted with permission from ref. 131. Copyright 2009 American Chemical Society.	39
Scheme 1.4. Reaction sequence of lipid peroxidation. ROO $\cdot$ represents a general peroxy radical derived from an initiator. LH is a polyunsaturated fatty acid in lipids, for example linoleate. The rate constant values are obtained from homogeneous solution studies (k_{ox} , ¹⁵⁵ k_p , ¹⁵⁶ k_t , ¹²⁴ k_{inh} , ¹⁵⁷ k_{coup} ¹⁵⁸).	41
Scheme 1.5. H-atom abstraction from a polyunsaturated fatty acid by a peroxy radical.	42
Scheme 1.6. Fragmentation patterns of hydrophilic (ABAP) and hydrophobic (DCP) radical initiators.	43
Scheme 2.1. Experimental Strategy.	84
Scheme 2.2: Production of peroxy radicals (ROO $\cdot$) following thermolysis of ABAP. ABAP initially decomposes in a first order reaction to yield two geminate carbon centered radicals which may either recombine (Equation 1) or diffuse away (Equation 2). In air equilibrated solutions radicals that escape cage recombination will readily trap molecular oxygen ³³ to yield two water soluble peroxy radicals.	97
Scheme 3.1. Oxidation of a BODIPY-based trap-reporter probe by ROS generated upon photolysis of dicumyl peroxide (DCP) or 2,2'-azobis(2-methylpropionamidine) dihydrochloride (ABAP) in air equilibrated solutions. ²⁰⁻²¹	117
Scheme 5.1. Excited state charge transfer pathways.	210
Scheme 5.2. Proposed degradation pathway for perylene fluorescent sensitizer.	211

List of Tables

Table 2.1. Effects of added antioxidants on ambient oxidation processes.	109
Table 3.1. Lipophilicity estimates from ensemble and single molecules techniques.	127
Table 3.2. Parameter used to determine % retention in membrane from confocal images.	153
Table 4.1. Spectroscopic and DLS characterization of MPS-PPV dispersions in H ₂ O/THF solutions.	168
Table 4.2. Spectroscopic and DLS characterization of MPS-PPV dispersions in H ₂ O/MeCN solutions.	191

List of Figures

Figure 1.1. Examples of fluorophores that have been used to study chemical reactions at the SM level. Reprinted with permission from Macmillan Publishers Ltd: Nature Chemistry, ref. 58, copyright 2013.	6
Figure 1.2. Visualization of quinone/hydroquinone redox cycling at the SM level. Adapted from ref. 80 with permission of the PCCP Owner Societies.	9
Figure 1.3. (A,B) Setups developed in Majima's research group to monitor diffusive reactive oxygen species (ROS) generated by photocatalysis on TiO ₂ surface. Panel C shows SM fluorescence images based on the setup shown in B. Adapted with permission from ref. 85. Copyright 2008 American Chemical Society.	10
Figure 1.4. Photophysical processes summarized in a Jablonski diagram. ⁸⁶ VR stands for vibrational relaxation, IC for internal conversion and ISC for intersystem crossing.	12
Figure 1.5. Effects of BME and Trolox on the photophysics of single DNA conjugated Cy5 molecules. (a) Fluorescence intensity traces in enzymatically deoxygenated solution without BME or Trolox. Notice the transient low intensity states ('blinks'). (b) Effect of addition of increasing concentration of BME (b). (c) Average time until the transition to the dark state versus BME concentration. (d) Fluorescence intensity traces (as in a) in the presence of 2 mM Trolox. Reprinted with permission from Macmillan Publishers Ltd: Nature Methods, ref. 96, copyright 2006.	19
Figure 1.6. Comparison of the ionization potential (IP) and electron affinity (EA) of a molecule in the ground state and excited state.	21
Figure 1.7. Examples of photoinduced electron transfers (PeTs) where the photoexcited molecule is the electron acceptor (Case I) or the electron donor (Case II).	22
Figure 1.8. (a) Marcus parabolas to show the relation between ΔG^0 , $\Delta G^\ddagger$ and the reorganization energy (λ). All parabolas have the same shape and therefore the λ are equal for the three different scenarios represented. (b) Experimental results showing the changes from the normal to the inverted Marcus regions. The maximal rate of electron transfer occurs for values near - 0.6 eV, where $\Delta G^0 = - \lambda$. Panel B was adapted with permission from Macmillan Publishers Ltd: Nature Chemistry, ref. 106, copyright 2014.	26
Figure 1.9. Representation of the photophysical cycles a fluorophore may enter when a PeT pathway is inactive (left, fluorescent) or active (right, non-fluorescent).	27
Figure 1.10. Visual representation of photooxidation and photoreduction of a dye by ICT to a semiconductor. Occupied electronic states are highlighted in red while empty states are represented in white. The density of states (DOS) of the semiconductor is shown as an exponential distribution, typical for metal oxide semiconductors such as TiO ₂ and SnO ₂	30
Figure 1.11. Schematic depiction of a functioning DSSC. The charge separated state is produced by ICT as the photoexcited sensitizer (D*) injects an electron into the mesoporous TiO ₂ electrode. Abbreviations are as follow: open circuit cell potential (ΔV_{oc}); driving force for oxidized dye regeneration (ΔV_{reg}); redox mediator (RM); rate constant for electron injection, oxidized dye regeneration and redox mediator reduction at the counter electrode (k_{inj} , k_{reg} , k_{CE} respectively).	31
Figure 1.12. State diagram representation of the kinetics of DSSC function. The forward (productive) processes of light absorption, electron injection, dye regeneration, and charge	

transport are indicated by blue arrows. The competing loss pathways of excited state decay to the ground state, and electron recombination with either dye cations or oxidized redox mediator are shown in black. Adapted with permission from ref. 113. Copyright 2008 Imperial College Press.

..... 32

Figure 1.13. The fluorogenic α -tocopherol probe **B-TOH** becomes brightly fluorescent upon oxidation of the chromanol moiety. R may be a wide range of hydrocarbon chains, such as a lipid chain in the case of lipid peroxy radicals..... 37

Figure 1.14. Chronological improvements in the fluorescence enhancement of fluorogenic α -tocopherol oxidation probes..... 39

Figure 1.15. Chemical structures of naturally-occurring (tocopherol) and artificial (Trolox, PMHC) phenolic antioxidants..... 46

Figure 1.16. SMS-EC setup as developed in Prof. Paul Barbara's research group. A) Schematic depiction of the electrochemical cell. B) Wide-field fluorescence image of the SMS-EC cell. C) Applied electrochemical potential. D) Normalized fluorescence trajectory of a BEH-PPV single molecule while applying the potential shown in C). E) Ensemble (810 molecules, red) and subensemble (75 molecules, blue) normalized fluorescence trajectories. F) Distribution of potentials at which the fluorescence is quenched by half. G) Ensemble cyclic voltammogram of a BEH-PPV film on an ITO electrode. Adapted with permission from ref. 181. Copyright 2009 American Chemical Society. 50

Figure 1.17. SMS-EC system developed by Prof. Peng Chen to study nanocatalytic electroreduction reactions. A) Experimental setup. B) TIRF image ($\sim 17 \times 19 \mu\text{m}^2$) showing fluorescent resorufin molecules. C) Fluorescence trajectory from the fluorescent spot marked by an arrow in B). D) Non-fluorescent resazurin is electrochemically reduced to fluorescent resorufin at the active sites of single-walled carbon nanotubes. Adapted with permission from 189. Copyright 2009 American Chemical Society. 53

Figure 1.18. Schematic of (a) confocal, (b) prism-based TIRF, and (c) epifluorescence microscopy setups. Objective-based TIRF microscopy is similar to (c) with the addition of a translatable optic component that allows to control at which angle the excitation beam exits the objective and reflects on the coverslip. Reprinted from ref. 198, Copyright 2004, with permission from Elsevier. 58

Figure 1.19. Schematic representation of a TIRF-based SMS-EC setup. RE stands for reference electrode, CE for counter electrode, ITO for indium tin oxide and WE for working electrode. In the bottom right is shown a picture of a sample mounted on an insulating plate which is then positioned on the microscope stage. 64

Figure 2.1. Fluorescence intensity images (top and middle rows) and intensity ratios of H₂B-PMHC/DiD channels (bottom row) before and during oxidation initiated by ABAP. Panels A (C) show images corresponding to the H₂B-PMHC channel and panels B (D) show those from the DiD channel, acquired before (during) oxidation, upon excitation with the 514 nm, 300 W/cm² output of an Ar⁺ laser. Scale bars are 6 μm . Panels E (before oxidation) and F (during oxidation) illustrate the correlation of H₂B-PMHC vs. DiD fluorescence intensity; also shown are linear fits as a guideline. Mean values of fluorescence intensity ratios obtained by Gaussian analyses are further listed..... 86

Figure 2.2. Panels A and B show fluorescence intensity images corresponding respectively to the H₂B-PMHC channel and the DiD channel, obtained for a sample prepared without Trolox and

PMHC antioxidants (- **PT**). Images were simultaneously acquired using the 514 nm output of an Ar⁺ laser (6,400 W/cm²). Linescan profiles are shown at the sides of the images. Scale bars are 2 μ m. Panels C (-**PT**) and D (+**PT**) illustrate the correlation of H₂B-PMHC vs. DiD fluorescence intensity in the absence and presence of antioxidants; also shown are linear fits as a guideline. Mean values of fluorescence intensity ratios, obtained by Gaussian analyses of data from 30 x 30 μ m² images, are further listed. **89**

Figure 2.3. Intensity-time trajectories recorded for individual liposomes prepared (A) without (- **PT**) and (B) with PMHC and Trolox (+**PT**). The black and red traces correspond to the emission from the H₂B-PMHC channel and the DiD channel. The data is binned at 5 ms. DiD traces are plotted every 10th point for clarity. **91**

Figure 2.4. Absorbance (dotted line) and fluorescence (solid line) spectra of H₂B-PMHC_(ox) (green) and DiD (red). H₂B-PMHC and DiD show good spectral separation and their emission can be split and detected by two separate channels (gray areas). The 514 nm output of an Ar⁺ laser was used to excite H₂B-PMHC near its absorbance maximum. This laser line further enables direct excitation of DiD at the blue edge of its absorbance spectrum. 7% H₂B-PMHC to DiD channel crosstalk and negligible DiD to H₂B-PMHC crosstalk was observed with our setup. **98**

Figure 2.5. H₂B-PMHC fluorescence intensity as a function of time under oxidative conditions. The representative average intensity is determined by averaging the intensity of all liposomes identified from the DiD channel. **99**

Figure 2.6. DiD fluorescence intensity as a function of time under oxidative conditions. The representative average intensity is determined by averaging the intensity of all liposomes identified from the DiD channel. **100**

Figure 2.7. H₂B-PMHC/DiD fluorescence intensity ratio as a function of time under oxidative conditions. The representative average value of H₂B-PMHC/DiD fluorescence intensity is determined from the mean value of Gaussian distributions of the H₂B-PMHC/DiD intensity ratio calculated for each liposome. **100**

Figure 2.8. A) Scatter plot of Intensities of H₂B-PMHC versus DiD for individual liposomes fitted with a linear regression. Outliers in red squares are shown. (B) Histogram of the H₂B-PMHC/DiD intensity ratios of individual liposomes constructed from the same data set as in (A) but now fitted to a Gaussian distribution. Both Gaussian fits (green lines) overlap perfectly. Outliers are shown as red data points on the right. **101**

Figure 2.9. Histogram of I_{H2B-PMHC}/I_{DiD} ratios for individual liposomes before (A) and during (B) exposure to ABAP. The number of liposomes analysed and the mean value of Gaussian fits (red lines) $\pm$ fitting error are also indicated. **102**

Figure 2.10. Histogram of I_{H2B-PMHC}/I_{DiD} ratios for individual liposomes imaged under ambient conditions and in the presence of different antioxidants added during sample preparation. The number of liposomes analysed and the mean value of Gaussian fits (red lines) $\pm$ fitting error are also indicated. **103**

Figure 2.11. Fluorescence intensity images corresponding respectively to the H₂B-PMHC channel and the DiD channel, obtained for a sample prepared (A) with no additional antioxidants (-**PT**); (B) with PMHC only (+**P**); (C) with Trolox only (+**T**); and (D) with both antioxidants (+**PT**). Linescans are shown for a single molecule beside the images and their positions on the images are indicated by a cyan line. White scale bars are 2 μ m. The right panel displays scatter plots showing

the correlation of H₂B-PMHC vs. DiD fluorescence intensity, where linear fits serve as a guideline. Mean values of fluorescence intensity ratios obtained by Gaussian analyses, and the total number of liposomes analysed are further listed. **104**

Figure 2.12. Stern-Volmer analysis of H₂B-PMHC_{ox} fluorescence intensity upon addition of increasing amounts of PMHC in acetonitrile solutions. Fluorescence intensities were integrated over the range of 500 - 675 nm, with $\lambda_{\text{ex}} = 480$ nm. Fluorescence lifetimes are the intensity-weighted averages of biexponential fits ($\chi^2 < 1.2$). The fluorescence lifetime measurements were carried out using a Picoquant Fluotime 200 time-correlated single photon counting setup employing an LDH 470 ps diode laser (Picoquant) with excitation wavelength at 467 nm as the excitation source. The laser was controlled by a PDL 800 B picosecond laser driver from Picoquant. The excitation rate was 10 MHz, and the detection frequency was less than 150 kHz. Photons were collected at the magic angle. **105**

Figure 2.13. Confocal images (90 x 90 μm^2 regions) acquired under 514 nm illumination. Top panels represent a region that has not been exposed to laser irradiation prior to the full region scan. The image in the bottom panel was acquired after the irradiation of predetermined regions along the diagonals for increasing periods of time, as depicted in the right grid. Irradiated sections in the top row of the grid were scanned once by laser, while the middle and bottom sections were scanned 2 times and 3 times, respectively. Scanning conditions: 1 ms dwell time/pixel, 256 x 256 pixels/scan; same as imaging conditions. Scale bars are 18 μm **106**

Figure 2.14. Ensemble irradiation of -PT sample. DiD was irradiated at the maximum of its absorbance (647 nm) for periods of 10 minutes. Emission spectra were acquired afterwards. Emission spectra were acquired with $\lambda_{\text{ex}} = 475$ nm. **107**

Figure 2.15. Fluorescence intensity-time trajectories recorded for single liposomes prepared with different antioxidant additives: (A) no antioxidants (-PT); (B) PMHC only (+P); (C) Trolox only (+T); and (D) both PMHC and Trolox (+PT). Trajectories were specifically chosen on the basis of having initial DiD intensities close to 50 kHz, i.e. they correspond to liposomes of similar size. The black lines display the intensity from the H₂B-PMHC channel and the red lines display the intensity from the DiD channel. Trajectories are from 3 minutes acquisitions while insets show a 5 s expanded region, indicated in full-length trajectories by a shaded orange area. The blue line in the insets corresponds to the threshold applied in order to count fluorescence burst events. Dashed black lines correspond to the observed intensity steps corresponding to 1, 2 or 3 H₂B-PMHC_{ox} simultaneously fluorescing. Data points are binned to 5 ms. **109**

Figure 3.1. Ensemble fluorescence quenching of liposome preparation containing H₂B-OH. Standard Stern-Volmer (A) and modified Stern-Volmer (B) analyses and corresponding linear fits are shown for MV (black triangle) and PMHC (red circles) quenchers. Full lines extend over the selected fitting range, while dashed lines prolong the fits as a visual guide. **126**

Figure 3.2. Fluorescence intensity measured in the dialysis buffer after 4-hour dialysis of a liposome suspension. The liposome solutions each contained on average 10 dyes per liposome (133 nM liposomes). **129**

Figure 3.3. Panels A and B show fluorescence intensity images corresponding to the BODIPY and DiD emission channels, respectively. Images were acquired for a Et₂B-CO₂C₉ liposome sample immediately after immobilization on the coverslip ($t = 0$ min). Images were simultaneously acquired using the 514 nm output of an Ar⁺ laser (5700 W cm⁻²). Scale bars are 6 μm . Panel C

shows the histogram constructed from 3 separate $30 \times 30 \mu\text{m}^2$ images and the Gaussian fitting used to determine the representative fluorescence ratio, shown above the plot. **130**

Figure 3.4. Representative BODIPY/DiD fluorescence ratios following Gaussian analyses of single liposome confocal images taken over time. Error bars indicate 95% confidence intervals as determined by Bootstrap error analysis (10k resamples) using bias corrected percentile intervals. Errors for H₂B-OH are within the square symbols. **131**

Figure 3.5. Panel A shows simulated normalized correlation function traces. Two components were simulated with different diffusion times (0.1 ms for free dye, 10 ms for liposome-embedded dye) and their ratios were varied according to the legend. Panel B shows normalized autocorrelation traces of selected samples acquired as quickly as possible after dilution ($t = 0$ h) (see Figure 3.10 for all traces). Dye only samples are depicted by a dashed line while dye/liposome samples are shown as full lines. Traces were obtained following a continuous 10-minute acquisition with the 70 kW/cm^2 output of a 514 nm laser. **135**

Figure 3.6. Dependence of the mole fraction of membrane-bound dye on the total lipid concentration and the partition constant of the system calculated from $x_m = K_m[\text{lipid}]/([\text{H}_2\text{O}] + K_m[\text{lipid}])$.³⁵ **138**

Figure 3.7. Dependence of x_m and A_{lipo} relationship on changes to then initial number of dyes per liposome ($\langle k \rangle_0$). Lower $\langle k \rangle_0$ gives better sensitivity for values of x_m closer to 1, while higher $\langle k \rangle_0$ gives better sensitivity for lower values of x_m **147**

Figure 3.8. Absorbance (A, top) and fluorescence (B, bottom) spectra of BODIPY dyes and probes and DiD. BODIPYs and DiD show good spectral separation and their emission can be split and detected by two separate channels (BODIPY channel 530 – 600 nm; DiD channel 645 – 725 nm). The 514 nm output of an Ar⁺ laser was used to excite the BODIPYs and also DiD at the blue edge of its absorbance spectrum. Crosstalk from BODIPYs to the DiD channel was 7% for H₂B-PMHC, 10% for Et₂B-CO₂C₉ and 3% for H₂B-OH. On the other hand, negligible DiD to BODIPY channel crosstalk was observed with our setup. **149**

Figure 3.9. Ensemble fluorescence quenching of liposome preparations containing BODIPY fluorophores. Standard Stern-Volmer (left) and modified Stern-Volmer (right) analyses and corresponding linear fits are shown for MV (black triangles) and PMHC (red circles) quenchers. Full lines extend over the selected fitting range, while dashed lines prolong the fits as a visual guide. **152**

Figure 3.10. Normalized autocorrelation traces of samples acquired as quickly as possible after dilution ($t = 0$ h). H₂B-OH dye only sample is depicted by a dashed line while dye/liposome samples are shown as full lines. Traces were calculated from a continuous 10-minute acquisition with 70 kW/cm^2 514 nm laser excitation. **154**

Figure 4.1. a) SEM image of MPS-PPV NPs (formed in 0.5% H₂O v/v in THF solution) dispersed on a glass coverslip. See sample preparation details in the text. Scale bar is 100 nm. Fluorescence emission properties of MPS-PPV dispersed in various H₂O/THF solutions (b) before (solid lines) and (c) after (dashed lines) addition of 100 mM Bu₄NClO₄ as a powder, $\lambda_{\text{ex}} = 450 \text{ nm}$ in both cases. Panel (c) also shows spectra of two samples before addition of Bu₄NClO₄ (solid lines) to allow for direct comparison. The water content (in % v/v) is indicated near the fluorescence maximum in each trace. The low water regime ($\leq 2.5\%$ H₂O by volume) is shown in red while the high water regime ($\geq 5\%$ H₂O by volume) is shown in blue. Fluorescence in water-only solution is shown in black. Shown in the inset is the chemical structure of MPS-PPV. **167**

Figure 4.2. Effect of applied bias on MPS-PPV NP fluorescence as measured by TIRFM based color-sensitive SMS-EC. a) Photograph of the SMS-EC cell. b) Cartoon illustrating the coverslip with four independent ITO working electrodes and the evanescent optical excitation. The resulting emission from individual nanoparticles is collected by the objective and relayed to the chip of an EM CCD camera for image capture. One acquired frame is shown below depicting real data in the green and red channels. The scale bar is 10 μm . c) Chromatically-resolved (green and red) SMS-EC single particle fluorescence intensity trajectories normalized to the initial fluorescence intensities. Panels d) (47% of the total) and e) (53% of the total) show normalized ensemble fluorescence intensity trajectories for two subsets of NPs showing differing enhancement behavior in the green channel under E_{app} in the region between + 0.03 V and + 0.54 V. Dashed lines corresponding to the initial intensities are visual guides to emphasize the changes in intensity. f) Non-normalized red-to-green fluorescence intensity ratios for NP subsets presented in d) and e). Peaks seen at E_{app} near + 1 V are artifacts resulting from the fluorescence intensity in the red channel dropping near background levels. A total of 68 NPs were analyzed. Time intervals where E_{app} was > + 0.54 V are highlighted in grey. 173

Figure 4.3. Distributions of $E_{1/2}$ values determined from single particle intensity-time trajectories. a) Representative chromatically-resolved (green and red) single particle fluorescence intensity trajectories recorded in our two-color SMS-EC setup. The I_f recorded at + 0.54 V, before the first oxidative quenching event, was taken to be 1. The $E_{1/2}$ is the applied bias where the intensity dropped by half. The center and width of the Gaussian distributions were taken to represent the mean and the standard deviation of $E_{1/2}$ values. Panels b) and c) display data extracted from the green and red channels for both NP subsets shown in Figure 4.2d and Figure 4.2e. 176

Figure 4.4. Cartoons illustrating: (a) state energy diagram for the MPS-PPV/ITO system. In SMS-EC experiments the energy of the ITO Fermi level (E_F) is modulated by an external bias. (b,c) cartoon rendering the hypothetical ordering of MPS-PPV NPs on an ITO electrode, where the polymer backbone is either (b) extended and aggregated or (c) coiled and deaggregated. 178

Figure 4.5. MPS-PPV NP fluorescence as measured by two-color SMS-EC showing the effect of negative applied biases ($-0.97 \leq E_{app} \leq +1.03$ V). (a and b) Average time trajectories normalized to the initial intensities of two subsets of NPs imaged in the same experiment and showing differing behavior at a potential scan rate of 100 mV/s. a) NPs where I_f is highly sensitive to changes in E_{app} . b) NPs that show an asymmetric I_f enhancement at an onset of ~ -0.68 V. c) Results for MPS-PPV NPs when the potential scan rate is reduced to 50 mV/s, normalized to the initial intensities. Only one type of NP behavior is seen ($n = 37$ particles). d) Non-normalized red-to-green fluorescence intensity ratios for NP subsets presented in a) and b). Peaks seen at E_{app} near + 1 V are artifacts resulting from the fluorescence intensity in the red channel dropping near background levels. e) Changes in normalized fluorescence intensity relative to the maximum I_f for the NP subsets shown in a) and b). The traces of subset 5b are offset by 0.2 units to increase clarity. e) Changes in normalized fluorescence relative to the minimum I_f recorded in the green channels at the first positive peak potential for NP subsets shown in a), b) and c). Data shown corresponds to time = 25 – 45 s for subsets 5a and 5b, and time = 50 – 70 s for subset 5c. Time intervals where E_{app} is > + 0.54 V are highlighted in grey, while those where E_{app} is < - 0.68 V are highlighted in purple. 182

Figure 4.6. Proposed effect of MPS-PPV/ITO interfacial ions on the electron transport in MPS-PPV NPs. k_{beT} is the rate of back electron transfer from a MPS-PPV radical anion segment (formed upon PeT from ITO to MPS-PPV) to the ITO electrode. k_{hop} is the self-exchange electron transfer

rate between MPS-PPV segments, leading to electron mobility in the CPE. The onset energy for reduction of quenching sites is indicated at -0.68 V ($E_{\text{onset quench site ox/red}}$) in accordance to experimental observations. Redox potentials for the MPS-PPV⁻ segment can be roughly approximated to the energy levels of the highest occupied and lowest unoccupied molecular orbitals (HOMO, LUMO) of ground state MPS-PPV. **185**

Figure 4.7. MPS-PPV particle size distribution obtained from SEM micrographs, particles were formed in 0.5% H₂O v/v in THF solution, deposited over a clean coverslip and gold coated, see main text's experimental section for details. A Gaussian distribution was used to extract the mean diameter and width of the distribution. **188**

Figure 4.8. Fluorescence emission spectra of MPS-PPV (12.5 µg/mL, 48 µM in polymer repeat units) dispersed in various H₂O/MeCN solutions (a) before (solid lines) and (b) after (dashed lines) addition of 100 mM Bu₄NClO₄ as a powder, $\lambda_{\text{ex}} = 450\text{ nm}$ in both cases. Panel (b) also shows spectra of two samples before addition of Bu₄NClO₄ (solid lines) to allow for direct comparison. The water content (in % v/v) is indicated near the fluorescence maxima in each trace. The low water regime ($\leq 2.5\%$ H₂O by volume) is indicated in red while the high water regime ($\geq 5\%$ H₂O by volume) is indicated in blue. Fluorescence in water-only solution is shown in black. **189**

Figure 4.9. Absorption spectra of MPS-PPV dispersions (12.5 µg/mL, 48 µM in polymer repeat unit) in a) H₂O/THF and b) H₂O/MeCN solutions. **190**

Figure 4.10. Non-normalized MPS-PPV NP fluorescence for NP subsets a) 2d and b) 5a of the main text. Time intervals where the applied bias is $> +0.54\text{ V}$ are highlighted in grey, while those where the applied bias is $< -0.68\text{ V}$ are highlighted in purple. **192**

Figure 4.11. MPS-PPV NP normalized fluorescence as measured by two-color SMS-EC showing the effect of both narrow applied biases ($+0.03 \leq E_{\text{app}} \leq +1.03\text{ V}$) cycles. First cycles were shown in Figure 4.2 of the main text: Panel a) corresponds to Figure 4.2c, b) to Figure 4.2d and c) to Figure 4.2e. Time intervals where the applied bias is $> +0.54\text{ V}$ are highlighted in grey. **193**

Figure 4.12. MPS-PPV NP normalized fluorescence as measured by two-color SMS-EC showing the effect of both negative applied biases ($-0.97 \leq E_{\text{app}} \leq +1.03\text{ V}$) cycles. First cycles were shown in Figure 4.5 of the main text: Panel a) corresponds to Figure 4.5a, b) to Figure 4.5b and c) to Figure 4.5c. Time intervals where the applied bias is $> +0.54\text{ V}$ are highlighted in grey, while those where the applied bias is $< -0.68\text{ V}$ are highlighted in purple. **194**

Figure 4.13. Subensemble fluorescence intensity trajectories normalized to the initial intensities under open circuit. Green and red channel fluorescence intensities are shown for the same subsets of NPs as in Figure 4.2d (solid line) and 4.2e (dashed line) of the main text. Time intervals where the applied bias is $> +0.54\text{ V}$ are highlighted in grey. **195**

Figure 4.14. Subensemble fluorescence intensity trajectories normalized to the initial intensities under open circuit. Green and red channel fluorescence intensities are shown for the same subsets of NPs as in Figure 4.5a (solid line) and 4.5b (dashed line) of the main text. Time intervals where the applied bias is $> +0.54\text{ V}$ are highlighted in grey, while those where the applied bias is $< -0.68\text{ V}$ are highlighted in purple. **195**

Figure 4.15. MPS-PPV NP fluorescence as measured by SMS-EC showing the effect at a 'narrow' applied biases range ($+0.03\text{ V} \leq E_{\text{app}} \leq +1.03\text{ V}$). a) Changes in normalized fluorescence intensity relative to the maximum I_f for the NP subset shown in Figure 4.2d and 4.2e of the main text. The traces for subset 2e is offset by 0.2 units for clarity. The peak maximum potentials are indicated

by the vertical blue dotted lines. b) Change in normalized fluorescence relative to the minimum I_f recorded in the green channels at the first maximal positive potential peak of both NP subsets shown in Figure 4.2d and 4.2e. Time intervals where the applied bias is $> +0.54$ V are highlighted in grey. 196

Figure 5.1. SMS-EC microscope setup and configuration of the samples studied. A) Photograph of the SMS-EC cell illustrating the electrodes and leads for the electrochemical chamber. B) Cartoon showing the coverslip with 4 independent L-shaped ITO electrodes and the evanescent optical excitation. The fluorescent emission from dyes bound to the SnO_2 film passes through the objective and is relayed to an EM-CCD camera for image capture. Shown to the right is a series of frames from a movie acquired on a perylene-sensitized region of the SnO_2 film under an applied bias of 0.83 V vs. NHE. The scale bar is 20 μm . C) Energy diagram showing the relevant energy levels of the SnO_2 and dyes utilized in this work (see also the chemical structures of the dyes). Occupied electronic states of the SnO_2 are highlighted in red. The ground state redox potentials are drawn in black (closed form of the perylene). The excited state redox potentials (excited state oxidation in red, excited state reduction in blue) are calculated taking into account the E_{00} energies of the sensitizers (opened form for the perylene). Excited state oxidation potentials are drawn in red and excited state reduction potentials are in blue. To the left is an example sensitizer depicting dye photooxidation (k^*_{ox}) and photoreduction (k^*_{red}) pathways. 208

Figure 5.2. Influence of applied bias on the photobleaching of the fluorescent sensitizer dyes under study. Shown to the left are the normalized fluorescence intensity trajectories of the perylene (A) and SQ2 (C) dyes. See Experimental section for details on excitation and emission wavelengths. Shown to the right are the photobleaching rates obtained from stretched exponential fits to the data (Equations (5.1) and (5.2)) and residual fluorescence after 80 s of illumination for the perylene (B) and SQ2 (D) dyes. The red lines in B) represent fits to the sigmoidal expressions depicted in Equations (5.3) and (5.4) below. The best fit parameters for E_0 are noted in the plot. 210

Figure 5.3. Comparison of the photocurrent measured upon excitation of perylene and SQ2 bound to SnO_2 . The laser excitation (488 nm, 14 μW for perylene; 647 nm, 396 μW for SQ2) was initiated at time $t = 0$ s. The applied bias was kept at 0.43 V before and during irradiation. Inset: Zoomed in view of the first 2 s of the photocurrent decay. 215

Figure 5.4. Influence of applied bias on the initial fluorescence intensity of the perylene and SQ2 dyes. A) The blue lines represent fits to the sigmoidal expression depicted in Equation (5.7). The k^*_{red} term is not observed in the case of SQ2 and was set to 0 for the fitting. The best fit E_0 parameters are noted in the plots. For the SQ2, the purple point at 0.73 V was excluded from the fitting since the fluorescence intensity decreases due to ground state oxidation at this E_{app} . B) Same data as in A) with normalized fluorescence intensity of both dyes, normalized to the values at 0.43 V. C) Relative charge transfer rates for the perylene as calculated from the fit parameters obtained in A). 218

Figure 5.5. Micrographs of SnO_2 film prepared by spin coating a NP dispersion on ITO coverslips followed by sintering at 450 $^\circ\text{C}$ for 1 hour as obtained by atomic force microscopy (AFM) for (A) 40 x 40 μm^2 and (B) 5 x 5 μm^2 areas. 223

Figure 5.6. Results of control experiments of sensitized ITO surfaces. All conditions were the same as those used in the experiments of sensitized SnO_2 films presented in the main text. See Figure 5.4 for comparisons of the intensities of dyes on bare ITO surfaces (shown here) and on the SnO_2 surface. 224

Figure 5.7. Photobleaching of the perylene and SQ2 dyes bound to an insulating SiO₂ support. We attribute the surprising fluorescence intensity increase observed for the perylene over time to photodegradation of quenching sites caused by aggregated dyes (e.g. non-emissive dimers). ... **225**

Figure 5.8. Additional representation of the photobleaching traces of the perylene dye bound to SnO₂ shown in Figure 5.2A of the main text. A and B) Normalized fluorescence intensity trajectories of the perylene at different timescales. Each data point is represented by a triangle. Stretched exponential fits (Equation (5.1)) are shown as gray lines overlaid on the fluorescence trajectories. C) Non-normalized fluorescence trajectory showing of the same data shown in B). The E_{app} under which the trajectories were acquired are noted in the plots. **225**

Figure 5.9 Comparison of the SnO₂ film DOS estimated by CV (A) or chronoamperometry (B) techniques. The film was cast from a 15% wt. SnO₂ NP solution. Electrochemical measurements were performed in argon-purged 0.1 M Bu₄NClO₄ acetonitrile solutions. The direction of the scans in the CV experiment (scan rate of 50 mV/s) is indicated by arrows. Exponential fits are shown in red with the extracted exponential constant indicated. **225**

Figure 6.1. A) SMS-EC setup depicting H₂B-PMHC molecules that are oxidized at the surface of the Pt/ITO WE and then diffuse through the confocal observation volume (dotted oval) and emit fluorescence. The distance between the electrode surface and the observation volume was controlled by a piezo nanopositioner. B) Oxidation of H₂B-PMHC to yield fluorescent H₂B-PMHC_{ox}. Addition of traces of H₂O may subsequently give rise to other oxidized species. C) Applied electrochemical potential. The potential scan rate was 20 mV/s. D) Fluorescence trajectories of chemically oxidized H₂B-PMHC (red), pristine H₂B-PMHC under open circuit conditions (black) and pristine H₂B-PMHC under influence of the bias shown in C). Times where $E_{app} \geq 0.88$ V are highlighted in gray. Trajectories were calculated using 100 ms time bins. The concentration of H₂B-PMHC was 0.5 nM in all cases. Trajectories were acquired with the observation volume 20 μ m above the electrode surface. **234**

Figure 6.2. SMS-EC of H₂B-PMHC under high positive E_{app} . A) Applied electrochemical potential. The potential scan rate was 20 mV/s. B) Fluorescence trajectory (black) and burst frequency (red) of H₂B-PMHC under influence of the bias shown in A). Trajectories were calculated using 100 ms time bins. C) and D) 100 ms segments of the fluorescence trajectories indicated in B) by the arrows. Time bins of 100 μ s were used, and high fluorescence bursts are counted for bins where counts/100 μ s ≥ 11 . E) Burst frequency as in B) but plotted against E_{app} instead of time. The potential scan towards 1.81 V is traced in red while the scan back towards 0.31 V is traced in black. Direction of potential scans are further indicated by arrows. Times where $E_{app} \geq 0.88$ V are highlighted in gray. Trajectories acquired with the observation volume 20 μ m above the electrode surface. **237**

Figure 6.3. SMS-EC determination of the concentration profile of electrochemically-generated H₂B-PMHC_{ox}. A) Applied electrochemical potential. The pulses were 10 s in duration. B) Fluorescence trajectories of H₂B-PMHC under influence of the bias shown in A) for confocal observation volumes located 20 μ m (black), 50 μ m (red) and 80 μ m (blue) above the electrode surface. The time axis was set to 0 s at the start of the + 1.26 V pulse that lasted for 10 s. C) Segment of the fluorescence trajectories shown in B) emphasizing the $E_{app} = + 1.26$ V region. Gray lines represent the best fit result of the model described by equation (6.2). **239**

Figure 6.4. Indirect fluorescence monitoring of the electroreduction of O₂. A) Applied electrochemical potential. B) Fluorescence trajectories of H₂B-PMHC under influence of the bias

shown in A) in air-equilibrated conditions. The red line represents the average fluorescence intensity prior to applying a bias. Trajectories were acquired with the observation volume 20 μm above the electrode surface. C) Scheme illustrating the oxidation of H₂B-PMHC initiated by electrochemically-generated O₂^{•-}. **242**

Figure 6.5. Fluorescence enhancement observed in acetonitrile solutions upon chemical oxidation of H₂B-PMHC (1 μM) by 2 eq. of cerium(IV) ammonium nitrate. An 83-fold fluorescence enhancement is calculated by integrating the intensities between 485 – 675 nm following emission correction for background. $\lambda_{\text{ex}} = 480 \text{ nm}$ **245**

Figure 6.6. Cyclic voltammogram of H₂B-PMHC (54 μM) in argon-purged 0.1 M Bu₄NClO₄ acetonitrile solution. The experiment was run in the SMS-EC cell: Pt/ITO served as the working electrode, AgCl-coated Ag wire as the quasireference electrode and a Pt wire as the counter electrode. The anodic peaks at 1.26 V and 1.61 V are assigned to the oxidation of the chromanol and BODIPY core, respectively. The cathodic peak at – 0.74 V is assigned to the reduction of the BODIPY core, in accordance with reduction potentials values for similar BODIPY dyes ($\sim -0.8 \text{ V}$).²⁷ The potential sweep rate was 0.1 V/s. **245**

Figure 6.7. Effect of the presence of oxygen under reductive conditions. A) Applied electrochemical potential. The potential scan rate was 20 mV/s. B and C) Fluorescence trajectories of H₂B-PMHC under influence of the bias shown in A). B) Argon-purged conditions. C) Air-equilibrated conditions. Times where $E_{\text{app}} \leq -0.6 \text{ V}$ are highlighted in purple. **246**

List of Abbreviations and Symbols

β	Electron transfer distance parameter ($\text{\AA}^{-1}$)
χ_m	Fraction of dye associated with lipid membrane
Δ	Heat
ΔE_{00}	Optical band gap
ΔG	Free energy change
ΔH	Enthalpy change
ΔS	Entropy change
η	Viscosity ($\text{Pa} \cdot \text{s}$)
ε	Dielectric constant
ε_0	Permittivity of vacuum
Φ_f	Quantum yield of fluorescence
Γ	Gamma function
λ	Wavelength; reorganization
ν	Frequency (s^{-1})
ρ	Density of states
τ	Lifetime (s^{-1})
ω	Coulombic correction term
[X]	Concentration (M)
A	Amplitude
ABAP	2,2'-Azobis-(2-methylpropionamide) dihydrochloride
Abs.	Absorbance
AFM	Atomic Force Microscopy
AH	Antioxidant
AOH	Phenolic Antioxidant
APD	Avalanche Photodiode Detector
Ar^+	Argon-ion laser
AuNP	Gold Nanoparticle
B	Brightness
BDE	Bond Dissociation Enthalpy
BeT	Back electron Transfer
Biotin-DPPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl) (sodium salt)
biotin-PEG-SVA	Biotinylated polyethylene glycol succinimidyl valerate
BODIPY	Boron-dipyrromethene (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)
BME	β -mercaptoethanol
BSA	Bovine Serum Albumin
B-TOH	8-(($\pm$) 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxyloxy)- methyl-2,6-diethyl-1,3,5,7-tetramethyl pyrromethene fluoroborate (Bodipy-Trolox adduct)
CCD	Charge-Coupled Device

CE	Counter Electrode
CPE	Conjugated Polyelectrolyte
CuNP	Copper Nanoparticle
CV	Cyclic Voltammetry
D	Diffusion coefficient ($\text{m}^2 \text{s}^{-1}$)
DCP	Dicumyl Peroxide
DiD	1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate
DiI	1,1'-dilinoleyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate
DNA	Deoxyribonucleic acid
DOPC	1,2-Dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DOS	Density of States
DSSC	Dye-Sensitized Solar Cell
e	Elemental charge
EA	Electron Affinity
EC	Electrochemistry
EMCCD	Electron Multiplied Charge-Coupled Device
E_F	Fermi Level
F	Faraday constant
FAD	Flavin Adenine Dinucleotide
Fc	Ferrocene
FCS	Fluorescence Correlation Spectroscopy
Fluo.	Fluorescence
FRET	Förster Resonance Energy Transfer
FTO	Fluorine-doped Tin Oxide
FVS	Fluorescence-Voltage Spectroscopy
F-V/SMS	Single Molecule Fluorescence-Voltage Spectroscopy
h	Planck constant
H ₂ B-PMHC	8-((6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)-methyl)-1,3,5,7-tetramethyl Pyrromethene Fluoroborate (H ₂ -Bodipy-PMHC adduct)
H ₂ B-PMHC _{ox}	Oxidized H ₂ B-PMHC
H ₂ B-TOH	8-((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxyloxy)-methyl-1,3,5,7-tetramethyl pyrromethene fluoroborate (H ₂ -Bodipy-Trolox adduct)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOMO	Highest Occupied Molecular Orbital
I	Fluorescence Intensity
IC	Internal Conversion
ICT	Intramolecular Charge Transfer
IP	Ionization Potential
IR	Infrared
ISC	Intersystem Crossing
ITO	Indium Tin Oxide
k	Rate constant

K	Equilibrium constant
k_B	Boltzmann constant
LDL	Low-Density Lipoprotein
LH	Lipid
LOOH	Lipid Hydroperoxide
LUMO	Lowest Occupied Molecular Orbital
<i>m</i> CPBA	<i>meta</i> -chloroperoxybenzoic
MeCN	Acetonitrile
MEH-PPV	Poly(2-methoxy-5-(2'-ethyl-hexyloxy)-1,4-phenylene vinylene)
MO	Molecular Orbital
mPEG-SVA	Polyethylene glycol succinimidyl valerate
MPS-PPV	Poly(5-methoxy-2-(3-sulfopropoxy)-1,4-phenylenevinylene)
MV ²⁺	N,N'-dimethyl-4,4'-bipyridinium dichloride (Methyl Viologen)
N	Number of molecules
N_A	Avogadro's constant
NA	Numerical Aperture
NHE	Normal Hydrogen Electrode
NP	Nanoparticle
PBS	Phosphate Buffer Saline
PEG	Polyethylene Glycol
PeT	Photoinduced electron Transfer
Phos.	Phosphorescence
PMHC	2,2,5,7,8-Pentamethyl-6-hydroxychroman
PMOH	8-Hydroxymethyl-2,6-diethyl -1,3,5,7-tetramethyl pyrromethene fluoroborate
PMT	Photomultiplier Tube
PUFA	Polyunsaturated Fatty Acids
q	Relative brightness
RE	Reference Electrode
Redox	Reduction-oxidation
RM	Redox Mediator
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
ROXS	Reducing and Oxidizing System
SEM	Scanning Electron Microscopy
SM	Single Molecule
smFRET	Single Molecule Förster Resonance Energy Transfer
SMS	Single Molecule Spectroscopy
SMS-EC	Single Molecule Spectroelectrochemistry
T	Triplet fraction
TEM	Transmission Electron Microscopy
TIRF	Total Internal Reflection Fluorescence
VR	Vibrational relaxation

WE
F-V/SMS

Working Electrode
Single Molecule Fluorescence-Voltage Spectroscopy

Abrégé

Cette thèse décrit nos avancés dans le domaine de la visualisation, de la détection, et du contrôle des processus d'oxydoréduction (redox) au niveau de la molécule unique. Deux thèmes distincts, inspirés par des systèmes biologiques et de matériaux, sont différenciés par l'étude de réactions chimiques ou électrochimiques. Les méthodes utilisées reposent sur l'utilisation de la spectroscopie de fluorescence de la molécule unique. L'extinction de la fluorescence, accomplie par un mécanisme de transfert électronique photo-induit, est au cœur de la détection par fluorescence de molécules/particules uniques de composés qui changent d'état redox. Nos contributions aux études de réactions chimiques prenant cour dans un environnement hétérogène au niveau de la molécule unique s'étendent du processus d'oxydation chimique de la peroxydation de lipides jusqu'aux systèmes sous contrôle électrochimique.

Nous avons initialement exploré l'utilisation d'antioxydants fluorogéniques afin de disséquer le processus de peroxydation de lipides. Nous avons déterminé que l'oxydation des liposomes procède de façon hétérogène, résultant en de grandes variations du niveau d'oxydation entre chaque liposome individuel. En utilisant des additifs antioxydants, nous avons pu déterminer qu'un certain niveau d'oxydation se produit durant la préparation standard de liposomes. Ces études initiales de molécules uniques ont aussi identifié un phénomène surprenant de clignotement de sondes fluorescentes activées que nous avons utilisées.

Nous avons ensuite investigué la localisation et la rétention de matière colorantes dans la membrane lipidique de liposomes sous les conditions hautement diluées requisent pour les expériences à molécule unique. Nous avons démontré qu'une considération judicieuse doit être portée quant au choix du colorant ou de la sonde utilisés dans des expériences de la molécule

unique. Tous les colorants BODIPY étudiés sont restés intégrés dans la membrane de lipides de liposome sous des conditions convenables pour les mesures traditionnelles d'un ensemble. La dilution jusqu'aux concentrations souhaitées pour des expériences à molécule unique apporte la distinction de lipophilicités différentes. Nous avons développé une méthode rapide basée sur la spectroscopie de corrélation de fluorescence (FCS) pour déterminer si une matière colorante ou une sonde sont appropriées pour mener des expériences de la molécule unique dans des membranes lipidiques. L'influence sur la répartition dans les lipides de l'adduit d'une molécule d'intérêt et une sonde activée chimiquement fût également constatée par cette méthode.

Par la suite, nous avons appliqué des techniques de spectroélectrochimie de la molécule unique (SMS-EC) afin de répondre des questions clés concernant l'hétérogénéité de systèmes d'intérêt aux chercheurs des matériaux. Premièrement, nous décrivons la préparation et la caractérisation de nanoparticules de polyélectrolytes conjugués qui donnent lieu à un environnement nanoconfiné. La réponse de la fluorescence de nanoparticules individuelles aux potentiels électrochimiques dévoile l'influence de la conformation et l'empilement de chaînes polymères et l'effet qui en résulte au sujet de la migration des ions. Nous avons couplé une imagerie bichrome à la SMS-EC pour la première fois, ce qui nous a permis de distinguer entre les propriétés électrochimiques de chaînes polymères non-agglomérées et agglomérées à l'intérieur de nanoparticules individuelles.

Ultérieurement, nous avons exploré la dynamique de transfert de charges interfacial qui prend place dans prototypes de cellules solaires à pigment photosensible. Avec la SMS-EC, la densité d'électrons dans un semi-conducteur était contrôlée en appliquant un potentiel électrochimique, et les changements de vitesse de transfert interfaciale de charges fussent inférés en mesurant l'intensité de la fluorescence émise. En mesurant la vitesse de photoblanchiment ainsi

que l'intensité de la fluorescence émise par deux pigments caractérisés par des potentiels d'oxydo-réduction d'états excités amplement différents, nous avons appris comment change la vitesse de transfert de charges interfacial sous l'action d'un potentiel électrochimique et quels sont les facteurs qui mènent à ces changements.

Nous avons également mis en valeur la SMS-EC comme étant un nouvel outil qui permet de déterminer optiquement le profil de concentration de produits générés par l'électrochimie. En prenant avantage encore une fois des avancés dans le domaine d'antioxydants fluorogéniques, nous avons observé des molécules de produits uniques sous contrôle électrochimique. Nous avons pu déterminer expérimentalement le transfert de masse qui se produit près de la surface d'une électrode plane. Nous avons élargi l'utilisation de la SMS-EC jusqu'aux petites molécules dissoutes dans des solvants organiques, ce qui enrichi la portée de réactions chimiques qui peuvent être étudiées au niveau de la molécule unique.

Nous concluons cette thèse avec des remarques finales et une perspective pour des recherches futures. Le progrès portant sur l'étude de processus redox hétérogènes au niveau de la molécule unique engendra de nouvelles avenues de recherche. Nous envisageons que la SMS-EC et d'autres techniques de la molécule unique gagneront une place importante comme outils dans la recherche contemporaine du domaine de la chimie.

Abstract

This thesis describes our advances in visualizing, monitoring and controlling oxidation-reduction (redox) processes at the single molecule level. Methods center on the use of single molecule fluorescence spectroscopy to understand two distinct themes. The two themes, inspired by biological and material systems, are differentiated by the study of chemical or electrochemical redox reactions. Fluorescence quenching via a photoinduced electron transfer (PeT) mechanism is at the heart of the fluorescence-based single molecule/particle detection of individual analytes that undergo changes in redox states in both cases. Our contributions to the study of heterogeneous single molecule chemical reactions spans the chemical oxidation process of lipid peroxidation to optoelectronic systems under electrochemical control.

We initially explored the use of lipophilic fluorogenic antioxidants to monitor the process of lipid peroxidation. We determined that oxidation taking place in liposomes is heterogeneous in nature, with large liposome-to-liposome variations in the extent of oxidation. Through the use of antioxidant additives we were able to establish that oxidation takes place during standard liposome preparation. These initial single molecule studies also revealed a surprising blinking behaviour in the activated fluorescent probe that was utilized.

We next investigated the localisation and retention of dyes in liposomes under the highly dilute conditions required for single molecule experiments. We showed that careful consideration of the dye or probe used in single molecule experiments is warranted. All BODIPY dyes studied remained embedded in the lipid membrane of liposomes at concentrations suitable for ensemble experiments. Upon dilution to single molecule conditions, differences in lipophilicity became apparent. We developed a rapid method based on fluorescence correlation spectroscopy (FCS) to

determine if a dye or probe of choice is suitable for single molecule experiments in lipid membranes. The influence on the lipid partitioning of the adduct between an analyte of interest and a chemically-activated probe was also revealed using this method.

We then applied single molecule spectroelectrochemistry (SMS-EC) techniques to answer key questions regarding heterogeneity in systems of great interest to material scientists. We first describe the preparation and spectroscopic characterization of nanoconfined conjugated polyelectrolyte nanoparticles. The fluorescence response of individual nanoparticles to applied biases shed light on the conformation and packing of polymer chains and the resulting effect on ion migration. We introduced two-color imaging to SMS-EC for the first time, which allowed us to discriminate between the electrochemical properties of deaggregated and aggregated polymer segments within individual nanoparticles.

We subsequently explored the interfacial charge transfer dynamics that take place in dye-sensitized solar cell prototypes. In a SMS-EC setup, the electron density in a semiconductor was controlled by applying an electrochemical potential, and the changes in the rates of interfacial charge transfers was inferred by monitoring the fluorescence intensity. By monitoring the rate of photobleaching in addition to the fluorescence intensity of two sensitizer dyes with distinct excited state potentials, we learned how the rates of interfacial charge transfers change with applied biases and which factors drive these changes.

We additionally showcased SMS-EC as a new tool to optically determine the concentration profile of electrochemically-generated products. Once more taking advantage of the advancements in fluorogenic antioxidants, we observed individual reaction products under electrochemical control. We were able to experimentally verify the mass transport process that occurs near a planar

electrode surface. We extended the use of SMS-EC to study small molecules in organic solvents, increasing the scope of chemical reactions that can be studied at the single molecule level.

We conclude this thesis with final remarks and an outlook for future research. The progress made in studying heterogeneous redox processes at the single molecule will spur new research avenues. We envisage that SMS-EC and other single molecule techniques will gain significant traction as important tools in contemporary chemical research.

1. Introduction

Reduction-oxidation (redox) processes are important for both biological functions and material applications. For example, homeostasis requires the proper control of the redox status of cells and in living organisms. Development of technologically advanced materials requires the fine tuning of the redox behaviour of optoelectronic systems where the redox state and luminescence are intertwined. Further improvements will require new methodologies and novel spectroscopies to elucidate fundamental complexities that are thus far indiscernible. We strongly believe that the development of single molecule (SM) fluorescence techniques will play a leading role in our future comprehension of redox processes. The advantage of single molecule spectroscopy (SMS) is its precision: in comparison, traditional measurement techniques average the contributions of many billions of molecules. Much information on the stochastic behaviour of individual molecules,¹⁻⁸ enzymes and proteins,⁹⁻²⁰ or the heterogeneity of nanomaterials²¹⁻³⁴ is lost in the average, obscuring the true behavior of molecules. The advent of novel imaging techniques such as super resolution imaging³⁵ and light sheet microscopy³⁶ in the last decade will continue to spur further advances in our understanding of molecular processes.

The work described in this thesis highlights our contributions to popularize fluorescence SM techniques for the study of redox processes. By developing methods and platforms using SM techniques that are applicable to study both chemical and electrochemical redox processes, we aim to contribute to the growth of the physical chemistry toolbox. The following introduction first exposes our motivation to use SMS as a tool to elucidate redox processes by underscoring the inherent advantages of SM techniques. We then focus on SM chemistry with an overview of the challenges that need to be surmounted and relevant past examples. Next, we cover the core concepts of fluorescence and fluorescence quenching needed to understand the process of

photoinduced electron transfer (PeT). PeT is the mechanism we chose to be able to distinguish the different redox states of a molecule based on the fluorescence intensity and is the key molecular process used to study the chemical systems of interest that are presented in this thesis. The following section introduces fluorescence probes, specifically those used to monitor oxidation processes, and the chemical oxidation process that occurs in lipid membranes (lipid peroxidation). These topics assist the reader to contextualize the results described in Chapters 2 to 6. Following this, we discuss state-of-the-art single molecule spectroelectrochemistry (SMS-EC), which not only allows us to discriminate between redox states of a molecule by monitoring the fluorescence emitted, but also enables electrochemical control of the redox processes. We took advantage of the unique possibilities offered by SMS-EC to carry out the experiments summarized in Chapters 4 to 6. A comprehensive review of the literature that involves optical SM observations of redox processes under electrochemical control is also described. Finally, we detail the instrumentation used to conduct the SMS experiments presented in this thesis.

1.1. Single Molecule Spectroscopy (SMS)

Motivated to understand the interactions of matter, scientists have strived to study materials at the smallest scale possible. A major breakthrough in optical microscopy occurred in 1989: the first observation of individual molecules.³⁷ This tour de force has spurred enormous efforts to perform studies one molecule at a time. Truly remarkable feats have been accomplished when observing individual molecules. We have gone from observing pentacene within p-terphenyl crystals at temperatures of 1.8 K,³⁷ to determining the walking motion of myosin,³⁸⁻³⁹ to gaining new insights on *in vivo* DNA replication.⁴⁰ We will see that while molecular biology has led the way in developing SMS, tremendous potential exists in applying SMS to challenging chemical systems.

SMS holds many advantages over traditional measurements that inspect an ensemble of molecules.⁴¹⁻⁴⁴ By experimentally detecting individuals as opposed to populations, the origin of complex behavior can be fully elucidated, from kinetics to deactivation processes. The observation of singular chemical species allows us to determine where the molecule is (down to nanometer precision), the path it took to get there, and its state. SM experiments additionally unlock the ability to probe the course of a molecule from reactant, to intermediate, and finally to product. It is also possible to correlate position or conformation to reactivity.⁴⁵⁻⁵³ One more advantage gained by the ability to detect individual molecules is the smaller amount of analytes used in a SM experiment. Typically only 10 μL of a 100 pM solution (i.e. 1×10^{-15} moles!) is needed for adequate surface coverage, an important consideration for expensive or hard-to-obtain substrates.

A principal benefit of SM techniques is the ability to measure dynamic and static heterogeneity.⁵⁴⁻⁵⁵ On the one hand, dynamic heterogeneity, for example, is the wavering changes in turnover rates of nanoparticle (NP) catalysts over time. On the other hand, static heterogeneity represents the particle-to-particle differences in average activity. These heterogeneities in reactivity are not discernable in ensemble measurements. Furthermore, as an imaging technique, SMS may be exploited to study the kinetics of biphasic systems, for example at solid-liquid interfaces. Complications that often arise in kinetic ensemble measurements when working with a mixture of phases are mitigated with SM techniques. All in all, SMS is a powerful tool that is particularly well-suited for the study of heterogeneous systems.⁵⁶

The field of molecular biology has led the way in adopting SM techniques.⁵⁷ Early SM experiments demonstrated that molecular machineries consisting of enzymes and proteins in motion have significant inherent heterogeneity.¹⁵⁻¹⁶ The heterogeneity arises from conformational changes that may lead to active or dormant states, dictate binding preferences and regulate gene

expression. Traditional measurements of an ensemble of molecules typically don't evaluate this heterogeneity and consequently do not always portray the complete molecular picture of the process under study.

Outside of molecular biology, however, adoption of SM techniques has been slow, particularly for chemistry.⁵⁸⁻⁵⁹ This is quite surprising considering the benefits of SM techniques to study heterogeneous systems and the ubiquitous use of nanomaterials in both research and industry. Additionally, a deep mechanistic understanding of chemical reactions is necessary for the development of novel synthetic strategies. SM will surely play a major role in future synthetic developments and has the potential to revolutionize our understanding of key chemical transformations in industrial and biological systems. By monitoring individual chemical reactions, we open the door to directly study the basis of kinetic models, that is, the elementary steps of a reaction.

1.2. Single Molecule Chemistry

Considering the unique insight made possible by SM experiments, the slow uptake of SM techniques by chemists is surprising. It seems likely that the experimental challenges tied to monitoring chemical reactions compared to biological systems represent a hurdle that needs to be overcome. After discussing these challenges, we will review the recent progress made in observing chemical reactions one at a time. Finally, examples that emphasize redox reactions will be highlighted in view of the focus of this thesis.

In order to monitor reactions with fluorescence-based SMS techniques, there needs to be a change in a fluorescence property to indicate that the reaction is taking place. The observable fluorescence properties include fluorescence anisotropy, fluorescence lifetime, emission color, and fluorescence intensity.⁶⁰ The simplest change to observe is one in fluorescence intensity, whereas

others may be more subtle and require larger amounts of collected photons (e.g. emission spectra). It thus comes as no surprise that fluorescence intensity is the main way that reactions at the SM level have been monitored. We will expand on the underlying photophysics of fluorescence in Section 1.3 and discuss strategies that control the fluorescence intensity, such as photoinduced electron transfer (PeT). The design of a fluorescence-based probe that relies on PeT as a fluorescence switching mechanism will then be covered in Section 1.4.1.

Since most chemical reactions involve non-fluorescent substrates, a reporting fluorescent segment must be appended in order to monitor changes in fluorescence. Even though fluorophore conjugation to biomolecules such as proteins or DNA can be accomplished through the use of commercially available kits, procedures to fluorescently label other types of molecules of interest have to be uniquely developed. This poses a challenge in applying SMS to chemical systems as significant time investment in the design and synthesis of a suitable platform is required before even attempting to monitor the reaction of interest.⁶¹ Another experimental challenge associated with chemical systems is the lower substrate affinity for organic and transition metal catalysts compared to enzymatic systems.⁶²⁻⁶³ It follows that issues in non-specifically bound fluorescent background are more problematic for chemical systems as larger amounts of fluorescent substrates are typically needed to promote binding. Chemical reactions also have different, sometimes restrictive environmental requirements. Rigorously dry and moisture-free imaging conditions are needed for some chemical transformations to proceed smoothly, but no commercial reaction cells are currently available to make imaging such reactions routine. The biophysical community now has over 20 years of experience to tackle common challenges such as fluorophore bleaching under aqueous conditions suitable for DNA and protein molecules.⁶⁴ However, this expertise is assuredly not extendable to significantly different imaging conditions (for instance the use organic solvents),

causing SM studies of chemical processes to often have unique requirements necessitating tailored conditions.

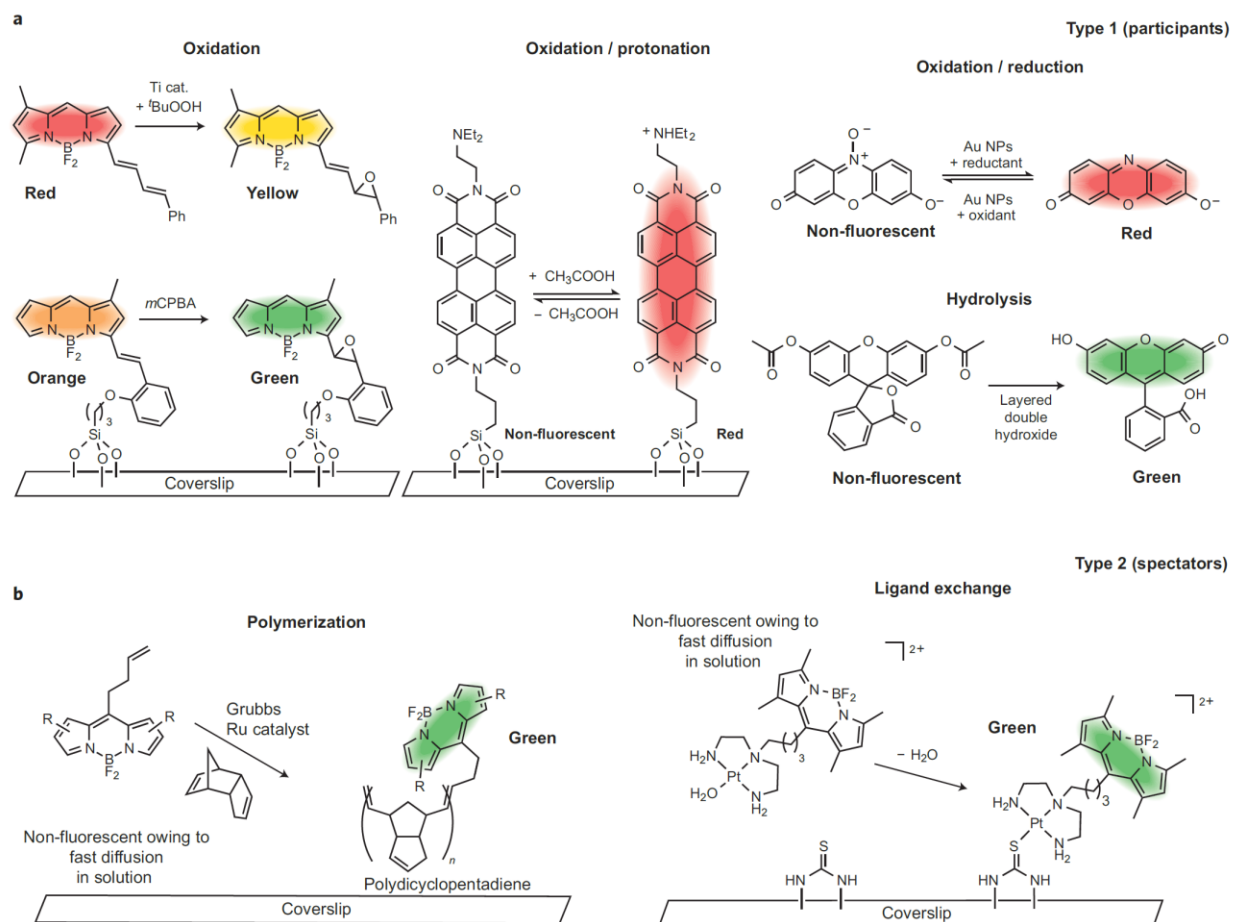


Figure 1.1. Examples of fluorophores that have been used to study chemical reactions at the SM level. Reprinted with permission from Macmillan Publishers Ltd: Nature Chemistry, ref. 58, copyright 2013.

Even in the face of such adversity, SM studies have been used to gain key insights into chemical reactivity.⁶⁵ For example, the Hofkens group has made significant contributions to the field of SMS. First, they identified crystal-face-dependant catalytic activity of $[\text{Li}^+ - \text{Al}^{3+}]$ layered double hydroxide particles.⁶⁶ Using SMS, they could discriminate between the similar reactions of transesterification (addition of 1-butanol) and hydrolysis (addition of H_2O) that take place on

different crystal faces; they found that transesterification occurred on the entire outer crystal surface while ester hydrolysis was preferentially observed at the crystal edges. Second, they presented a strategy to sense protons or metal ions at the SM level.⁶⁷ Surface-immobilized perylene diimide with a pendent amine functionality was non-fluorescent when the amine was uncoordinated, but emitted bright fluorescence once a source of protons was added, resulting in the protonation of the amine. This research paves the way to determine whether or not a catalytic center is bound by modulating the fluorescence of the perylene reporter.

The power of SM is also evident when considering studies of heterogeneous vs. homogeneous catalysis. Heterogeneous catalysis is where the catalyst is present in a different phase of matter (typically a solid) than the reactants (typically in liquid solution). Homogeneous catalysis implies that both the catalyst and reactants are present in the same phase, i.e. all in liquid solution. SM techniques confirmed that polymerization using Grubbs II catalysis occurs solely by homogeneous means,⁶⁸ whereas CuNP-catalysed ‘click’ cycloaddition only took place on the surface of the nanoparticles (i.e. heterogeneous catalysis).⁶⁹ These conclusions are virtually impossible to reach by traditional ensemble setups, such as cuvette-based measurements in a fluorimeter. Another interesting case was the observation of a two distinct reaction pathways that yielded the same product. The epoxidation of a BODIPY probe by *meta*-chloroperoxybenzoic acid (*m*CPBA) was shown to not only occur through the accepted, concerted ‘butterfly’ transition state, but also via a distinct pathway where an intermediate species is formed.⁷⁰ Similar discovery of a reaction intermediate was made possible by SM techniques that were applied to the catalytic oxidation of amplex red by AuNPs.⁷¹ Another remarkable report described the acceleration of bond breaking in conjugated polymers due to collisions that induce bending and weaken chemical bonds.⁵³ The increased catalytic heterogeneity of acid mordenite zeolites after dealumination was

inferred by the oligomerization of furfuryl alcohol into a fluorescent product.⁷² Other examples of studied chemical reactions include metal complex formation⁷³ and ligand exchange reactions.⁷⁴⁻⁷⁶

Specifically important examples, given the focus of this thesis, are those that observed redox cycling at the SM level. A first report showed temporal fluctuations of a Cu site within a fluorescently-labelled nitrite reductase enzyme.⁷⁷ The oxidation of the Cu active site ($\text{Cu}^+ \rightarrow \text{Cu}^{2+}$) concomitant with the reduction of nitrite (NO_2^-) to nitric oxide (NO^*) led to fluorescence quenching of the Atto647N reporter dye. Related studies of nitrite reductase provided further insight in the reaction mechanism⁷⁸ and disorder in the catalytic site.⁷⁹ Another interesting publication showed that fluorescent immobilized hydroquinone-coupled Rhodamine B was oxidized by Cu^{2+} in air and led to quenched fluorescence in the resulting oxidized quinone state (see Figure 1.2). Individual fluorophore intensity trajectories showed that subsequent reduction by cysteine restored the fluorescence in some of the molecules, while others were unreactive, revealing dynamic processes and the formation of intermediate states upon reaction.⁸⁰

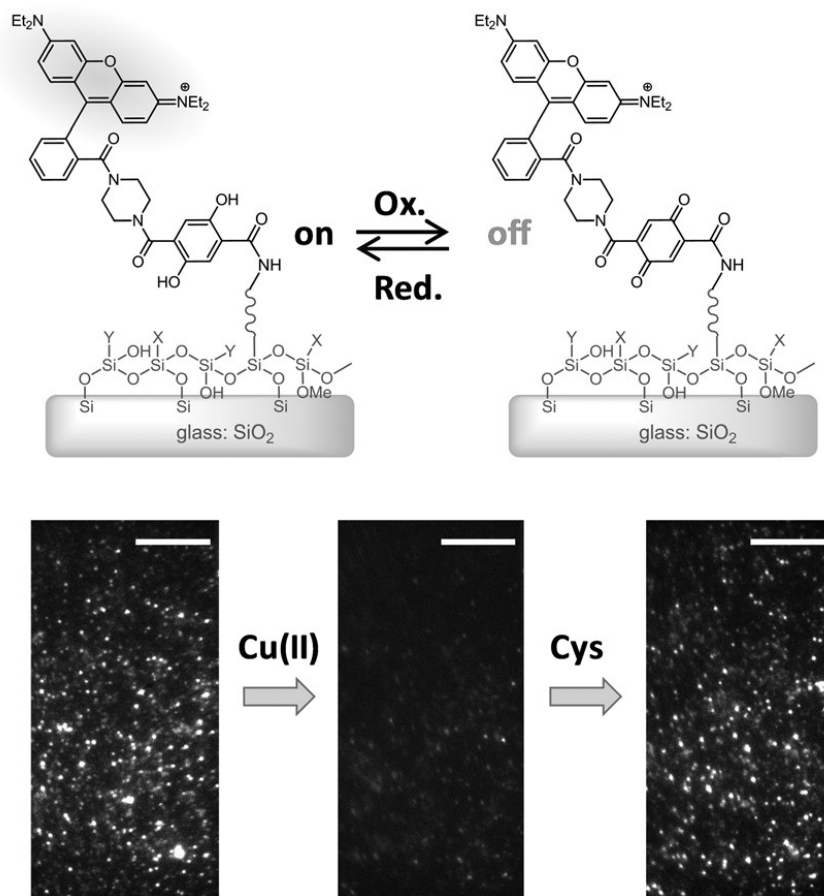


Figure 1.2. Visualization of quinone/hydroquinone redox cycling at the SM level. Adapted from ref. 80 with permission of the PCCP Owner Societies.

Equally of interest is the body of work that Majima's group has provided in the SM sensing of photocatalytic processes of TiO₂.⁸¹⁻⁸² The spatial and temporal distributions of ROS such as hydroxyl radical (HO[•])⁸³ and singlet oxygen (¹O₂)⁸⁴ produced during TiO₂ photocatalytic reactions is inferred from the observed changes in fluorescence upon UV irradiation of TiO₂.⁸⁵ Again utilizing SM detection, the photocatalytic reduction of aromatic nitro groups to amines could be observed on individual TiO₂ crystals.²⁵ The 101 crystal face of anatase TiO₂ showed much higher photocatalytic activity compared to the 001 face, a result not evident from bulk measurements.

These studies established the significance of SM methods in observing individual redox reactions in the presence of a source of ROS and photocatalytic processes.

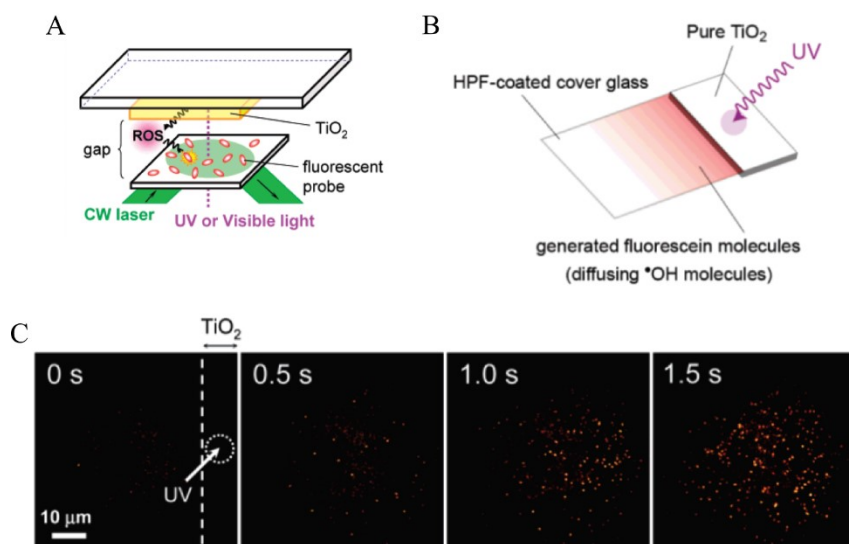


Figure 1.3. (A,B) Setups developed in Majima's research group to monitor diffusive reactive oxygen species (ROS) generated by photocatalysis on TiO₂ surface. Panel C shows SM fluorescence images based on the setup shown in B. Adapted with permission from ref. 85. Copyright 2008 American Chemical Society.

1.3. Fluorescence

A key requirement of SM detection is the collection of a sufficient number of photons to be able to confidently discriminate signal from background. Photons are emitted from photoexcited molecules by the process of fluorescence. In order to be able to take advantage of mechanisms that can modulate fluorescence intensity and the amount of photons emitted, we first need to understand the photophysics behind fluorescence and the processes that may interfere with photon emission. This section will explain the nature of competing deactivation pathways once a molecule reaches the first excited singlet state. Here the competing pathways may cause a decrease in the observed fluorescence intensity, in other words, result in fluorescence quenching. These topics are tied to the overarching theme of this PhD dissertation that is the visualization of chemical reactions at the

SM level by taking advantage of photophysical and photochemical reactions, mainly photoinduced electron transfer (PeT). We will then define PeT and its thermodynamic requirements, followed by the related process of interfacial charge transfer (ICT). While PeT and ICT are fundamentally the same process (the transfer of an electron that occurs upon photoexcitation), they are here considered separately to highlight the different reaction partners in both cases. PeT involves intra- or intermolecular electron transfer between small molecules in solution while the electron transfer in ICT takes place between a small molecule and the solid it is bound to.

1.3.1. Excited State Processes

Upon light absorption, molecules are promoted to an electronic excited state where they may take many different pathways to return to the ground state, two of which are fluorescence and PeT. In order to understand the interplay of the pathways competing to deactivate the excited state of a molecule, we will first describe the photophysical processes a molecule undergoes upon photon absorption. These processes are described in detail below and can be summarized in a Jablonski diagram, as seen in Figure 1.4.

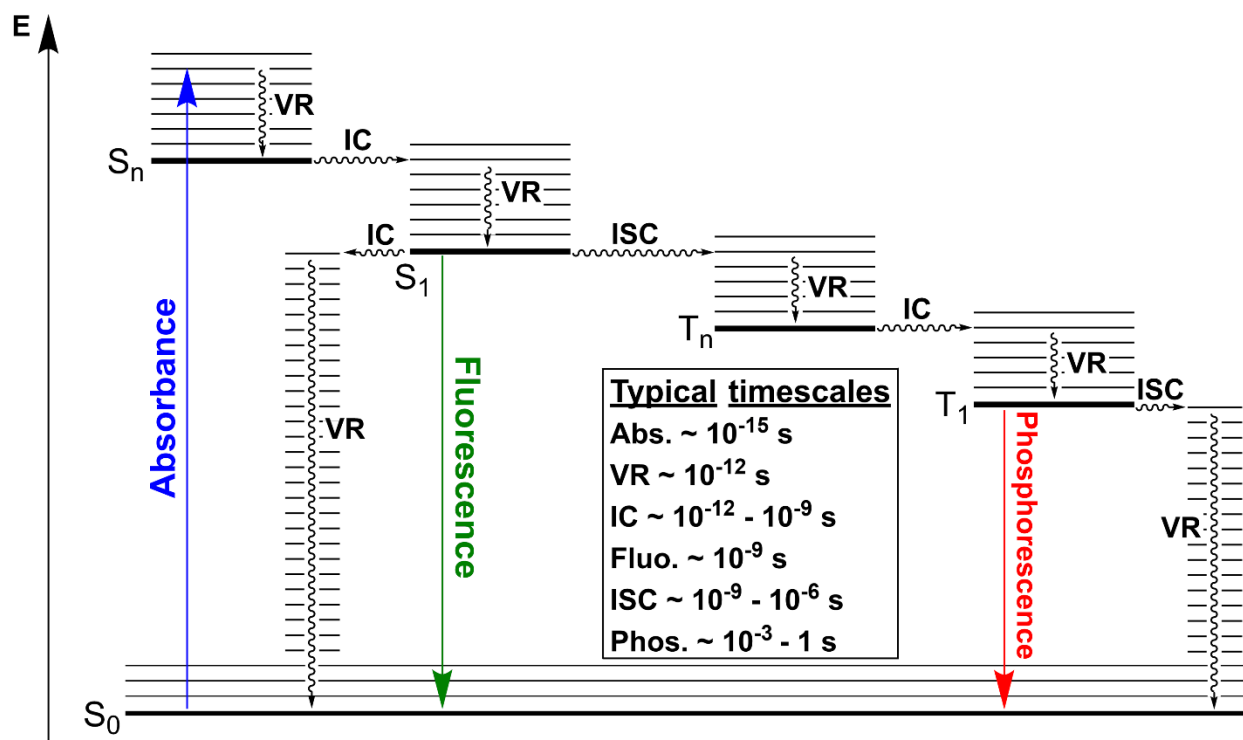


Figure 1.4. Photophysical processes summarized in a Jablonski diagram.⁸⁶ VR stands for vibrational relaxation, IC for internal conversion and ISC for intersystem crossing.

An initial excited state is generated by the absorption of light. The absorption of a photon represents an electric dipole transition that excites one electron from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) in the absorbing molecule. Transition rules state that the spin multiplicity of the system does not change for this allowed process. Ground state closed-shell organic molecules have no unpaired electrons and correspondingly are singlets since they possess a spin multiplicity of 1. The excited electron therefore retains its spin, antiparallel to that of the electron in the HOMO, and an excited singlet state is formed. The excited singlet states are labelled $S_{1,2,\dots}$ in increasing order of energy whereas the ground state is labelled S_0 .

Dissipation of energy follows absorption of light. Coupled to the electronic states are vibrational states that are involved in the dissipation of energy and have much smaller energy gaps.

Two rapid processes initially take place as the molecule relaxes to the lowest vibrational level of the first excited singlet state, S_1 . The first is vibrational relaxation (VR) between vibrational levels of the same electronic state, and the second is internal conversion (IC) between electronic states S_n and $S_{n-1, n-2, \dots}$. These non-radiative processes occur on the order of picoseconds or faster. It follows that most molecules rapidly reach the lowest vibrational level of S_1 wherefrom deactivation of the excited state to S_0 takes place.

After relaxing to S_1 , the excited state can then be depopulated by a number of pathways, including fluorescence, intersystem crossing (ISC) to the triplet state (spin multiplicity of 3; T_n), IC to S_0 , intramolecular excited state reactions and intermolecular deactivation. Fluorescence is the radiative emission of a photon associated with, in almost all cases, an $S_1 \rightarrow S_0$ electronic transition. Molecules that emit fluorescence are called fluorophores. Typical fluorescence lifetimes (τ_f) for organic fluorophores are a few nanoseconds, making fluorescence a relatively fast excited state deactivation process.

An alternative pathway to consider is ISC. This is a transition from an excited singlet state (S_1) to an excited triplet state (T_n). The resultant triplet spin state requires spin inversion of an electron in the singlet excited state. Although ISC is formally forbidden from transition rules it can be accelerated by other factors. For example, ISC is favored when vibrational energy levels of a triplet state overlap with the lowest energy level of S_1 . Two more processes are known to facilitate ISC. The transitions rules can be relaxed and ISC made 'less forbidden' by coupling the change in electron spin state with either spin-orbit coupling in heavy atoms (called heavy atom effect)⁸⁷ or a change in the orbital angular momentum of the fluorophore (El-Sayed's rule).⁸⁸ The triplet excited state may then relax through IC to lower triplet states, reactions, or a radiative $T_1 \rightarrow S_0$ transition that is termed phosphorescence. Relaxation of the triplet excited state to the singlet

ground state is spin forbidden and correspondingly both phosphorescence and ISC pathways to the ground singlet state have low rates. As a result, the excited triplet state lifetime is on the order of milliseconds to seconds, much longer than for the singlet excited state.

1.3.2. Fluorescence Quenching

An important concept in fluorescence-based spectroscopies is that all of the singlet excited state deactivation processes are in competition with each other. Fluorescence is the process we can observe by monitoring emitted photons, and hence the introduction or acceleration of other deactivation pathways will lead to a reduction in fluorescence intensity, termed fluorescence quenching. Quenching mechanisms are separated into two classes: dynamic and static. Dynamic quenching takes place when a fluorophore in its excited state diffuses and collides with a quencher. On the contrary, no diffusion takes place in static quenching. In the latter case a ground-state complex is formed or chemical tethering of quencher and fluorophore is performed to ensure their close proximity and ‘instantaneous’ quenching upon photoexcitation. Importantly, long tethers holding together a quencher and fluorophore will also lead to dynamic quenching as the molecule explores its conformational space.

Fluorescence quenching is a versatile tool that has been used as a method to report on chemical reactions, changes in the environment, accessibility to small molecules, the formation of complexes, and on many other phenomena.

1.3.2.1. Fluorescence Quantum Yield and Fluorescence Lifetime

The intensity of the observed fluorescence depends on two main intrinsic parameters: the extinction coefficient at the excitation wavelength and the rate constant of the fluorescence process (radiative rate constant, k_r). The extinction coefficient is a property of a fluorophore and is a measure of the probability that a photon is absorbed by the fluorophore. It cannot be altered without

structural modifications to the fluorophore that affect the electronic structure. Although the fluorescence rate constant k_r is also dictated by the structure of the fluorophore and is directly related to the extinction coefficient,⁸⁹ the observed fluorescence intensity will ultimately depend on both the radiative rate constant *and* its competition with other possible decay pathways. Therefore, the fluorescence intensity will be affected by environmental parameters that lead to fluorescence quenching, as will be discussed further in this section.

It is convenient to define a concentration-independent parameter of a fluorophore called the fluorescence quantum yield (Φ_f). The fluorescence quantum yield is the ratio of the number of emitted photons to the number of absorbed photons. It can also be viewed as the probability of excited state deactivation via the fluorescence pathway, reflecting the competitive nature of excited state deactivation. The quantum yield can be written in terms of rate constants of transitions:

$$\Phi_f = \frac{k_r}{k_r + k_{nr}} \quad (1.1)$$

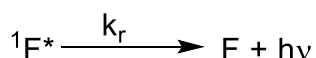
$$k_{nr} = k_{IC} + k_{ISC} + k_q[Q] \quad (1.2)$$

where k_r is the rate constant for radiative decay (i.e. fluorescence) and k_{nr} represents the sum of the first order rate constants for all non-radiative deactivation pathways: internal conversion (k_{IC}), intersystem crossing (k_{ISC}) and any other additional quenching ($k_q[Q]$, where the product is a pseudo-first order rate constant) (see Scheme 1.1). Note that the rate constants and lifetimes of transitions are inversely related to one another ($k = 1/\tau$). The quantum yield tends to a maximal value of 1 as the non-radiative rate constants get smaller compared to the radiative rate constant (cases where $k_r \gg k_{nr}$). Changes in the quantum yield of fluorescence are directly proportional to changes in the fluorescence intensity.

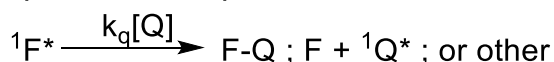
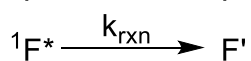
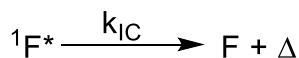
The decay rate constant of the singlet excited state is the sum of all deactivation pathways (shown in Scheme 1.1) and is thus given by equation (1.3).

$$k_{\text{dec}} = k_r + k_{\text{nr}} = k_r + k_{\text{IC}} + k_{\text{ISC}} + k_q[\text{Q}] \quad (1.3)$$

Radiative decay pathway



Non-radiative decay pathways



Scheme 1.1. Radiative and non-radiative singlet excited state deactivation pathways.

In turn, the rate equation for the consumption of the excited fluorophore is:

$$\frac{\delta[\text{F}^*]}{\delta t} = -k_{\text{dec}}[\text{F}^*] \quad (1.4)$$

Integration with respect to time (t) yields a first order kinetic expression:

$$[\text{F}^*] = [\text{F}^*]_0 e^{-k_{\text{dec}} t} \quad (1.5)$$

Where $[\text{F}^*]_0$ is the initial concentration of excited fluorophores immediately following excitation (time = 0), and $[\text{F}^*]$ is the concentration of excited fluorophores at time = t.

The population of excited molecules thus decays exponentially with time while all deactivation pathways take place. The fluorescence intensity (I) is proportional to the

concentration of excited molecules, and also decreases exponentially with time from the initial fluorescence intensity (I_0).

$$I_t = I_0 e^{-k_{\text{dec}} t} \quad (1.6)$$

The lifetime of an excited state is the average time a molecule spends in the excited state before deactivation. The deactivation processes are either unimolecular or bimolecular. Fluorescence, IC, ISC and intramolecular chemical reactions are unimolecular deactivation processes. Bimolecular deactivation involves the presence of a second molecule, a quencher (Q). The fluorescence lifetime can then be defined as equivalent to the inverse of the decay rate of the excited state.

$$\tau = \frac{1}{k_{\text{dec}}} = \frac{1}{k_r + k_{\text{IC}} + k_{\text{ISC}} + k_{\text{rxn}} + k_q[\text{Q}]} \quad (1.7)$$

The maximal fluorescence intensity possible is achieved when every excited state produced deactivates via emission of a photon, or in other words, when $\Phi_{\text{fl}} = 1$. This case is achieved when no non-radiative pathways effectively compete with fluorescence ($k_r \gg k_{\text{nr}}$). We can notice from inspection of equations (1.1) and (1.7) that additional quenching pathways with rate constants comparable to or larger than k_r will lead to a decrease in fluorescence intensity in addition to a decrease in fluorescence lifetime.

1.3.2.2. Fluorescence Quenching by O_2

A typical concern with fluorescence experiments is production of singlet oxygen ($^1\text{O}_2$) that arises when triplet excited states of fluorophores are quenched by molecular oxygen (O_2). O_2 is a triplet paramagnetic diradical in the ground state ($^3\text{O}_2$) and is a very efficient quencher of excited states.⁹⁰⁻⁹¹ Quenching of singlet excited states is usually insignificant as these excited states usually

only live for a few nanoseconds, too short to be efficiently quenched by collisional diffusion with O_2 . In contrast, triplet excited states are long lived ($\sim$ milliseconds) and therefore are prone to diffusional quenching by O_2 . Quenching of excited triplet states by O_2 is accompanied by the formation of singlet oxygen ($^1O_2$) due to spin conservation. An energetically excited oxygen molecule is thus formed that may oxidize the fluorophore, amongst other reactive routes.

$^1O_2$ is part of the family of reactive oxygen species (ROS). ROS are highly reactive chemical species that may also be formed upon incomplete reduction of oxygen in the mitochondrial respiratory chain.⁹² Contrary to $^3O_2$, $^1O_2$ readily adds to unsaturated C-C bonds. For $^3O_2$ to react quickly with other atoms or molecules, spin considerations make it necessary to accept a pair of electrons with the same spin. The vast majority of closed-shell organic molecules are diamagnetic (singlet ground state), with pairs of electrons that have opposite spins. Chemical reaction of $^3O_2$ with a typical fluorophore is therefore spin forbidden. However, $^1O_2$ can accept two electrons of opposite spins, such as two electrons from a C-C π bond. Fluorophores typically possess a number of unsaturations to create a delocalized π system, making them highly susceptible to destruction upon $^1O_2$ addition, especially since $^1O_2$ is formed in close proximity to the fluorophore following triplet excited state quenching.

Quenching by O_2 poses even greater complications in single molecule experiments. In order to collect enough photons to discern individual molecules, an extremely high excitation rate is used, ca. 10^6 Hz. Each molecule cycles through excitation and deactivation pathways hundreds or thousands of times per millisecond. For every cycle, there is a finite chance that the excited state is quenched by $^3O_2$, produces $^1O_2$, and next reacts with the fluorophore to produce non-fluorescent products. For this reason, rapid photobleaching of dyes is seen in single molecule experiments performed in the presence of O_2 . Therefore, measures are typically taken to remove O_2 from the

imaging solution. This is most often accomplished by imaging under a flow of buffer that contains enzyme solutions that carry out enzymatic processes which consume oxygen and ensure anaerobic conditions. Examples of such enzymatic systems are glucose oxidase⁹³ or pyranose oxidase⁹⁴ in combination with catalase, and protocatechuate-dioxygenase.⁹⁵

As mentioned above, $^3\text{O}_2$ is a fantastic triplet excited state quencher. Without dynamic quenching by $^3\text{O}_2$, lifetimes of triplet excited states of fluorophores can be as long as a few milliseconds. During the triplet lifetime, the fluorophore is neither getting excited nor emitting photons. Long-lived dark states are formed as a result. This phenomena is referred to as blinking since the detector measures/sees dark states interspersed by fluorescent bursts (Figure 1.5), similar to what our own photon detector (eye) experiences when we blink.

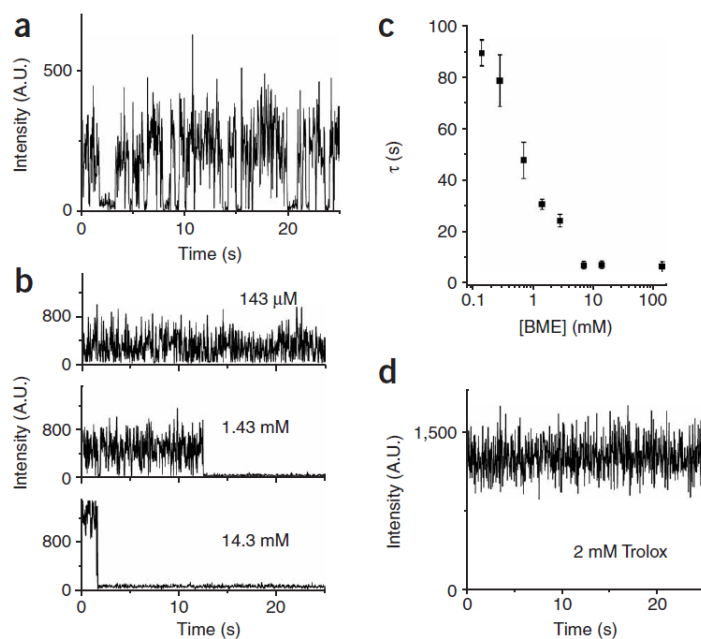


Figure 1.5. Effects of BME and Trolox on the photophysics of single DNA conjugated Cy5 molecules. (a) Fluorescence intensity traces in enzymatically deoxygenated solution without BME or Trolox. Notice the transient low intensity states ('blinks'). (b) Effect of addition of increasing concentration of BME (b). (c) Average time until the transition to the dark state versus BME concentration. (d) Fluorescence intensity traces (as in a) in the presence of 2 mM Trolox. Reprinted with permission from Macmillan Publishers Ltd: Nature Methods, ref. 96, copyright 2006.

Triplet-associated blinking is an unwanted process since it complicates data analysis and obscures transitions between fluorescent and non-fluorescent states that are triggered by chemical reactions of interest, as discussed below. To counteract the removal of oxygen, exogenous triplet quenchers are added to the imaging solution. Most popular is the use of β -mercaptoethanol (BME), a thiol molecule that assists in rapid deactivation of the triplet excited state. The mechanism of action is suggested to be electron transfer from the deprotonated thiolate form.⁹⁷ Other strategies employ the antioxidant Trolox⁹⁶ or a mixture of reducing and oxidizing compounds (ROXS)⁹⁸ to rapidly quench the triplet excited state.

The addition of an enzymatic mixture and an exogenous triplet quencher has the potential to interfere with the system under study. For example, this thesis will describe sensing of oxidation at the single molecule level in Chapters 2 and 6. Thiols and deprotonated thiolates are strong reducing agents, and their presence would clearly interfere with the redox reaction of interest. It is noteworthy that the experiments presented in Chapters 2 and 3 were conducted in ambient conditions, in the presence of oxygen, without the use of ‘imaging’ buffers or cocktails to enhance photostability. Unexpected interactions between the reaction of interest and the usual additives that improve photostability are thus eliminated, increasing the relevance of the obtained results to physiological conditions.

1.3.3. Fluorescence Quenching by Photoinduced Electron Transfer (PeT)

PeT is one of the many possible chemical reactions a molecule may undergo upon photoexcitation, and may be harnessed as an effective pathway to modulate the fluorescence of individual fluorophores.¹⁰ It is a ubiquitous process which requires a suitable electron acceptor/donor partner and leads to the oxidation/reduction of the photoexcited molecule. We have identified PeT as a strategic way to modulate the fluorescence intensity of a fluorophore upon

changes in redox state, leading to the development of fluorogenic probes as will be discussed in Section 1.4.1. We now first present the energetic considerations of PeT as a fluorescence quenching mechanism followed by a discussion of the kinetics.

We first consider the effect absorption of light has on the gas phase redox properties of a molecule. When a molecule absorbs light, an electron is promoted from the HOMO to the LUMO. A high energy electron is now present in the molecule, in addition to a high energy hole caused by the electron vacancy in the HOMO. As a result, the excited molecule is both better at accepting an electron (a stronger oxidant due to the hole in the HOMO) and better at donating an electron (a stronger reductant due to the electron in the LUMO; see Figure 1.6).⁹⁹ In other words, the energy released from binding an electron (electron affinity, EA) is larger in the excited state compared to the ground state, resulting in a more favorable electron acceptance process. Similarly, the energy required to remove an electron from an excited molecule (ionization potential, IP) is less than that needed for a molecule in the ground state, favoring electron donation.

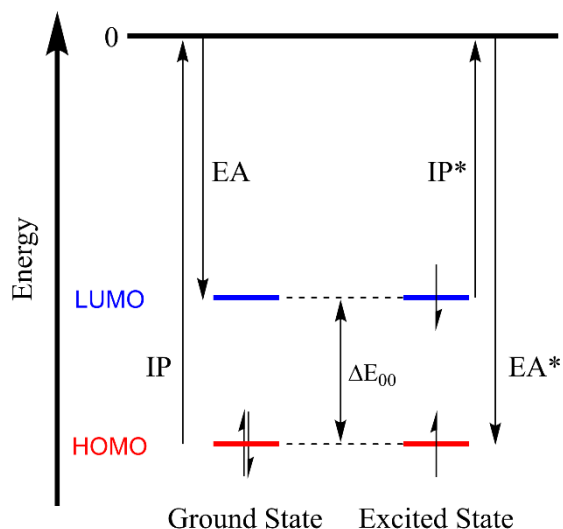


Figure 1.6. Comparison of the ionization potential (IP) and electron affinity (EA) of a molecule in the ground state and excited state.

For PeT to occur, the transfer of an electron between reaction partners must be thermodynamically favored upon the photoexcitation of a dye ($\Delta G_{\text{eT}} < 0$). The role of the excited molecule as an electron acceptor (oxidant) or electron donor (reductant) depends on the energetics of the excited molecule and the electron transfer partner. To anticipate the direction of the electron transfer, it is insightful to compare the energy levels of the HOMO and LUMO of both molecules participating in the electron transfer. The energy released through PeT and the rate of electron transfer will depend on the energy levels of the appropriate MOs of the electron acceptor (A) and the electron donor (D). We first consider the case of photoinduced oxidation of a dye molecule (Figure 1.7, Case I). The electron is transferred from the photoexcited fluorophore, acting here as the electron donor, to an acceptor in close proximity. The excited electron in the dye (LUMO or D) must therefore be higher in energy than the accepting energy level (LUMO of A) for the transfer to be exergonic and PeT to occur. The alternate case where the excited dye is the electron acceptor (photoinduced reduction), is also possible if the energy level of the half-filled HOMO of the excited dye (A^*) is lower than the HOMO energy level of the ground state donor (D).

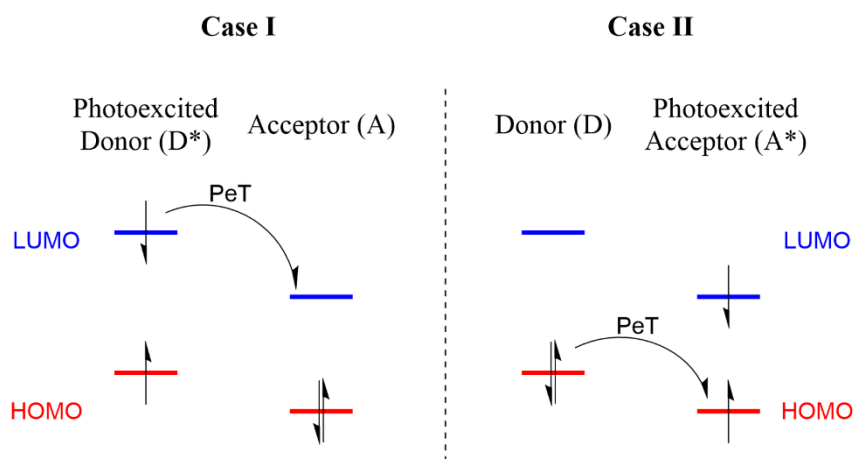


Figure 1.7. Examples of photoinduced electron transfers (PeTs) where the photoexcited molecule is the electron acceptor (Case I) or the electron donor (Case II).



Scheme 1.2. Photoinduced electron transfers (PeTs) where the photoexcited molecule is the electron acceptor (Case I) or the electron donor (Case II).

The driving force of the electron transfer processes illustrated in Scheme 1.2 will depend on the optical band gap of the absorber (ΔE_{00}), the ionization potential of the donor and the electron affinity of the acceptor:

$$\Delta G_{eT}^{\circ} = (IP)_D - (EA)_A - \Delta E_{00} \quad (1.8)$$

Equation (1.8) is applicable in the case of gas phase reactions. Here ΔE_{00} is the energy of the $S_0 \rightarrow S_1$ transition of the fluorophore, D in Case I of Figure 1.7 presented above. The photon energy absorbed by the fluorophore provides the energy to drive charge separation.

IP and EA only offer limited information as they do not take into account solvation of the charged species. Instead, the reduction potential of the acceptor ($E_{A/A^{\bullet-}}^{\circ}$) and the oxidation potential of the donor ($-E_{D^{\bullet+}/D}^{\circ}$) are used to describe the energy changes associated with the transfer of electrons in solution. The correlation between IP (E_A) and the oxidation (reduction) potential is often linear, keeping the validity of the concepts developed above for gas phase reactions. For PeT taking place in solution, the solvation of the ion pair formed upon electron transfer also affects the change in free energy ΔG_{eT}° . A correction term is therefore added to account for solvation. The energy change (in units of eV) is now given by the Gibbs energy of photoinduced electron transfer equation:¹⁰⁰⁻¹⁰¹

$$\Delta G_{eT}^{\circ} = (E_{D^{\bullet+}/D}^{\circ} - E_{A/A^{\bullet-}}^{\circ})nF - \Delta E_{00} + \varpi \quad (1.9)$$

where n is the number of transferred electrons and F is the Faraday constant (96 485 C/mol). For single electron transfer, $nF = 1$ eV/V and conversion of the redox potentials (given in V) to energy (in units of eV) is thus straightforward. The term ϖ is the Coulombic correction term that takes into account the electrostatic energy released by bringing two oppositely charged particles together. It is given by

$$\varpi = -N_A \frac{e^2}{4\pi\epsilon_0\epsilon r} \quad (1.10)$$

or

$$\varpi = -\frac{14.4}{\epsilon r} \quad \text{for } \varpi \text{ in eV and } r \text{ in } \text{\AA} \quad (1.11)$$

N_A is Avogadro's constant ($6.022 \times 10^{23} \text{ mol}^{-1}$), e is the elementary charge ($1.602 \times 10^{-19} \text{ C}$), ϵ_0 is the permittivity of vacuum ($8.854 \times 10^{-12} \text{ C V}^{-1} \text{ m}^{-1}$) and ϵ is the dielectric constant of the solvent.

1.3.3.1. Marcus Theory

Having discussed the thermodynamics, we now tackle the kinetics of electron transfer. In this regard, the relationship between the Gibbs free energy for electron transfer (ΔG_{eT}°) and the Gibbs free energy required to reach the transition state ($\Delta G_{eT}^\ddagger$) is interesting. The link between thermodynamics and kinetics of electron transfer was described by Marcus in the 1950's and is known as the Marcus theory of electron transfer; see equation (1.12).¹⁰²⁻¹⁰³

$$\Delta G_{eT}^\ddagger = \frac{(\Delta G_{eT}^\circ + \lambda)^2}{4\lambda} \quad (1.12)$$

In the above equation λ is the reorganization energy accounting for changes in electronic state reorganization and solvation during the electron transfer. The term λ includes both the inner-

sphere and the outer-sphere reorganization energies. Inner-sphere reorganization refers to the energy changes caused by modifications in bond lengths and bond angles during the electron transfer step. Outer-sphere reorganization instead refers to the energy changes as the solvent shells surrounding the reactants rearrange. It follows from the transition state theory that the rate of electron transfer is given by:

$$k_{\text{eT}} = \nu_{\text{N}} \kappa_{\text{el}} \exp\left(-\frac{\Delta G_{\text{eT}}^{\ddagger}}{k_{\text{B}} T}\right) \quad (1.13)$$

Here the term ν_{N} is the nuclear vibrational frequency ($\sim 10^{12}$ to 10^{14} s^{-1}) and κ_{el} is the electronic factor. κ_{el} determines the probability of electron transfer at the intersection of diabatic curves. The classical treatment of solution-based electron transfer theory usually neglects electronic barriers, such that $\kappa_{\text{el}} \sim 1$.¹⁰⁴ Importantly, equations (1.9), (1.12) and (1.13) provide us with the framework necessary to predict changes in the rate of electron transfer based on the variation of parameters such as the redox potentials of the electron transfer partners and the reorganization energy associated with the transfer of charge.

A surprising result comes out of equation (1.12). The smallest activation barrier, $\Delta G_{\text{eT}}^{\ddagger} = 0$, corresponding to the fastest rate of electron transfer (k_{eT}) is obtained when $\Delta G_{\text{eT}}^{\circ} = -\lambda$ rather than for arbitrarily large driving forces. In fact, k_{eT} *decreases* for more exergonic electron transfers ($\Delta G_{\text{eT}}^{\circ} < -\lambda$). Reactions where $\Delta G_{\text{eT}}^{\circ} < -\lambda$ are said to occur in the Marcus Inverted Region. In this region, a more exergonic reaction proceeds with a smaller rate constant. If instead $\Delta G_{\text{eT}}^{\circ} > -\lambda$, the electron transfer is said to take place in the Marcus Normal Region. In this region, a more endergonic reaction proceeds with a smaller rate constant. The importance of the Marcus Theory was highlighted in 1992 when Marcus was awarded a Nobel Prize for these theoretical

developments.¹⁰⁵ The different scenarios of normal vs. inverted regions are visually represented in Figure 1.8.

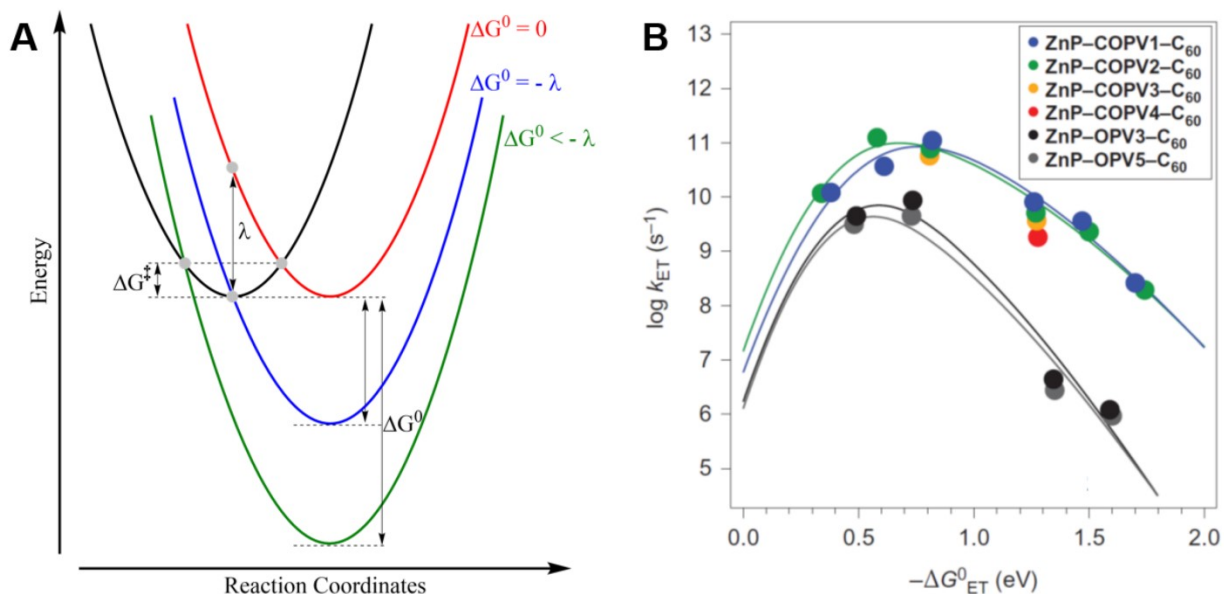


Figure 1.8. (a) Marcus parabolas to show the relation between ΔG^0 , $\Delta G^\ddagger$ and the reorganization energy (λ). All parabolas have the same shape and therefore the λ are equal for the three different scenarios represented. (b) Experimental results showing the changes from the normal to the inverted Marcus regions. The maximal rate of electron transfer occurs for values near - 0.6 eV, where $\Delta G^0 = - \lambda$. Panel B was adapted with permission from Macmillan Publishers Ltd: Nature Chemistry, ref. 106, copyright 2014.

The rate of electron transfer also depends exponentially on the spatial separation between donor and acceptor as shown in equation (1.14).

$$k_{\text{eT}} = k_0 \exp[-\beta(R_{\text{DA}} - R_{\text{DA}}^0)] \quad (1.14)$$

Here k_0 is the rate of electron transfer when the reaction partners are in Van der Waals contact, that is, when separated by R_{DA}^0 . R_{DA} is the distance between the donor and acceptor and finally β is a parameter that represents the sensitivity of k_{eT} to distance. Typical values of β are on the order of $1 \text{ \AA}^{-1}$, meaning that small changes in the separation can induce significant changes in the rate of

electron transfer. For example, an increase in the donor-acceptor distance of 1 Å would reduce the k_{eT} by a factor of $1/e$ (reduce in value by about 2/3).

Following PeT and in the absence of secondary charge transfers or other reactions, the charge separated state is rapidly quenched. Fast back electron transfer (BeT) takes place from the negatively charged acceptor moiety to the positively charged donor moiety. The driving force for BeT is typically larger compared to the forward electron transfer. As a result, if BeT takes place within the normal region it will be characterized by a much larger rate constant than the forward electron transfer. The effectiveness of BeT is important to consider in the of the excited state deactivation process as it regenerates the ground states of both species and ultimately renders PeT a non-destructive and very versatile fluorescence quenching mechanism (see Figure 1.9).

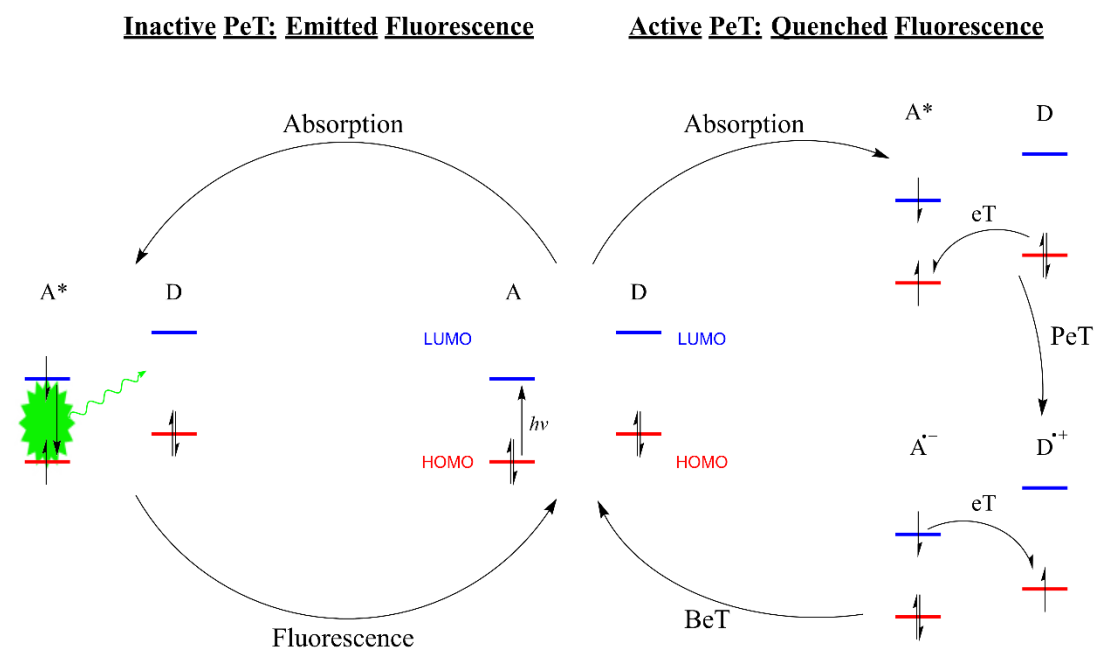


Figure 1.9. Representation of the photophysical cycles a fluorophore may enter when a PeT pathway is inactive (left, fluorescent) or active (right, non-fluorescent).

1.3.4. Interfacial Charge Transfer

The projects presented in Chapters 4 and 5 involve PeT between fluorophores (particle or small molecule) and solid inorganic conductors or semiconductors. In these cases, PeT occurs between fluorophores and the solid they are bound to, as opposed to an inter- or intramolecular partner in solution. PeT at heterogeneous interfaces follows similar rules to those in homogeneous solution, but with some particularities that are described below. Throughout this work the typical nomenclature of interfacial *charge* transfer (ICT) is used since it can be formalized as the transfer of negative electrons or positive holes. This is sometimes an important distinction because of the different mobilities of electrons and holes in the semiconductor.

1.3.4.1. Effect of Density of States

For the case of a molecule adsorbed on the surface of a solid metal or semiconductor, the rates of ICT may be expressed as the sum of charge transfer rates to (from) all possible accepting (donating) sites in the semiconductor:¹⁰⁷

$$k_{\text{ox}}^* = A \int V^2 (1 - f(E, E_F)) \rho(E) \exp\left(\frac{-(E - E_{D^{+\bullet}/D^*}^\circ + \lambda)^2}{4\pi\lambda k_B T}\right) dE \quad (1.15)$$

and

$$k_{\text{red}}^* = A \int V^2 f(E, E_F) \rho(E) \exp\left(\frac{-(E - E_{A^{\bullet-}/A^*}^\circ - \lambda)^2}{4\pi\lambda k_B T}\right) dE \quad (1.16)$$

Here k_{ox}^* and k_{red}^* represent the rate of photooxidation and photoreduction, respectively, of the dye. E stands for energy, E_F for the Fermi level of the semiconductor, $E_{D^{+\bullet}/D^*}^\circ$ for the excited state oxidation potential of the dye, $E_{A^{\bullet-}/A^*}^\circ$ for the excited state reduction potential of the dye, λ for the reorganization energy, k_B for the Boltzmann constant (8.617×10^{-5} eV/K) and T for the

temperature. The terms of the integrals, reading from left to right, account for the electronic coupling between the dye and the solid (V^2), the electronic occupancy determined by Fermi-Dirac statistics and the Fermi level ($f(E, E_F)$), the density of states (DOS) of the semiconductor or metal ($\rho(E)$), and finally an energetic term derived from the Marcus theory. The Marcus term in ICT has the same energy dependence as the one found for molecular PeT (Section 1.3.3.1). However, the DOS term is a new concept and is now detailed.

In a simple view, in going from PeT to ICT, we have replaced a small molecule partner with a semiconductor. A molecule has well defined molecular orbital energy levels while semiconductors possess a certain number of electronic states at specific energies as represented by the DOS. Therefore, the electronic occupancy of the DOS of the semiconductor or metal will additionally influence the rate of ICT compared to molecular PeT. If the adsorbed dye undergoes photooxidation through donation of an electron, the charge transfer rate will be faster for higher levels of unoccupied states since it translates to a larger concentration of accepting energy. If the dye instead undergoes photoreduction by accepting an electron, higher electronic occupancy will lead to higher charge transfer rates from the greater concentration of electrons. Conceptually, we can rationalize the changes in charge transfer rates by thinking of the changes in concentration of electrons in the solid. The changes in occupancy effectively modulate the number of available states that can participate in the ICT.

Another distinction between ICT and molecular PeT is the fact that in ICT we must consider all of the available pathways for the charge transfer. The integral in the expression for k^*_{ox} or k^*_{red} denotes that the total rate of ICT is the sum of the rates at each energy. According to equations (1.15) and (1.16), the rate of ICT at a specific energy is dependent on the DOS and its occupancy in addition to the energetic contribution. This is visually represented in Figure 1.10.

The interplay between these factors is still a subject of intense research, as discussed in the next section.¹⁰⁸⁻¹⁰⁹

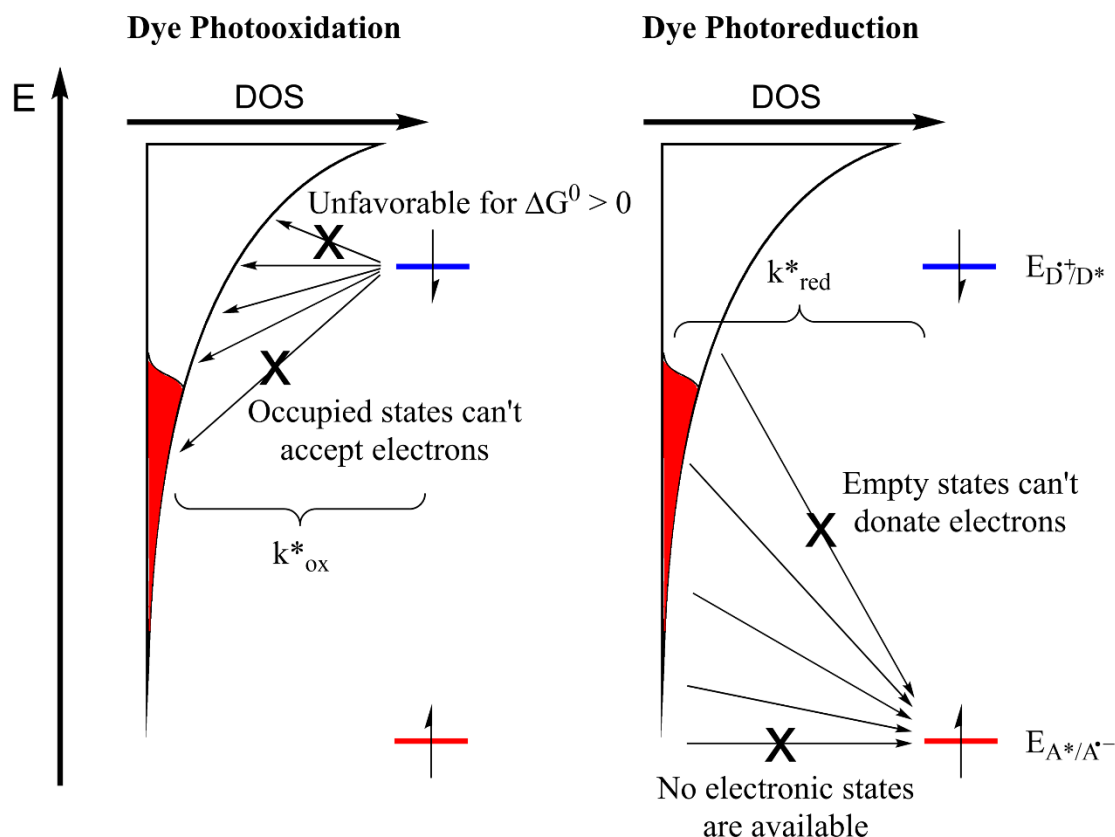


Figure 1.10. Visual representation of photooxidation and photoreduction of a dye by ICT to a semiconductor. Occupied electronic states are highlighted in red while empty states are represented in white. The density of states (DOS) of the semiconductor is shown as an exponential distribution, typical for metal oxide semiconductors such as TiO_2 and SnO_2 .

1.3.4.2. ICT in Dye-Sensitized Solar Cells

A significant source of motivation for studying ICT has been to understand the complex charge transfer pathways in dye-sensitized solar cells (DSSCs, Figure 1.11).¹¹⁰ DSSCs are photovoltaic devices that show promise for widespread adoption due to their ease of processing and low manufacturing costs. Typical DSSCs consist of a mesoporous TiO_2 electrode covered with a monolayer of dye sensitizers.¹¹¹ For decades, ruthenium-based dyes have shown superior

performance in DSSCs. However, DSSCs will not provide a sustainable solution to the growing world energy demands until they can be easily manufactured on large scales with the use of inexpensive and Earth-abundant materials. A significant step towards widespread adoption was recently made: the most efficient DSSC to date was prepared with organic porphyrin dyes, containing only carbon, nitrogen, oxygen and hydrogen atoms.¹¹²

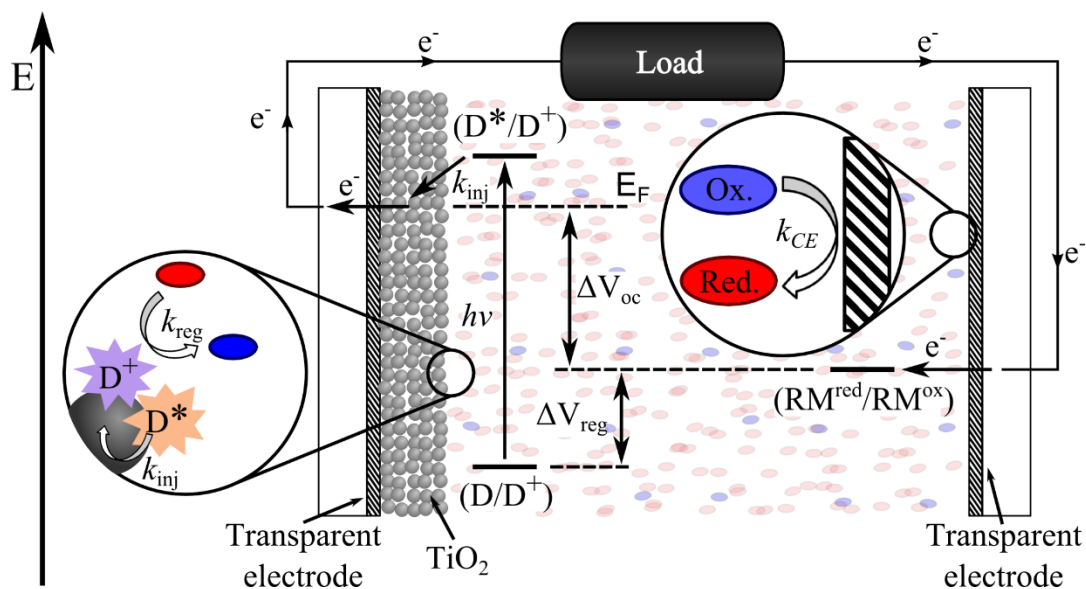


Figure 1.11. Schematic depiction of a functioning DSSC. The charge separated state is produced by ICT as the photoexcited sensitizer (D^*) injects an electron into the mesoporous TiO_2 electrode. Abbreviations are as follow: open circuit cell potential (ΔV_{oc}); driving force for oxidized dye regeneration (ΔV_{reg}); redox mediator (RM); rate constant for electron injection, oxidized dye regeneration and redox mediator reduction at the counter electrode (k_{inj} , k_{reg} , k_{CE} respectively).

The role of the dye sensitizer is to absorb a large fraction of the available sunlight and to then generate charge separated states by electron injection into the TiO_2 semiconductor (i.e. ICT). The process of charge separation is crucial in converting solar energy to electricity in DSSCs (Figure 1.12). An important distinction is made here between PeT in a fluorogenic probe and ICT in DSSCs. In probes, the role of PeT is to efficiently deactivate the excited state of the fluorophore before emission takes place. Therefore, a cycle where PeT and BeT successively occur to dissipate

the energy gained by absorption of a photon is ideal. In contrast, DSSCs aim to convert photon energy into electrical energy. In consequence, BeT in DSSCs is a detrimental pathway as it destroys the charge separated state and wastes the sunlight energy before it can be harnessed.

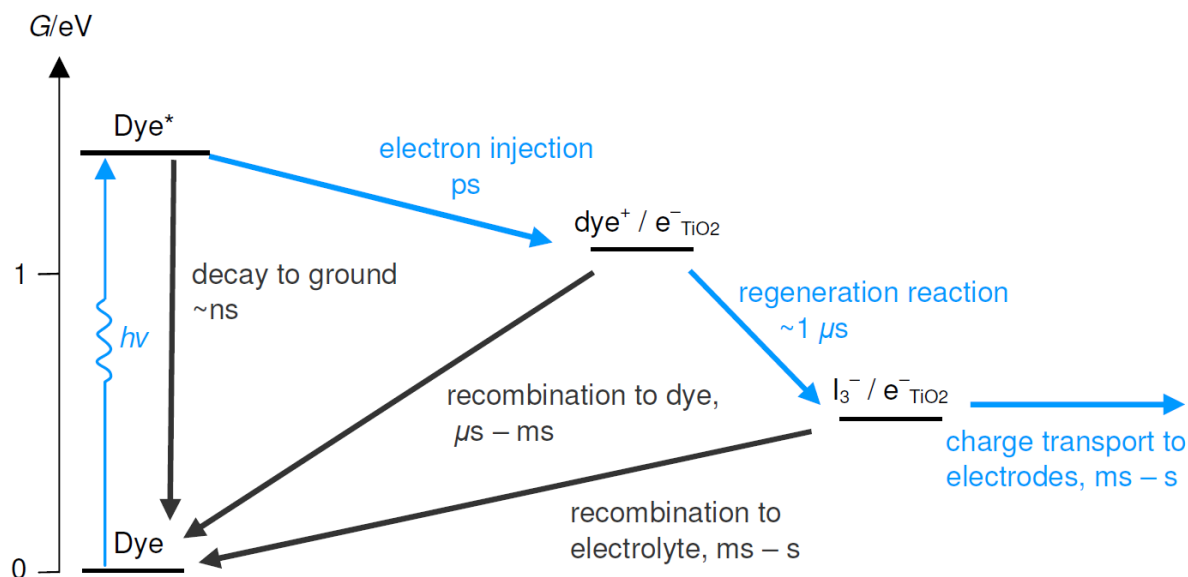


Figure 1.12. State diagram representation of the kinetics of DSSC function. The forward (productive) processes of light absorption, electron injection, dye regeneration, and charge transport are indicated by blue arrows. The competing loss pathways of excited state decay to the ground state, and electron recombination with either dye cations or oxidized redox mediator are shown in black. Adapted with permission from ref. 113. Copyright 2008 Imperial College Press.

The charge transfer dynamics at the dye-semiconductor interface are highly complex and heterogeneous.¹¹⁴ For example, the injected electron can either diffuse away from the interface, getting a step closer to collection at the back contact and generating electrical current, or can return to the photooxidized sensitizer via BeT. A fast rate of BeT has been identified as an important energy loss pathway. BeT from the semiconductor to the dye sensitizer leads to charge recombination, incurring energy loss and decrease in cell efficiency. The BeT pathway is especially problematic when organic dyes are used. Ruthenium-based sensitizers have the advantage that the forward and backward electron transfer involve different molecular orbitals

(MO) which means that a spatial discrimination of rates is inherently built-in. Engineering of dyes has shown promise in reducing the rate of BeT by tethering a hole donor to the sensitizer dye in order to increase the spatial separation between the electron injected into the semiconductor and the MO where the hole is located.¹¹⁵ The development of the next generation of solar cells that utilize Earth abundant materials (i.e. not ruthenium) with higher conversion efficiencies requires a complete understanding of ICT occurring in nanostructured semiconductor electrodes so as to reduce energy loss pathways. It is with this goal in mind that studies of ICT between organic dyes and SnO₂ semiconductor NPs have been undertaken as part of this thesis work.

1.4. Oxidation at the Single Molecule Level

Fluorescent probes are molecules designed to change their fluorescent properties upon sensing of specific chemical interaction or reactions.¹¹⁶ The methodology is broadly applicable and as a result fluorescent probes have been synthesized to detect ions and molecules such as Cu²⁺, cysteine and HOCl,¹¹⁷⁻¹¹⁸ biomolecules such as DNA, RNA¹¹⁹ and proteins,¹²⁰ amongst a plethora of other examples. When coupled with fluorescence imaging, information with high spatiotemporal resolution of processes occurring on the surface of materials or within biological systems can be gathered. Extension to SMS is a natural step in the development of more sensitive methods to fully elucidate complex systems.

The most effective probe design involves an ‘off-on’ switching mechanism. Probes based on this design are named fluorogenic probes. In this case, the initial state of the probe is dark and non-fluorescent. Once in contact with the analyte of interest, activation occurs and a brightly fluorescent state is formed.¹²¹ This approach is inherently more sensitive than alternative ‘on-off’ probes, where the initial state is the fluorescent state. We can turn to the analogy of candles in a dark room to illustrate why this is the case. Imagine 100 unlit candles in a room. The room is

completely dark, and it will be very easy to notice when a single candle is lit. In contrast, if all 100 of them are lit, the room will be brightly illuminated and it will be difficult to notice when one candle is blown out. Furthermore, producing a bright state (off-on or fluorogenic) is generally more difficult to accomplish than quenching of the fluorescence. Off-on fluorescent probes are therefore extremely sensitive, show high specificity, and are less prone to false positives.

PeT is widely exploited in the pursuit of fluorescence switching mechanisms in probes.¹²²⁻
¹²³ PeT-based fluorogenic probes separate the fluorophore segment (reporter) from the reactive segment (receptor or trap). A vital aspect of rational probe design is the selectivity of the probe to the analyte(s) or reaction(s) of interest imparted by the choice of the receptor/trap segment. The choice of receptor, with its specific redox levels, will in part dictate which fluorophore is best suited for the design of the probe. Above and beyond the crucial fluorophore parameters such as the extinction coefficient, quantum yield and photostability, the redox levels of the reporter segment also need to be taken into consideration for the PeT-based fluorescence switching mechanism to be effective. The ultimate goal is to promote PeT between the fluorophore and the unmodified receptor segment with a suitable driving force for electron transfer (see Section 1.3.3) and maximum rate of PeT. Activation of the probe then modifies the redox levels of the receptor segment so as to deactivate the PeT pathway.

Chapters 2 and 3 describe our efforts towards monitoring oxidation reactions that take place within lipid membranes using SMS. We aim to develop a general platform to decipher individual chemical oxidation processes. We chose lipid peroxidation as our first model heterogeneous oxidation system to demonstrate the capabilities of our fluorescent probe at the SM level. This choice was motivated by the fact that lipid peroxidation is relatively well understood from a chemical standpoint and has important biological implications.¹²⁴ This section will thus briefly

introduce the fluorescent probes that are central to our method of detecting, visualizing and quantifying oxidation and were critical in investigating lipid peroxidation taking place within individual liposomes. This section will end with a description of the chemical steps involved in lipid peroxidation. While we have not yet conclusively determined if we are observing individual oxidation events, the results in Chapters 2 and 3 represent a step towards the ambitious goal of monitoring discrete oxidation reactions in heterogeneous media. We hope that the approach described in this thesis serves to stimulate interest in deciphering oxidation of heterogeneous systems at the SM level.

1.4.1. Oxidation-sensitive Fluorescent Probes

Our research group is highly interested in monitoring and visualizing oxidation processes with the use of SMS. We wish to develop a platform to intimately study the propagation of oxidation reactions and ultimately observe discrete oxidation events of individual molecules in heterogeneous systems. Oxidation of organic materials in heterogeneous systems, such as micelles, aerosols, low density lipoproteins and lipid membranes, is an important degradation process having biological, environmental and industrial importance.¹²⁵ It further offers unique challenges in understanding the pathways at play. Learning through which route oxidation takes place in different environments will allow us to improve protective approaches that aim to limit (inhibit) oxidation and further develop methodologies where increasing oxidation is beneficial, such as photodynamic therapy.¹²⁶

Over the past decade, significant progress has been made in the development of fluorogenic probes that target oxidation and reactive oxygen species (ROS).¹²⁷ A range of probes have been developed to detect various ROS with high sensitivity: superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical ($\bullet OH$) and peroxy radical ($ROO^{\bullet}$).¹²⁸

These probes typically rely on fluorescence intensity changes upon chemical reaction with the ROS of interest, although exclusive reactivity with a single ROS and quantification remains challenging.¹²⁹ Due to their application in SMS, it is worthwhile to mention again the probes developed in Majima's research group. In addition to the observation of fluorescence enhancement of individual molecules upon reaction with ROS (Section 1.2), they have shown direct oxidation of a fluorogenic probe by reaction with photogenerated holes on the surface of a single titanosilicate zeolite particle.²⁵

In order to further advance the field of oxidation sensing, our laboratory has recently developed oxidation-sensitive fluorogenic probes that comprise an α -tocopherol mimic (trap/receptor segment) covalently coupled to a BODIPY fluorophore (reporter segment) as seen in Figure 1.13.¹³⁰ The chromanol moiety was chosen as a mimic of the naturally-occurring lipophilic antioxidant α -tocopherol because the structural aspects impacting reactivity are unchanged. PeT here acts as the fluorescence switching mechanism. An active PeT in the initial state of the molecule (reduced state) quenches the fluorescence of the BODIPY. The chromanol is electron rich and possesses a HOMO level above that of the BODIPY HOMO. Rapid PeT from the chromanol to the photoexcited BODIPY quenches the excited state and the fluorescence, resulting in a dark reduced state of the probe. The chromanol thus acts as the electron donor while the BODIPY is the acceptor.

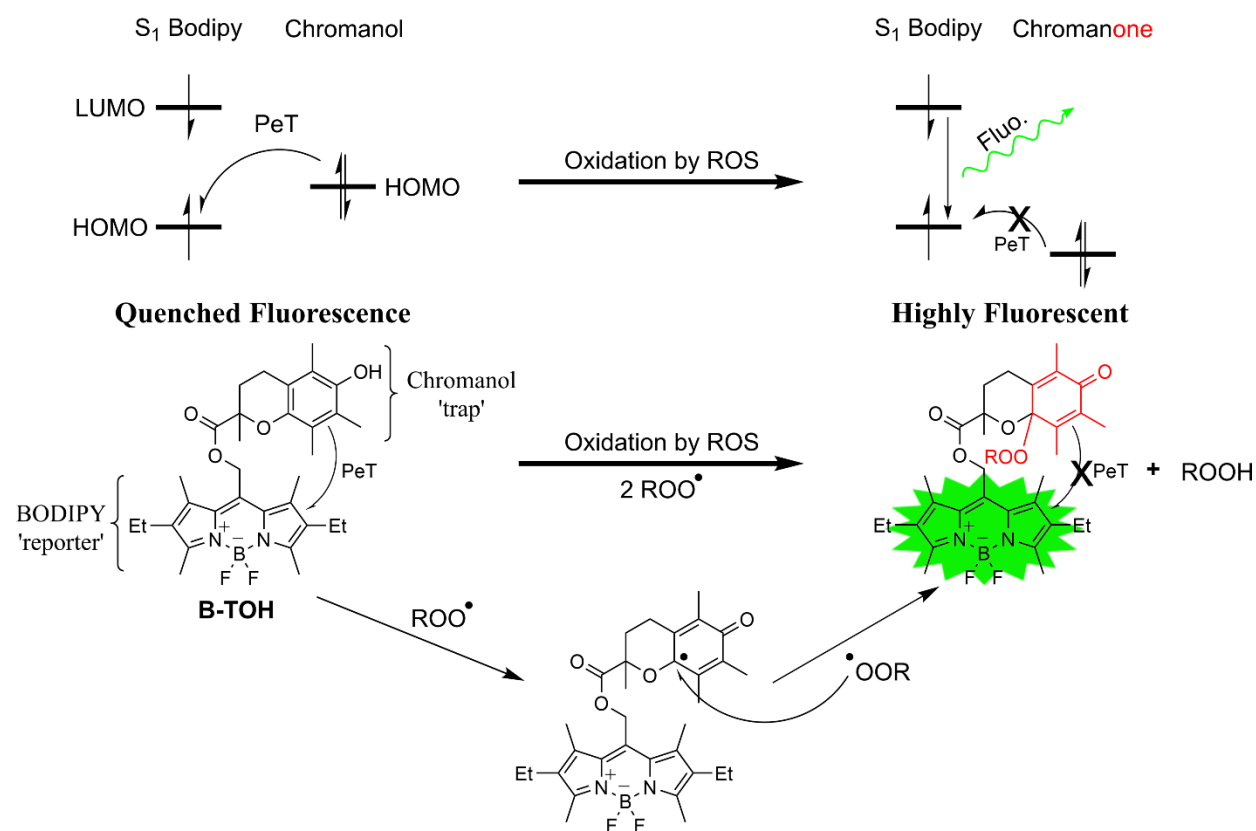
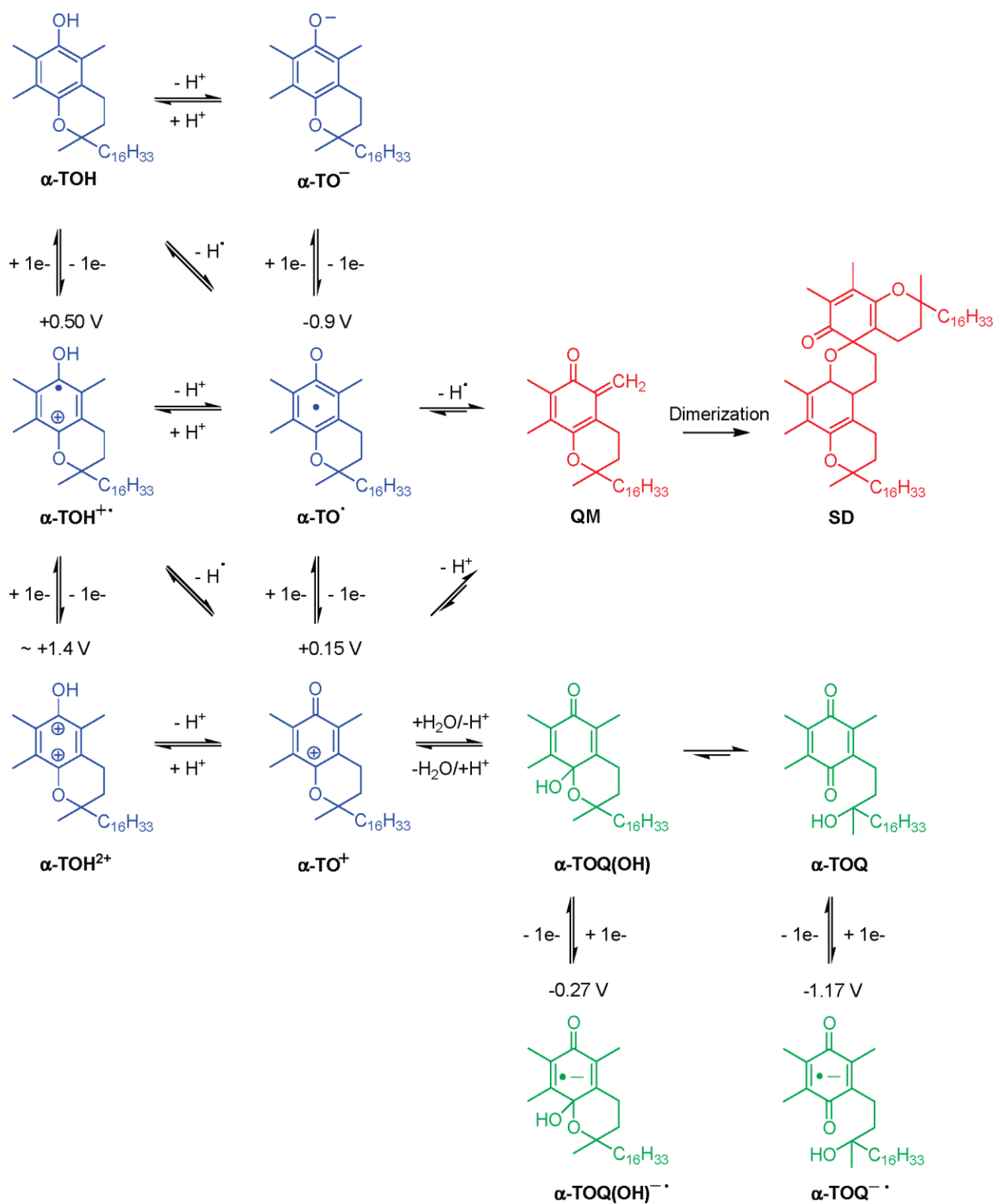


Figure 1.13. The fluorogenic α -tocopherol probe **B-TOH** becomes brightly fluorescent upon oxidation of the chromanol moiety. R may be a wide range of hydrocarbon chains, such as a lipid chain in the case of lipid peroxy radicals.

Akin to α -tocopherol, the chromanol moiety in **B-TOH** and other related probes is oxidized by ROS. Scheme 1.3 shows the various redox pathways and states of α -tocopherol. Oxidation of the chromanol by H-atom abstraction leads to the formation of electron poor products, such as a conjugated ketone (chromanone), shown in Figure 1.13. The HOMO energy level of the oxidized quinone form is accordingly much lower than that for the reduced phenolic form. The PeT pathway is deactivated since electron transfer between the quinone and BODIPY becomes thermodynamically unfavorable. Consequently, the bright fluorescence of the BODIPY is restored. By following the fluorescence enhancement over time, the probe can be used to track

oxidation reactions and informs us how α -tocopherol would be consumed in the system, telling us to what extent oxidation is occurring.



Scheme 1.3. Mechanism of electrochemical and chemical oxidation of α -tocopherol (α -TOC). Redox potentials indicated are referenced to the ferrocene/ferrocenium couple. Reprinted with permission from ref. 131. Copyright 2009 American Chemical Society.

The fluorescence enhancement of the 1st generation probe upon oxidation has subsequently been improved on two fronts.¹³² Firstly, the original ethyl groups at the 2 and 8 side positions have been replaced by hydrogens. This structural change has the effect of lowering the HOMO energy level of the BODIPY and making the PeT more exergonic.¹³³ The larger driving force accelerates the rate of PeT and results in a less fluorescent reduced state and a higher sensitivity for the probe. Secondly, the spatial separation between the BODIPY and the chromanol was shortened by turning to a methylene bridge. The reduced distance between PeT partners once again results in a faster rate of PeT, a reduced state with negligible emission and ultimately highly improved sensitivity in the 2nd generation probe **H₂B-PMHC**.

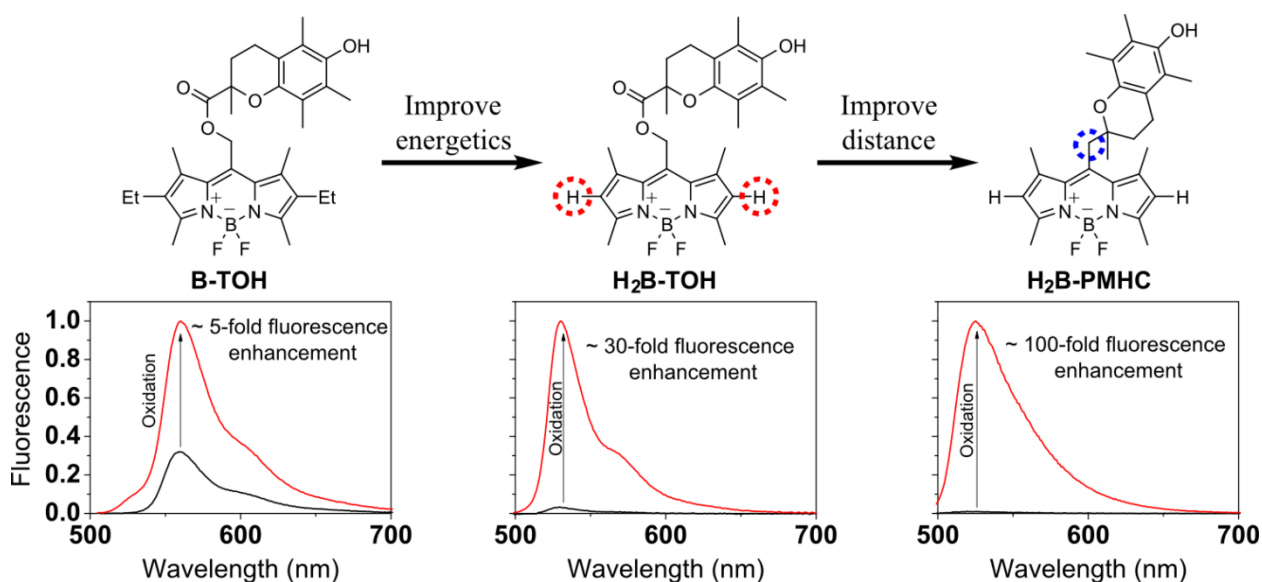


Figure 1.14. Chronological improvements in the fluorescence enhancement of fluorogenic α -tocopherol oxidation probes.

1.4.2. Lipid Peroxidation

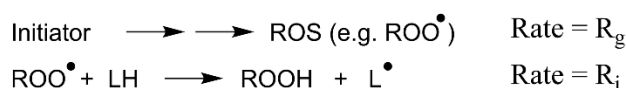
Lipid peroxidation is a free-radical mediated chain reaction that oxidizes the lipids of cellular membranes. The physiological role of lipid peroxidation has long been considered deleterious.¹³⁴ Chemical changes of lipids have dramatic effects on the structure, fluidity, permeability and associated functions of biomembranes.¹³⁵⁻¹³⁶ Furthermore, many of the decomposition products of oxidized lipids are highly reactive towards vital biomolecules such as proteins and DNA bases. Lipid peroxidation has been linked to aging¹³⁷⁻¹³⁸ and numerous disorders and diseases: cancer,¹³⁹⁻¹⁴⁰ cardiovascular and liver diseases,¹⁴¹ as well as neurodegenerative disorders such as Alzheimer's and Parkinson's.¹⁴²⁻¹⁴⁴ In contrast, there is also mounting evidence suggesting that oxidatively modified lipids are involved in various signaling pathways and in the regulation of biological pathways. Lipid peroxidation products and ROS are now thought to be implicated in signal transduction, regulation of gene expression, stress response, adaptive responses, and activation of receptors and nuclear transcription factors.¹⁴⁵⁻¹⁵² Given the detrimental, but also potentially key signalling role of lipid peroxidation, substantial efforts are being invested into developing the field of oxidative lipidomics in order to bridge the gap between lipid biochemistry, free radical chemistry and cell biology.¹⁵³

The ability to precisely follow oxidation taking place in membranes is evidently crucial for our understanding of lipid peroxidation and ROS pathways in biological systems. Therefore, it is of great interest to develop probes for monitoring the oxidation process in lipid membranes to be able to fully understand the complex roles of ROS. In this regard, the fluorogenic α -tocopherol probes previously developed in our research group and described in Section 1.4.1 represent a significant step forward.¹³⁰ The reactive α -tocopherol mimic possesses similar reactivity to

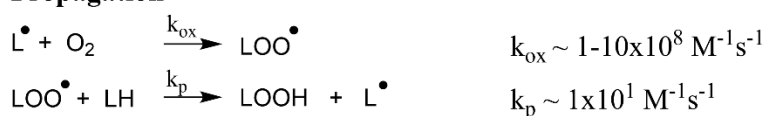
naturally-occurring α -tocopherol and thus ensures that conclusions obtained with the probe can be extended to physiological systems.

The process of lipid peroxidation can be divided into 4 reaction sequences: 1) initiation, 2) propagation, 3) termination, and in the presence of antioxidants, 4) inhibition.¹²⁴ These steps are also found in the analogous free radical chain oxidation of organic material, classically termed autoxidation.¹⁵⁴ The primary reactions involved in the autoxidation of lipids include H-atom abstraction by peroxy or alkoxy radicals (i.e. ROS), oxygen addition to lipid carbon-centered radicals to give lipid peroxy radicals, and peroxy-peroxy chain termination.

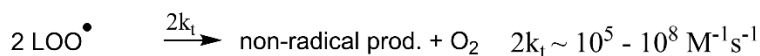
Initiation



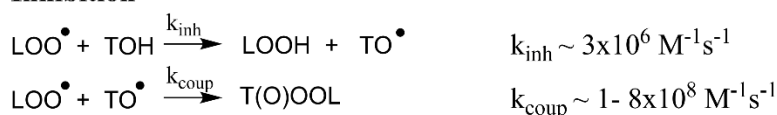
Propagation



Termination



Inhibition

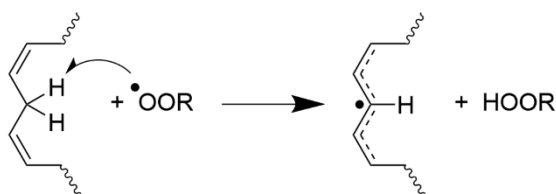


Scheme 1.4. Reaction sequence of lipid peroxidation. ROO[•] represents a general peroxy radical derived from an initiator. LH is a polyunsaturated fatty acid in lipids, for example linoleate. The rate constant values are obtained from homogeneous solution studies (k_{ox} ,¹⁵⁵ k_p ,¹⁵⁶ k_t ,¹²⁴ k_{inh} ,¹⁵⁷ k_{coup} ¹⁵⁸).

1.4.2.1. Initiation

The first step of lipid peroxidation is the formation of a lipid carbon-centered radical, L[•]. The lipid radical is typically formed by H-atom abstraction from a lipid molecule (LH) by ROS.

The H-atom is typically abstracted from the relatively weak C-H bonds in bis-allylic positions of polyunsaturated fatty acids (PUFAs) for which the bond dissociation energy (BDE) is ca. 80 kcal/mol (see Scheme 1.5).¹⁵⁹ For reference, the BDE of C-H bonds in saturated hydrocarbon chains is ~ 100 kcal/mol. The low C-H BDE of PUFAs is explained in terms of stabilization of the $L^\bullet$ radical product by conjugation with the adjacent unsaturations. In contrast, the lack of extended radical delocalization in monounsaturated lipids leads to a C-H BDE that is about 10 kcal/mol higher in energy, ca. 90 kcal/mol.¹²⁴ As a result, the activation energy is raised and the rate of H-atom abstraction lowered.¹⁶⁰ The C-H BDE of saturated lipid chains is ~ 100 kcal/mol and in consequence they generally do not participate in lipid peroxidation.¹⁶¹⁻¹⁶²

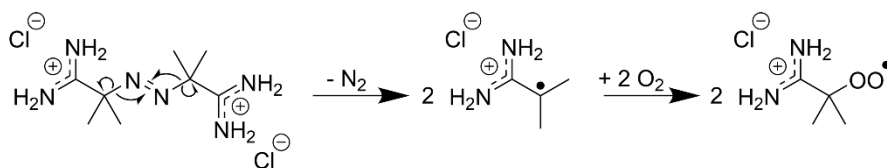


Scheme 1.5. H-atom abstraction from a polyunsaturated fatty acid by a peroxy radical.

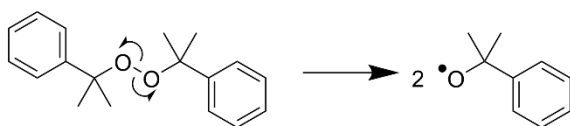
In detailed studies, the use of radical initiators as the source of ROS allows control of the rate at which ROS are formed and oxidation takes place. Azo free-radical initiators¹⁶³ have been developed for use in free radical polymerization, and as such, their properties as initiators are well established.¹⁶⁴⁻¹⁶⁶ Azo initiators thermally decompose following first order kinetics to give two carbon-centered radicals and release one molecule of N_2 . Subsequent to fragmentation, the pair of carbon-centered radicals ($R^\bullet$) that are formed react at diffusion controlled rates with molecular oxygen to form oxygen-centered peroxy radicals ($ROO^\bullet$).¹⁶⁷ Non-azo initiators are also available, most commonly in the form of organic peroxides.

Light is a stimulus with high spatiotemporal resolution that may be used to promote bond cleavage in the initiators and accelerate the production of ROS. In the case of 2,2'-azobis(2-methylpropionamidine) dihydrochloride (ABAP), irradiation at ~ 360 nm accelerates C-N bond fragmentation and thus increases the rate of formation of $R^\bullet$ and $ROO^\bullet$. Similarly, dicumyl peroxide (DCP) undergoes fragmentation under UV light ($\lambda \sim 260$ nm) by homolytic bond cleavage of the O-O peroxide bond and leads to the formation of two alkoxy radicals ($RO^\bullet$). Notably, ROS are formed without the need for molecular oxygen in the dicumyl peroxide system.

2,2'-azobis(2-methylpropionamidine) dihydrochloride (**ABAP**)



Dicumyl peroxide (**DCP**)



Scheme 1.6. Fragmentation patterns of hydrophilic (ABAP) and hydrophobic (DCP) radical initiators.

Additionally, the structure of the radical initiator plays a role in the localization of the generated ROS.¹³² The positively charged amidinium groups of ABAP render it hydrophilic and as a result, cause it to preferentially localize in the aqueous phase. Conversely, the hydrocarbon groups of dicumyl peroxide make it lipophilic and consequently the radicals will be generated within the lipid milieu. As discussed in Chapter 3, the side groups of the radical initiator may further affect the localization of any adduct formed upon reaction with the generated ROS. ABAP produces hydrophilic peroxy radicals whereas dicumyl peroxide produces hydrophobic alkoxy radicals.

radicals (Scheme 1.6). The preference of these radicals to partition within the aqueous or lipid phase is transferred to the oxidation reaction product.

1.4.2.2. Propagation

Lipid peroxidation is propagated in two steps. In the first, the carbon-centered $L^{\bullet}$ radical reacts at approximately diffusion-controlled rates with O_2 to yield $LOO^{\bullet}$. This same reaction was described above for the azo initiators. In the second step, $LOO^{\bullet}$ abstracts $H^{\bullet}$ from another lipid molecule (LH). The rate for this process is typically low compared to other reactions in the chain. In consequence, $LOO^{\bullet}$ is the dominant radical species present in the chain reaction and the rate constant k_p for H-atom abstraction is rate limiting and found in the overall rate expression.¹⁶⁸ The newly-formed $L^{\bullet}$ in turn reacts with O_2 to give $LOO^{\bullet}$, and continues the chain reaction. The lipid hydroperoxide, $LOOH$, can propagate the chain reaction further by, for example, undergoing fragmentation to $LO^{\bullet}$ and $HO^{\bullet}$, two highly reactive species that can abstract H-atoms from LH.

1.4.2.3. Termination

The chain reaction ends when two lipid peroxy radicals ($LOO^{\bullet}$) either couple or disproportionate to form closed-shell products. Other radical species may couple to form non-radical products, yet they may undergo fragmentation to revert to the radical species. Termination is not accomplished in this case and the chain reaction will continue.

1.4.2.4. Inhibition

Specific compounds called antioxidants (AH) protect against oxidative degradation of organic materials in both living organisms and industrial applications. Two types of antioxidants can either slow down or prevent oxidation. The first group covers preventative antioxidants. These reduce the rate of initiation to limit the extent of oxidation. The downfall is that if radicals are formed by initiation, they can still oxidize the material through propagation. The second class

comprises chain-breaking antioxidants, and is arguably more important. Chain-breaking antioxidants interfere with the propagation steps of peroxidation to stop the chain reaction. In a simplified view, the addition of chain-breaking antioxidants increases the rate of termination and reduces the concentration of intermediate radical species in solution.

For a compound to act as an effective chain-breaking antioxidant, it has to fulfill a number of conditions:¹⁶⁹⁻¹⁷¹

- 1) The rate of H-atom transfer to a peroxy radical must be much higher than H-abstraction from the molecule that is to be protected (i.e. LH)
- 2) The antioxidant radical $A^{\bullet}$ does not react with molecular oxygen
- 3) The radical $A^{\bullet}$ does not react with LH
- 4) The antioxidant AH does not react directly with molecular oxygen by H-atom abstraction
- 5) The antioxidant has a high ionization potential abstraction to prevent proton-coupled electron transfer with O_2

Phenolic compounds (AOH), such as those illustrated in Figure 1.15, are the classic example of chain-breaking antioxidants, and with good reason. Not only do phenols exhibit high inhibition rate constants and little ill side effects, they also are prevalent in living organisms. Tocopherols are a class of natural, lipid-soluble, antioxidants. The most active form, α -tocopherol, is also known as Vitamin E.¹⁵⁷

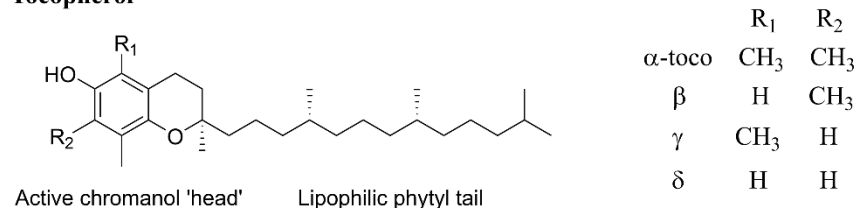
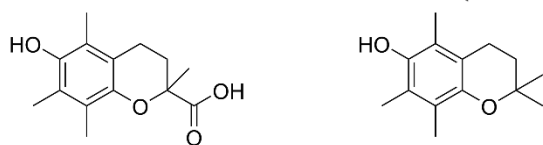
Tocopherol**Trolox**2,2,5,7,8-Pentamethyl-6-chromanol
(PMHC)

Figure 1.15. Chemical structures of naturally-occurring (tocopherol) and artificial (Trolox, PMHC) phenolic antioxidants.

The phenolic OH group is at the center of the high reactivity of phenols with peroxy radicals and other ROS. The bond dissociation energy (BDE) of the O-H bond in α-tocopherol and other related compounds is low. Typical values are ~ 80 kcal/mol, with PMHC specifically having a BDE value of 78.9 kcal/mol.¹⁷² In comparison, the BDE of the lipid hydroperoxide LOO-H bond is on the order of 90 kcal/mol.¹⁶² It then follows that H-atom abstraction from Vitamin E by a lipid peroxy radical is energetically favored.

The phenoxyl radical (AO•) formed by H-abstraction from α-tocopherol or other phenols is stabilized by conjugation with the aromatic ring. Importantly, AO• does not react with molecular oxygen, in contrast to related semiquinone compounds. AO• does however react efficiently with a second LOO• to form closed-shelled adducts. Overall, one molecule of α-tocopherol can scavenge two chain-carrying lipid peroxy radicals, and effectively inhibit oxidation.

The role of α-tocopherol in living organisms has been under study for many years. It is clear that α-tocopherol plays a beneficial role in mitigating oxidative stress incurred in chronic diseases such as cardiovascular and neurodegenerative disorders,¹⁷³⁻¹⁷⁴ cancer and atherosclerosis.

Vitamin E is also involved in promoting plasma membrane repair, and fills non-antioxidant roles in cellular signalling, kinase activity modulation and gene regulation.¹⁷⁵ Nonetheless, the therapeutic role of Vitamin E is far from certain.¹⁷⁶ Under certain circumstances, α -tocopherol can counterproductively promote autoxidation through tocopherol-mediated peroxidation. This occurs in low-density lipoproteins (LDL) because of the compartmentalization of tocopherol radical within the nanoparticle and the lack of termination pathways.¹⁷⁷⁻¹⁷⁸

1.5. Single Molecule Spectroelectrochemistry

In addition to developing methods for monitoring oxidation processes occurring in heterogeneous media, the focus of this thesis revolves around establishing single molecule spectroelectrochemistry (SMS-EC) as a suitable method to examine redox reactions in heterogeneous systems. SMS-EC allows for ultrasensitive ‘indirect’ electrochemical measurements on the basis of the observed changes in fluorescence properties that are correlated to changes in redox states. Monitoring electron transfer of single fluorescent molecules or aggregates, one at a time, opens the possibility to access the distribution of values for key electrochemical variables, such as the half-wave potential. The combination of detecting the fluorescence of individual molecules under controlled electrochemical conditions makes SMS-EC a unique tool to study the interplay between photophysical properties and redox states. A distinctive aspect of SMS-EC is the ability to detect single electron processes, not only single molecules, as opposed to electrochemical techniques that amplify the signal of individual molecules. In addition, SMS-EC is versatile, the only true restriction being the use of a transparent substrate, and applicable to a wide range of optoelectronic systems. For instance, SMS-EC has the potential to enable a deeper understanding of the delicate balance between high charge mobility and high luminescence of conjugated polymers, and elucidate the intricacies of the charge

dynamics at the sensitizer-semiconductor interface that are central to efficient solar cell function. SMS-EC additionally offers the opportunity to visualize individual electrochemical reactions taking place on nanocatalysts, where it may potentially identify small populations of highly active sites and thus dictate future catalyst development. In similar fashion, SMS-EC also offers the opportunity to determine the distribution of electrochemically-generated species to determine how factors such as electrode geometry and diffusion through irregular-sized pores affects mass transport, particularly when the parameters or boundary conditions for the mass transport equation are ill-defined, in order to ultimately optimize industrial applications of electrocatalytic processes. The necessity of fluorescent or fluorogenic substrates sometimes require substantial efforts in order to design suitable systems to study with SMS-EC. Further development of fluorogenic probes such as those described in section 1.4.1 is important for broader applications of SMS-EC.

Section 1.3.3 showed us how changes in either distance or driving force for electron transfer will influence the rate, and therefore the efficiency, of PeT. In the case of ICT, in Section 1.3.4.1 we saw that the occupancy of the density of states (DOS) of a metal or semiconductor influences the rate of charge transfer. Using SMS-EC, we control the electronic occupancy of the states by applying an external electrical bias to manipulate the Fermi level (E_F). The electrochemical aspect within SMS-EC thus allows us to dictate the E_F of a semiconductor while the SM aspect enables us to simultaneously monitor of the fluorescence intensity. Since ICT competes with emission to deactivate the excited state of a bound sensitizer, the fluorescence intensity observed is modulated by changes in the way charges flow within the system in response to setting the E_F at a certain energy. The present section now describes previous research undertaken with SMS-EC and other SM fluorescence techniques.

Palacios *et al.* first demonstrated the spectroelectrochemical study of individual molecules with their elegant SM setup (Figure 1.16).¹⁷⁹ Collaboration between two world renowned scientists, Prof. Allen Bard and the late Prof. Paul F. Barbara at the University of Texas at Austin, spurred the development of SMS-EC to visualize electrochemical processes at the SM level using fluorescence detection. In the first SMS-EC study they studied the fluorescence of conjugated polymers under applied biases. The technique initially allowed the distinction between excited state and ground state oxidation processes, and the observation of a much narrower distribution of single-molecule half-wave potential values for the ground state process. Reports that followed¹⁸⁰⁻¹⁸¹ provided insights into the formation of deeply trapped holes and the screening of injected charges. These results highlight the heterogeneity found in conjugated polymers, a concept echoed in Chapter 4. Further optimization of conjugated polymer devices will be aided by understanding how this heterogeneity impacts operating conditions.

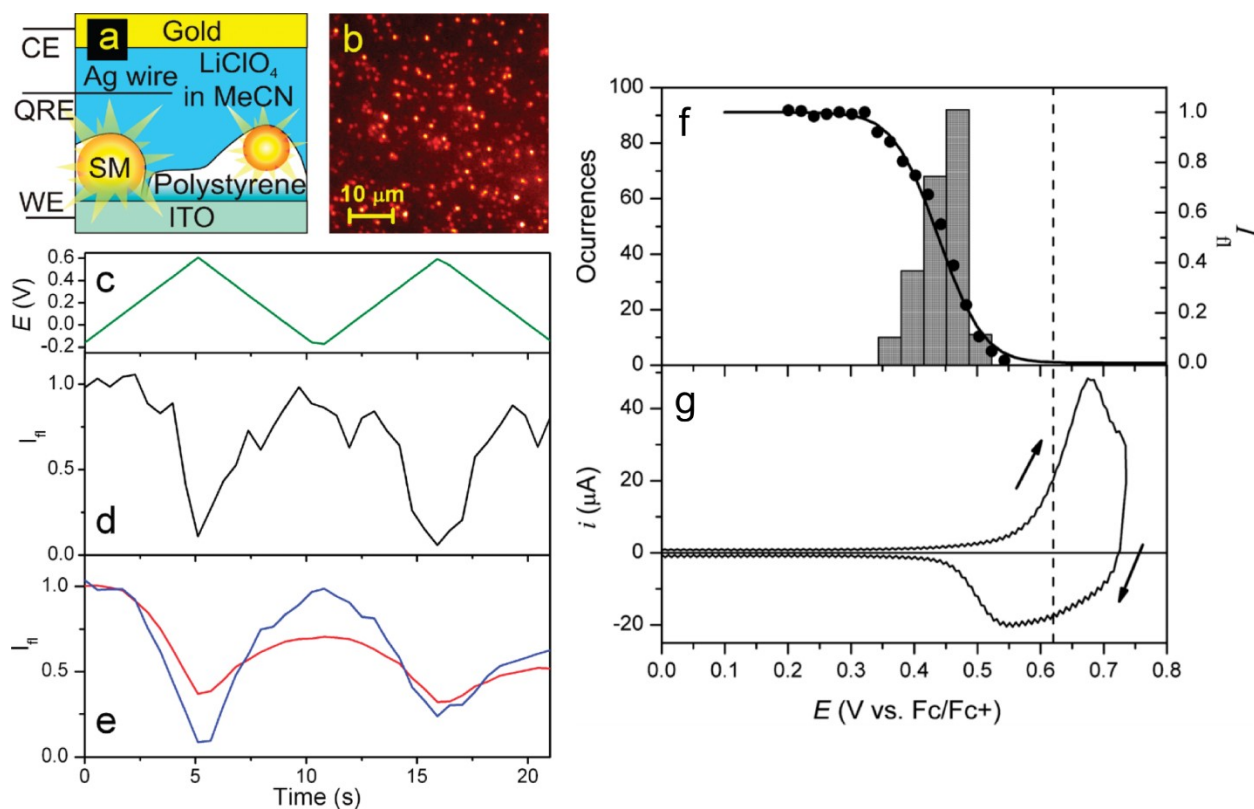


Figure 1.16. SMS-EC setup as developed in Prof. Paul Barbara's research group. A) Schematic depiction of the electrochemical cell. B) Wide-field fluorescence image of the SMS-EC cell. C) Applied electrochemical potential. D) Normalized fluorescence trajectory of a BEH-PPV single molecule while applying the potential shown in C). E) Ensemble (810 molecules, red) and subensemble (75 molecules, blue) normalized fluorescence trajectories. F) Distribution of potentials at which the fluorescence is quenched by half. G) Ensemble cyclic voltammogram of a BEH-PPV film on an ITO electrode. Adapted with permission from ref. 181. Copyright 2009 American Chemical Society.

Guided by our long-lasting collaboration with Prof. Rodrigo Palacios, we have developed in house state-of-the-art SMS-EC setups to fully exploit the opportunities that SMS-EC provides for studying redox processes in heterogeneous systems of interest. The experimental setups we used are described in Section 1.6.3. We identified SMS-EC as the technique of choice to gain insights into three different systems:

- 1) The effect of polymer chain conformation on the optoelectronic properties of conjugated polyelectrolyte NPs (Chapter 4)
- 2) The intricacies of ICT between dyes and nanostructured semiconductor (Chapter 5)
- 3) Electrocatalysis of both oxidation and reduction reactions that result in fluorescence enhancement of fluorogenic probes (Chapter 6)

The chosen systems highlight two distinct uses of SMS-EC. In 2), the electrochemical setup controlled the E_F of a semiconductor, thus influencing the ICT rates. Here we monitored how the effect of changes in ICT rates affected the competing fluorescence and photophysics of dye sensitizers. In 3), SMS-EC was used to provide the electrochemical potential required for observing single electrochemical products in an electrocatalytic redox chemical system. System 1) showed aspects of both ICT and redox reactions, highlighting the fact that control of the E_F and electrocatalysis are not mutually exclusive, but may actually introduce complexities due to their interrelation.

1.5.1. Other combined SM and EC System

Even though the field of SMS-EC is in its infancy, there are a few other examples where optical SMS has been applied to systems under electrochemical control.¹⁸²⁻¹⁸³ We can divide fluorescence spectroelectrochemistry systems into four groups:

- 1) Fluorescent molecules: The molecule of interest is innately fluorescent and the fluorescence properties of the redox states of fluorophore are distinguishable.
- 2) Chemiluminescence: Redox events produce photons via chemiluminescence.
- 3) Fluorescent labeling: The compound of interest is fluorescently labeled and the fluorescent probe reports on the redox state of the compound.
- 4) Electrocatalysis: Fluorescent products are produced from non-fluorescent reactants.

In relation to the systems described in this thesis, case 1) applies to Chapters 4 and 5, while cases 3) and 4) apply to Chapter 6.

A few other systems proved successful in electrochemically controlling the redox state of individual molecules as determined by SM fluorescence (i.e. SMS-EC). To appreciate the precise information that can be garnered by SMS-EC, we will briefly describe examples from the literature. To the best of our knowledge, the examples presented here and the work from the Barbara group discussed above represent a comprehensive review of SMS-EC studies.

The first report using SMS-EC outside of the Barbara group involved the SM observation of oxidized and reduced cresyl violet molecules performed on a confocal microscope setup.¹⁸⁴ The frequency of fluorescent bursts from individual molecules diffusing in the observation volume depended on the potential applied at the ITO WE. The observations suggest that fluorescent cresyl violet molecules accept an electron from the WE to produce reduced molecules whose

fluorescence is completely quenched. In comparison, ensemble measurements were not able to conclusively attribute residual fluorescence to either low emission from the reduced state of the fluorophore or to a remaining fraction of non-reduced fluorophores. In a subsequent report, the system was modified to immobilize cresyl violet molecules within the 3D arrangement of a clay-modified ITO electrode.¹⁸⁵ Stochastic on-off behaviour could be observed for individual molecules, and was ascribed to reversible oxidation/reduction processes. Interestingly, the electron transfer rate of individual molecules could be extracted from analysis of their intensity trajectories, which proved to be in line with the value obtained from ensemble measurements.

Successful SMS-EC experiments were also performed on flavin adenine dinucleotide (FAD) molecules.¹⁸⁶ The experimental strategy involved a nanoarray of 60-150-nm wide holes forged into a 100-nm thick Au layer that acted as the WE. Thoughtful consideration of the FAD concentration during immobilization was key in obtaining pores that contained at most one FAD molecule bound to the Au electrode. The holes functioned as zero-mode waveguides¹⁸⁷ and temporal fluorescence trajectories of individual FAD molecules were obtained. Analysis of tens of individual molecules revealed a spread in the observed midpoint potentials and electron transfer rates.

A recent study proved fruitful in observing the modulation of ICT rates between 1,10-dioctadecyl-3,3,30,30-tetramethylindo-dicarbocyanine (DiD) molecules and their ITO support.¹⁸⁸ The experimental setup allowed the authors to flow different current densities through an ITO WE on which DiD molecules were immobilized. Larger applied currents were found to correlate with higher rates of ICT (i.e. lower fluorescence). The measurement of the fluorescence lifetime further enabled discrimination between changes in the forward, backward and ground state electron transfers.

Recent work by Peng Chen and coworkers demonstrated single molecule nanoelectrocatalytic turnovers (Figure 1.17).¹⁸⁹⁻¹⁹⁰ The electrochemical reduction of non-fluorescent resazurin to fluorescent resorufin was shown to take place at discrete sites rather than along the whole sidewall of single-walled carbon nanotubes. The active sites displayed large reactivity heterogeneity, likely due to their heterogeneous electronic properties following from their distinct chemical nature or from different nanotube electronic properties.

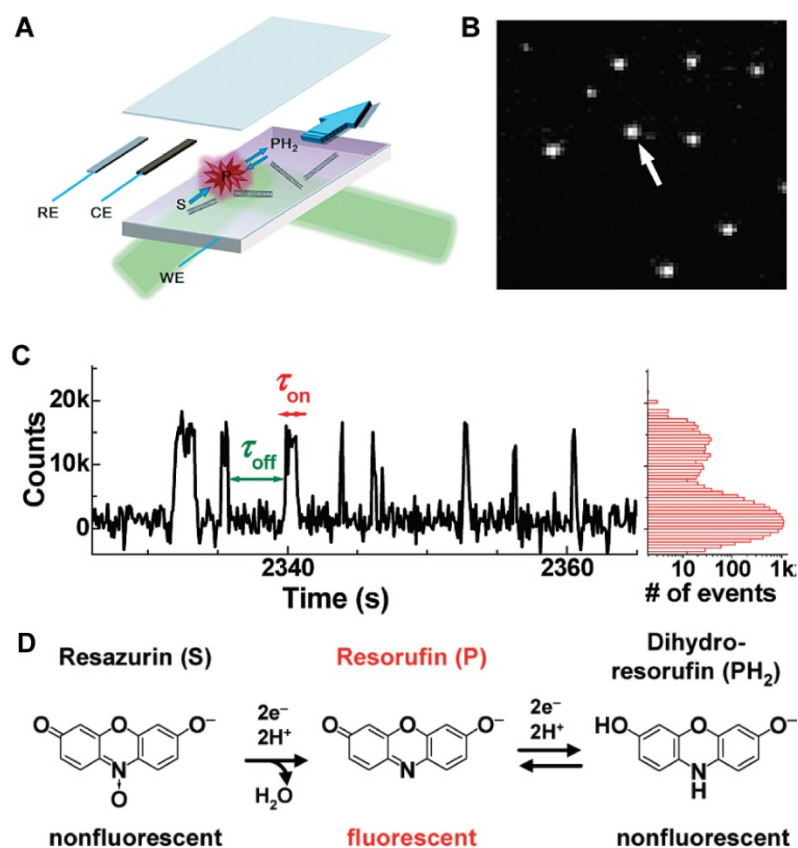


Figure 1.17. SMS-EC system developed by Prof. Peng Chen to study nanocatalytic electroreduction reactions. A) Experimental setup. B) TIRF image ($\sim 17 \times 19 \mu\text{m}^2$) showing fluorescent resorufin molecules. C) Fluorescence trajectory from the fluorescent spot marked by an arrow in B). D) Non-fluorescent resazurin is electrochemically reduced to fluorescent resorufin at the active sites of single-walled carbon nanotubes. Adapted with permission from 189. Copyright 2009 American Chemical Society.

We close this section by mentioning the sister technique of fluorescence-voltage spectroscopy (FVS) first developed at the SM level in the Barbara group¹⁹¹⁻¹⁹² and more recently extended as part of Prof. Vanden Bout's solar cell research program.¹⁹³⁻¹⁹⁴ While strictly not under electrochemical control, the voltage applied in the capacitor-style devices may induce the injection of charges in conjugated polymer materials, allowing for correlation between redox and fluorescent states. The SM variant of FVS (F-V/SMS) showed that transient non-emissive states were formed by photooxidation of single conjugated polymer chains. These states could then be 'repaired' by removing holes from the polymer.¹⁹¹⁻¹⁹² The quenching/restoration phenomena was exploited to create a SM image capture write-read-erase device.¹⁹⁵ Subsequent F-V/SMS studies visualized exciton quenching (induced by hole polarons) at subdiffraction resolution by mapping the movement of the emission centroid as the applied biases were swept.¹⁹⁶ The Vanden Bout research group has made advances in understanding the role of interfacial dipoles on charge traps in inorganic-organic (i.e. inorganic TiO₂ and organic polythiophene conjugated polymer) solar cells. Hysteresis in the fluorescence response of the polymer to changes in applied bias was determined to be indicative of charge traps at the interface with TiO₂. Modifying the interface by adding carboxylic acid side groups to the polythiophene altered the pathways of charge motion by eliminating charge traps. No hysteresis was then observed.¹⁹³ The use of oligomers instead of polymers enabled molecular precision in carboxylation of the thiophenes.¹⁹⁴ This study also showcased the imaging aspect of FVS to determine the amount of aggregation and to demonstrate which organic sensitizer gives the best interfaces.

1.6. Single Molecule Experimental Setup

Central to successful SM studies are the experimental setup, fine tuning of the system, and thorough sample preparation. Before discussing the details of the instrumentation, the two modes

of sample arrangement used in the experiments described in the later chapters of this thesis will be quickly reviewed. The sample is usually presented in one of two ways in SM experiments. The analyte is either freely diffusing in a solvent or is immobilized by tethering to a surface or by inclusion in a matrix. In diffusion experiments, photons emitted by the fluorophore are detected in transient bursts, which are generated as the molecules pass through the small excitation/observation volume. This sample geometry was used to perform the fluorescence correlation spectroscopy (FCS) experiments described in Chapter 3 and the SMS-EC studies of Chapter 6. For experiments involving immobilized molecules, the fluorescence emitted following photoexcitation can be detected over an extended period of time whose duration is limited only by photobleaching. The continuous monitoring of fluorescence from individual molecules allows for detailed analysis of any process that results in a change of detectable fluorescence properties: intensity, emission wavelength, polarization, etc. Immobilization by inclusion in a matrix may take various forms including dispersion in a polymer glass and liposome encapsulation. Most of the experiments described in Chapters 2, 4 and 5 were carried out on immobilized analytes, either embedded in the lipid membrane of liposomes or bound to a surface via covalent linking or physisorption.

For the immobilization of liposomes, we utilized a self-assembled strategy. First, microscope coverslips were scrupulously cleaned by Piranha treatment (1:3 30% H₂O₂ solution: concentrated sulfuric acid by volume) and subsequent water and acetone rinsing. Second, the surface hydroxyl groups reacted with an amine-terminated silane (Vectabond®) to accomplish aminosilanization of the surface. Third, the surface was functionalized with a mixture of polyethylene glycol (PEG) and biotin-modified PEG, both bearing activated carboxylic acids in the form of succinimidyl esters, that formed covalent bonds to the amine groups installed in the

previous step. The PEGylation of the coverslip minimizes non-specific binding of unwanted emitters. Fourth, a solution of streptavidin was flowed on the coverslip. Streptavidin has 4 binding sites for biotin characterized by a $K_D \approx 1 \times 10^{-15}$ M ensuring a tight, long-lasting binding with the biotin handles.¹⁹⁷ Finally, liposomes containing embedded biotinylated lipids were flowed over the coverslip binding irreversibly to the streptavidin found on the surface.

The immobilization procedure was more straightforward for the inorganic systems studied. Solutions of the desired NPs were simply spin coated onto clean coverslips. Typically, 40 μ L of solution was dropped on a coverslip rotating at 3000 rpm. The rotation was then slowed to 2000 rpm and the sample spun for 50 s to ensure complete evaporation of the solution. Subsequent sintering (450 °C, 1 hour) ensured good contact between the coverslip and NPs, and between different NPs to form an interconnected network. Next, the nanostructured semiconductor films were immersed overnight in solutions of dye sensitizer with a μ M to mM concentrations range. Carboxylic acid moieties on the sensitizers form covalent bonds to the metal oxide surface. Subsequent rinsing and sonication in the immersion solvent (e.g. ethanol) and the solvent used during the measurements (acetonitrile) afforded monolayers of dye sensitizers bound to the surface of metal oxides.

We now turn our attention to the microscope setups. The basic instrumentation required for SM experiments is a fluorescence microscope equipped with a powerful excitation source (i.e. laser) and an ultrasensitive detector with single photon detection capabilities. The microscope holds all of the components in the optimum optical geometry that is needed to reach the diffraction limit and maximize the ability to spatially distinguish between individual molecules immobilized on the surface of a coverslip. Inverted microscopes equipped with high numerical aperture ($NA \sim$

1.4) objectives are used. A high NA is desirable as it translates to a greater photon collection efficiency stemming from the wider light acceptance cone.

There are two main classes of fluorescence microscopies used in SM: Confocal microscopy and Total Internal Reflection Fluorescence (TIRF) microscopy (Figure 1.18). The configuration of the excitation beam is different in both cases, as is the detector used, and both are described in the following sections. Both types of microscopies have been used in this thesis work. On one hand, the advantages of SM confocal setups lie their enhanced time resolution, high signal-to-noise ratio and the straightforward implementation of fluorescence correlation spectroscopy (FCS) techniques for freely diffusing samples. On the other hand, TIRF setups boast simultaneous monitoring of hundreds of individual molecules for rapid acquisition, and selective excitation only a few hundred nanometers above the coverslip surface, reduces the background fluorescence. The decisions regarding which microscope to use for an experiment also involved spectral considerations and limitations specific to our own SM setups. For example, one of our confocal microscopes was the only setup that possessed both a 514 nm excitation line and dual-color emission detection. After detailing confocal and TIRF microscopes, the present section ends with a description of the single molecule spectroelectrochemistry (SMS-EC) setups that were used for the experiments described in Chapters 4 to 6.

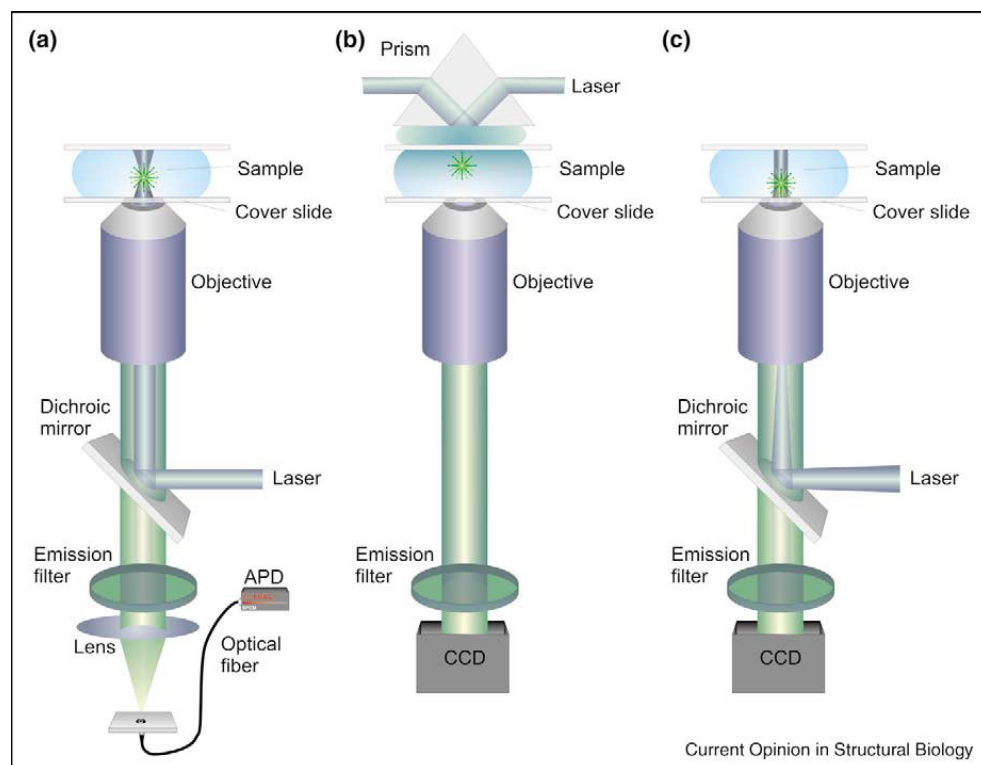


Figure 1.18. Schematic of (a) confocal, (b) prism-based TIRF, and (c) epifluorescence microscopy setups. Objective-based TIRF microscopy is similar to (c) with the addition of a translatable optic component that allows to control at which angle the excitation beam exits the objective and reflects on the coverslip. Reprinted from ref. 198, Copyright 2004, with permission from Elsevier.

1.6.1. Confocal Microscopy

All of the SM experiments performed in Chapters 2, 3 and 6 were performed on confocal microscopes. The specific laser excitation sources equipped on the confocal microscopes and the need to perform FCS experiments (Chapter 3) led us to the decision to use a confocal geometry. In a confocal microscope, the excitation light is focused to a diffraction-limited Gaussian-shaped spot on the sample. This is done by projecting a collimated beam with a diameter of a few millimeters into the objective back aperture. The size of the illumination volume depends on the NA of the objective used (itself dependant on the refractive index of the immersion medium (η); $NA = \eta \sin \theta$) and the wavelength of light (λ). The radius of the focused spot in the plane perpendicular to the optical axis is given by:

$$r_{xy} = \frac{\lambda}{2 \times \text{NA}} \quad (1.17)$$

and the radius in the direction parallel to the optical axis is:

$$r_z = \frac{2\lambda}{\text{NA}^2} \quad (1.18)$$

As an example, using an excitation wavelength of 488 nm and an objective with NA = 1.4, we expect a roughly cylindrical confocal excitation volume of ~ 0.1 femtoliter with lateral and axial spot radii of 175 nm and 500 nm, respectively. In practice, larger spots are obtained due to the effect of various optical aberrations. The observation volume is then experimentally determined by analysing the FCS trace obtained using a standard dye with a known diffusion coefficient.¹⁹⁹

The fluorescence image is reconstructed by raster scanning the sample over the fixed excitation spot. The fluorescence at each area (each pixel in the final image) passes through the objective and is recorded by a single point high-sensitivity detector that lacks spatial resolution. The home built acquisition software then assigns the measured intensity to the position of the sample. For SM experiments, avalanche photodiode detectors (APDs) are ideal. Compared to photomultiplier tubes (PMTs), APDs display excellent linearity and have lower dark counts, features that are critical for distinguishing individual fluorophores from the background. The small active area of APDs (circle of diameter ~ 130 µm) effectively rejects out of focus light. The acquisition of a complete image, typically 256 x 256 pixels, takes about 5 minutes and is much slower than the acquisition time of our TIRF setups. However, the signal to noise ratio is significantly better for confocal setups and increased time resolutions (as short as ~ 10 µs) are accessible.²⁰⁰

Two-color detection is useful to spectrally separate the emission from different fluorophores and enables SM Förster Resonance Energy Transfer (smFRET) studies.⁹³ For confocal setups, adding two-color detection is straightforward. A dichroic mirror that reflects only light below a certain wavelength is placed in the detection light path. The short wavelength reflected light is directed to a second APD detector at a 90° angle while the long wavelength emission is detected by the APD in ‘straight-through’ configuration. Selective long pass or band pass emission filters are positioned in front of the APDs to reduce background and improve the spectral discrimination of the two channels. Alternatively, spectrally-narrow Raman notch filters may be utilized to only block the scattered excitation from reaching the APD. The acquisition software handles the data in such a way to produce two images each corresponding to the short or long wavelength emission.

1.6.2. Total Internal Reflection Fluorescence Microscopy

All of the SM experiments conducted in Chapters 4 and 5 were performed on TIRF microscopes. We chose a TIRF setup for these experiments due to the statistical and time advantages of simultaneously imaging hundreds of fluorophores. For TIRF microscopes, the excitation beam is launched towards the top interface of the glass coverslip (glass-air or glass-solution interface) past the critical angle. The light is thus reflected in what is called total internal reflection, hence the name Total Internal Reflection Fluorescence (TIRF) Microscopy. This process generates an evanescent wave that penetrates a few hundred nanometers into the solution, exciting fluorophores near the surface of the coverslip. The excited area, in contrast to confocal setups, is relatively large in the lateral direction – typically $> 150 \times 150 \mu\text{m}^2$. Two distinct excitation geometries are possible in TIRF setups. In prism-based TIRF the excitation beam does not pass through the objective. The setups available for the experiments described herein are

objective-based TIRFs. In this case, the laser light exits the objective collimated, which requires the excitation beam to be focused at the back aperture of the objective. This is usually performed by placing a mobile focusing lens in the beam path before the objective. Objective-based TIRF is simpler to align and set up, but the difficulties in using prism-based setups are compensated by the better signal-to-background ratios achieved.

Regardless of the excitation scheme, the fluorescence is collected through the objective and is relayed to an electron multiplying charge coupled device (EM-CCD) camera. The two-dimensional 512 x 512 array of $16 \times 16 \mu\text{m}^2$ pixels of the camera can capture images of regions ca. $80 \times 80 \mu\text{m}^2$ if using a 100x magnification objective. An entire image is thus recorded at once as a frame. Movies of hundreds to thousands of frames are constructed by continuously capturing images over time, where the fluorescence of a few hundred fluorophores is monitored simultaneously in each frame. Typical frame acquisition rates are approximately 20 Hz, giving a time resolution of ~ 50 ms. Reducing the size of the region of interest, to 128×128 pixels for example, enables faster image capture with a time resolution of ~ 1 ms.

A benefit of TIRF setups is that one may analyze the fluorescence intensity-time trajectories of hundreds of molecules from a single stack of images. This allows for the rapid acquisition of the large number of trajectories needed for robust statistical analysis of the stochastic behavior of individual molecules. The use of image processing software is essential in order to extract information from the image sequences. For two-color experiments, the analysis is performed using a software package first developed in Prof. Ha's group⁹³ and subsequently modified in house according to our specific needs. Single color experiments can be processed either by the same software package, or with the help of Fiji/ImageJ software.²⁰¹ For both cases, and also for data acquired on a confocal microscope, processed data is generated using the same

general method: (1) Fluorescence peaks are identified, and a predefined area around the peak is considered to originate from the emission of a single molecule/particle (i.e. the signal). (2) The signal intensity is background corrected by subtracting the intensity due to background. One method to do so is to take the intensity in a ring of pixels around the signal area as the background intensity. (3) Intensity-time trajectories for each individual molecule are constructed with the signal intensity at each time point.

For wide-field microscopy setups such as TIRF, two-color acquisition is more complex to implement compared to a confocal microscope. A single EM-CCD is still used, but two different spectrally separated images are projected onto the camera. The image coming out of the microscope is spatially truncated by a slit positioned at the image plane of the microscope tube lens. This step is needed to avoid overlap between the two images on the imaging sensor of the camera. The narrowed image is then separated by wavelength and relayed to the camera with the use of a lens, mirrors and dichroic mirrors. Emission filters are also used to reduce background signal and improve spectral discrimination between the two channels. Chromatic aberrations and the distinct light paths that the two spectrally separated images take complicate the assignment of the same spatial area to pixels in the short and long wavelength images. Therefore the two channels of the image must be ‘mapped’ to one another in order to associate where a given molecule appears in both the short and long wavelength images. The mapping is typically accomplished by imaging a sparse sample of fluorescent beads under the same conditions as the sample. With the help of three reference peaks chosen by the user, processing software extracts the calibration needed to align the two different spectral channels. This is done by fitting the three reference peaks to a plane for both channels and determining the geometrical transformation (image warping) that leads to

parallel planes. During processing of the sample movie, the bead calibration is called by the software and the fluorescent peaks in the sample images are appropriately linked to each other.

The importance of SM techniques, and subsequent interest in performing SM experiments, has spurred development in commercial instrumentation. Gone are the days where each laboratory was required to build their own custom SM microscope. Many universities have microscope facilities with SM-ready instruments. Full imaging solutions are available commercially and the costs of the components required for SM experiments are steadily going down as technology improves. As a result, SM spectroscopy is transitioning from a highly specialized field to becoming much more user friendly and accessible. Nonetheless, the expertise needed to apply SM techniques to monitor chemical transformations, particularly redox processes, is severely lacking. The work described in this thesis will hopefully catalyze progress in developing chemists' SM know-how.

1.6.3. Single Molecule Spectroelectrochemistry (SMS-EC)

The most popular experimental setup is similar to SM TIRF microscopy (described in Section 1.6.2) with modifications to accommodate the electrochemical cell (Figure 1.19). TIRF geometry is in no way a prerequisite. The experiments described in Chapter 6 have been performed on a confocal-based SMS-EC setup, and other researchers have also used a confocal geometry.¹⁸⁴⁻

185

The electrochemical cell consists of a three-electrode system: the working electrode (WE), the reference electrode (RE) and the counter electrode (CE). The reaction of interest (i.e. electron transfer) takes place at the surface of the WE. A potential is imposed to the WE vs. the RE by a potentiostat and provides the energy input to drive electrochemical processes. The composition of a good RE remains effectively constant for the duration of an experiment, in order to provide a stable potential for controlled regulation of the WE potential. Ag wire can be used as an easy to

implement quasi-RE. In this thesis, the RE of choice was a non-aqueous Ag/Ag^+ RE (Ag wire submersed in 10 mM AgNO_3 solution with supporting electrolyte), externally calibrated to the ferrocene/ferrocenium redox couple. The direction of the current (reversed compared to the movement of electrons) at the CE is opposite to the direction of the current at the WE. For example, when current flows into the WE (anodic current), current will be flowing out of the CE (cathodic current), and vice versa. We used a common Pt wire as the CE in our own spectroelectrochemistry experiments.

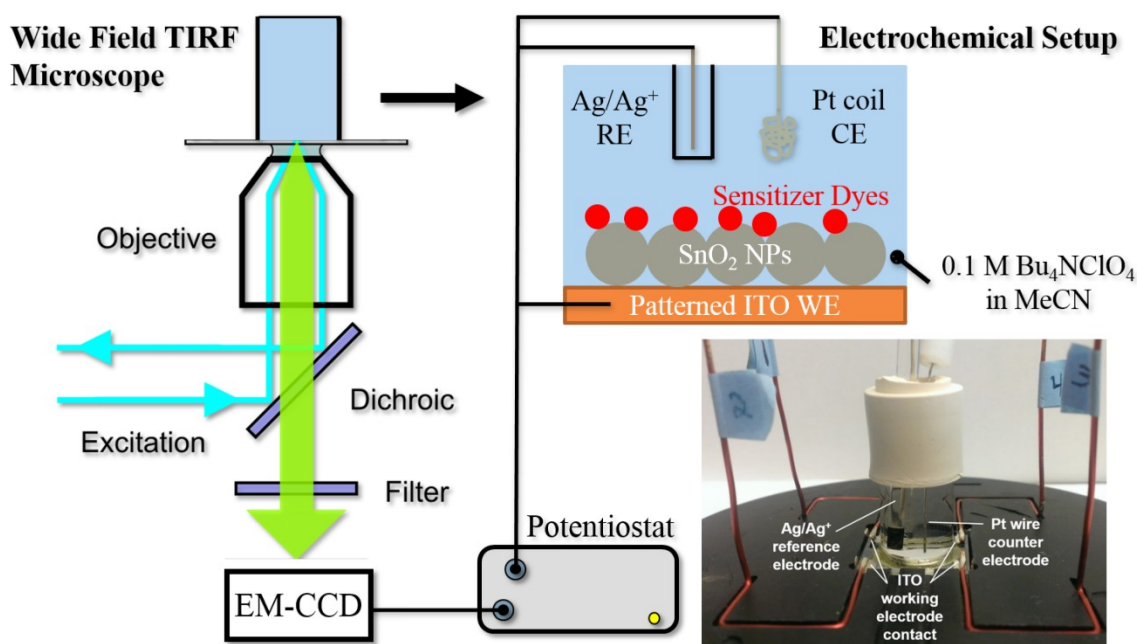


Figure 1.19. Schematic representation of a TIRF-based SMS-EC setup. RE stands for reference electrode, CE for counter electrode, ITO for indium tin oxide and WE for working electrode. In the bottom right is shown a picture of a sample mounted on an insulating plate which is then positioned on the microscope stage.

The electrochemical cell was self-contained and mounted on the microscope for SMS-EC experiments. A glass coverslip was coated with a transparent conducting oxide (typically indium tin oxide, ITO, or fluorine-doped tin oxide, FTO) that acts as the WE. Connection to the potentiostat was accomplished using copper wire leads attached to the WE with the help of a

conducting silver paint. A glass tube was then glued to the top of the ITO-covered coverslip to form the sample chamber that is filled with a solution of supporting electrolyte (e.g. 0.1 M tetrabutylammonium perchlorate in anhydrous acetonitrile). The chamber was finally sealed by a rubber septum, and both the RE and CE are introduced in the cell through this septum. Prior to measurements, the air-tight electrochemical cell is purged with argon to remove any oxygen present that may interfere with the electrochemical process. Experiments were typically conducted under inert argon atmosphere provided by an argon-filled balloon that kept a positive argon pressure in the electrochemical cell.

1.7. Research Goals

This thesis depicts our efforts to demonstrate and expand SM techniques as useful tools for chemical research. Two distinct types of systems were chosen to display the scope of SM techniques to visualize chemical redox reactions. The first system studied was the biologically relevant chemical process of lipid peroxidation. Key to study lipid peroxidation was the development of fluorogenic α -tocopherol mimics. These oxidation-sensitive probes were previously used in ensemble experiments and shed light on how the chemical structure of an antioxidant can affect its reactivity and selectivity.¹³²

Building on these results, Chapter 2 discusses the first observation of oxidation reactions occurring in lipid membranes at the SM level. Oxidation taking place in individual liposomes was heterogeneous, with some liposomes showing high levels of oxidation and others showing little. The unsurpassed sensitivity of SM techniques was instrumental in establishing that standard liposome preparations caused oxidation and in quantifying the relative effectiveness of water-soluble antioxidants vs. lipid-soluble antioxidants.

Initial efforts to observe oxidation processes in individual liposomes were hampered by chemically-activated dyes leaking out of the lipid membrane. This led to the research described in Chapter 3. The dye retention in the lipid membrane was dependent on the structure of the dye, as expected. Significantly and particularly important for imaging in lipid membranes, the lipophilicity of fluorogenic probes after chemical activation was strongly dependant on the activation reaction partner. A technique to quickly determine the fraction of dye bound to the lipid membrane under dilute conditions was developed.

The second set of chemical systems studied as part of this thesis revolved around the use of SMS-EC. SMS-EC holds tremendous potential in monitoring electrochemical processes of individual molecules to decipher heterogeneity in reactivity, reaction mechanism, and interfacial charge transfers rates.

Chapter 4 discusses the influence of external applied biases on the optoelectronic properties of conjugated polyelectrolyte NPs. Two types of fluorescence responses were observed which were linked to different degrees of alignment and packing of the polymer backbones. Of note, two-color imaging was combined with SMS-EC for the first time, which unearthed differential behavior for spectrally distinct emitting sites.

In Chapter 5, the topic is the modulation of the rate of ICT between dyes and a nanostructured semiconductor when the Fermi level of the semiconductor is changed. We determined that photoreduction of the sensitizer dyes was an active pathway that deactivates the excited state, and should thus be taken into consideration in future designs of DSSCs. Two different dyes were immobilized on the same semiconductor surface and allowed for evaluation of the relative importance of DOS and energetic effects. The instability of the oxidized form of one

of the dyes proved useful in ascertaining changes in the rate of electron injection into the semiconductor.

Finally, Chapter 6 reports the visualization of individual electrocatalytic turnovers. Both oxidation and reduction reactions occurring on the surface of a platinized ITO electrode could be used to produce fluorescence bursts via separate reaction pathways of the same fluorogenic probe. Additionally, the concentration profile of the fluorescent species could be monitored over time, confirming that mass transport occurred solely by a diffusion mechanism in the case of direct electrochemical oxidation, but not for the coupled reduction reaction pathway.

1.8. References

- (1) Wustholz, K. L.; Bott, E. D.; Kahr, B.; Reid, P. J., Memory and Spectral Diffusion in Single-Molecule Emission. *J. Phys. Chem. C* **2008**, *112*, 7877-7885.
- (2) Dertinger, T.; Heilemann, M.; Vogel, R.; Sauer, M.; Weiss, S., Superresolution Optical Fluctuation Imaging with Organic Dyes. *Angew. Chem. Int. Ed.* **2010**, *49*, 9441-9443.
- (3) Lee, J.; Lee, S.; Ragunathan, K.; Joo, C.; Ha, T.; Hohng, S., Single-Molecule Four-Color FRET. *Angew. Chem. Int. Ed.* **2010**, *49*, 9922-9925.
- (4) Sabir, T.; Schröder, G. F.; Toulmin, A.; McGlynn, P.; Magennis, S. W., Global Structure of Forked DNA in Solution Revealed by High-Resolution Single-Molecule FRET. *J. Am. Chem. Soc.* **2011**, *133*, 1188-1191.
- (5) Vogelsang, J.; Bizard, J.; Adachi, T.; Bolinger, J. C.; Barbara, P. F., Watching the Annealing Process One Polymer Chain at a Time. *Angew. Chem. Int. Ed.* **2011**, *50*, 2257-2261.
- (6) Dill, M.; Baier, M. C.; Mecking, S.; Wöll, D., Enhanced Accuracy of Single-Molecule Diffusion Measurements with a Photocleavable Energy-Transfer Dyad. *Angew. Chem. Int. Ed.* **2013**, *52*, 12435-12438.
- (7) Rybina, A.; Lang, C.; Wirtz, M.; Großmayer, K.; Kurz, A.; Maier, F.; Schmitt, A.; Trapp, O.; Jung, G.; Hertel, D.-P., Distinguishing Alternative Reaction Pathways by Single-Molecule Fluorescence Spectroscopy. *Angew. Chem. Int. Ed.* **2013**, *52*, 6322-6325.
- (8) Keller, B. G.; Kobitski, A.; Jäschke, A.; Nienhaus, G. U.; Noé, F., Complex RNA Folding Kinetics Revealed by Single-Molecule FRET and Hidden Markov Models. *J. Am. Chem. Soc.* **2014**, *136*, 4534-4543.
- (9) Rothwell, P. J.; Berger, S.; Kensch, O.; Felekyan, S.; Antonik, M.; Wöhrle, B. M.; Restle, T.; Goody, R. S.; Seidel, C. A. M., Multiparameter Single-Molecule Fluorescence Spectroscopy Reveals Heterogeneity of HIV-1 Reverse Transcriptase:Primer/Template Complexes. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 1655-1660.
- (10) Yang, H.; Luo, G.; Karnchanaphanurach, P.; Louie, T.-M.; Rech, I.; Cova, S.; Xun, L.; Xie, X. S., Protein Conformational Dynamics Probed by Single-Molecule Electron Transfer. *Science* **2003**, *302*, 262-266.

- (11) English, B. P.; Min, W.; van Oijen, A. M.; Lee, K. T.; Luo, G.; Sun, H.; Cherayil, B. J.; Kou, S. C.; Xie, X. S., Ever-Fluctuating Single Enzyme Molecules: Michaelis-Menten Equation Revisited. *Nat. Chem. Biol.* **2006**, *2*, 87-94.
- (12) Smiley, R. D.; Hammes, G. G., Single Molecule Studies of Enzyme Mechanisms. *Chem. Rev.* **2006**, *106*, 3080-3094.
- (13) Abbondanzieri, E. A.; Bokinsky, G.; Rausch, J. W.; Zhang, J. X.; Le Grice, S. F. J.; Zhuang, X., Dynamic Binding Orientations Direct Activity of HIV Reverse Transcriptase. *Nature* **2008**, *453*, 184-189.
- (14) Liu, S.; Abbondanzieri, E. A.; Rausch, J. W.; Grice, S. F. J. L.; Zhuang, X., Slide into Action: Dynamic Shuttling of HIV Reverse Transcriptase on Nucleic Acid Substrates. *Science* **2008**, *322*, 1092-1097.
- (15) Blank, K.; De Cremer, G.; Hofkens, J., Fluorescence-Based Analysis of Enzymes at the Single-Molecule Level. *Biotech. J.* **2009**, *4*, 465-479.
- (16) Claessen, V. I.; Engelkamp, H.; Christianen, Peter C.M.; Maan, J. C.; Nolte, R. J. M.; Blank, K.; Rowan, A. E., Single-Biomolecule Kinetics: The Art of Studying a Single Enzyme. *Annu. Rev. Anal. Chem.* **2010**, *3*, 319-340.
- (17) Madden, C.; Vaughn, M. D.; Díez-Pérez, I.; Brown, K. A.; King, P. W.; Gust, D.; Moore, A. L.; Moore, T. A., Catalytic Turnover of [FeFe]-Hydrogenase Based on Single-Molecule Imaging. *J. Am. Chem. Soc.* **2012**, *134*, 1577-1582.
- (18) Terentyeva, T. G.; Hofkens, J.; Komatsuzaki, T.; Blank, K.; Li, C.-B., Time-Resolved Single Molecule Fluorescence Spectroscopy of an A-Chymotrypsin Catalyzed Reaction. *J. Phys. Chem. B* **2013**, *117*, 1252-1260.
- (19) Holmstrom, E. D.; Polaski, J. T.; Batey, R. T.; Nesbitt, D. J., Single-Molecule Conformational Dynamics of a Biologically Functional Hydroxocobalamin Riboswitch. *J. Am. Chem. Soc.* **2014**, *136*, 16832-16843.
- (20) Rabe, M.; Tabaei, S. R.; Zetterberg, H.; Zhdanov, V. P.; Höök, F., Hydrolysis of a Lipid Membrane by Single Enzyme Molecules: Accurate Determination of Kinetic Parameters. *Angew. Chem. Int. Ed.* **2015**, *54*, 1022-1026.
- (21) Zhang, K.; Chang, H.; Fu, A.; Alivisatos, A. P.; Yang, H., Continuous Distribution of Emission States from Single CdSe/Zns Quantum Dots. *Nano Lett.* **2006**, *6*, 843-847.
- (22) Xu, W.; Kong, J. S.; Yeh, Y.-T. E.; Chen, P., Single-Molecule Nanocatalysis Reveals Heterogeneous Reaction Pathways and Catalytic Dynamics. *Nat Mater* **2008**, *7*, 992-996.
- (23) Zheng, H.; Smith, R. K.; Jun, Y.-w.; Kisielowski, C.; Dahmen, U.; Alivisatos, A. P., Observation of Single Colloidal Platinum Nanocrystal Growth Trajectories. *Science* **2009**, *324*, 1309-1312.
- (24) Tachikawa, T.; Wang, N.; Yamashita, S.; Cui, S.-C.; Majima, T., Design of a Highly Sensitive Fluorescent Probe for Interfacial Electron Transfer on a TiO₂ Surface. *Angew. Chem. Int. Ed.* **2010**, *49*, 8593-8597.
- (25) Tachikawa, T.; Yamashita, S.; Majima, T., Probing Photocatalytic Active Sites on a Single Titanosilicate Zeolite with a Redox-Responsive Fluorescent Dye. *Angew. Chem. Int. Ed.* **2010**, *49*, 432-435.
- (26) Hu, Z.; Gesquiere, A. J., Charge Trapping and Storage by Composite P3HT/PC60BM Nanoparticles Investigated by Fluorescence-Voltage/Single Particle Spectroscopy. *J. Am. Chem. Soc.* **2011**, *133*, 20850-20856.

- (27) Andoy, N. M.; Zhou, X.; Choudhary, E.; Shen, H.; Liu, G.; Chen, P., Single-Molecule Catalysis Mapping Quantifies Site-Specific Activity and Uncovers Radial Activity Gradient on Single 2D Nanocrystals. *J. Am. Chem. Soc.* **2013**, *135*, 1845-1852.
- (28) Liu, Y.; Lin, X.-M.; Sun, Y.; Rajh, T., In Situ Visualization of Self-Assembly of Charged Gold Nanoparticles. *J. Am. Chem. Soc.* **2013**, *135*, 3764-3767.
- (29) Qin, H.; Niu, Y.; Meng, R.; Lin, X.; Lai, R.; Fang, W.; Peng, X., Single-Dot Spectroscopy of Zinc-Blende CdSe/CdS Core/Shell Nanocrystals: Nonblinking and Correlation with Ensemble Measurements. *J. Am. Chem. Soc.* **2013**, *136*, 179-187.
- (30) Tachikawa, T.; Ohsaka, T.; Bian, Z.; Majima, T., Single-Molecule Fluorescence Detection of Effective Adsorption Sites at the Metal Oxide–Solution Interface. *J. Phys. Chem. C* **2013**, *117*, 11219-11228.
- (31) Ha, J. W.; Ruberu, T. P. A.; Han, R.; Dong, B.; Vela, J.; Fang, N., Super-Resolution Mapping of Photogenerated Electron and Hole Separation in Single Metal–Semiconductor Nanocatalysts. *J. Am. Chem. Soc.* **2014**, *136*, 1398-1408.
- (32) Lee, Y. K.; Kim, S.; Oh, J.-W.; Nam, J.-M., Massively Parallel and Highly Quantitative Single-Particle Analysis on Interactions between Nanoparticles on Supported Lipid Bilayer. *J. Am. Chem. Soc.* **2014**, *136*, 4081-4088.
- (33) Ristanović, Z.; Kerssens, M. M.; Kubarev, A. V.; Hendriks, F. C.; Dedecker, P.; Hofkens, J.; Roeffaers, M. B. J.; Weckhuysen, B. M., High-Resolution Single-Molecule Fluorescence Imaging of Zeolite Aggregates within Real-Life Fluid Catalytic Cracking Particles. *Angew. Chem. Int. Ed.* **2015**, *54*, 1836-1840.
- (34) Yamashita, S.-i.; Hamada, M.; Nakanishi, S.; Saito, H.; Nosaka, Y.; Wakida, S.-i.; Biju, V., Auger Ionization Beats Photo-Oxidation of Semiconductor Quantum Dots: Extended Stability of Single-Molecule Photoluminescence. *Angew. Chem. Int. Ed.* **2015**, *54*, 3892-3896.
- (35) Huang, B.; Bates, M.; Zhuang, X., Super-Resolution Fluorescence Microscopy. *Annu. Rev. Biochem.* **2009**, *78*, 993-1016.
- (36) Hu, Y. S.; Zimmerley, M.; Li, Y.; Watters, R.; Cang, H., Single-Molecule Super-Resolution Light-Sheet Microscopy. *ChemPhysChem* **2014**, *15*, 577-586.
- (37) Moerner, W. E.; Kador, L., Optical Detection and Spectroscopy of Single Molecules in a Solid. *Phys. Rev. Lett.* **1989**, *62*, 2535-2538.
- (38) Yildiz, A.; Forkey, J. N.; McKinney, S. A.; Ha, T.; Goldman, Y. E.; Selvin, P. R., Myosin V Walks Hand-over-Hand: Single Fluorophore Imaging with 1.5-nm Localization. *Science* **2003**, *300*, 2061-2065.
- (39) Snyder, G. E.; Sakamoto, T.; Hammer III, J. A.; Sellers, J. R.; Selvin, P. R., Nanometer Localization of Single Green Fluorescent Proteins: Evidence That Myosin V Walks Hand-over-Hand Via Telemark Configuration. *Biophys. J.* **2004**, *87*, 1776-1783.
- (40) Stratmann, S. A.; van Oijen, A. M., DNA Replication at the Single-Molecule Level. *Chem. Soc. Rev.* **2014**, *43*, 1201-1220.
- (41) Nie, S.; Zare, R. N., Optical Detection of Single Molecules. *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 567-596.
- (42) Xie, X. S.; Trautman, J. K., Optical Studies of Single Molecules at Room Temperature. *Annu. Rev. Phys. Chem.* **1998**, *49*, 441-480.
- (43) Moerner, W. E.; Orrit, M., Illuminating Single Molecules in Condensed Matter. *Science* **1999**, *283*, 1670-1676.
- (44) Zander, C.; Enderlein, J.; Keller, R. A., *Single Molecule Detection in Solution: Methods and Applications*. Wiley-VCH Berlin, 2002; p 363.

- (45) Weiss, S., Fluorescence Spectroscopy of Single Biomolecules. *Science* **1999**, 283, 1676-1683.
- (46) Zhuang, X.; Bartley, L. E.; Babcock, H. P.; Russell, R.; Ha, T.; Herschlag, D.; Chu, S., A Single-Molecule Study of Rna Catalysis and Folding. *Science* **2000**, 288, 2048-2051.
- (47) Boukobza, E.; Sonnenfeld, A.; Haran, G., Immobilization in Surface-Tethered Lipid Vesicles as a New Tool for Single Biomolecule Spectroscopy. *J. Phys. Chem. B* **2001**, 105, 12165-12170.
- (48) Borisenko, V.; Loughheed, T.; Hesse, J.; Füreder-Kitzmüller, E.; Fertig, N.; Behrends, J. C.; Woolley, G. A.; Schütz, G. J., Simultaneous Optical and Electrical Recording of Single Gramicidin Channels. *Biophys. J.* **2003**, 84, 612-622.
- (49) Rhoades, E.; Gussakovsky, E.; Haran, G., Watching Proteins Fold One Molecule at a Time. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, 100, 3197-3202.
- (50) Cosa, G.; Harbron, E. J.; Zeng, Y.; Liu, H.-W.; O'Connor, D. B.; Eta-Hosokawa, C.; Musier-Forsyth, K.; Barbara, P. F., Secondary Structure and Secondary Structure Dynamics of DNA Hairpins Complexed with HIV-1 NC Protein. *Biophys. J.* **2004**, 87, 2759-2767.
- (51) Liu, H.-W.; Cosa, G.; Landes, C. F.; Zeng, Y.; Kovaleski, B. J.; Mullen, D. G.; Barany, G.; Musier-Forsyth, K.; Barbara, P. F., Single-Molecule FRET Studies of Important Intermediates in the Nucleocapsid-Protein-Chaperoned Minus-Strand Transfer Step in HIV-1 Reverse Transcription. *Biophys. J.* **2005**, 89, 3470-3479.
- (52) Lee, Y. K.; Kim, S.; Oh, J.-W.; Nam, J.-M., Massively Parallel and Highly Quantitative Single-Particle Analysis on Interactions between Nanoparticles on Supported Lipid Bilayer. *J. Am. Chem. Soc.* **2014**.
- (53) Tian, Y.; Kuzimenkova, M. V.; Xie, M.; Meyer, M.; Larsson, P.-O.; Scheblykin, I. G., Watching Two Conjugated Polymer Chains Breaking Each Other When Colliding in Solution. *NPG Asia Mater* **2014**, 6, e134.
- (54) Kulzer, F.; Xia, T.; Orrit, M., Single Molecules as Optical Nanoprobes for Soft and Complex Matter. *Angew. Chem. Int. Ed.* **2010**, 49, 854 - 866.
- (55) Roeffaers, M. B. J.; De Cremer, G.; Uji-i, H.; Muls, B.; Sels, B. F.; Jacobs, P. A.; De Schryver, F. C.; De Vos, D. E.; Hofkens, J., Single-Molecule Fluorescence Spectroscopy in (Bio)Catalysis. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, 104, 12603-12609.
- (56) Chen, P.; Zhou, X.; Andoy, N. M.; Han, K.-S.; Choudhary, E.; Zou, N.; Chen, G.; Shen, H., Spatiotemporal Catalytic Dynamics within Single Nanocatalysts Revealed by Single-Molecule Microscopy. *Chem. Soc. Rev.* **2014**, 43, 1107-1117.
- (57) Joo, C.; Balci, H.; Ishitsuka, Y.; Buranachai, C.; Ha, T., Advances in Single-Molecule Fluorescence Methods for Molecular Biology. *Annu. Rev. Biochem* **2008**, 77, 51-76.
- (58) Cordes, T.; Blum, S. A., Opportunities and Challenges in Single-Molecule and Single-Particle Fluorescence Microscopy for Mechanistic Studies of Chemical Reactions. *Nat. Chem.* **2013**, 5, 993-999.
- (59) Blum, S. A., Location Change Method for Imaging Chemical Reactivity and Catalysis with Single-Molecule and -Particle Fluorescence Microscopy. *PCCP* **2014**, 16, 16333-16339.
- (60) Weidtkamp-Peters, S.; Felekyan, S.; Bleckmann, A.; Simon, R.; Becker, W.; Kuhnemuth, R.; Seidel, C. A. M., Multiparameter Fluorescence Image Spectroscopy to Study Molecular Interactions. *Photochem. Photobiol. Sci.* **2009**, 8, 470-480.
- (61) Jung, G.; Schmitt, A.; Jacob, M.; Hinkeldey, B., Fluorescent Probes for Chemical Transformations on the Single-Molecule Level. *Ann. N.Y. Acad. Sci.* **2008**, 1130, 131-137.

- (62) Friguet, B.; Chaffotte, A. F.; Djavadi-Ohanian, L.; Goldberg, M. E., Measurements of the True Affinity Constant in Solution of Antigen-Antibody Complexes by Enzyme-Linked Immunosorbent Assay. *J. Immunol. Methods* **1985**, *77*, 305-319.
- (63) Yatsimirsky, A. K.; Mirsky, V. M., Quantitative Affinity Data on Selected Artificial Receptors. In *Artificial Receptors for Chemical Sensors*, Wiley-VCH Verlag GmbH & Co. KGaA: 2010; pp 439-460.
- (64) Ha, T.; Tinnefeld, P., Photophysics of Fluorescent Probes for Single-Molecule Biophysics and Super-Resolution Imaging. *Annu. Rev. Phys. Chem.* **2012**, *63*, 595-617.
- (65) Chen, P.; Zhou, X.; Shen, H.; Andoy, N. M.; Choudhary, E.; Han, K.-S.; Liu, G.; Meng, W., Single-Molecule Fluorescence Imaging of Nanocatalytic Processes. *Chem. Soc. Rev.* **2010**, *39*, 4560-4570.
- (66) Roeffaers, M. B. J.; Sels, B. F.; Uji-i, H.; De Schryver, F. C.; Jacobs, P. A.; De Vos, D. E.; Hofkens, J., Spatially Resolved Observation of Crystal-Face-Dependent Catalysis by Single Turnover Counting. *Nature* **2006**, *439*, 572-575.
- (67) Ameloot, R.; Roeffaers, M.; Baruah, M.; De Cremer, G.; Sels, B.; De Vos, D.; Hofkens, J., Towards Direct Monitoring of Discrete Events in a Catalytic Cycle at the Single Molecule Level. *Photochem. Photobiol. Sci.* **2009**, *8*, 453-456.
- (68) Esfandiari, N. M.; Blum, S. A., Homogeneous Vs Heterogeneous Polymerization Catalysis Revealed by Single-Particle Fluorescence Microscopy. *J. Am. Chem. Soc.* **2011**, *133*, 18145-18147.
- (69) Decan, M. R.; Impellizzeri, S.; Marin, M. L.; Scaiano, J. C., Copper Nanoparticle Heterogeneous Catalytic 'Click' Cycloaddition Confirmed by Single-Molecule Spectroscopy. *Nat Commun* **2014**, *5*.
- (70) Rybina, A.; Lang, C.; Wirtz, M.; Grubmayer, K.; Kurz, A.; Maier, F.; Schmitt, A.; Trapp, O.; Jung, G.; Herten, D.-P., Distinguishing Alternative Reaction Pathways by Single-Molecule Fluorescence Spectroscopy. *Angew. Chem. Int. Ed.* **2013**, *52*, 6322-6325.
- (71) Shen, H.; Zhou, X.; Zou, N.; Chen, P., Single-Molecule Kinetics Reveals a Hidden Surface Reaction Intermediate in Single-Nanoparticle Catalysis. *J. Phys. Chem. C* **2014**, *118*, 26902-26911.
- (72) Liu, K.-L.; Kubarev, A. V.; Van Loon, J.; Uji-i, H.; De Vos, D. E.; Hofkens, J.; Roeffaers, M. B. J., Rationalizing Inter- and Intracrystal Heterogeneities in Dealuminated Acid Mordenite Zeolites by Stimulated Raman Scattering Microscopy Correlated with Super-Resolution Fluorescence Microscopy. *ACS Nano* **2014**, *8*, 12650-12659.
- (73) Kiel, A.; Kovacs, J.; Mokhir, A.; Krämer, R.; Herten, D.-P., Direct Monitoring of Formation and Dissociation of Individual Metal Complexes by Single-Molecule Fluorescence Spectroscopy. *Angew. Chem. Int. Ed.* **2007**, *46*, 3363-3366.
- (74) Esfandiari, N. M.; Wang, Y.; Bass, J. Y.; Cornell, T. P.; Otte, D. A. L.; Cheng, M. H.; Hemminger, J. C.; McIntire, T. M.; Mandelshtam, V. A.; Blum, S. A., Single-Molecule Imaging of Platinum Ligand Exchange Reaction Reveals Reactivity Distribution. *J. Am. Chem. Soc.* **2010**, *132*, 15167-15169.
- (75) Esfandiari, N. M.; Wang, Y.; Bass, J. Y.; Blum, S. A., Deconvoluting Subensemble Chemical Reaction Kinetics of Platinum-Sulfur Ligand Exchange Detected with Single-Molecule Fluorescence Microscopy. *Inorg. Chem.* **2011**, *50*, 9201-9203.
- (76) Esfandiari, N. M.; Wang, Y.; McIntire, T. M.; Blum, S. A., Real-Time Imaging of Platinum-Sulfur Ligand Exchange Reactions at the Single-Molecule Level Via a General Chemical Technique. *Organometallics* **2011**, *30*, 2901-2907.

- (77) Goldsmith, R. H.; Tabares, L. C.; Kostrz, D.; Dennison, C.; Aartsma, T. J.; Canters, G. W.; Moerner, W. E., Redox Cycling and Kinetic Analysis of Single Molecules of Solution-Phase Nitrite Reductase. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 17269-17274.
- (78) Tabares, L. C.; Kostrz, D.; Elmalk, A.; Andreoni, A.; Dennison, C.; Aartsma, T. J.; Canters, G. W., Fluorescence Lifetime Analysis of Nitrite Reductase from *Alcaligenes xylosoxidans* at the Single-Molecule Level Reveals the Enzyme Mechanism. *Chem. Eur. J.* **2011**, *17*, 12015-12019.
- (79) Kuznetsova, S.; Zauner, G.; Aartsma, T. J.; Engelkamp, H.; Hatzakis, N.; Rowan, A. E.; Nolte, R. J. M.; Christianen, P. C. M.; Canters, G. W., The Enzyme Mechanism of Nitrite Reductase Studied at Single-Molecule Level. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 3250-3255.
- (80) Rybina, A.; Thaler, B.; Kramer, R.; Herten, D. P., Monitoring Hydroquinone-Quinone Redox Cycling by Single Molecule Fluorescence Spectroscopy. *PCCP* **2014**, *16*, 19550-19555.
- (81) Tachikawa, T.; Majima, T., Single-Molecule Detection of Reactive Oxygen Species: Application to Photocatalytic Reactions. *J. Fluoresc.* **2007**, *17*, 727-738.
- (82) Tachikawa, T.; Majima, T., Single-Molecule Fluorescence Imaging of TiO₂ Photocatalytic Reactions. *Langmuir* **2009**, *25*, 7791-7802.
- (83) Naito, K.; Tachikawa, T.; Fujitsuka, M.; Majima, T., Single-Molecule Fluorescence Imaging of the Remote TiO₂ Photocatalytic Oxidation. *J. Phys. Chem. B* **2005**, *109*, 23138-23140.
- (84) Naito, K.; Tachikawa, T.; Cui, S.-C.; Sugimoto, A.; Fujitsuka, M.; Majima, T., Single-Molecule Detection of Airborne Singlet Oxygen. *J. Am. Chem. Soc.* **2006**, *128*, 16430-16431.
- (85) Naito, K.; Tachikawa, T.; Fujitsuka, M.; Majima, T., Real-Time Single-Molecule Imaging of the Spatial and Temporal Distribution of Reactive Oxygen Species with Fluorescent Probes: Applications to TiO₂ Photocatalysts. *J. Phys. Chem. C* **2008**, *112*, 1048-1059.
- (86) Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*. 3rd ed.; Springer US: 2006.
- (87) Kasha, M., Collisional Perturbation of Spin-Orbital Coupling and the Mechanism of Fluorescence Quenching. A Visual Demonstration of the Perturbation. *J. Chem. Phys.* **1952**, *20*, 71-74.
- (88) El-Sayed, M. A., Phosphorescence Microwave Multiple Resonance Studies in Determining the Radiative and Nonradiative Properties of the Triplet State. *Acc. Chem. Res.* **1971**, *4*, 23-31.
- (89) Strickler, S. J.; Berg, R. A., Relationship between Absorption Intensity and Fluorescence Lifetime of Molecules. *J. Chem. Phys.* **1962**, *37*, 814-822.
- (90) Kautsky, H., Quenching of Luminescence by Oxygen. *Trans. Faraday Soc.* **1939**, *35*, 216-219.
- (91) Kikuchi, K.; Sato, C.; Watabe, M.; Ikeda, H.; Takahashi, Y.; Miyashi, T., New Aspects of Fluorescence Quenching by Molecular Oxygen. *J. Am. Chem. Soc.* **1993**, *115*, 5180-5184.
- (92) Dröse, S.; Brandt, U., Molecular Mechanisms of Superoxide Production by the Mitochondrial Respiratory Chain. In *Mitochondrial Oxidative Phosphorylation*, Kadenbach, B., Ed. Springer New York: 2012; Vol. 748, pp 145-169.
- (93) Roy, R.; Hohng, S.; Ha, T., A Practical Guide to Single-Molecule FRET. *Nat Methods* **2008**, *5*, 507-516.
- (94) Swoboda, M.; Henig, J.; Cheng, H.-M.; Brugger, D.; Haltrich, D.; Plumeré, N.; Schlierf, M., Enzymatic Oxygen Scavenging for Photostability without pH Drop in Single-Molecule Experiments. *ACS Nano* **2012**, *6*, 6364-6369.
- (95) Aitken, C. E.; Marshall, R. A.; Puglisi, J. D., An Oxygen Scavenging System for Improvement of Dye Stability in Single-Molecule Fluorescence Experiments. *Biophys. J.* **2008**, *94*, 1826-1835.

- (96) Rasnik, I.; McKinney, S. A.; Ha, T., Nonblinking and Long-Lasting Single-Molecule Fluorescence Imaging. *Nat Meth* **2006**, *3*, 891-893.
- (97) Holzmeister, P.; Gietl, A.; Tinnefeld, P., Geminate Recombination as a Photoprotection Mechanism for Fluorescent Dyes. *Angew. Chem. Int. Ed.* **2014**, *53*, 5685-5688
- (98) Vogelsang, J.; Kasper, R.; Steinhauer, C.; Person, B.; Heilemann, M.; Sauer, M.; Tinnefeld, P., A Reducing and Oxidizing System Minimizes Photobleaching and Blinking of Fluorescent Dyes. *Angew. Chem. Int. Ed.* **2008**, *47*, 5465-5469.
- (99) Turro, N. J.; Ramamurthy, V.; Scaiano, J. C., *Principles of Molecular Photochemistry: An Introduction*; University Science Books: Sausalito, Calif., 2009.
- (100) Rehm, D.; Weller, A., Kinetics of Fluorescence Quenching by Electron and H-Atom Transfer. *Isr. J. Chem.* **1970**, *8*, 259-271.
- (101) IUPAC, *Compendium of Chemical Terminology*, 2nd ed.; Blackwell Scientific Publications: Oxford 1997. On-line corrected version: <http://goldbook.iupac.org> (2006-) created by M. Nic, J. Jirat, B. Kosata; updates compiled by A. Jenkins. ISBN 0-9678550-9-8. doi:10.1351/goldbook.
- (102) Marcus, R. A., On the Theory of Oxidation-Reduction Reactions Involving Electron Transfer. I. *J. Chem. Phys.* **1956**, *24*, 966-978.
- (103) Marcus, R. A., Electron Transfer Theory and Its Inception. *PCCP* **2012**, *14*, 13729-13730.
- (104) Kavarnos, G. J., *Fundamentals of Photoinduced Electron Transfer*; VCH Publishers: New York, 1993.
- (105) The Nobel Prize in Chemistry 1992. http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1992/ (accessed Web. 3 Apr 2015.).
- (106) Sukegawa, J.; Schubert, C.; Zhu, X.; Tsuji, H.; Guldi, D. M.; Nakamura, E., Electron Transfer through Rigid Organic Molecular Wires Enhanced by Electronic and Electron-Vibration Coupling. *Nat. Chem.* **2014**, *6*, 899-905.
- (107) Gao, Y. Q.; Marcus, R. A., On the Theory of Electron Transfer Reactions at Semiconductor/Liquid Interfaces. II. A Free Electron Model. *J. Chem. Phys.* **2000**, *113*, 6351-6360.
- (108) Prezhdo, O. V.; Duncan, W. R.; Prezhdo, V. V., Dynamics of the Photoexcited Electron at the Chromophore-Semiconductor Interface. *Acc. Chem. Res.* **2008**, *41*, 339-348.
- (109) Ardo, S.; Meyer, G. J., Photodriven Heterogeneous Charge Transfer with Transition-Metal Compounds Anchored to TiO₂ Semiconductor Surfaces. *Chem. Soc. Rev.* **2009**, *38*, 115-164.
- (110) Oregan, B.; Grätzel, M., A Low-Cost, High-Efficiency Solar-Cell Based on Dye-Sensitized Colloidal TiO₂ Films. *Nature* **1991**, *353*, 737-740.
- (111) Hagfeldt, A.; Boschloo, G.; Sun, L.; Kloo, L.; Pettersson, H., Dye-Sensitized Solar Cells. *Chem. Rev.* **2010**, *110*, 6595-6663.
- (112) Yella, A.; Lee, H.-W.; Tsao, H. N.; Yi, C.; Chandiran, A. K.; Nazeeruddin, M. K.; Diau, E. W.-G.; Yeh, C.-Y.; Zakeeruddin, S. M.; Grätzel, M., Porphyrin-Sensitized Solar Cells with Cobalt (II/III)-Based Redox Electrolyte Exceed 12 Percent Efficiency. *Science* **2011**, *334*, 629-634.
- (113) Grätzel, M.; Durrant, J. R., Dye-Sensitized Mesoscopic Solar Cells. In *Nanostructured and Photoelectrochemical Systems for Solar Photon Conversion*, pp 503-536.
- (114) Listorti, A.; O'Regan, B.; Durrant, J. R., Electron Transfer Dynamics in Dye-Sensitized Solar Cells. *Chem. Mater.* **2011**, *23*, 3381-3399.
- (115) Haque, S. A.; Handa, S.; Peter, K.; Palomares, E.; Thelakkat, M.; Durrant, J. R., Supramolecular Control of Charge Transfer in Dye-Sensitized Nanocrystalline TiO₂ Films:

Towards a Quantitative Structure–Function Relationship. *Angew. Chem. Int. Ed.* **2005**, *44*, 5740-5744.

(116) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E., Signaling Recognition Events with Fluorescent Sensors and Switches. *Chem. Rev.* **1997**, *97*, 1515-1566.

(117) Yuan, L.; Lin, W.; Zheng, K.; Zhu, S., FRET-Based Small-Molecule Fluorescent Probes: Rational Design and Bioimaging Applications. *Acc. Chem. Res.* **2013**, *46*, 1462-1473.

(118) Manjare, S. T.; Kim, Y.; Churchill, D. G., Selenium- and Tellurium-Containing Fluorescent Molecular Probes for the Detection of Biologically Important Analytes. *Acc. Chem. Res.* **2014**, *47*, 2985-2998.

(119) Martí, A. A.; Jockusch, S.; Stevens, N.; Ju, J.; Turro, N. J., Fluorescent Hybridization Probes for Sensitive and Selective DNA and RNA Detection. *Acc. Chem. Res.* **2007**, *40*, 402-409.

(120) Mizukami, S.; Hori, Y.; Kikuchi, K., Small-Molecule-Based Protein-Labeling Technology in Live Cell Studies: Probe-Design Concepts and Applications. *Acc. Chem. Res.* **2014**, *47*, 247-256.

(121) Eun Jun, M.; Roy, B.; Han Ahn, K., "Turn-on" Fluorescent Sensing with "Reactive" Probes. *Chem. Commun.* **2011**, *47*, 7583-7601.

(122) Bissell, R.; Prasanna de Silva, A.; Nimal Gunaratne, H. Q.; Mark Lynch, P. L.; Maguire, G. M.; McCoy, C.; Samankumara Sandanayake, K. R. A., Fluorescent PET (Photoinduced Electron Transfer) Sensors. In *Photoinduced Electron Transfer V*, Mattay, J., Ed. Springer Berlin Heidelberg: 1993; Vol. 168, pp 223-264.

(123) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E., *Advances in Supramolecular Chemistry*, 1 ed.; Elsevier: Burlington, 1997; Vol. 4, p 338.

(124) Yin, H.; Xu, L.; Porter, N. A., Free Radical Lipid Peroxidation: Mechanisms and Analysis. *Chem. Rev.* **2011**, *111*, 5944-5972.

(125) Clerici, M. G.; Kholdeeva, O. A., *Liquid Phase Oxidation Via Heterogeneous Catalysis: Organic Synthesis and Industrial Applications*. John Wiley & Sons: Somerset, NJ, USA, 2013; p 546.

(126) Dolmans, D. E. J. G. J.; Fukumura, D.; Jain, R. K., Photodynamic Therapy for Cancer. *Nat. Rev. Cancer* **2003**, *3*, 380-387.

(127) Macia, N.; Heyne, B., Using Photochemistry to Understand and Control the Production of Reactive Oxygen Species in Biological Environments. *J. Photochem. Photobiol. A: Chem.* **2015**, *306*, 1-12.

(128) Gomes, A.; Fernandes, E.; Lima, J. L. F. C., Fluorescence Probes Used for Detection of Reactive Oxygen Species. *J. Biochem. Biophys. Methods* **2005**, *65*, 45-80.

(129) Winterbourn, C. C., The Challenges of Using Fluorescent Probes to Detect and Quantify Specific Reactive Oxygen Species in Living Cells. *Biochim. Biophys. Acta, Gen. Subj.* **2014**, *1840*, 730-738.

(130) Oleynik, P.; Ishihara, Y.; Cosa, G., Design and Synthesis of a Bodipy- α -Tocopherol Adduct for Use as an Off/on Fluorescent Antioxidant Indicator. *J. Am. Chem. Soc.* **2007**, *129*, 1842-1843.

(131) Wei Yao, W.; Mei Peng, H.; Webster, R. D., Electrochemistry of α -Tocopherol (Vitamin E) and α -Tocopherol Quinone Films Deposited on Electrode Surfaces in the Presence and Absence of Lipid Multilayers. *J. Phys. Chem. C* **2009**, *113*, 21805-21814.

- (132) Krumova, K.; Friedland, S.; Cosa, G., How Lipid Unsaturation, Peroxyl Radical Partitioning, and Chromanol Lipophilic Tail Affect the Antioxidant Activity of α -Tocopherol: Direct Visualization Via High-Throughput Fluorescence Studies Conducted with Fluorogenic α -Tocopherol Analogues. *J. Am. Chem. Soc.* **2012**, *134*, 10102-10113.
- (133) Krumova, K.; Cosa, G., Bodipy Dyes with Tunable Redox Potentials and Functional Groups for Further Tethering: Preparation, Electrochemical, and Spectroscopic Characterization. *J. Am. Chem. Soc.* **2010**, *132*, 17560-17569.
- (134) Esterbauer, H., Cytotoxicity and Genotoxicity of Lipid-Oxidation Products. *Am. J. Clin. Nutr.* **1993**, *57*, 779S-785S.
- (135) Chatgililoglu, C.; Ferreri, C.; Ballestri, M.; Mulazzani, Q. G.; Landi, L., Cis-Trans Isomerization of Monounsaturated Fatty Acid Residues in Phospholipids by Thiyl Radicals. *J. Am. Chem. Soc.* **2000**, *122*, 4593-4601.
- (136) Chatgililoglu, C.; Ferreri, C., Trans Lipids: The Free Radical Path. *Acc. Chem. Res.* **2005**, *38*, 441-448.
- (137) Finkel, T.; Holbrook, N. J., Oxidants, Oxidative Stress and the Biology of Ageing. *Nature* **2000**, *408*, 239-247.
- (138) Uchida, K., 4-Hydroxy-2-Nonenal: A Product and Mediator of Oxidative Stress. *Prog. Lipid Res.* **2003**, *42*, 318-343.
- (139) Dianzani, M. U., Lipid Peroxidation and Cancer. *Crit. Rev. Oncol./Hematol.* **1993**, *15*, 125-147.
- (140) Wu, R. P.; Hayashi, T.; Cottam, H. B.; Jin, G.; Yao, S.; Wu, C. C. N.; Rosenbach, M. D.; Corr, M.; Schwab, R. B.; Carson, D. A., NRF2 Responses and the Therapeutic Selectivity of Electrophilic Compounds in Chronic Lymphocytic Leukemia. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 7479-7484.
- (141) Poli, G.; Biasi, F.; Leonarduzzi, G., 4-Hydroxynonenal-Protein Adducts: A Reliable Biomarker of Lipid Oxidation in Liver Diseases. *Molecular Aspects of Medicine* **2008**, *29*, 67-71.
- (142) Simonian, N. A.; Coyle, J. T., Oxidative Stress in Neurodegenerative Diseases. *Annu. Rev. Pharmacol. Toxicol.* **1996**, *36*, 83-106.
- (143) Barnham, K. J.; Masters, C. L.; Bush, A. I., Neurodegenerative Diseases and Oxidative Stress. *Nat. Rev. Drug Discov.* **2004**, *3*, 205-214.
- (144) Lin, M. T.; Beal, M. F., Mitochondrial Dysfunction and Oxidative Stress in Neurodegenerative Diseases. *Nature* **2006**, *443*, 787-795.
- (145) Sen, C. K.; Packer, L., Antioxidant and Redox Regulation of Gene Transcription. *FASEB J.* **1996**, *10*, 709-20.
- (146) Dröge, W., *Free Radicals in the Physiological Control of Cell Function*, 2002; Vol. 82, p 47-95.
- (147) Apel, K.; Hirt, H., Reactive Oxygen Species: Metabolism, Oxidative Stress, and Signal Transduction. *Annu. Rev. Plant Biol.* **2004**, *55*, 373-399.
- (148) Mittler, R.; Vanderauwera, S.; Gollery, M.; Van Breusegem, F., Reactive Oxygen Gene Network of Plants. *Trends Plant Sci.* **2004**, *9*, 490-498.
- (149) Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T. D.; Mazur, M.; Telser, J., Free Radicals and Antioxidants in Normal Physiological Functions and Human Disease. *Intl. J. Biochem. Cell Biol.* **2007**, *39*, 44-84.
- (150) Noguchi, N., Role of Oxidative Stress in Adaptive Responses in Special Reference to Atherogenesis. *J. Clin. Biochem. Nutr.* **2008**, *43*, 131-138.

- (151) Poli, G.; Schaur, R. J.; Siems, W. G.; Leonarduzzi, G., 4-Hydroxynonenal: A Membrane Lipid Oxidation Product of Medicinal Interest. *Med. Res. Rev.* **2008**, *28*, 569-631.
- (152) Niki, E., Lipid Peroxidation: Physiological Levels and Dual Biological Effects. *Free Radical Biol. Med.* **2009**, *47*, 469-484.
- (153) Kagan, V. E.; Quinn, P. J., Toward Oxidative Lipidomics of Cell Signaling. *Antioxid. Redox Signaling* **2004**, *6*, 199-202.
- (154) Ingold, K. U., Peroxy Radicals. *Acc. Chem. Res.* **1969**, *2*, 1-9.
- (155) Hasegawa, K.; Patterson, L. K., Pulse Radiolysis Studies in Model Lipid Systems: Formation and Behavior of Peroxy Radicals in Fatty Acids. *Photochem. Photobiol.* **1978**, *28*, 817-823.
- (156) Niki, E.; Noguchi, N., Dynamics of Antioxidant Action of Vitamin E. *Acc. Chem. Res.* **2004**, *37*, 45-51.
- (157) Burton, G. W.; Ingold, K. U., Vitamin E: Application of the Principles of Physical Organic Chemistry to the Exploration of Its Structure and Function. *Acc. Chem. Res.* **1986**, *19*, 194-201.
- (158) Jonsson, M.; Lind, J.; Reitberger, T.; Eriksen, T. E.; Merenyi, G., Free Radical Combination Reactions Involving Phenoxyl Radicals. *J. Phys. Chem.* **1993**, *97*, 8229-8233.
- (159) Pratt, D. A.; Mills, J. H.; Porter, N. A., Theoretical Calculations of Carbon–Oxygen Bond Dissociation Enthalpies of Peroxyl Radicals Formed in the Autoxidation of Lipids. *J. Am. Chem. Soc.* **2003**, *125*, 5801-5810.
- (160) Porter, N. A.; Mills, K. A.; Carter, R. L., A Mechanistic Study of Oleate Autoxidation: Competing Peroxyl H-Atom Abstraction and Rearrangement. *J. Am. Chem. Soc.* **1994**, *116*, 6690-6696.
- (161) Korcek, S.; Chenier, J. H. B.; Howard, J. A.; Ingold, K. U., Absolute Rate Constants for Hydrocarbon Autoxidation. XXI. Activation Energies for Propagation and the Correlation of Propagation Rate Constants with Carbon–Hydrogen Bond Strengths. *Can. J. Chem.* **1972**, *50*, 2285-2297.
- (162) Blanksby, S. J.; Ellison, G. B., Bond Dissociation Energies of Organic Molecules. *Acc. Chem. Res.* **2003**, *36*, 255-263.
- (163) Niki, E., [3] Free Radical Initiators as Source of Water- or Lipid-Soluble Peroxyl Radicals. In *Methods Enzymol.*, Lester Packer, A. N. G., Ed. Academic Press: 1990; Vol. Volume 186, pp 100-108.
- (164) Chiefari, J., et al., Living Free-Radical Polymerization by Reversible Addition–Fragmentation Chain Transfer: The Raft Process. *Macromolecules* **1998**, *31*, 5559-5562.
- (165) Moad, G.; Solomon, D. H., 3 - Initiation. In *The Chemistry of Radical Polymerization (Second Edition)*, Solomon, G. M. H., Ed. Elsevier Science Ltd: Amsterdam, 2005; pp 49-166.
- (166) Smith, M. B.; March, J., *March's Advanced Organic Chemistry : Reactions, Mechanisms, and Structure (7th Edition)*; John Wiley & Sons: Somerset, NJ, USA, 2013.
- (167) Maillard, B.; Ingold, K. U.; Scaiano, J. C., Rate Constants for the Reactions of Free Radicals with Oxygen in Solution. *J. Am. Chem. Soc.* **1983**, *105*, 5095-5099.
- (168) Williams, G. H., In *Advances in Free Radical Chemistry*, Logos Press Ltd.: London, 1972; Vol. IV, p 49.
- (169) Zhang, H.-Y.; Sun, Y.-M.; Wang, X.-L., Substituent Effects on O-H Bond Dissociation Enthalpies and Ionization Potentials of Catechols: A Dft Study and Its Implications in the Rational Design of Phenolic Antioxidants and Elucidation of Structure–Activity Relationships for Flavonoid Antioxidants. *Chem. Eur. J.* **2003**, *9*, 502-508.

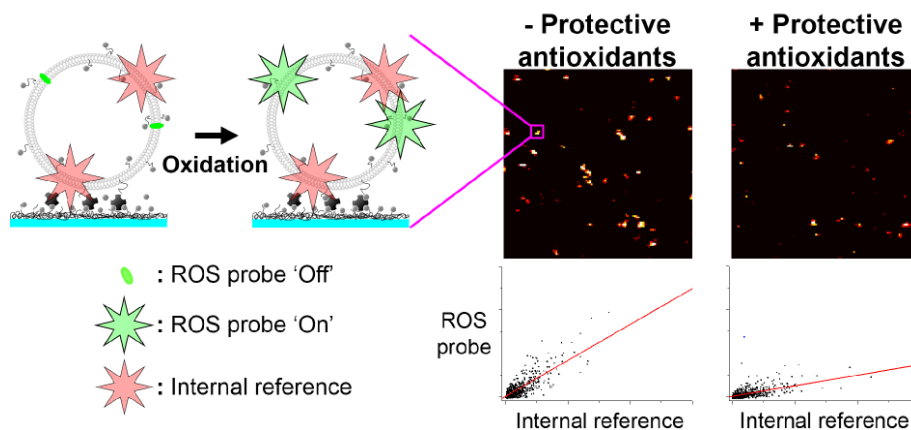
- (170) Mulder, P.; Korth, H.-G.; Ingold, K. U., Why Quantum-Thermochemical Calculations Must Be Used with Caution to Indicate 'a Promising Lead Antioxidant'. *Helv. Chim. Acta* **2005**, *88*, 370-374.
- (171) Korth, H.-G., Carbon Radicals of Low Reactivity against Oxygen: Radically Different Antioxidants. *Angew. Chem. Int. Ed.* **2007**, *46*, 5274-5276.
- (172) Burton, G. W.; Doba, T.; Gabe, E.; Hughes, L.; Lee, F. L.; Prasad, L.; Ingold, K. U., Autoxidation of Biological Molecules. 4. Maximizing the Antioxidant Activity of Phenols. *J. Am. Chem. Soc.* **1985**, *107*, 7053-7065.
- (173) Sano, M., et al., A Controlled Trial of Selegiline, Alpha-Tocopherol, or Both as Treatment for Alzheimer's Disease. *New Engl. J. Med.* **1997**, *336*, 1216-1222.
- (174) Singh, U.; Devaraj, S.; Jialal, I., Vitamin E, Oxidative Stress, and Inflammation. *Annu. Rev. Nutr.* **2005**, *25*, 151-174.
- (175) Boscoboinik, D.; Szewczyk, A.; Hensey, C.; Azzi, A., Inhibition of Cell Proliferation by Alpha-Tocopherol. Role of Protein Kinase C. *J. Biol. Chem.* **1991**, *266*, 6188-6194.
- (176) Miller, I. I. E. R.; Pastor-Barriuso, R.; Dalal, D.; Riemersma, R. A.; Appel, L. J.; Guallar, E., Meta-Analysis: High-Dosage Vitamin E Supplementation May Increase All-Cause Mortality. *Ann. Intern. Med.* **2005**, *142*, 37-46.
- (177) Bowry, V. W.; Ingold, K. U.; Stocker, R., Vitamin-E in Human Low-Density-Lipoprotein - When and How This Antioxidant Becomes a Prooxidant. *Biochem. J* **1992**, *288*, 341-344.
- (178) Bowry, V. W.; Stocker, R., Tocopherol-Mediated Peroxidation. The Prooxidant Effect of Vitamin E on the Radical-Initiated Oxidation of Human Low-Density Lipoprotein. *J. Am. Chem. Soc.* **1993**, *115*, 6029-6044.
- (179) Palacios, R. E.; Fan, F.-R. F.; Bard, A. J.; Barbara, P. F., Single-Molecule Spectroelectrochemistry (SMS-EC). *J. Am. Chem. Soc.* **2006**, *128*, 9028-9029.
- (180) Palacios, R. E.; Fan, F.-R. F.; Grey, J. K.; Suk, J.; Bard, A. J.; Barbara, P. F., Charging and Discharging of Single Conjugated-Polymer Nanoparticles. *Nat. Mater.* **2007**, *6*, 680-685.
- (181) Palacios, R. E.; Chang, W.-S.; Grey, J. K.; Chang, Y.-L.; Miller, W. L.; Lu, C.-Y.; Henkelman, G.; Zepeda, D.; Ferraris, J.; Barbara, P. F., Detailed Single-Molecule Spectroelectrochemical Studies of the Oxidation of Conjugated Polymers. *J. Phys. Chem. B* **2009**, *113*, 14619-14628.
- (182) Audebert, P.; Miomandre, F., Electrofluorochromism: From Molecular Systems to Set-up and Display. *Chem. Sci.* **2013**, *4*, 575-584.
- (183) Mathwig, K.; Aartsma, T. J.; Canters, G. W.; Lemay, S. G., Nanoscale Methods for Single-Molecule Electrochemistry. *Annu. Rev. Anal. Chem.* **2014**, *7*, 383-404.
- (184) Lei, C.; Hu, D.; Ackerman, E. J., Single-Molecule Fluorescence Spectroelectrochemistry of Cresyl Violet. *Chem. Commun.* **2008**, 5490-5492.
- (185) Lei, C.; Hu, D.; Ackerman, E., Clay Nanoparticle-Supported Single-Molecule Fluorescence Spectroelectrochemistry. *Nano Lett.* **2009**, *9*, 655-658.
- (186) Zhao, J.; Zaino Iii, L. P.; Bohn, P. W., Potential-Dependent Single Molecule Blinking Dynamics for Flavin Adenine Dinucleotide Covalently Immobilized in Zero-Mode Waveguide Array of Working Electrodes. *Faraday Discuss.* **2013**, *164*, 57-69.
- (187) Levene, M. J.; Korlach, J.; Turner, S. W.; Foquet, M.; Craighead, H. G.; Webb, W. W., Zero-Mode Waveguides for Single-Molecule Analysis at High Concentrations. *Science* **2003**, *299*, 682-686.

- (188) Zhang, G.; Xiao, L.; Chen, R.; Gao, Y.; Wang, X.; Jia, S., Single-Molecule Interfacial Electron Transfer Dynamics Manipulated by an External Electric Current. *PCCP* **2011**, *13*, 13815-13820.
- (189) Xu, W.; Shen, H.; Kim, Y. J.; Zhou, X.; Liu, G.; Park, J.; Chen, P., Single-Molecule Electrocatalysis by Single-Walled Carbon Nanotubes. *Nano Lett.* **2009**, *9*, 3968-3973.
- (190) Shen, H.; Xu, W.; Chen, P., Single-Molecule Nanoscale Electrocatalysis. *PCCP* **2010**, *12*, 6555-6563.
- (191) Gesquiere, A. J.; Park, S.-J.; Barbara, P. F., F-V/Sms: A New Technique for Studying the Structure and Dynamics of Single Molecules and Nanoparticles. *J. Phys. Chem. B* **2004**, *108*, 10301-10308.
- (192) Park, S.-J.; Gesquiere, A. J.; Yu, J.; Barbara, P. F., Charge Injection and Photooxidation of Single Conjugated Polymer Molecules. *J. Am. Chem. Soc.* **2004**, *126*, 4116-4117.
- (193) Reeja-Jayan, B.; Adachi, T.; Ono, R. J.; Vanden Bout, D. A.; Bielawski, C. W.; Manthiram, A., Effect of Interfacial Dipoles on Charge Traps in Organic-Inorganic Hybrid Solar Cells. *J. Mater. Chem. A Mater. Energy Sustain.* **2013**, *1*, 3258-3262.
- (194) Reeja-Jayan, B.; Koen, K. A.; Ono, R. J.; Vanden Bout, D. A.; Bielawski, C. W.; Manthiram, A., Oligomeric Interface Modifiers in Hybrid Polymer Solar Cell Prototypes Investigated by Fluorescence Voltage Spectroscopy. *PCCP* **2015**, *17*, 10640-10647.
- (195) Bolinger, J. C.; Fradkin, L.; Lee, K.-J.; Palacios, R. E.; Barbara, P. F., Light-Assisted Deep-Trapping of Holes in Conjugated Polymers. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 1342-1346.
- (196) Bolinger, J. C.; Traub, M. C.; Adachi, T.; Barbara, P. F., Ultralong-Range Polaron-Induced Quenching of Excitons in Isolated Conjugated Polymers. *Science* **2011**, *331*, 565-567.
- (197) Michael Green, N., [5] Avidin and Streptavidin. In *Methods Enzymol.*, Meir, W.; Edward, A. B., Eds. Academic Press: 1990; Vol. Volume 184, pp 51-67.
- (198) Haustein, E.; Schwille, P., Single-Molecule Spectroscopic Methods. *Curr. Opin. Struct. Biol.* **2004**, *14*, 531-540.
- (199) Gao, Y.; Zhong, Z.; Geng, L. M., Calibration of Probe Volume in Fluorescence Correlation Spectroscopy. *Appl. Spectrosc.* **2007**, *61*, 956-962.
- (200) Campos, L. A.; Liu, J.; Wang, X.; Ramanathan, R.; English, D. S.; Munoz, V., A Photoprotection Strategy for Microsecond-Resolution Single-Molecule Fluorescence Spectroscopy. *Nat. Methods* **2011**, *8*, 143-146.
- (201) Schindelin, J., et al., Fiji: An Open-Source Platform for Biological-Image Analysis. *Nat. Methods* **2012**, *9*, 676-682.

2. Ambient Condition Oxidation in Individual Liposomes Observed at the Single Molecule Level

Robert Godin, Hsiao-Wei Liu and Gonzalo Cosa, *Chemical Science*, 2014, 5, 2525 – 2529.

Reproduced by permission of The Royal Society of Chemistry



2.1. Preface

Our laboratory has developed fluorogenic antioxidant indicators that rely on deactivation of photoinduced electron transfer (PeT) to provide an off/on molecular switch of fluorescence. These molecules consist of two segments (reporter and trap) and are intrinsic antioxidants that become emissive following oxidation of the α -tocopherol mimic trap segment. In this Chapter, we used our 2nd generation fluorogenic antioxidant (H₂B-PMHC) in order to study heterogeneous oxidation occurring in individual liposomes. Lipid peroxidation was chosen as a well-defined model system in accordance with our interest in developing methods to probe oxidation within the heterogeneous environment of lipid membranes. We demonstrate the use of H₂B-PMHC as a probe to monitor oxidation events that take place within lipid membranes at the SM level. A ratiometric method determined that oxidation varied liposome-to-liposome, which is indicative of heterogeneous processes. While conducting these experiments, we noticed that the initial H₂B-PMHC fluorescence intensity was higher than what would be expected for a pristine, unoxidized H₂B-PMHC. We performed ambient condition experiments where antioxidant additives were added prior to the preparation of liposomes to verify whether oxidation occurred during standard liposome preparation procedure. Subsequent imaging of individual liposomes confirmed that oxidation does indeed occur during liposome preparation. We also found that addition of a water-soluble antioxidant was more effective at preventing the oxidation. Our results suggest that water-soluble antioxidants should be used to inhibit oxidation, a concept important to the food and drug industry where oxidation may lead to decreasing quality or adverse health effects. Finally, we describe the intriguing blinking behaviour of oxidized H₂B-PMHC under laser excitation. While the intermittent fluorescence complicates the assignment of fluorescent bursts to discrete oxidation

events, this behaviour may be exploited in super resolution imaging techniques that rely on stochastic changes in fluorescence intensity.

2.2. Abstract

Single particle imaging studies on liposomes stained with lipophilic, fluorogenic, peroxy radical-trapping probes show oxidation taking place during standard liposome preparation conditions. A ratiometric assay involving the fluorogenic probe enables quantifying oxidative events at the single particle level. The assay provides a general strategy to monitor reactions on individual liposomes.

2.3. Introduction

Free radical-mediated lipid chain autoxidation of polyunsaturated fatty acids¹ (PUFA) is a prevalent chemical process with diverse ramifications given the widespread distribution of lipids in nature and in man-made materials. PUFA peroxidation is associated not only with a range of pathologies²⁻⁴ but also cellular signaling.⁵⁻⁸ It is further directly involved in food quality decay,⁹ and has the potential to impart structural alterations in lipid based products and materials such as biosensors, medical nano-devices for e.g. imaging, and liposome formulations for e.g. personal care or drug delivery.

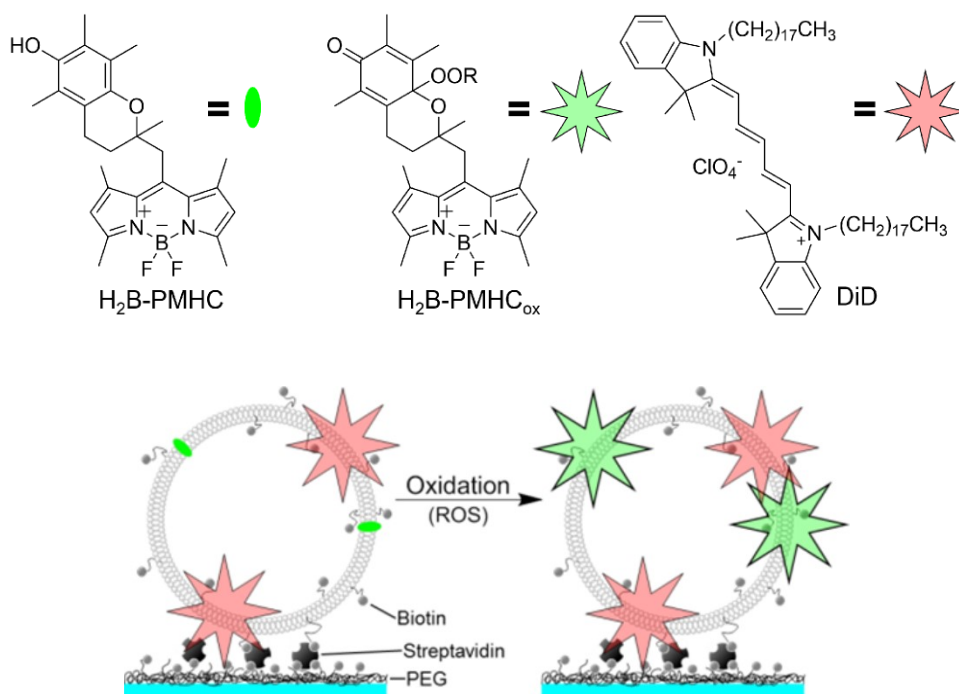
The kinetics of lipid chain autoxidation and the effects of lipophilic antioxidants within the nano-confined boundaries of lipid membranes and lipid nanoparticles has attracted enormous attention over the years.¹ Liposomes have been the system of choice to study PUFA chain autoxidation and to better understand the antioxidant properties of various lipophilic compounds,¹⁰ in particular α -tocopherol, the most active naturally occurring lipid soluble antioxidant.¹¹ Liposomes have been additionally sought after as surrogate models of peroxidation in low density lipoproteins, small ca. 20 nm diameter lipid nanoparticles with a non-polar lipid core and a polar lipid shell.¹²

Here we report our ability to visualize the oxidation of single fluorogenic α -tocopherol analogues (trap-reporter probes that mimic the peroxy radical-scavenging activity of α -tocopherol¹³⁻¹⁸) embedded within the lipid membrane of surface-immobilized liposomes at the single molecule level (see Scheme 2.1). The tremendous sensitivity provided by our single molecule/particle assay shows that oxidative chemical events take place in individual liposomes under ambient conditions in the absence of additives intended as a source of reactive oxygen species (ROS). Our assay underscores the possible occurrence of lipid degradation taking place under standard liposome preparation conditions. It may also provide new approaches to decipher reaction mechanisms in a lipid membrane given its physical-chemical heterogeneity as a reaction medium. In general the assay further provides a simple sensitive way to monitor single chemical reactions¹⁹⁻²¹ at the level of individual liposomes.

2.4. Results and Discussion

Scheme 2.1 illustrates the experimental strategy used. The sample consisted of 100 nm diameter liposomes prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (75,000 DOPC lipids/liposome on average).²² The liposomal membrane contained the fluorogenic α -tocopherol analogue H₂B-PMHC (ca. 100 molecules per liposome, i.e., 0.13 mol% vs. DOPC). Following scavenging of peroxy or alkoxy radicals with rate constants akin to those of α -tocopherol,¹¹ oxidized emissive H₂B-PMHC (H₂B-PMHC_{ox})¹⁵ is generated and the emission intensity undergoes a ca. 30-fold enhancement in liposomes upon deactivation of an intramolecular photoinduced electron transfer quenching pathway.¹³⁻¹⁷ The liposomes also contained the red-emitting lipophilic dye DiD (ca. 200 molecules per liposome, 0.27 mol% vs. DOPC). We utilized DiD as a spectrally orthogonal emissive dye to independently label and identify single surface-anchored liposomes²³ before 2,2'-azobis(2-amidinopropane) (ABAP) was flowed and H₂B-

PMHC_{ox} was generated (see Figure 2.1).[§] The dual labeling strategy further enabled ratiometric analysis (see below).



Scheme 2.1. Experimental Strategy.

In order to anchor the liposomes on PEG-coated glass coverslips *via* biotin-streptavidin interactions, liposome formulations included 1 mol% vs. DOPC of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol-amine-N-(biotinyl) (biotin-DPPE).²³⁻²⁶ Glass coverslips assembled with a flow chamber were mounted on a piezoelectric nanopositioner. A confocal microscope was utilized to image the samples, where the green (H₂B-PMHC) and red (DiD) emissions were simultaneously collected in two parallel channels with two avalanche photodiode detectors. This setup provides optimum signal-to-background ratio. The circularly polarized 514 nm output of a CW Ar⁺ laser was utilized to simultaneously excite the H₂B-PMHC dye near its absorption maximum and the DiD dye at the blue edge of its absorption spectrum (see Figure 2.4).

We initially explored highly oxidative conditions in order to establish whether H₂B-PMHC can report oxidation events in individual liposomes. Aqueous solution of the azo initiator ABAP (0.25 M) in aerated phosphate buffered saline (PBS) were flowed over surface-immobilized liposomes. ABAP thermally decomposes into 2 positively charged carbon centered radicals that upon reaction with oxygen yield 2 peroxy radicals (see Scheme 2.2). The rate of generation of these peroxy radicals is estimated to be $3 \times 10^{-8} \text{ M s}^{-1}$ at 23°C under our conditions.[‡]

Figure 2.1 displays images of the green channel recorded before (A) and during (C) flowing of ABAP. A 6-fold emission enhancement was recorded when comparing the total fluorescence intensity of all liposomes ($n = 159$) in the H₂B-PMHC emission channel from images acquired before and during flow of ABAP solution (see also Figure 2.5 to Figure 2.7). A 1.4-fold increase in the red channel was also recorded as ABAP was introduced, that we assign to energy transfer from emissive H₂B-PMHC_{ox} to DID. An increase in energy transfer was also observed in ensemble experiments upon oxidation of H₂B-PMHC. A ca. 3-fold increase in the background of the green channel was also observed throughout the image arising from impurities introduced with ABAP. Comparison of Figure 2.1A and Figure 2.1C reveals that not all liposomes were observable in the H₂B-PMHC channel before oxidative conditions. Thus, on a per liposome basis, a large range of enhancements was detected.

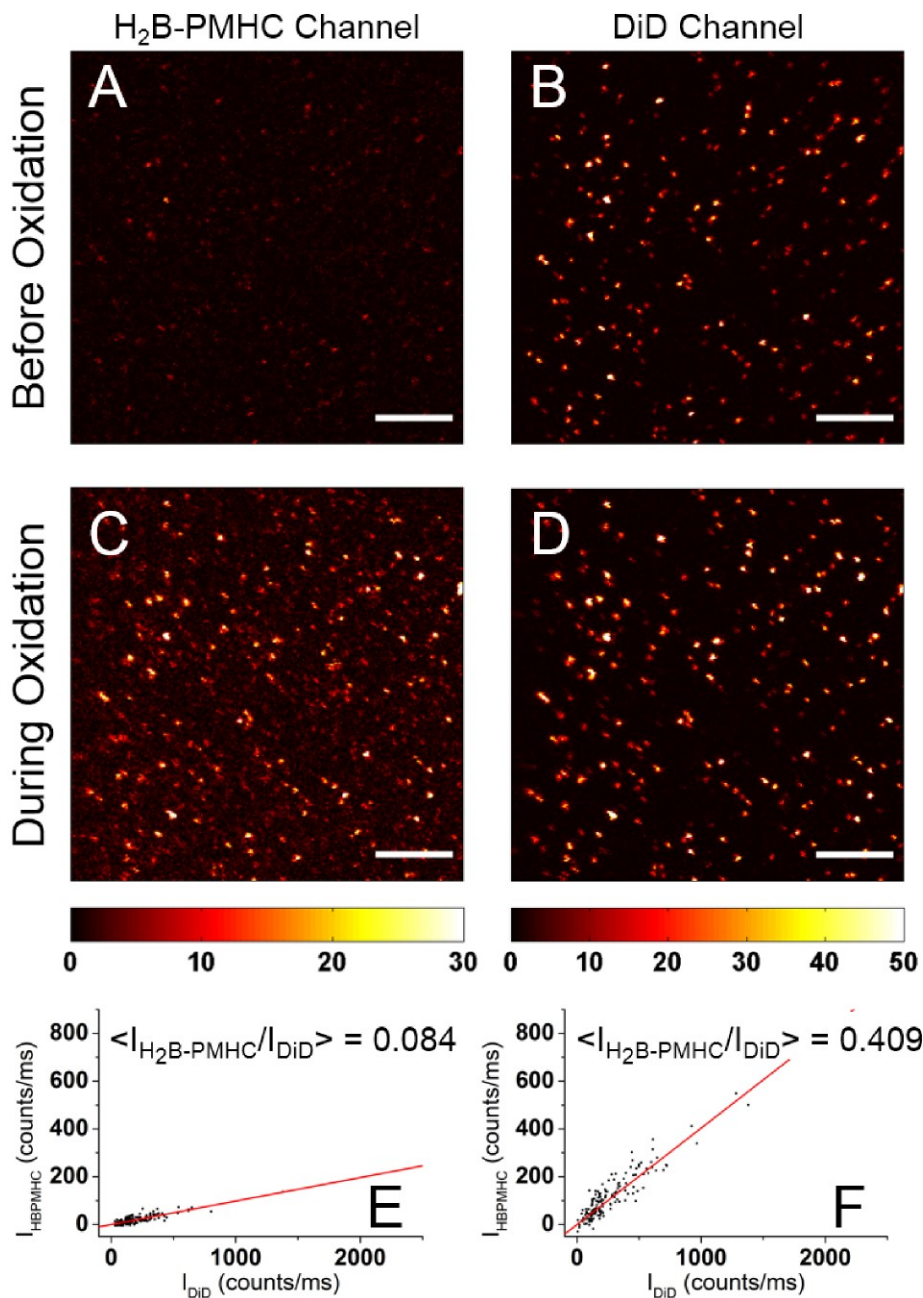


Figure 2.1. Fluorescence intensity images (top and middle rows) and intensity ratios of H₂B-PMHC/DiD channels (bottom row) before and during oxidation initiated by ABAP. Panels A (C) show images corresponding to the H₂B-PMHC channel and panels B (D) show those from the DiD channel, acquired before (during) oxidation, upon excitation with the 514 nm, 300 W/cm² output of an Ar⁺ laser. Scale bars are 6 μm . Panels E (before oxidation) and F (during oxidation) illustrate the correlation of H₂B-PMHC vs. DiD fluorescence intensity; also shown are linear fits as a guideline. Mean values of fluorescence intensity ratios obtained by Gaussian analyses are further listed.

The dual labeling scheme incorporating DiD as an internal standard enabled us to quantify the change in H₂B-PMHC fluorescence intensity in a ratiometric analysis that minimizes the influence of liposomal size distribution.^{23, 27} DiD incorporation further enabled us to account for all liposomes in the sample, even those not showing initial H₂B-PMHC intensity. Figure 2.1E and Figure 2.1F display the observed linear correlation of the green (H₂B-PMHC) vs. the red (DiD) fluorescence intensity, recorded for each liposome before and during ABAP addition, respectively. The larger slope for the latter illustrates the fluorescence enhancement caused upon peroxy radical reactions with H₂B-PMHC on individual liposomes.

For a quantitative treatment we resorted to the distribution of the H₂B-PMHC/DiD fluorescence intensity ratios. This treatment is less sensitive to outliers in comparison to a linear fit of the correlation of green to red intensity (Figure 2.9 and Figure 2.10).²⁷⁻²⁸ Histograms of intensity ratios were compiled for all liposomes in a 30 x 30 μm^2 area and fit to a Gaussian distribution. The mean value of the distribution was taken as the representative H₂B-PMHC/DiD fluorescence intensity ratio of the sample (see Figure 2.10). We observed a maximal 4.9-fold fluorescence enhancement during oxidation. After longer exposure to oxidative conditions, fluorescence intensities in both H₂B-PMHC and DiD channels decreased, consistent with oxidative degradation of the sample.¹⁵

Fluorescence enhancements of ca. 30-fold are typically recorded under thermal ABAP-initiated oxidation conditions executed in ensemble experiments.¹⁵ Presumably, under our imaging conditions the adduct of H₂B-PMHC_{ox} and a positively charged peroxy radical during the image acquisition and diffuse away from the confocal observation volume. This is supported by confocal microscopy experiments that reveal that oxidation of H₂B-PMHC by lipophilic alkoxyl radicals (generated from dicumyl peroxide) yields a more lipophilic product (5-fold larger H₂B-

PMHC/DiD intensity ratio) than the one formed upon ABAP-initiated oxidation. FRET from H₂B-PMHC_{ox} to DiD and photobleaching of H₂B-PMHC_{ox} during imaging (albeit low excitation powers of 300 W/cm² were used) may also explain the smaller enhancement. Probe degradation upon oxidation of the BODIPY core is additionally expected from the high ABAP concentrations used. These conditions are needed in order to observe the H₂B-PMHC fluorescence intensity enhancement under short (5-10 minutes) monitoring periods.[¶]

We were initially surprised to see bright emission from single molecules in some of the “pristine” liposomes prior to ABAP addition, arguably the result of oxidative events during sample preparation. This prompted us to investigate the extent of ambient oxidation during standard liposome preparation in the absence of additives intended as a source of ROS (Figure 2.2). Four different sample preparations with different antioxidant loads were monitored utilizing the H₂B-PMHC/DiD fluorescence intensity ratio as a marker of ambient oxidation. Two samples contained either the lipophilic antioxidant 2,2,5,7,8-pentamethyl-6-hydroxy-chroman-6-ol (PMHC) (+P) or the hydrophilic antioxidant 6-hydroxy-2,5,7,8-tetramethyl-2H-chromene-2-carboxylic acid (Trolox) (+T). Two more samples were also studied with no antioxidant (-PT), and with both antioxidants (+PT) included in the preparation. Given the stronger laser powers used (6,400 W/cm²) to facilitate the acquisition of intensity-time trajectories (see below), DOPC liposomes were prepared with a reduced load of DiD molecules (50, 0.07 mol%). Imaging was always performed under HEPES buffer flow with no added Trolox in order to isolate sample preparation effects.

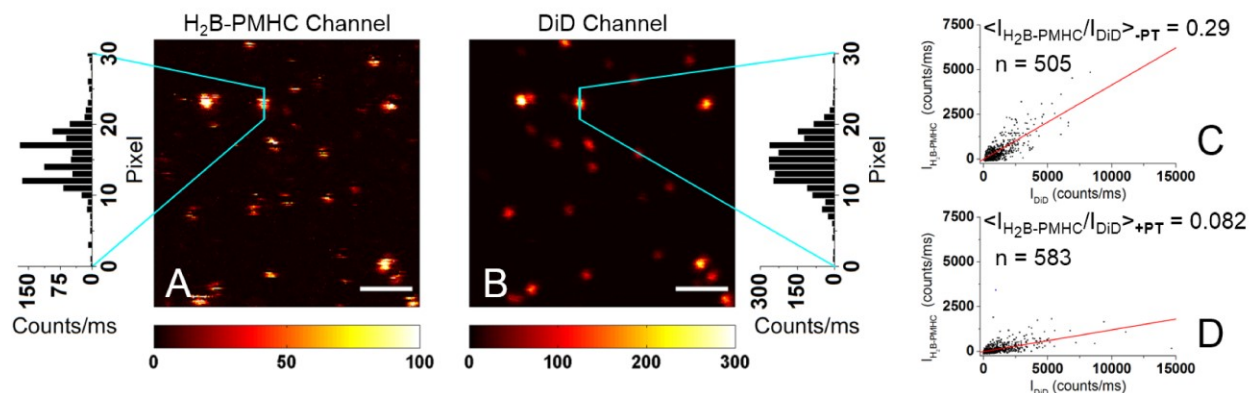


Figure 2.2. Panels A and B show fluorescence intensity images corresponding respectively to the H₂B-PMHC channel and the DiD channel, obtained for a sample prepared without Trolox and PMHC antioxidants (-PT). Images were simultaneously acquired using the 514 nm output of an Ar⁺ laser (6,400 W/cm²). Linescan profiles are shown at the sides of the images. Scale bars are 2 μ m. Panels C (-PT) and D (+PT) illustrate the correlation of H₂B-PMHC vs. DiD fluorescence intensity in the absence and presence of antioxidants; also shown are linear fits as a guideline. Mean values of fluorescence intensity ratios, obtained by Gaussian analyses of data from 30 x 30 μ m² images, are further listed.

Based on the images we obtained with the above samples, we concluded that there is significant, quantifiable fluorogenic probe oxidation occurring during typical liposome sample preparation in resulting from ABAP decomposition may readily escape the liposome ambient conditions. This is reflected by the 3.5-fold higher H₂B-PMHC/DiD fluorescence intensity ratio in the sample lacking any antioxidant (-PT) compared to the sample with both PMHC and Trolox (+PT) (Figure 2.2, panels C and D). The observed intensity ratio for our samples in increasing order was +PT $\lesssim$ +T $\ll$ +P $\lesssim$ -PT (Figure 2.10, Figure 2.11 and Table 2.1). Under our conditions, Trolox clearly has a more potent protective antioxidant effect than PMHC. This could indicate that species responsible for ambient oxidation in liposomes originate within the water phase.

The alternative to probe oxidation occurring during liposome preparation (described in the above paragraph) is that preoxidized H₂B-PMHC existing as a trace contaminant in e.g. the dye batch was incorporated during the liposome preparation. Our experiments with antioxidants rule

out this possibility, as neither Trolox nor PMHC may reduce preoxidized H₂B-PMHC_{ox} (Figure 2.12). The activity of these antioxidants is thus preventive, arising from trapping ROS in solution, rather than “curative” or healing.

Importantly, a control experiment described in Figure 2.13 shows that the potential photosensitization of ROS by DiD is not a prevalent phenomenon under our imaging conditions but may be observed under exhaustive irradiation conditions (Figure 2.14). Accordingly, the contribution of photogenerated ROS to the observed intensity enhancement of H₂B-PMHC is negligible.

We note that the unrivalled sensitivity of single molecule spectroscopy plays a major factor in enabling us to measure the small differences in fluorescence intensities under different antioxidant loading. Importantly, given the similar antioxidant activity of H₂B-PMHC and α -tocopherol,^{15, 29} our observations would imply that there is a significant degradation of antioxidant during sample preparation. This would have detrimental effects in the lifetime of lipid based products and materials enriched with α -tocopherol as a protective agent. The degradation of protective α -tocopherol in lipid formulations in turn underscores the potential lipid degradation under ambient conditions.

Close inspection of confocal images recorded under ambient conditions showed fluctuations in the liposome intensity over time in the green channel, manifested as pixelated rather than smooth profiles as observed in the DiD channel. The difference in intensity profiles between both channels may be better appreciated from the image linescans for 10 x 10 μm^2 regions in Figure 2.2 (see also Figure 2.11). The highly dynamic green emission profile arises from fluctuations in the amount of emissive H₂B-PMHC_{ox} with time in an individual liposome as the sample is raster scanned resulting in a distorted Gaussian shape. Overlaid in Figure 2.3 are the

fluorescence intensity-time trajectories recorded on individual liposomes, without (**-PT**, Figure 2.3A) and with (**+PT**, Figure 2.3B) PMHC and Trolox. The fluorescence time trajectories highlight the on-off “blinking” of the H₂B-PMHC probe (ca. 100 molecules/liposome). Conversely, the emission intensity arising upon DiD illumination (ca. 50 molecules/liposome) follows an exponential decay due to photobleaching, with a lifetime of approx. 10 s under our aerated imaging conditions. In the former case, the measured intensity counts per second recorded for H₂B-PMHC_{ox} are those expected to arise from zero to a few dyes simultaneously emitting. In the case of DiD, the low excitation probability due to its small cross section at 514 nm results in a comparable intensity when all DiD dyes in a liposome are emissive.

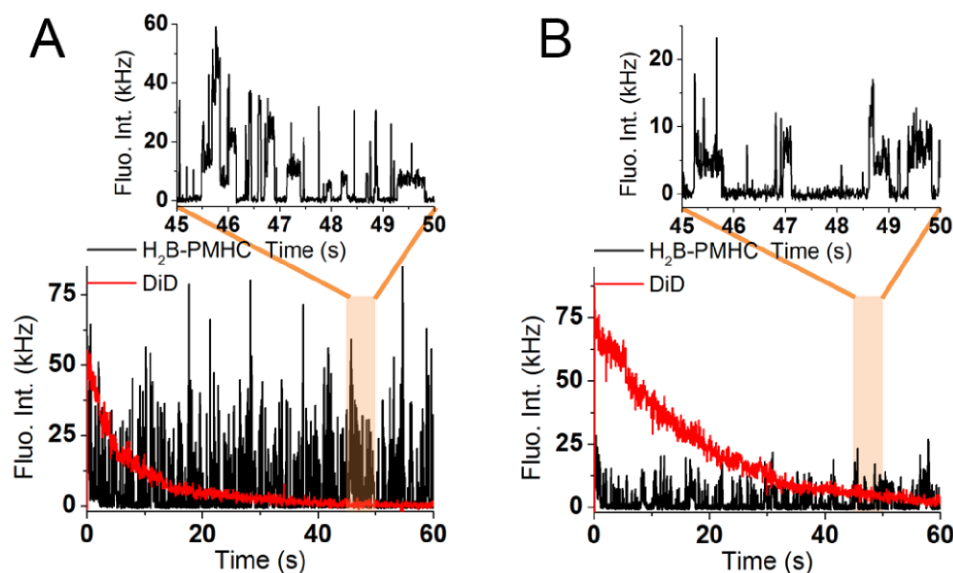


Figure 2.3. Intensity-time trajectories recorded for individual liposomes prepared (A) without (**-PT**) and (B) with PMHC and Trolox (**+PT**). The black and red traces correspond to the emission from the H₂B-PMHC channel and the DiD channel. The data is binned at 5 ms. DiD traces are plotted every 10th point for clarity.

While tempting to postulate that the emission dynamics are the result of the generation of H₂B-PMHC_{ox} and its subsequent photobleaching, we may not currently preclude reversible photobleaching/blinking also operating on individual H₂B-PMHC_{ox} molecules within the

liposomes. A few hundreds of fluorescent bursts are observed within 3 minutes when focusing on liposomes, which is much greater than the average of 100 H₂B-PMHC dyes per liposome. In contrast, typically less than 15 events are seen when looking at a region with no liposomes (see also Figure 2.15 and Table 2.1). Regardless of whether the dynamic emission profile results solely from reaction upon oxidation of the trap segment in H₂B-PMHC or further involves photochemical/photophysical processes intrinsic to H₂B-PMHC_{ox}, it may prove uniquely powerful towards super resolution imaging of lipid substrates relying on stochastic processes.³⁰⁻³²

2.5. Conclusions

In summary, we have successfully detected reactions with peroxy radicals within individual liposomes utilizing the fluorogenic antioxidant probe H₂B-PMHC. The ratiometric assay we have developed has the sensitivity to observe oxidation events in individual liposomes under ambient conditions (typical laboratory temperature and lighting). These results stress the potential prominence of lipid degradation during their formulation. The new assay further provides a simple sensitive way to monitor chemical reactions at the level of individual liposomes. Our assay may become a powerful methodology towards studying oxidative phenomena taking place in nanoreactors such as liposomes and low density lipoproteins, as well as other systems of biological importance. We further believe that the emission dynamics may prove uniquely powerful towards super resolution imaging of lipid substrates.

2.6. Notes

§ The spectroscopic properties of the dyes used combined with the emission filters employed in our setup (SI) gave rise to 7% H₂B-PMHC to DiD channel crosstalk and negligible DiD to H₂B-PMHC crosstalk.

‡ Calculated from its activation energy ($124 \text{ kJ} \cdot \text{mol}^{-1}$) and 10 hour half-life temperature (56°C).

¶ We note that by working with a single lipid rather than a mixture of lipids in the liposome formulation, the heterogeneities in dye concentration reported to occur with lipid mixtures, are expected to be minimal. The number of fluorescent dyes are a true marker of individual liposome size, and inhomogeneities in dye distribution, if any, will not introduce a systematic bias in liposome size determination. See reference 27.

¶ These conditions were chosen to limit the number of confocal images needed to observe the maximal fluorescence intensity to 1 or 2 images, in an effort to minimize photobleaching of $\text{H}_2\text{B-PMHC}_{\text{ox}}$.

2.7. Supporting Information

The Supporting Information contains detailed experimental of individual liposome imaging, oxidation experimental conditions, spectra characteristics of the dyes used, additional results for the $\text{H}_2\text{B-PMHC}$ fluorescence enhancement within individual liposomes, comparison of fits to extract the characteristic $\text{H}_2\text{B-PMHC/DiD}$ ratios, Gaussian fits results, confocal images for the four different antioxidants conditions studied, the effect of PMHC on the oxidation state of $\text{H}_2\text{B-PMHC}_{\text{ox}}$, light irradiation effects, and a summary of the antioxidant additive effects.

2.7.1. Materials

All chemicals were used as received without additional purification. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(biotinyl) sodium salt (biotin-DPPE) lipids were purchased from Avanti Polar Lipids (Alabaster, AL). 2,2,5,7,8-Pentamethyl-6-chromanol (PMHC), ($\pm$)- α -tocopherol, ($\pm$)-6-Hydroxy-2,5,7,8-

tetramethylchromane-2-carboxylic acid (Trolox) and 2,2'-azobis(2-methylpropionamidine) dihydrochloride (ABAP) were purchased from Sigma-Aldrich. Chloroform and acetone (HPLC grade) and HEPES buffer were purchased from Fischer Scientific. 5 M NaCl stock solution was purchased from Life Technologies (Ambion® brand). Water (molecular biology grade) was acquired from Thermo Scientific HyClone. (South Logan, UT). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) was purchased from Invitrogen Canada (Burlington, ON). Vectabond™ was purchased from Vector Laboratories (Burlington, ON). Poly(ethylene glycol) succinimidyl valerate, MW 5000 (mPEG-SVA) and biotin-PEG-SVA were purchased from Laysan Bio Inc. (Arab, AL). Streptavidin protein was purchased from Life Technologies (Burlington, ON). H₂B-PMHC was prepared as previously reported.¹⁸

2.7.2. Liposome Preparation

Lipid films were prepared by mixing lipid solutions of DOPC and biotin-DPPE lipids (1 mol% vs. DOPC), with (±)- α -tocopherol in the case of the ABAP oxidation experiment, all dissolved in chloroform. Dye solutions (H₂B-PMHC in acetonitrile or DiD in EtOH) were also added to the solution. When liposomes containing PMHC were prepared, H₂B-PMHC was dissolved in an acetonitrile solution containing 2 mM PMHC to prevent premature probe oxidation. Concentrations of antioxidants and dyes per liposomes were calculated by assuming 75000 DOPC lipid molecules per liposome.²² The lipid solution with additives was then dried under a stream of argon gas to produce a thin film and solvents were completely removed *in vacuo*. The dry lipid film was hydrated by the addition of HEPES buffer (10 mM HEPES, 150 mM NaCl, pH = 7.3) which contained 200 μ M of Trolox when desired, to give 20 mM DOPC solutions. After five freeze-thaw cycles (freeze in dry ice, thaw in a sonicator bath) the hydrated lipids were extruded by passing 11 times through a 100 nm polycarbonate membrane to yield small

unilamellar vesicles. Care was taken to keep the liposomes under dark conditions during the longest steps, namely removal of trace solvents and the freeze/thaw cycles. Exposure to ambient light did not exceed 10 minutes during liposome preparation. Liposome solutions were diluted 2000x with the appropriate HEPES buffer and immediately flowed onto prepared coverslips for confocal imaging.

2.7.3. Coverslip Preparation

The coverslips were prepared as described previously.²³ Coverslips (Fischerfinest #1, Fisher Scientific) were first cleaned in piranha solution (1:3 30% H₂O₂:concentrated H₂SO₄ by volume) for 1 h, followed by rinsing with water and acetone. Clean coverslips were then immediately treated with VectabondTM/acetone 2% v/v solutions for 5 min. Amino-modified coverslips were then rinsed with H₂O and stored for usage within a week. Further surface modifications were made by a mixture of mPEG-SVA and biotin-PEG-SVA in order to prevent non-specific binding and create a platform onto which liposomes could be immobilized. More precisely, clean coverslips were masked with patterned silicone films (Grace Bio-Labs Inc., Bend, OR) and the unprotected area was exposed to a 25% w/w mPEG-SVA aqueous solution containing 0.25% w/w biotin-PEG-SVA and 0.1 M sodium bicarbonate for 3-8 hours. The silicone templates were removed, the excess PEG solution rinsed with water, and the coverslips dried under a N₂ stream. Sample chambers were formed by depositing a pre-drilled adhesive polycarbonate gasket (Grace Bio-Labs Inc.) on the PEG-functionalized side of a coverslip. Silicone inlet and outlet ports (Grace Bio-Labs Inc.) were glued on top of the chambers. Streptavidin (0.2 mg/mL) was flowed in the assembled chamber, and then rinsed with the appropriate HEPES buffer. Finally, a small volume (typically ~ 20 µL) of the liposome sample (ca. 130 pM liposome concentration) was then flowed in the sample chamber and rinsed when adequate surface density was achieved.

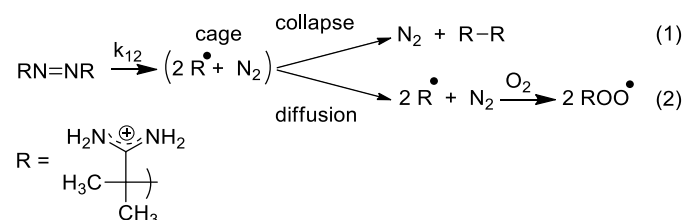
2.7.4. Scanning Confocal Microscopy Imaging of Single Liposomes

Confocal imaging was performed on an Olympus IX-71 inverted microscope equipped with a closed-loop nanopositioning stage (Nano LP100, Mad City Labs, Madison, WI) used for imaging and sample positioning. Samples were excited by CW laser emission from either the 514 nm line from an Ar⁺ laser (43 series, Melles Griot) or the 633 nm output from a HeNe laser (Melles Griot). The linearly polarized laser beams introduced *via* a single mode fibre optic were circularly polarized by a quarter-wave plate and directed by a dichroic beamsplitter (z514-633rdc, Chroma, Rockingham, VT) to the sample through a high numerical aperture oil immersion objective (Olympus U PLAN SAPO 100X, N.A. = 1.40). Fluorescence emission was collected through the same objective, split into two channels by a second dichroic beam splitter (640DCXR Chroma, Rockingham, VT) and directed to two avalanche photodiode detectors (Perkin Elmer Optoelectronics SPCM-AQR-14, Vaudreuil, QC). A 514nm or 633nm holographic Raman notch filter (Kaiser Optical Systems, Ann Arbor, MI) was placed in front of the APDs to block residual laser excitation. The H₂B-PMHC channel emission ($\lambda < 640$ nm) was cleaned by a 565/70nm bandpass filter (Chroma, Rockingham, VT) while the DiD channel emission ($\lambda > 640$ nm) was cleaned by a 685/80nm bandpass filter (Chroma, Rockingham, VT). Images were acquired under ambient conditions, with no added imaging buffers.

2.7.5. ABAP Oxidation Experiment

Following the procedure outlined in the Liposome Preparation section, liposomes containing 100 (0.13 mol% vs. DOPC) H₂B-PMHC, 200 (0.27 mol%) DiD, 1600 (2.13 mol%) PMHC, 200 (0.27 mol%) ($\pm$)- α -tocopherol and 750 (1 mol%) biotin-DPPE were prepared. The dried lipid film was hydrated with HEPES buffer to which 200 μ M of Trolox was added. Following immobilization on the microscope coverslip, HEPES-only buffer was flowed over the sample to

remove Trolox from the solution. A buffer consisting of 10 mM PBS (5 mM KH_2PO_4 , 5 mM K_2HPO_4 , 140 mM NaCl, pH = 7.2) buffer was then flowed to remove HEPES from the solution before inducing oxidation by flowing a 0.25 M ABAP solution in PBS. PBS buffer was chosen as solvent for the ABAP-induced oxidation as HEPES is a free radical scavenger. The salt concentrations of the PBS buffer were chosen to yield an isotonic solution to the HEPES buffer solution. Images were acquired using laser powers of 300 W/cm² at 514 nm and 4 W/cm² at 633 nm.



Scheme 2.2: Production of peroxy radicals ($\text{ROO}^\bullet$) following thermolysis of ABAP. ABAP initially decomposes in a first order reaction to yield two geminate carbon centered radicals which may either recombine (Equation 1) or diffuse away (Equation 2). In air equilibrated solutions radicals that escape cage recombination will readily trap molecular oxygen³³ to yield two water soluble peroxy radicals.

2.7.6. Ambient Oxidation Experiments

Following the procedure outlined in the Liposome Preparation section, liposomes containing 100 (0.13 mol% vs. DOPC) H₂B-PMHC, 50 (0.07 mol%) DiD and 750 (1 mol%) biotin-DPPE molecules per liposome were prepared. 1600 (2.13 mol%) PMHC molecules per liposome were also added in samples that contained PMHC. The dried lipid film was hydrated with HEPES buffer, which may also contain 200 μ M of Trolox depending on the desired conditions. Images were acquired using laser line powers of 6400 W/cm² at 514 nm and 200 W/cm² at 633 nm, when needed. The images used for determining the center of Gaussian distributions of fluorescence

intensity ratios (see Figure 2.10) were only scanned once with the 514 nm laser excitation during data acquisition.

2.7.7. *H₂B-PMHC and DiD Spectral Discrimination*

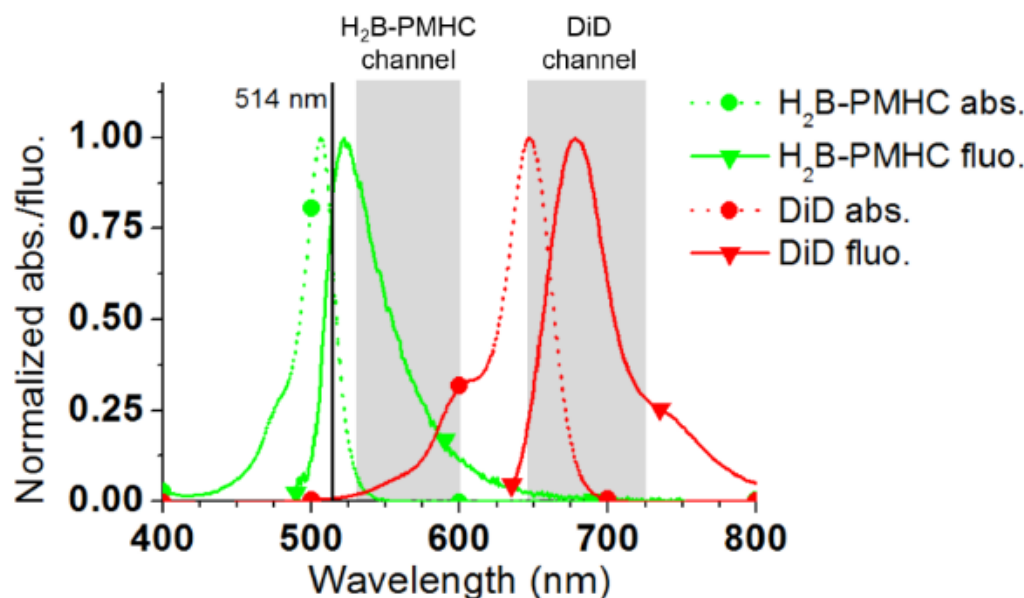


Figure 2.4. Absorbance (dotted line) and fluorescence (solid line) spectra of $H_2B-PMHC_{(ox)}$ (green) and DiD (red). $H_2B-PMHC$ and DiD show good spectral separation and their emission can be split and detected by two separate channels (gray areas). The 514 nm output of an Ar^+ laser was used to excite $H_2B-PMHC$ near its absorbance maximum. This laser line further enables direct excitation of DiD at the blue edge of its absorbance spectrum. 7% $H_2B-PMHC$ to DiD channel crosstalk and negligible DiD to $H_2B-PMHC$ crosstalk was observed with our setup.

2.7.8. *H₂B-PMHC Fluorescence Enhancement over Time under Oxidative Conditions*

To determine the fluorescence intensity enhancement of $H_2B-PMHC$, we first compared the $H_2B-PMHC$ emission intensity from all individual liposomes before and during ABAP exposure. Liposomes were identified from their DiD emission in the red channel with a custom MATLAB routine.

Alternatively, we also calculated the fluorescence intensity enhancement from the average of the intensity ratio of $H_2B-PMHC$ versus DiD ($I_{H_2B-PMHC}/I_{DiD}$) recorded for each individual

liposome, determined before and during ABAP exposure (see below). Importantly, the same pixel regions (same liposomes) were used in the green and red channels to calculate the intensity ratio of H₂B-PMHC versus DiD. This ratiometric method is preferred as it corrects for effects such as dye partitioning heterogeneity due to liposome size distribution. The immobilized liposomes were initially incubated in HEPES buffer which was rinsed away by PBS buffer and subsequently 0.25 M ABAP in PBS was flowed over the liposomes at a rate of 5 μ L/min to initiate the oxidation of H₂B-PMHC. The fluorescence intensity ratios are determined by the mean value of a distribution of $I_{\text{H}_2\text{B-PMHC}}/I_{\text{DiD}}$ fitted by a Gaussian function (Figure 2.8B). Time points correspond to the onset of each image acquisition, which takes about 5 minutes, and time = 0 minutes corresponds to when the ABAP flow was started.

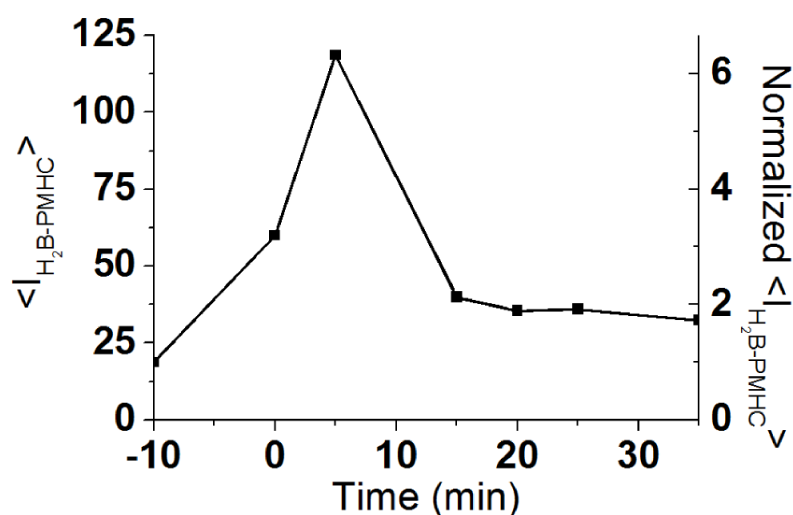


Figure 2.5. H₂B-PMHC fluorescence intensity as a function of time under oxidative conditions. The representative average intensity is determined by averaging the intensity of all liposomes identified from the DiD channel.

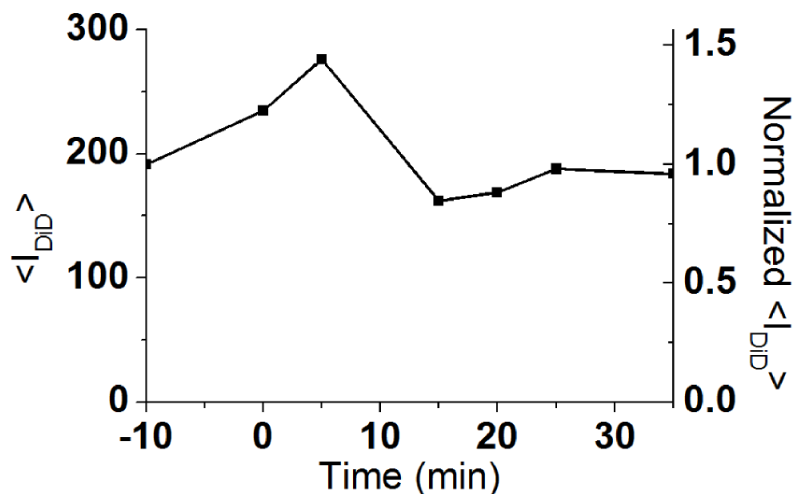


Figure 2.6. DiD fluorescence intensity as a function of time under oxidative conditions. The representative average intensity is determined by averaging the intensity of all liposomes identified from the DiD channel.

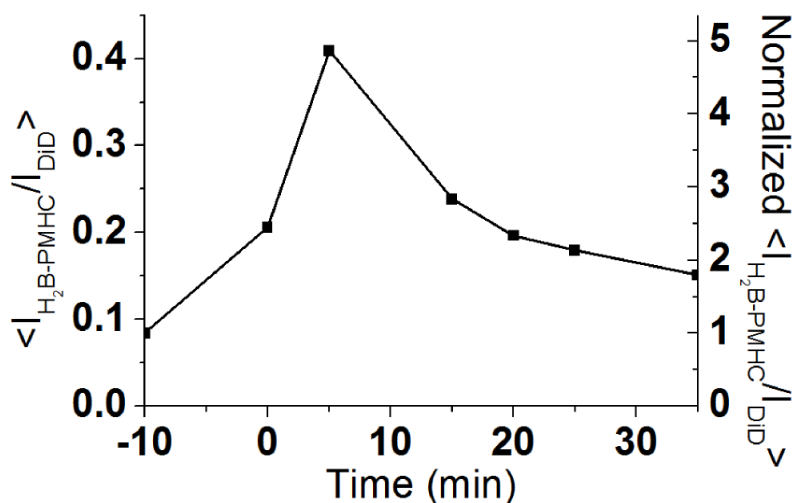


Figure 2.7. H₂B-PMHC/DiD fluorescence intensity ratio as a function of time under oxidative conditions. The representative average value of H₂B-PMHC/DiD fluorescence intensity is determined from the mean value of Gaussian distributions of the H₂B-PMHC/DiD intensity ratio calculated for each liposome.

2.7.9. Comparison of Linear and Gaussian Fitting of Individual Liposome Data

To determine the ratio of H₂B-PMHC/DiD intensity, we initially plotted intensities of H₂B-PMHC versus those of DiD for individual liposomes (583 liposomes in total, +PT case). However,

this analysis is sensitive to outliers. The initial data was fit by a linear regression model, yielding a slope of 0.163. The slope of the linear fit when excluding 3 outliers decreased 39% to 0.117.

We preferred a Gaussian fitting as the method to extract representative averages for all the different samples. Here the H_2B -PMHC/DiD intensity ratios of individual liposomes were used to construct a histogram fitted with a Gaussian function. The mean value of the H_2B -PMHC/DiD intensity ratio resulting from the Gaussian fits did not vary regardless of whether outliers are included or excluded.

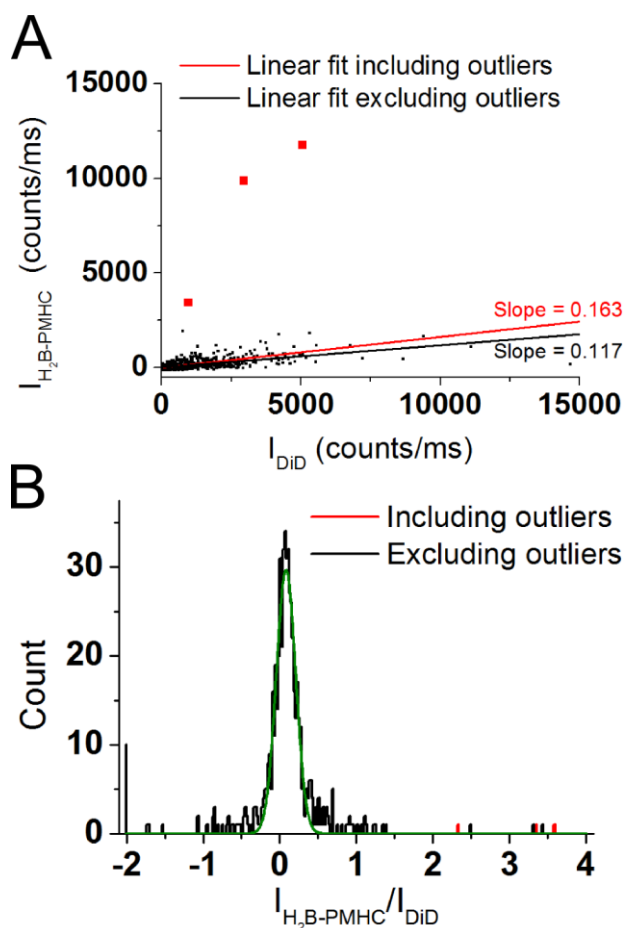


Figure 2.8. A) Scatter plot of Intensities of H_2B -PMHC versus DiD for individual liposomes fitted with a linear regression. Outliers in red squares are shown. (B) Histogram of the H_2B -PMHC/DiD intensity ratios of individual liposomes constructed from the same data set as in (A) but now fitted to a Gaussian distribution. Both Gaussian fits (green lines) overlap perfectly. Outliers are shown as red data points on the right.

2.7.10. Gaussian Fitting of Fluorescence Intensity Ratios Recorded Under Oxidation

Conditions Induced by ABAP

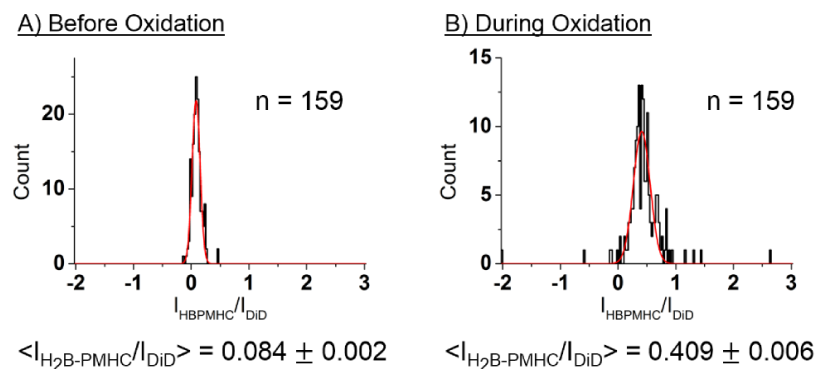


Figure 2.9. Histogram of $I_{\text{H}_2\text{B-PMHC}}/I_{\text{DiD}}$ ratios for individual liposomes before (A) and during (B) exposure to ABAP. The number of liposomes analysed and the mean value of Gaussian fits (red lines) $\pm$ fitting error are also indicated.

2.7.11. Gaussian Fitting of Fluorescence Intensity Ratios Recorded Under Ambient

Conditions and Different Antioxidant Loads

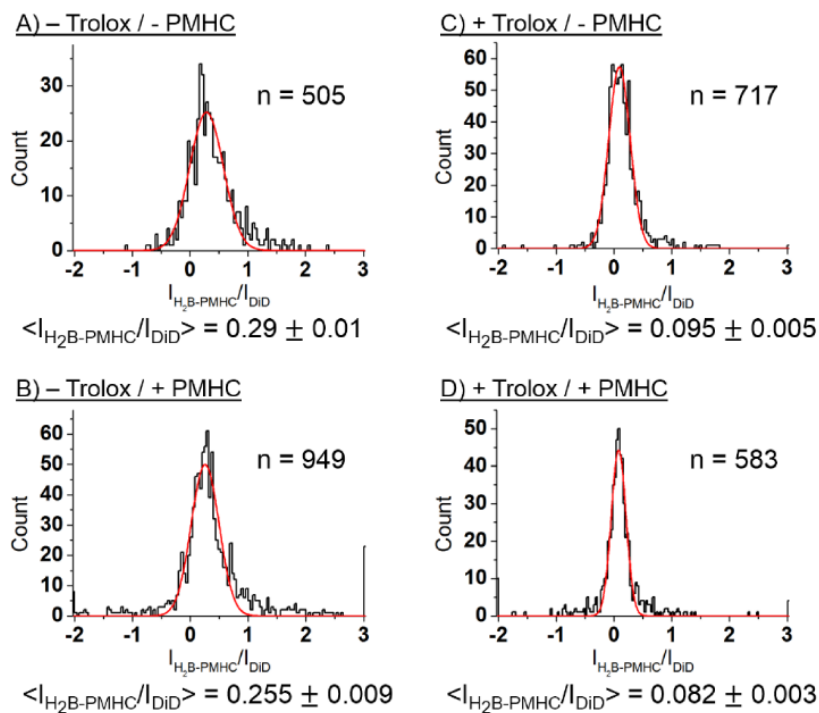
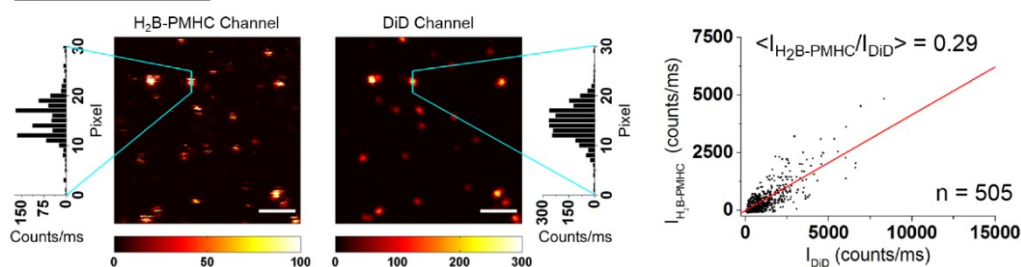


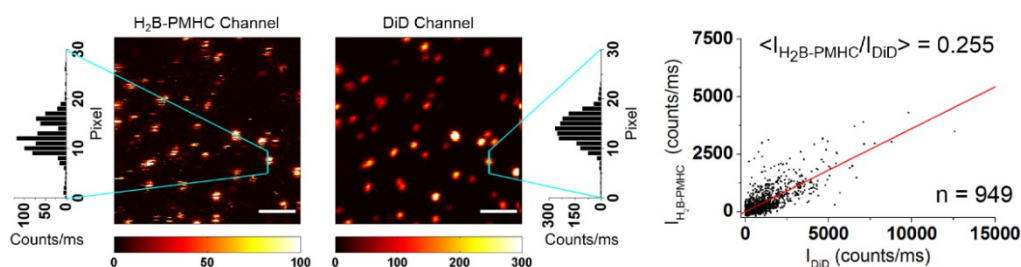
Figure 2.10. Histogram of $I_{H_2B-PMHC}/I_{DiD}$ ratios for individual liposomes imaged under ambient conditions and in the presence of different antioxidants added during sample preparation. The number of liposomes analysed and the mean value of Gaussian fits (red lines) $\pm$ fitting error are also indicated.

2.7.12. Confocal Fluorescence Intensity Images Recorded Under Ambient Conditions and Different Antioxidant Loads

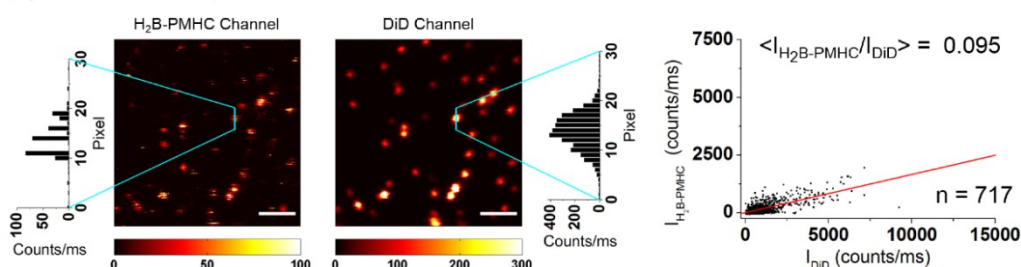
A) – Trolox / – PMHC



B) – Trolox / + PMHC



C) + Trolox / – PMHC



D) + Trolox / + PMHC

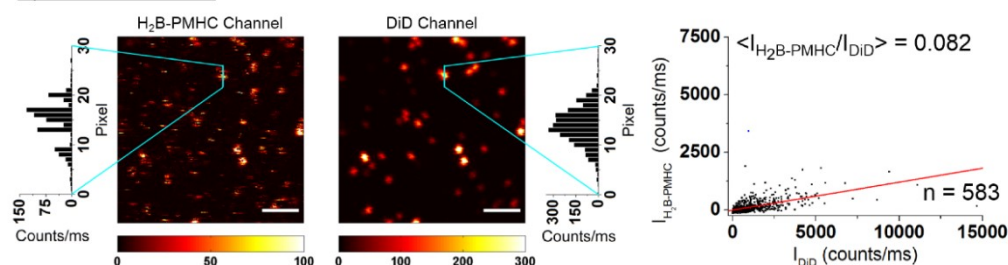


Figure 2.11. Fluorescence intensity images corresponding respectively to the H₂B-PMHC channel and the DiD channel, obtained for a sample prepared (A) with no additional antioxidants (-PT); (B) with PMHC only (+P); (C) with Trolox only (+T); and (D) with both antioxidants (+PT). Linescans are shown for a single molecule beside the images and their positions on the images are indicated by a cyan line. White scale bars are 2 μ m. The right panel displays scatter plots showing the correlation of H₂B-PMHC vs. DiD fluorescence intensity, where linear fits serve as a guideline. Mean values of fluorescence intensity ratios obtained by Gaussian analyses, and the total number of liposomes analysed are further listed.

2.7.13. Effect of PMHC on H₂B-PMHC_{ox}: Preoxidized H₂B-PMHC Is Not Reduced By PMHC

We conducted fluorescence intensity measurements of H₂B-PMHC_{ox} in the presence of a large excess of PMHC to establish whether preoxidized, emissive dye may be reduced by PMHC regenerating non-emissive H₂B-PMHC. Such a process would lead to a drop in the emission intensity reflected as a static (not dynamic) quenching. Steady-state and time-resolved emission experiments were conducted to establish whether fluorescence quenching was static or dynamic (the latter is expected based on intermolecular photoinduced electron transfer from the chromanol moiety in PMHC to the photoexcited BODIPY dye in H₂B-PMHC_{ox}).

Following addition of increasing amounts of PMHC we observed a drop in the fluorescence intensity of preoxidized H₂B-PMHC. The H₂B-PMHC_{ox} solution was prepared by oxidation of 1.7 μ M H₂B-PMHC with cumyloxyl radicals generated upon the irradiation at 263 nm of a 100 μ M dicumyl peroxide solution in acetonitrile. The initial intensity divided by the intensity in the presence of increasing amount of quencher PMHC (I_0/I), when plotted vs the PMHC concentration, followed a Stern-Volmer (linear) correlation as expected from a quenching process, with $K_{SV} = 29.1 \text{ M}^{-1}$ (Figure 2.12 below). The same correlation was observed when the analysis involved the ratio of initial fluorescence decay lifetime over the fluorescence decay lifetime in the presence of increasing amount of quencher PMHC, that is τ_0/τ plotted vs. [PMHC]. These results points to a purely dynamic fluorescence quenching mechanism. The lack of static quenching (drop in H₂B-

PMHC_{ox} intensity with no change in its observed fluorescence decay lifetime) rules out the occurrence of any thermal reduction of H₂B-PMHC_{ox} by PMHC. In summary, there is no evidence of redox reactions under these conditions.

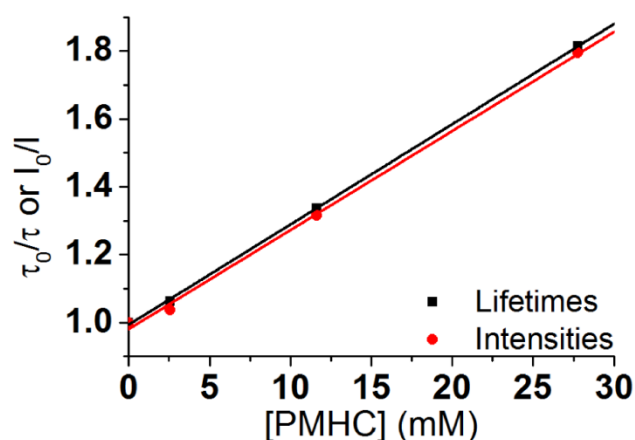


Figure 2.12. Stern-Volmer analysis of H₂B-PMHC_{ox} fluorescence intensity upon addition of increasing amounts of PMHC in acetonitrile solutions. Fluorescence intensities were integrated over the range of 500 - 675 nm, with $\lambda_{\text{ex}} = 480$ nm. Fluorescence lifetimes are the intensity-weighted averages of biexponential fits ($\chi^2 < 1.2$). The fluorescence lifetime measurements were carried out using a Picoquant Fluotime 200 time-correlated single photon counting setup employing an LDH 470 ps diode laser (Picoquant) with excitation wavelength at 467 nm as the excitation source. The laser was controlled by a PDL 800 B picosecond laser driver from Picoquant. The excitation rate was 10 MHz, and the detection frequency was less than 150 kHz. Photons were collected at the magic angle.

2.7.14. Confocal Imaging of Regions That Have Been Pre-Irradiated with 633 nm Laser Light

We conducted imaging experiments in order to rule out photosensitization of ROS upon direct excitation of DiD occurring either when absorbing ambient light during liposome preparation. Specifically, we imaged a large 90 x 90 μm^2 area once it had been pre-irradiated in predefined regions with the 633 nm (200 W/cm²) or the 514 nm 6400 W/cm²) laser output in the absence of any external antioxidant (-PT). Three sectors of 30 x 30 μm^2 along one diagonal were irradiated for increasing periods of time (increasing number of scans from 1 to 3) with 633 nm

laser light. Three sectors of $30 \times 30 \mu\text{m}^2$ along the other diagonal were irradiated for increasing periods of time with 514 nm laser light (increasing number of scans from 1 to 3). Next the whole $90 \times 90 \mu\text{m}^2$ area was imaged upon exciting at 514 nm. We did not observe increased emission arising from oxidized probes in regions excited with 633 nm laser light only. In turn, regions excited with increasing amounts of 514 nm showed a drop in intensity arising from sample photobleaching. We conclude that exposure to 633 nm laser excitation has no significant effects under our experimental conditions. The potential photosensitization of ROS by DiD is not a prevalent phenomenon, thus the contribution of photogenerated ROS to the observed intensity enhancement of H₂B-PMHC is negligible.

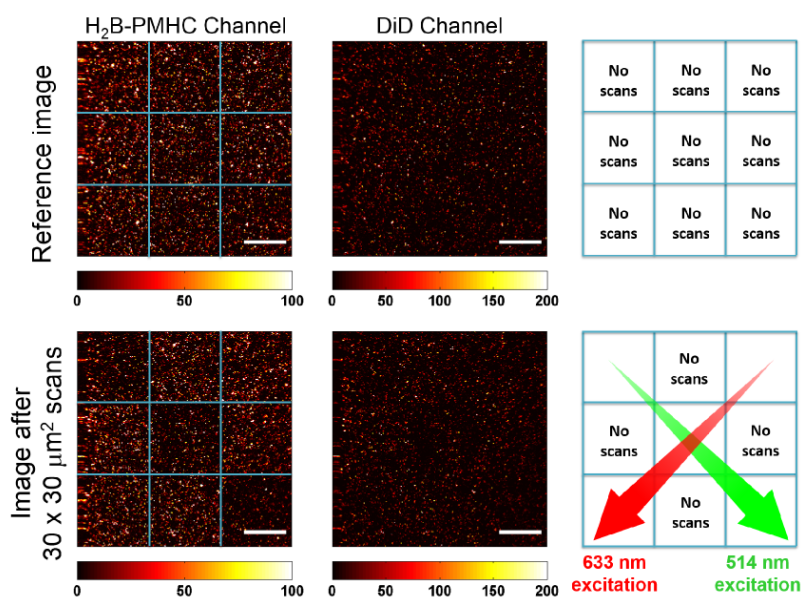


Figure 2.13. Confocal images ($90 \times 90 \mu\text{m}^2$ regions) acquired under 514 nm illumination. Top panels represent a region that has not been exposed to laser irradiation prior to the full region scan. The image in the bottom panel was acquired after the irradiation of predetermined regions along the diagonals for increasing periods of time, as depicted in the right grid. Irradiated sections in the top row of the grid were scanned once by laser, while the middle and bottom sections were scanned 2 times and 3 times, respectively. Scanning conditions: 1 ms dwell time/pixel, 256×256 pixels/scan; same as imaging conditions. Scale bars are $18 \mu\text{m}$.

2.7.15. Ensemble Irradiation of DiD Under Maximum Power

Liposomes containing H₂B-PMHC and DiD, without added antioxidants (-PT), were irradiated in a fluorimeter with fully open excitation slits (5 mm wide, gives 20 nm bandpass), exciting at the DiD absorbance maximum. We observed that extended (up to 30 minutes) irradiation results in an increase (ca. 2 fold) of H₂B-PMHC fluorescence intensity. No changes were observed in a sample that was kept in the dark for the same amount of time. This suggests the potential influence of, presumably, photogenerated ROS species in oxidation of liposome suspensions. The illumination intensity at 647 nm (4.5 mW/cm²) used was roughly 40-fold higher than the ambient lighting in our laboratory (0.12 mW/cm²).

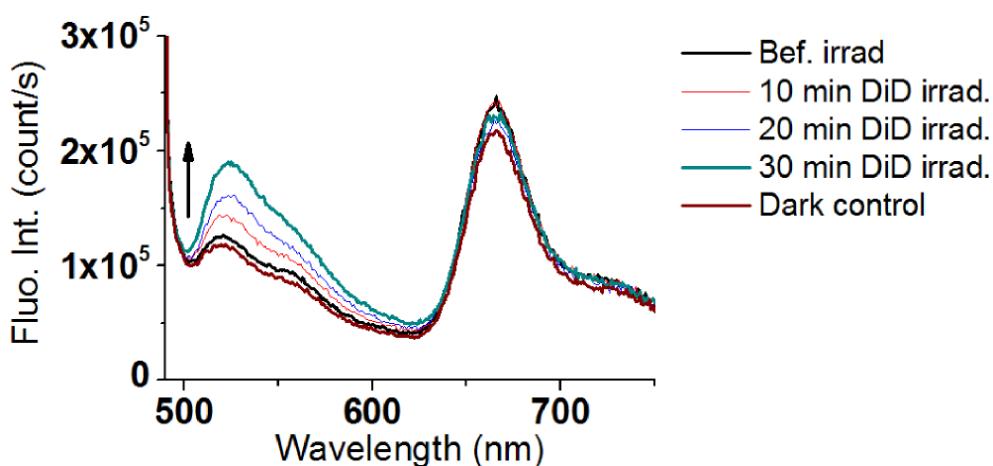


Figure 2.14. Ensemble irradiation of -PT sample. DiD was irradiated at the maximum of its absorbance (647 nm) for periods of 10 minutes. Emission spectra were acquired afterwards. Emission spectra were acquired with $\lambda_{\text{ex}} = 475$ nm.

2.7.16. Number of Fluorescent Bursts in Individual Liposomes

Monitoring the H₂B-PMHC fluorescence over time allowed us to further investigate the observed blinking dynamics. Interestingly, in all cases many more fluorescence bursts were recorded within a 3 minutes observation period than the expected number of embedded H₂B-PMHC molecules (i.e. 100) per liposome (Table 2.1). A MATLAB algorithm was written to

quantify fluorescence bursts from time trajectories binned at 5 ms per data point. Fluorescence burst events were counted as such only if the fluorescence intensity in the H₂B-PMHC channel crossed a threshold and remained above it for at least two consecutive data points. The threshold used for analysis was the average of the sum of the mean background intensity (lowest intensity level) plus five times its standard deviation under each conditions. The background intensity-time trajectory was acquired in a region with no liposomes. Importantly, only one event is counted when the intensity above the threshold drops for one or two data points below the threshold and returns. This avoids counting as multiple events a noisy fluorescence burst that lies near the threshold, similar to the ‘interpeak’ correction method used by Terentyeva et al.³⁴ The number of fluorescence bursts counted provides a lower estimate since any jump in the fluorescence intensity that crosses the threshold is counted as a single event, regardless of the fluorescence intensity change (e.g. many dyes becoming simultaneously fluorescent).

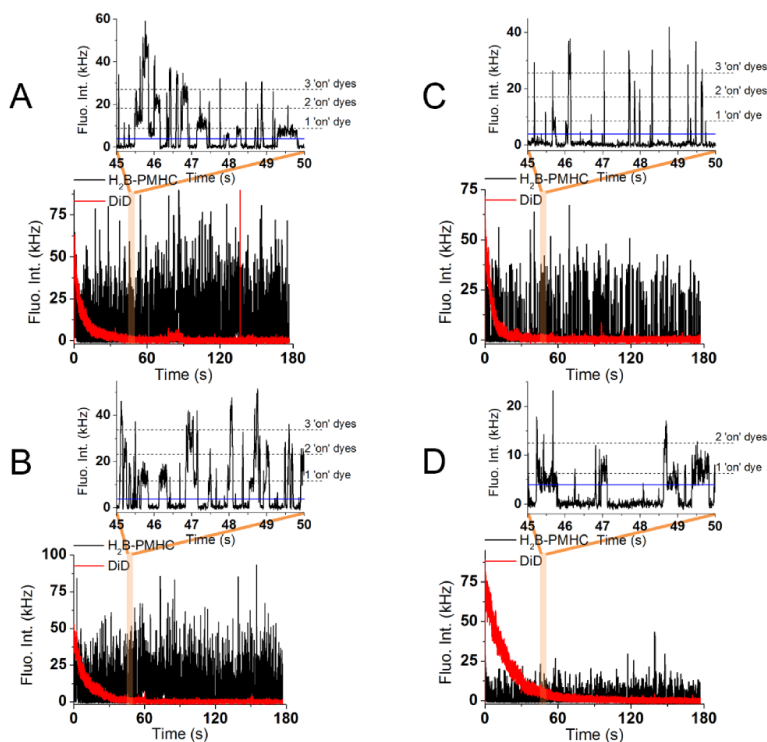


Figure 2.15. Fluorescence intensity-time trajectories recorded for single liposomes prepared with different antioxidant additives: (A) no antioxidants (-PT); (B) PMHC only (+P); (C) Trolox only (+T); and (D) both PMHC and Trolox (+PT). Trajectories were specifically chosen on the basis of having initial DiD intensities close to 50 kHz, i.e. they correspond to liposomes of similar size. The black lines display the intensity from the H₂B-PMHC channel and the red lines display the intensity from the DiD channel. Trajectories are from 3 minutes acquisitions while insets show a 5 s expanded region, indicated in full-length trajectories by a shaded orange area. The blue line in the insets corresponds to the threshold applied in order to count fluorescence burst events. Dashed black lines correspond to the observed intensity steps corresponding to 1, 2 or 3 H₂B-PMHC_{ox} simultaneously fluorescing. Data points are binned to 5 ms.

2.7.17. Summary of Antioxidant Additive Effects

Table 2.1. Effects of added antioxidants on ambient oxidation processes.

	Antioxidants			
	- PT	+ P	+ T	+ PT
$\langle I_{\text{H2B-PMHC}}/I_{\text{DiD}} \rangle^{\text{a}}$	0.29	0.255	0.095	0.082
	[0.25 0.32]	[0.24 0.28]	[0.08 0.11]	[0.07 0.10]
Fluorescence bursts in 3 min representative intensity-time trajectory of a liposome ^b	638	659	282	364
$\langle \text{Fluorescence bursts} \rangle$ in 3 min intensity-time trajectory of background ^c	3	75	5	11

^aMean values of Gaussian distribution of individual liposome intensity ratios obtained from confocal images. 95% confidence intervals as determined by Bootstrap analysis (10k resamples) using the bias corrected percentile method in MATLAB are indicated in square brackets. ^bNumber of fluorescence bursts in liposomes of similar sizes (similar initial DiD intensity) were determined from a single 3 minute long trajectory for each condition (shown in Figure 2.14), where the laser was parked on a region containing a liposomes as determined from the DiD channel image under 633 nm excitation. ^cThe average number of bursts was calculated for 1 or 2 separate 3 minutes trajectories acquired by parking the laser on a region containing no liposomes as determined from the absence of DiD emission under 633 nm excitation.

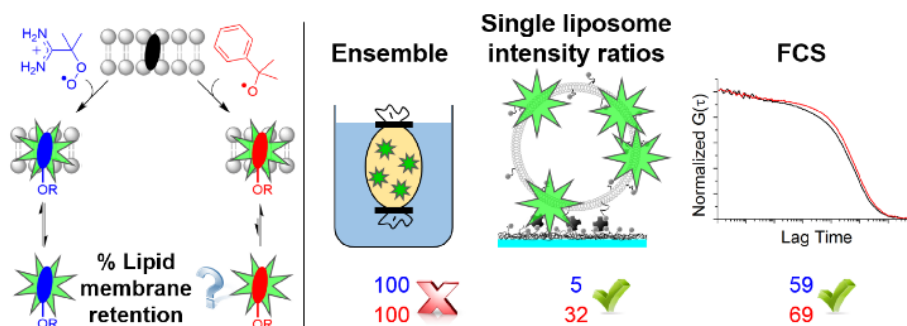
2.8. References

- (1) Yin, H.; Xu, L.; Porter, N. A., Free Radical Lipid Peroxidation: Mechanisms and Analysis. *Chem. Rev.* **2011**, *111*, 5944-5972.
- (2) Barnham, K. J.; Masters, C. L.; Bush, A. I., Neurodegenerative Diseases and Oxidative Stress. *Nat. Rev. Drug Discov.* **2004**, *3*, 205-214.
- (3) Bowry, V. W.; Ingold, K. U., The Unexpected Role of Vitamin E (α -Tocopherol) in the Peroxidation of Human Low-Density Lipoprotein. *Acc. Chem. Res.* **1999**, *32*, 27-34.
- (4) Kagan, V. E.; Fabisiak, J. P.; Shvedova, A. A.; Tyurina, Y. Y.; Tyurin, V. A.; Schor, N. F.; Kawai, K., Oxidative Signaling Pathway for Externalization of Plasma Membrane Phosphatidylserine During Apoptosis. *FEBS Lett.* **2000**, *477*, 1-7.
- (5) Schopfer, F. J.; Cipollina, C.; Freeman, B. A., Formation and Signaling Actions of Electrophilic Lipids. *Chem. Rev.* **2011**, *111*, 5997-6021.
- (6) West, R.; Panagabko, C.; Atkinson, J., Synthesis and Characterization of Bodipy- α -Tocopherol: A Fluorescent Form of Vitamin E. *J. Org. Chem.* **2010**, *75*, 2883-2892.
- (7) Winterbourn, C. C., Reconciling the Chemistry and Biology of Reactive Oxygen Species. *Nat Chem. Bio.* **2008**, *4*, 278-286.
- (8) Rudolph, T. K.; Freeman, B. A., Transduction of Redox Signaling by Electrophile-Protein Reactions. *Sci. Signal.* **2009**, *2*, re7.
- (9) Waraho, T.; McClements, D. J.; Decker, E. A., Mechanisms of Lipid Oxidation in Food Dispersions. *Trends Food Sci. Tech.* **2011**, *22*, 3-13.
- (10) Niki, E.; Noguchi, N., Dynamics of Antioxidant Action of Vitamin E. *Acc. Chem. Res.* **2003**, *37*, 45-51.
- (11) Burton, G. W.; Ingold, K. U., Vitamin E: Application of the Principles of Physical Organic Chemistry to the Exploration of Its Structure and Function. *Acc. Chem. Res.* **1986**, *19*, 194-201.
- (12) Alessi, M.; Paul, T.; Scaiano, J. C.; Ingold, K. U., The Contrasting Kinetics of Peroxidation of Vitamin E-Containing Phospholipid Unilamellar Vesicles and Human Low-Density Lipoprotein1. *J. Am. Chem. Soc.* **2002**, *124*, 6957-6965.
- (13) Oleynik, P.; Ishihara, Y.; Cosa, G., Design and Synthesis of a Bodipy- α -Tocopherol Adduct for Use as an Off/on Fluorescent Antioxidant Indicator. *J. Am. Chem. Soc.* **2007**, *129*, 1842-1843.
- (14) Krumova, K.; Oleynik, P.; Karam, P.; Cosa, G., Phenol-Based Lipophilic Fluorescent Antioxidant Indicators: A Rational Approach. *J. Org. Chem.* **2009**, *74*, 3641-3651.
- (15) Krumova, K.; Friedland, S.; Cosa, G., How Lipid Unsaturation, Peroxyl Radical Partitioning, and Chromanol Lipophilic Tail Affect the Antioxidant Activity of α -Tocopherol: Direct Visualization Via High-Throughput Fluorescence Studies Conducted with Fluorogenic α -Tocopherol Analogues. *J. Am. Chem. Soc.* **2012**, *134*, 10102-10113.
- (16) Krumova, K.; Cosa, G., Bodipy Dyes with Tunable Redox Potentials and Functional Groups for Further Tethering: Preparation, Electrochemical, and Spectroscopic Characterization. *J. Am. Chem. Soc.* **2010**, *132*, 17560-17569.
- (17) Khatchadourian, A.; Krumova, K.; Boridy, S.; Ngo, A. T.; Maysinger, D.; Cosa, G., Molecular Imaging of Lipid Peroxyl Radicals in Living Cells with a Bodipy α -Tocopherol Adduct. *Biochemistry* **2009**, *48*, 5658-5668.
- (18) Krumova, K.; Greene, L. E.; Cosa, G., Fluorogenic α -Tocopherol Analogue for Monitoring the Antioxidant Status within the Inner Mitochondrial Membrane of Live Cells. *J. Am. Chem. Soc.* **2013**, *135*, 17135-17143.

- (19) Zang, L.; Liu, R.; Holman, M. W.; Nguyen, K. T.; Adams, D. M., A Single-Molecule Probe Based on Intramolecular Electron Transfer. *J. Am. Chem. Soc.* **2002**, *124*, 10640-10641.
- (20) Naito, K.; Tachikawa, T.; Fujitsuka, M.; Majima, T., Single-Molecule Fluorescence Imaging of the Remote TiO₂ Photocatalytic Oxidation. *J. Phys. Chem. B* **2005**, *109*, 23138-23140.
- (21) Cordes, T.; Blum, S. A., Opportunities and Challenges in Single-Molecule and Single-Particle Fluorescence Microscopy for Mechanistic Studies of Chemical Reactions. *Nat. Chem.* **2013**, *5*, 993-999.
- (22) Torchilin, V. P.; Weissig, V., *Liposomes*. 2nd ed.; Oxford University Press Inc. : NY, 2003; Vol. 264.
- (23) Ngo, A. T.; Karam, P.; Fuller, E.; Burger, M.; Cosa, G., Liposome Encapsulation of Conjugated Polyelectrolytes: Toward a Liposome Beacon. *J. Am. Chem. Soc.* **2008**, *130*, 457-459.
- (24) Boukobza, E.; Sonnenfeld, A.; Haran, G., Immobilization in Surface-Tethered Lipid Vesicles as a New Tool for Single Biomolecule Spectroscopy. *J. Phys. Chem. B* **2001**, *105*, 12165-12170.
- (25) Cisse, I.; Okumus, B.; Joo, C.; Ha, T., Fueling Protein-DNA Interactions inside Porous Nanocontainers. *Proc. Natl. Acad. Sci. U. S. A* **2007**, *104*, 12646-12650.
- (26) Stamou, D.; Duschl, C.; Delamarche, E.; Vogel, H., Self-Assembled Microarrays of Attoliter Molecular Vessels. *Angew. Chem. Int. Ed. Engl.* **2003**, *42*, 5580-5583.
- (27) Larsen, J.; Hatzakis, N. S.; Stamou, D., Observation of Inhomogeneity in the Lipid Composition of Individual Nanoscale Liposomes. *J. Am. Chem. Soc.* **2011**, *133*, 10685-10687.
- (28) Elizondo, E.; Larsen, J.; Hatzakis, N. S.; Cabrera, I.; Bjørnholm, T.; Veciana, J.; Stamou, D.; Ventosa, N., Influence of the Preparation Route on the Supramolecular Organization of Lipids in a Vesicular System. *J. Am. Chem. Soc.* **2011**, *134*, 1918-1921.
- (29) Li, B.; Harjani, J. R.; Cormier, N. S.; Madarati, H.; Atkinson, J.; Cosa, G.; Pratt, D. A., Besting Vitamin E: Sidechain Substitution Is Key to the Reactivity of Naphthyridinol Antioxidants in Lipid Bilayers. *J. Am. Chem. Soc.* **2012**, *135*, 1394-1405.
- (30) De Cremer, G.; Sels, B. F.; De Vos, D. E.; Hofkens, J.; Roefsaers, M. B. J., Fluorescence Micro(Spectro)Scopy as a Tool to Study Catalytic Materials in Action. *Chem. Soc. Rev.* **2010**, *39*, 4703-4717.
- (31) Rust, M. J.; Bates, M.; Zhuang, X., Sub-Diffraction-Limit Imaging by Stochastic Optical Reconstruction Microscopy (STORM). *Nat. Methods* **2006**, *3*, 793-796.
- (32) Tachikawa, T.; Majima, T., Single-Molecule, Single-Particle Approaches for Exploring the Structure and Kinetics of Nanocatalysts. *Langmuir* **2012**, *28*, 8933-8943.
- (33) Maillard, B.; Ingold, K. U.; Scaiano, J. C., Rate Constants for the Reactions of Free Radicals with Oxygen in Solution. *J. Am. Chem. Soc.* **1983**, *105*, 5095-5099.
- (34) Terentyeva, T. G.; Engelkamp, H.; Rowan, A. E.; Komatsuzaki, T.; Hofkens, J.; Li, C.-B.; Blank, K., Dynamic Disorder in Single-Enzyme Experiments: Facts and Artifacts. *ACS Nano* **2011**, *6*, 346-354.

3. Dye Lipophilicity and Retention in Lipid Membranes: Implications for Single-Molecule Spectroscopy

Reprinted with permission from Robert Godin, Hsiao-Wei Liu, Laura Smith and Gonzalo Cosa, *Langmuir*, **2014**, 30, 11138–11146. Copyright 2014 American Chemical Society.



3.1. Preface

In Chapter 2 we showed that H₂B-PMHC is a useful probe to monitor oxidation taking place in heterogeneous lipid membranes. When those experiments were initially conducted, we were using H₂B-TOH as the fluorogenic antioxidant since H₂B-PMHC had not yet been developed. While the structural differences are minor (the methylene bridge in H₂B-PMHC is replaced by an ester link in H₂B-TOH), attempts to visualize oxidation and fluorescence enhancement in individual liposomes using H₂B-TOH were unsuccessful. We determined that there were significant differences in the lipid partitioning of H₂B-PMHC and H₂B-TOH that impacted the observation of fluorescence enhancement under SM conditions.

Here in Chapter 3 we describe the development of a rapid fluorescence correlation spectroscopy (FCS) method to determine the fraction of dye that remains associated with the lipid membrane of a liposome under highly dilute conditions. We compared the lipid partitioning of our fluorogenic antioxidants (H₂B-PMHC, H₂B-TOH, and the ethyl-substituted B-TOH) to BODIPYs with hydrophilic substituents (the alcohol H₂B-OH) and hydrophobic substituents (Et₂B-CO₂C₉ bearing a long alkyl chain). A total of four methods to assess the lipid partitioning were explored. Two were based on ensemble techniques that require concentrated samples (fluorescence quenching and dialysis) while two were performed under dilute conditions appropriate for single molecule experiments (single particle confocal imaging and FCS). On one hand, little distinction between dyes was possible with the ensemble methods. On the other hand, significant differences in dye retention in liposomes were seen with the single liposome techniques. These results highlight the fact that the fraction of lipid-associated dyes is dependent on the lipid concentration and that FCS provides a robust, rapid method to establish partition ratios of fluorescent compounds. We additionally conducted experiments using different initiators to generate either

hydrophilic or hydrophobic reactive oxygen species (ROS). The SM methods reported clear differences in the retention of activated probes in the liposomes, indicating transfer of the lipid partitioning preference of the various ROS utilized to the oxidized probe. We thus show that the reaction partner can influence the localization of a chemical probe after activation. This result has significance for chemical sensing in lipid environments, particularly cellular imaging where parallel reaction pathways for probe activation may exist.

3.2. Abstract

Fluorescence studies of individual lipid vesicles rely on the proper positioning of probes in the lipid milieu. This is true for both positional tags and chemoselective fluorogenic probes that undergo chemical modification following reaction with an analyte of interest within the lipid environment. The present report describes lipophilicity and localization estimations for a series of BODIPY dyes bearing substituents of varying hydrophobicity. We also studied fluorogenic trap-reporter probes that undergo fluorescence emission enhancement upon trapping of reactive oxygen species (ROS), including lipid peroxy radicals. We show that caution has to be taken to extrapolate ensemble partition measurements of dyes to the single-molecule regime as a result of the dramatically different lipid concentration prevailing in ensemble versus single-molecule experiments. We show that the mole fraction of dyes that remains embedded in liposomes during a typical single-molecule experiment may be accurately determined from a ratiometric single-particle imaging analysis. We further demonstrate that fluorescence correlation spectroscopy (FCS) provides a very rapid and reliable estimate of the lipophilic nature of a given dye under highly dilute single-molecule-like conditions. Our combined single-particle spectroscopy and FCS experiments suggest that the minimal mole fraction of membrane-associated dyes (x_m) as determined from FCS experiments is about 0.5 for adequate dye retention during single molecule imaging in lipid membranes. Our work further highlights the dramatic effect that chemical modifications can have on chemoselective fluorogenic probe localization.

3.3. Introduction

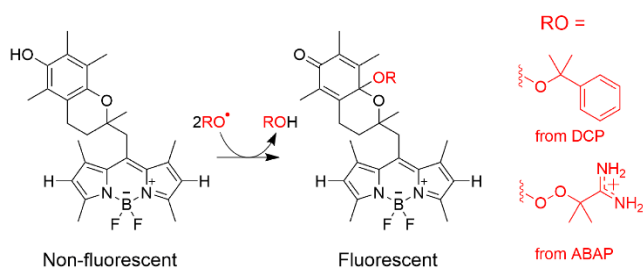
Lipid membranes play crucial roles in the segregation of cellular components, are actively involved in sensory and signaling pathways, provide the scaffolding where key cellular processes such as energy production take place, and also tune the activity of membrane-bound proteins.¹⁻²

Additionally, lipid membranes have multiple applications in drug delivery³ and as nanoreactors.⁴ Despite the major advances in studying heterogeneous lipid structures, many challenges arise because of the nature of solute partitioning within these structures. In this regard, fluorescence microscopy has provided tremendous insight on the nature of membrane heterogeneity.⁵ In particular, single-particle studies conducted on giant unilamellar vesicles have highlighted the preferential partitioning of dyes based on the lipid phase.⁶ More recently, the inhomogeneity of the lipid composition in single liposomes was demonstrated by single-particle studies on surface-immobilized liposomes incorporating two different fluorescent dyes.⁷⁻⁸ The above studies relied on positional tags, essentially emissive fluorophores that reported their preferential localization within different regions in the membrane, or acted as beacons that provided a reliable count of dye distribution.

The advent of chemoselective probes for bioimaging that rely on the induction/enhancement of fluorescence upon reaction with a molecular entity⁹⁻¹¹ provide new opportunities for exploring the lipid membrane environment. In combination with single-molecule spectroscopy techniques, the new probes offer the opportunity to decipher relevant reaction mechanisms in a lipid membrane. Experiments may be carried out with infinitesimal amounts of probe, minimizing the potential impact on membrane structures and biological processes.¹² The new probes and single-molecule methodologies however introduce new challenges in the data interpretation. In this regard, our group has developed a number of fluorogenic trap-reporter probes to monitor the presence of reactive oxygen species (ROS) at the molecular (or nano-) and cellular levels with unprecedented spatiotemporal resolution and sensitivity, at the single-molecule level (see Scheme 3.1 for the overall reaction mechanism in the presence of peroxy and alkoxy free radicals).¹³⁻¹⁹ Initial studies intended to monitor ROS under single-molecule conditions

however showed a disappointing outcome for some of our probes. Explicitly, limited fluorescence enhancement was recorded at the single-molecule level in contrast to the large fluorescence enhancements recorded under ensemble conditions.¹⁹ We suspected that the single molecule conditions, where fluorophore and liposome concentrations are in the picomolar range and where the ratio of $[H_2O]/[lipid]$ is above 1×10^7 , favored probe dissociation from the lipid membrane. We reasoned that, at these low concentrations, the probes may tend to leak out of the lipid membrane and diffuse into the bulk aqueous phase. Diffusion away from the detection volume would result in the loss of detected fluorescence intensity. This observation prompted us to further investigate fluorescent solute retention in liposomal membranes under single-molecule conditions.

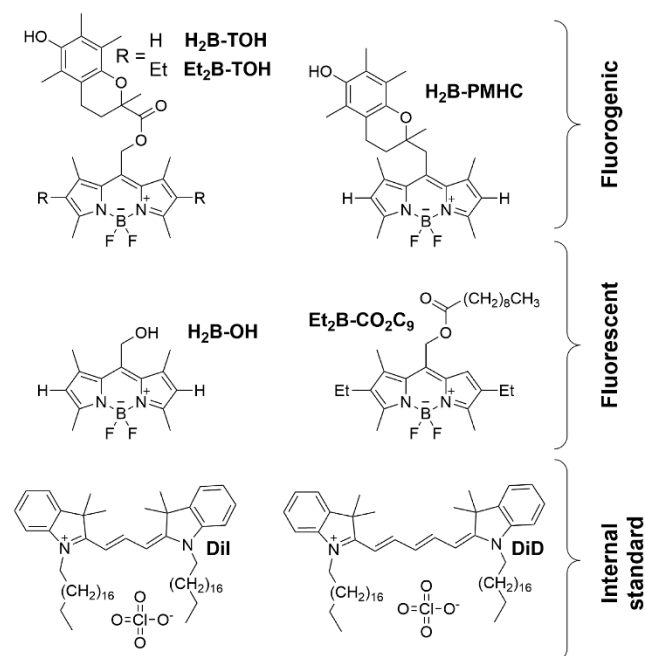
Scheme 3.1. Oxidation of a BODIPY-based trap-reporter probe by ROS generated upon photolysis of dicumyl peroxide (DCP) or 2,2'-azobis(2-methylpropanimidine) dihydrochloride (ABAP) in air equilibrated solutions.²⁰⁻²¹



In this report, we describe our lipophilicity and localization estimations for a series of BODIPY dyes, including fluorogenic trap-reporter probes prepared with a BODIPY dye (reporter segment) tagged through different linkers to a chromanol moiety (trap segment), see Chart 3.1. Our motivation was to find a simple, straightforward way to verify if a given probe has adequate lipophilic character to be used under single-molecule conditions without ambiguous interpretation. In particular, high dye retention in the lipid membrane is desirable to ensure that, when working with fluorogenic chemoselective probes, the sensitivity is not diminished because of leakage of the fluorescent product from the lipid environment. Two ensemble techniques, namely,

fluorescence quenching and dialysis, were applied. The results were compared to those acquired under single-molecule conditions, where we exploited a ratiometric analysis to reveal the underlying tendency of each dye to associate with lipid membranes. Fluorescence correlation spectroscopy (FCS) data were also acquired as a mean to gain a reliable estimate of the lipophilic nature of a given dye under highly dilute single-molecule-like conditions.

Chart 3.1. Names and structures of the dyes and probes used.



On the basis of our work reported herein, we suggest the use of FCS to rapidly and properly assess the tendency of a probe to remain embedded in a lipid membrane under particularly dilute conditions. We highlight the contrast between ensemble and single-molecule conditions toward retention of fluorophores in the liposomal membrane, a result of the dramatically different lipid concentration prevailing in both types of experiments. Additionally, we report the significant influence that chemical transformations of a trap segment in a fluorogenic probe can have on the lipophilicity and localization of trap-reporter-type probes.

3.4. Experimental Section

3.4.1. Materials

All chemicals were used as received without further purification. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(biotinyl) sodium salt (biotin-DPPE) lipids were purchased from Avanti Polar Lipids (Alabaster, AL). 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (ABAP), bovine serum albumin (BSA), ($\pm$)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), methyl viologen (MV), and 2,2,5,7,8-pentamethyl-6-chromanol (PMHC) were obtained from Sigma-Aldrich (St. Louis, MO). Chloroform and acetone [high-performance liquid chromatography (HPLC) grade] and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer were bought from Fischer Scientific (Waltham, MA). NaCl stock solution (5 M, Ambion brand) and streptavidin were purchased from Life Technologies (Burlington, Ontario, Canada). Molecular-biology-grade water was acquired from Thermo Scientific HyClone (South Logan, UT). 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiI) were purchased from Invitrogen Canada (Burlington, Ontario, Canada). Vectabond was purchased from Vector Laboratories (Burlington, Ontario, Canada). Poly(ethylene glycol) succinimidyl valerate (molecular weight = 5000; mPEG-SVA) and biotin-PEG-SVA were purchased from Laysan Bio, Inc. (Arab, AL). Materials for sample chambers were purchased from Grace Bio-Labs (Bend, OR). Et₂B-CO₂C₉ was synthesized according to the procedure described in the Supporting Information. Other BODIPY dyes were synthesized according to previously published procedures (H₂B-OH,¹⁶ Et₂B-TOH,¹³ H₂B-TOH, and H₂B-PMHC¹⁷).

3.4.2. Liposome Preparation

Typical liposome preparation involved adding appropriate dye solutions in acetonitrile or ethanol to DOPC and biotin-DPPE (1 mol % versus DOPC) lipids dissolved in chloroform. The total BODIPY dye loading per liposome (assuming 75 000 DOPC molecules per 100 nm diameter liposome)²² was 10 for ensemble dye retention, confocal imaging, and FCS studies or 30 for ensemble quenching studies. This yielded a dye/lipid molar ratio of ~ 0.01 and 0.04% , respectively. Samples prepared for confocal imaging experiments additionally contained 50 DiD molecules per liposome. The lipids and additives were first dried *in vacuo* and then rehydrated in HEPES buffer (10 mM HEPES and 150 mM NaCl at pH 7.3) to yield 20 mM DOPC solutions. Small unilamellar vesicles were then formed by 5 consecutive freeze/thaw (dry ice/sonicator bath) cycles and extrusion through a 100 nm pore diameter polycarbonate membrane. We note that, for ensemble quenching experiments, Et₂B–TOH and H₂B–TOH were added to the liposomes after extrusion, as opposed to the lipid mixture before drying and rehydration.

3.4.3. Activation of Fluorogenic BODIPY Probes

The fluorogenic trap–reporter probes were oxidized/activated by photolysis of either dicumyl peroxide (DCP, 100 μ M) or the azo-initiator ABAP (1–10 mM) to a 20 $\times$ diluted suspension of liposomes (see also Scheme 3.1). Irradiation was performed within a fluorometer to generate ROS upon exciting with ultraviolet (UV) light (263 nm for DCP and 263 or 370 nm for ABAP; 2.4 mW/cm² at 263 nm and 7.1 mW/cm² at 370 nm). The fluorescence intensity at the emission maxima of the probes was monitored over time. UV irradiation was stopped when the fluorescence intensity enhancement (ca. 30-fold for H₂B–TOH, 3-fold for Et₂B–TOH, and up to 100-fold for H₂B–PMHC) reached its maximum. The fluorescence intensity of activated probes was the same when using ABAP or DCP as the initiator.

3.4.4. Fluorescence Quenching Experiments

Fluorescence emission spectra were obtained for ensemble liposome solutions (diluted 20× to a final concentration of 1 mM in lipids) in $1 \times 1 \text{ cm}^2$ quartz cuvettes before and after the addition of either a hydrophilic (MV) or hydrophobic (PMHC) quencher. The fluorescence intensity was calculated by integration of the emission spectra in each case and corrected for dilution when the quencher was added. A Stern–Volmer analysis was initially used to interpret fluorescence quenching results (see equation (3.1))

$$\frac{I_0}{I} = 1 + K_{SV}[Q] \quad (3.1)$$

where I_0 is the initial fluorescence intensity, I is the fluorescence intensity at any given quencher concentration $[Q]$, and K_{SV} is the Stern–Volmer constant. A linear fit to the data yields a straight line with a slope of K_{SV} when either static or dynamic quenching is the sole operative mechanism. The observation of a plateau is expected when there exists an unquenchable fraction of fluorophores. In this case, the quencher accessible fraction (f_a) may be extracted from the intercept in a modified Stern–Volmer analysis,²³ recommended when a plateau (saturation) is not clearly distinguishable (see equation (3.2)).

$$\frac{I_0}{I_0 - I} = \frac{1}{f_a K_a [Q]} + \frac{1}{f_a} \quad (3.2)$$

This method was applied to extract the fraction of dye accessible to either lipophilic or hydrophilic quenchers for the BODIPY dyes under study. We note that deviation from linearity (downward curvature) in the modified Stern–Volmer analysis suggests that the inaccessible fraction is in fact affected by the addition of the quencher. This leads to a higher apparent f_a . Downward curvatures are observed in most cases and are attributed to heterogeneous localization of the fluorophores associated with the lipid membrane, which gives rise to a range of quenching

efficiencies. Consequently, the aim of the ensemble quenching experiments is to give a qualitative idea of the localization of the fluorophores relative to the lipid membrane of liposomes.

3.4.5. Dye Retention during Dialysis

Initial liposome preparations (20 mM lipid, 10 dyes/liposome) were diluted 20× and divided into two 2 mL portions. One part was loaded in a small dialysis device (20 kDa molecular weight cutoff, retained liposomes) and dialyzed against 44.5 mL of HEPES buffer, while the other was kept in a cuvette in the dark as a control. Dye retention in liposomes under ensemble conditions (1 mM DOPC and 133 nM liposomes) was calculated by the difference in fluorescence intensity between the dialyzed liposome solution and the control. The loss of fluorescence in the dialyzed sample, corrected for dilution because of osmotic pressure, was attributed to BODIPY dyes escaping from the liposomal membrane and migrating to the bulk aqueous phase.

3.4.6. Single-Particle Confocal Fluorescence Imaging

The retention of BODIPY dyes within a liposomal membrane under single-molecule conditions was determined using a dual-channel single-particle imaging strategy. To minimize the effect of liposome size distribution, a ratiometric approach was chosen.¹⁹ A red lipophilic stain (DiD) was present in all samples as the internal standard. The BODIPY emission channel covered the spectral range of 530–600 nm, while it was 645–725 nm for DiD. The ratio of the BODIPY/DiD fluorescence intensities was measured for each individual liposome, and histograms were built next to extract an average from the Gaussian fitting. To avoid photobleaching effects, each image was acquired on a fresh new area of the sample. Typically three different regions were imaged for each condition, yielding ca. 550 liposomes that were next combined in the analysis. The fluorescence intensity ratio was converted to percent retention by calibration with a highly lipophilic BODIPY compound (Et₂B–CO₂C₉), for which we assumed quantitative retention in the

lipid membrane. Retention was calculated for the other BODIPY dyes taking into account their spectral properties to correct for different emission intensities in the BODIPY channel upon excitation with the 514 nm laser line (see Table 3.2). Further corrections were applied to take into account green to red channel crosstalk (Figure 3.8) and fluorescence resonance energy transfer (FRET) between BODIPY dyes and DiD. FRET efficiencies of 29 and 13% were measured with Et₂B–CO₂C₉ and H₂B–PMHC_{ox}. No FRET correction was applied to H₂B–OH because it dissociates from the lipid membrane under these experimental conditions.

A suitable bottom-up self-assembly strategy was used to immobilize the liposomes for confocal imaging. The strategy involved the tethering of liposomes containing biotinylated lipids (biotin-DPPE, 1 mol % versus DOPC) to a streptavidin-coated coverslip. Briefly, coverslips were submerged in Piranha solution (1:3 30% H₂O₂/concentrated H₂SO₄ by volume) for 1 h; next, they were rinsed with water and acetone. Subsequently, they were treated with Vectabond [2% (v/v) in acetone, 5 min reaction] to yield an aminosilanized surface. In the following step, we proceeded to the covalent tethering of a mixture of mPEG-SVA (25% by weight in H₂O) and biotin-PEG-SVA (0.25% by weight) to the aminosilanized surface. The PEG solution was rinsed after 3 h of reaction, and the sample chamber was closed by affixing a polycarbonate cap with two small openings. PEG-treated coverslips were used within a week of preparation. On the day of the experiment, a solution of streptavidin (0.2 mg/mL) flowed over the coverslip and the excess protein was rinsed off with HEPES buffer after 5 min of incubation. The liposome sample (2000× dilution, 10 μM in lipids) containing on average 10 BODIPY fluorophores, which were activated in the case of the fluorogenic probes, and 50 DiD stains per liposome, was next flowed through the sample chamber at a rate of 2 μL/min until adequate liposome surface binding was observed (10 min or less). The chamber was finally rinsed again with HEPES buffer to remove any non-

tethered liposomes, and a constant flow of buffer (2 $\mu\text{L}/\text{min}$) was maintained during imaging. Typically, 30 min elapsed between liposome extrusion and acquisition of the first image ($t = 0$ h). Images of the BODIPY and DiD channels were acquired simultaneously with two independent detectors following excitation with a single laser line (514 nm). More details are provided in the Supporting Information (Single Particle Confocal Microscopy Imaging of Individual Liposomes).

We note that, by working with a single lipid rather than a mixture of lipids in the liposome formulation, we expect the number of either DiD or BODIPY dyes found in each liposome to follow a Poissonian distribution. Any heterogeneity in liposome-to-liposome dye concentration, manifested as deviations from Poissonian distributions observed with lipid mixtures,⁷ is expected to be minimal in our case.

3.4.7. FCS Measurements

Sample chambers were made by sticking a spacer on top of the coverslip, followed by a polycarbonate cap with two small openings. A solution of bovine serum albumin (BSA, 1 mg/mL) was then flowed through the sample chamber to passivate the surface to prevent non-specific binding of liposomes. Thorough rinsing with HEPES buffer was performed before the liposome solution was loaded in the chamber. Intensity–time trajectories of freely diffusing 1 nM liposome solutions were acquired by focusing the 514 nm laser excitation (70 kW/cm^2 output at the objective) 50 μm above the glass–water interface. The emission (530–600 nm window) was collected by the same objective and split onto two avalanche photodiode (APD) detectors with a 50:50 non-polarizing beamsplitter in pseudo-cross-correlation format.²⁴ This configuration was used to observe microsecond dynamics that are obscured by detector afterpulsing in a single-detector setup. The autocorrelation function was then software-calculated from a 10 min long point acquisition. The shape of the confocal observation volume was experimentally determined using

a 10 nM rhodamine B solution in HEPES buffer. The structural parameter extracted from fitting the FCS trace of rhodamine B in our setup was fixed for the fitting of all other samples. Photobleaching in the laser focus was observed for liposome-associated dyes, which resulted in faster apparent diffusion times. Importantly, the amplitude of FCS traces and, thus, the calculation of the mole fraction of dyes associated with the lipid membrane (x_m) were not affected by photobleaching.

3.5. Results and Discussion

3.5.1. Ensemble Fluorescence Quenching

We initially conducted fluorescence quenching experiments to assess the location of the various fluorescent compounds, i.e., lipid versus aqueous environment. The fluorescence intensity of the probes was recorded upon increasing amounts of either a hydrophobic quencher (PMHC) or a hydrophilic quencher (MV). We expect PMHC to embed within the lipid membrane and quench the fluorescence of fluorophores that are associated with the membrane, while MV will only quench the fluorescence of fluorophores free in the bulk aqueous solution or sufficiently close to the lipid/water interface. We used a modified Stern–Volmer analysis²³ to determine the quencher accessible fraction (f_a) for each fluorophore under study, which is the fraction of emission intensity that is quenched once the sample is saturated with a quencher.

Figure 3.1 displays the results that we obtained with H₂B–OH in a liposome formulation. Similar plots for the other BODIPY fluorophores studied are found in Figure 3.9. On the basis of the results, which show a quantitative quenching by MV ($f_a \sim 1$) and 60% quenching by PMHC ($f_a \sim 0.59$), we concluded that H₂B–OH is found both in the bulk aqueous solution and weakly associated with the lipid membrane. H₂B–OH is more efficiently quenched by MV and quenched to a lesser extent by PMHC compared to the other fluorophores studied, namely, Et₂B–CO₂C₉,

oxidized H₂B–TOH (H₂B–TOH_{ox}), Et₂B–TOH_{ox}, and H₂B–PMHC_{ox} (see Table 3.1). This reflects the lack of a substitution pattern rich in hydrocarbons for H₂B–OH compared to the other BODIPYs (see Chart 3.1).

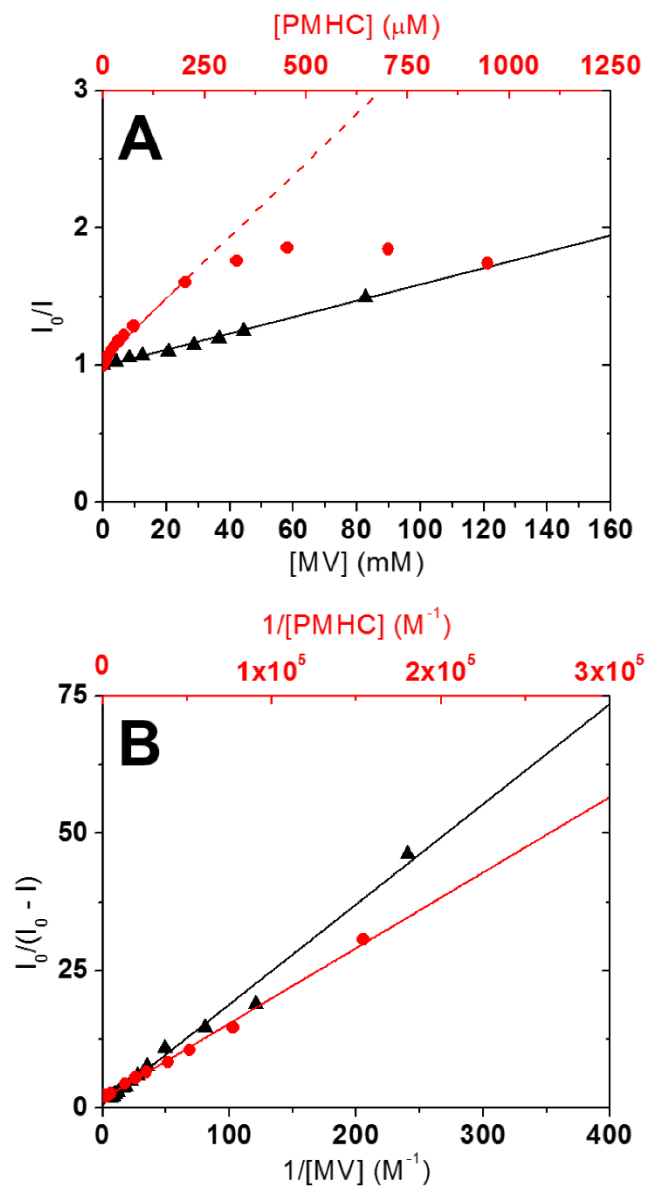


Figure 3.1. Ensemble fluorescence quenching of liposome preparation containing H₂B–OH. Standard Stern-Volmer (A) and modified Stern-Volmer (B) analyses and corresponding linear fits are shown for MV (black triangle) and PMHC (red circles) quenchers. Full lines extend over the selected fitting range, while dashed lines prolong the fits as a visual guide.

Table 3.1. Lipophilicity estimates from ensemble and single molecules techniques.

Dye	Quenching		Dialysis	Confocal Imaging	FCS
	MV f_a (%)	PMHC f_a (%)	% retention ^a	% retention ^{a,b}	x_m (%) ^{c,d}
H ₂ B-OH	~ 100	59	92	~ 0	13 ± 1
H ₂ B-TOH _{ox} ABAP	-	-	-	-	33 ± 1
H ₂ B-TOH _{ox} DCP	8	~ 100	-	-	32 ± 1
Et ₂ B-TOH _{ox} ABAP	-	-	-	-	36 ± 3
Et ₂ B-TOH _{ox} DCP	~ 0	~ 100	-	-	~ 100
H ₂ B-PMHC _{ox} ABAP	16	90	~ 100	5 ± 2	59 ± 2
H ₂ B-PMHC _{ox} DCP	9	~ 100	~ 100	32 ± 6	69 ± 2
Et ₂ B-CO ₂ C ₉	~ 0	~ 100	~ 100	~ 100	~ 100

^a % of dyes which remain associated to the lipid membrane after 4 hours. ^bErrors estimated from Bootstrap analysis (10k resamples) of Gaussian fitting of histograms. ^cMole fraction of dyes (represented as %) associated to the lipid membrane as determined by FCS amplitudes. ^dErrors were estimated from the standard deviation of x_m values recovered from fitting of three independent 3-minute sections of the acquired 10-minute time trajectory.

The fluorescence intensity of Et₂B–CO₂C₉ was, in turn, completely quenched by PMHC ($f_a \sim 1$), while no fluorescence quenching was observed upon addition of increasing amounts of MV ($f_a \sim 0$). Presumably Et₂B–CO₂C₉ lies deep within the lipid membrane, with no apparent diffusion in the bulk aqueous phase. This observation is in line with expectations that a long alkyl substituent will greatly enhance the lipophilicity of the BODIPY dye anchoring the fluorophore to the lipid membrane.

For the activated H₂B–TOH (H₂B–TOH_{ox} DCP) and Et₂B–TOH (Et₂B–TOH_{ox} DCP) probes, the quencher accessible fraction with PMHC was $f_a \sim 1$. No quenching of Et₂B–TOH_{ox} DCP ($f_a \sim 0$) was observed with MV in the liposome formulation. However, when MV was used as the quencher with H₂B–TOH_{ox} DCP, non-negligible quenching was observed ($f_a \sim 0.1$). Subtle changes

are thus observed in going from a H substitution to a ethyl substitution in the BODIPY segment of reporter-trap probes.

Results obtained with $\text{H}_2\text{B-PMHC}_{\text{ox DCP}}$ also show quantitative quenching with increasing concentrations of PMHC ($f_a \sim 1$). The MV accessible fraction was $\sim 9\%$ in this case, comparable to what was found for $\text{H}_2\text{B-TOH}_{\text{ox DCP}}$. Diffusion of $\text{H}_2\text{B-PMHC}_{\text{ox DCP}}$ into the bulk aqueous phase is inconsistent with the observed quantitative PMHC quenching, which leads us to propose that the activated probe stays relatively close to the lipid headgroups, accessible to MV, but still associated with the lipid membrane. Quenching experiments were also performed on $\text{H}_2\text{B-PMHC}$ oxidized by ABAP-derived ROS ($\text{H}_2\text{B-PMHC}_{\text{ox ABAP}}$). In comparison to results where the probe was oxidized by the use of DCP, we found a larger MV-accessible fraction ($f_a \sim 0.16$) and a lower PMHC-accessible fraction ($f_a \sim 0.9$). We argue that the hydrophilicity of the ABAP-generated peroxy radical, which couples to $\text{H}_2\text{B-PMHC}$ during oxidation yields a more hydrophilic adduct compared to that formed upon reaction with a DCP-generated alkoxyl radical (see Scheme 3.1). This point is further demonstrated by the single-particle spectroscopy experiments (see below).

3.5.2. Ensemble Dye Retention

We next conducted ensemble dye retention studies that confirm the relative low lipophilicity of $\text{H}_2\text{B-OH}$ compared to the other BODIPY fluorophores. Water/liposome partitioning was evaluated by dialyzing a DOPC liposome solution (1 mM DOPC and 133 nM liposome) containing 10 dyes per liposome against a blank HEPES buffer for 4 h. Only the sample with $\text{H}_2\text{B-OH}$ showed BODIPY fluorescence in the dialysis buffer after the 4 h experiment (Figure 3.2). $\text{Et}_2\text{B-CO}_2\text{C}_9$, $\text{H}_2\text{B-PMHC}_{\text{ox DCP}}$, and $\text{H}_2\text{B-PMHC}_{\text{ox ABAP}}$ all behaved in the same manner during dialysis: no fluorophores leaked into the aqueous fraction, while an 8% drop in fluorescence intensity of the liposome solution was observed in comparison to a non-dialyzed control sample.

The 8% drop is rationalized by the osmotic pressure arising from the liposomes, causing buffer to flow into the liposome compartment, diluting the sample. Taking into account the dilution effect, it was established that 8% of the $\text{H}_2\text{B-OH}$ fluorophores were found in the aqueous phase after dialysis based on the decrease in fluorescence intensity of the liposome sample. The ensemble dye retention studies, summarized in Table 3.1, highlight the difficulty in distinguishing dyes with high lipophilicity from each other ($\text{Et}_2\text{B-CO}_2\text{C}_9$, $\text{H}_2\text{B-PMHC}_{\text{ox DCP}}$, and $\text{H}_2\text{B-PMHC}_{\text{ox ABAP}}$) based on ensemble techniques.

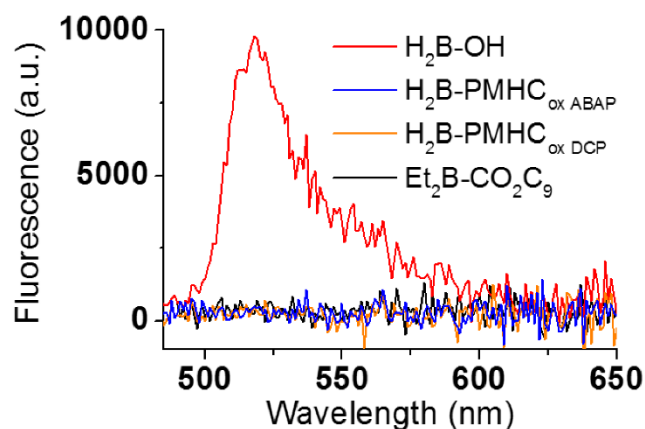


Figure 3.2. Fluorescence intensity measured in the dialysis buffer after 4-hour dialysis of a liposome suspension. The liposome solutions each contained on average 10 dyes per liposome (133 nM liposomes).

3.5.3. Single-Particle Confocal Fluorescence Imaging

We subsequently performed single-particle confocal imaging studies to visualize and quantify the propensity of fluorophores to leak out of liposomes under single-molecule conditions (see Figure 3.3). Our experimental strategy resorted on a ratiometric analysis, where the highly lipophilic fluorophore DiD^{25} is introduced in the lipid fraction together with any given BODIPY dye under study. By simultaneously monitoring both fluorophores in an individual, surface-immobilized liposome in parallel channels [a green emission channel and a red emission channel

where only BODIPY and DiD are detected, respectively (see also Figure 3.8)], we may then report the relative amount of BODIPY to DiD in liposomes over time. Given that BODIPY dyes may leak from the liposome membrane, individual liposomes were identified on the basis of the presence of suitably emissive diffraction-limited spots in the red (DiD) channel. Following imaging by raster scanning of the sample (panels A and B of Figure 3.3 for the green and red emission channels, respectively), the BODIPY/DiD fluorescence intensity ratios for individual liposomes were calculated and compiled into a histogram (Figure 3.3C). The mean value of the resulting Gaussian distribution was taken as the representative BODIPY/DiD fluorescence intensity ratio of the sample.

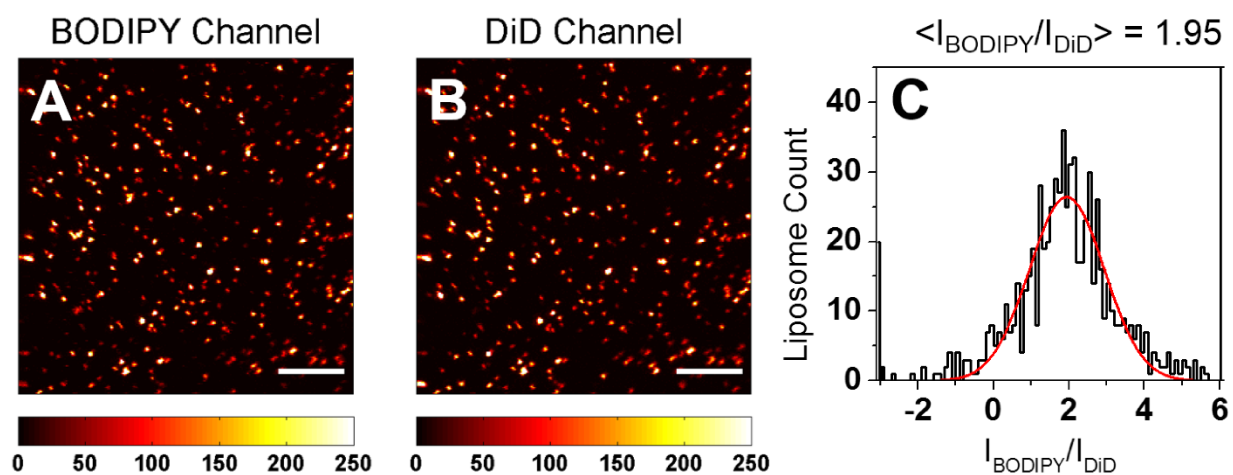


Figure 3.3. Panels A and B show fluorescence intensity images corresponding to the BODIPY and DiD emission channels, respectively. Images were acquired for a $\text{Et}_2\text{B-CO}_2\text{C}_9$ liposome sample immediately after immobilization on the coverslip ($t = 0$ min). Images were simultaneously acquired using the 514 nm output of an Ar^+ laser (5700 W cm^{-2}). Scale bars are $6 \mu\text{m}$. Panel C shows the histogram constructed from 3 separate $30 \times 30 \mu\text{m}^2$ images and the Gaussian fitting used to determine the representative fluorescence ratio, shown above the plot.

Figure 3.4 and Table 3.1 summarize single-liposome confocal imaging results acquired periodically for up to 4 h. We recorded the highest BODIPY/DiD fluorescence intensity ratio of ~ 1.95 when $\text{Et}_2\text{B-CO}_2\text{C}_9$ was used. We further observed no decrease in either intensity channel

throughout the 4 h single-particle experiment. This latter observation is consistent with both dyes remaining anchored in the lipid membrane and draws a parallel with results from the ensemble experiments that show a high affinity of Et₂B–CO₂C₉ for the lipid environment.

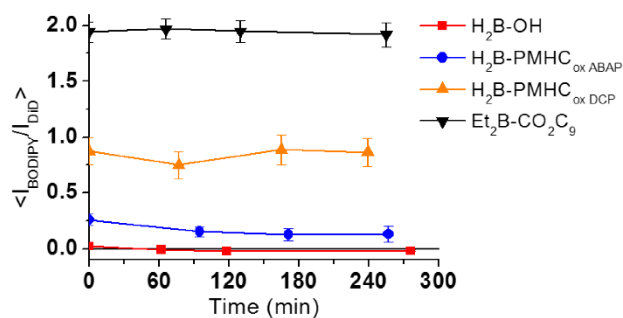


Figure 3.4. Representative BODIPY/DiD fluorescence ratios following Gaussian analyses of single liposome confocal images taken over time. Error bars indicate 95% confidence intervals as determined by Bootstrap error analysis (10k resamples) using bias corrected percentile intervals. Errors for H₂B–OH are within the square symbols.

We used the fluorescence ratio of 1.95 as a benchmark value to calculate the percentage of other BODIPY dyes remaining in the lipid membrane in single-particle studies, where we assumed 100% for Et₂B–CO₂C₉. To compare the Et₂B–CO₂C₉/DiD fluorescence ratio to that of other dyes, the relative brightness in the BODIPY channel of each BODIPY dye studied by single-particle imaging was measured. From these values, the observed BODIPY/DiD ratio can be converted to percent retention in the lipid membrane for each dye (see Table 3.2).

H₂B–OH shows low initial fluorescence intensity in the BODIPY channel (30 min after dilution). The intensity dropped to background levels; i.e., no H₂B–OH was found in the liposome membrane, within 2 h of acquiring the first image. Evidently, H₂B–OH dissociated from the lipid membrane and diffused into the aqueous bulk phase. Ensemble fluorescence quenching experiments revealed that a fraction of 60% of the dye was near the lipid/water interface and was readily quenched by lipophilic PMHC. This discrepancy in ensemble versus single-molecule

results may be easily reconciled upon recognizing that, under single-molecule conditions, where the lipid fraction is minute (immobilized liposome and lipid concentrations of ca. 1 pM and 75 nM, respectively), the equilibrium shifts to freely diffusing dye. For comparison, the liposome and lipid concentrations are 13 nM and 1 mM under ensemble conditions used for quenching and dialysis experiments.

Turning our attention to oxidized H₂B–PMHC, a marked difference in fluorescence ratios is observed for H₂B–PMHC_{ox} depending upon the nature of the oxidizing agent employed. The reaction of the amidinium peroxy radical and H₂B–PMHC yielded an adduct that is more hydrophilic (positively charged at pH 7.3) than the corresponding H₂B–PMHC and cumyloxyl radical adduct (see Scheme 3.1). Thus, when oxidation occurred from DCP-derived alkoxyl radicals, a third of the oxidized probe remained anchored in the membrane. In contrast, initially only 9% of the oxidized probe was associated with liposomal membrane when ABAP-derived peroxy radicals reacted with H₂B–PMHC. This fraction decreased to 5% after about 3 h, at which point it remained constant.

Interestingly, most of the change in the H₂B–PMHC_{ox} in the membrane occurred under short time scales (< 30 min), with relatively little sample evolution afterward. This suggests initial dissociation of more hydrophilic species from the liposomes upon dilution, followed by slower leakage of less hydrophilic species. We postulate that the 5% fraction of H₂B–PMHC_{ABAP ox}, which remains in the membrane at long time scales, arises from the reaction of the fluorogenic probe with a lipid-derived peroxy radical formed upon reaction of the amidinium peroxy radical with DOPC. Although a minor pathway, the amidinium peroxy radicals can react directly with DOPC, instead of the fluorogenic antioxidant, to yield lipid peroxy radicals in the presence of O₂.²⁶⁻²⁷ The lipid chain in the product would greatly enhance the lipophilicity of the activated probe, anchoring the

molecule in the liposomal membrane. A similar situation is plausible in the case of H₂B-PMHC_{DCP ox}, where some of the oxidized probe may bear a lipid chain.

3.5.4. FCS

We finally conducted FCS experiments²⁸⁻³⁰ to evaluate the potential of this technique to provide a rapid assessment of the tendency of dyes or probes to remain in the lipid membrane under single-molecule conditions. The concentrations used in FCS (low nanomolar to picomolar) are similar to those used during single-molecule imaging. FCS relies on the observation of a small volume, typically a few femtoliters, which is defined by the excitation and detection optics. Diffusing fluorophores are detected by single-photon counting detectors as they traverse the observation volume. This leads to fluctuations in the observed photon counts because of the variation of the number of fluorophores present in the confocal volume at any given time. The fluctuations are time-correlated, and their analysis provides information on diffusion times (size related, millisecond time scale) and excited state dynamics (fluorophore related, nanosecond to microsecond time scale). Additionally, the amplitude of the correlation curve provides information on the concentration of species whose intensity fluctuates with a given relaxation time.

We reasoned that FCS would be extremely powerful in quantifying the extent of free dye and membrane-embedded dye for any given BODIPY under study.³¹⁻³³ Dyes embedded within the liposome membrane will diffuse at a much slower rate than free dyes in solution because of the large size discrepancy of individual molecules (roughly 1 nm length) versus liposomes (100 nm diameter). The different diffusion rates characterizing these two populations (membrane-embedded and free) will, in turn, result in experimentally distinguishable FCS amplitudes.³⁴

Figure 3.5A displays simulated FCS traces, where fluctuations are due to two different diffusive processes: rapid (free dye) and slow (dye embedded in liposome) diffusion. The change

in shape of the autocorrelation function at different free/liposome-embedded dye ratios is demonstrated. Figure 3.5B shows normalized autocorrelation traces experimentally acquired with the various BODIPY dyes under study. The acquisitions were performed on HEPES solutions of either dye or dye/liposome preparations. Contrary to the single-particle experiments, no surface immobilization was employed in FCS experiments. The amplitude and relaxation time characteristic to the autocorrelation fluctuations were extracted by fitting the traces assuming one photophysical process (triplet state population) and two diffusion processes (small molecules and large particles). The triplet state dynamics were observed in the microsecond time domain, while solution-based diffusion of free dyes and liposome-embedded dyes were recorded in the sub-millisecond and millisecond time domains, respectively. The mole fraction of dyes associated with the lipid membrane (x_m) was calculated from the amplitude parameters of the diffusive processes (see Section 3.8.3).^{23, 33} We remark that the sensitivity in determining x_m by FCS and the associated errors will be influenced both by the value of x_m and the initial concentration of dyes per liposome. Values of x_m closer to 1 will be harder to distinguish, while the initial concentration of dyes will dictate if greater sensitivity will be observed for low or high values of x_m , as presented in Figure 3.7.

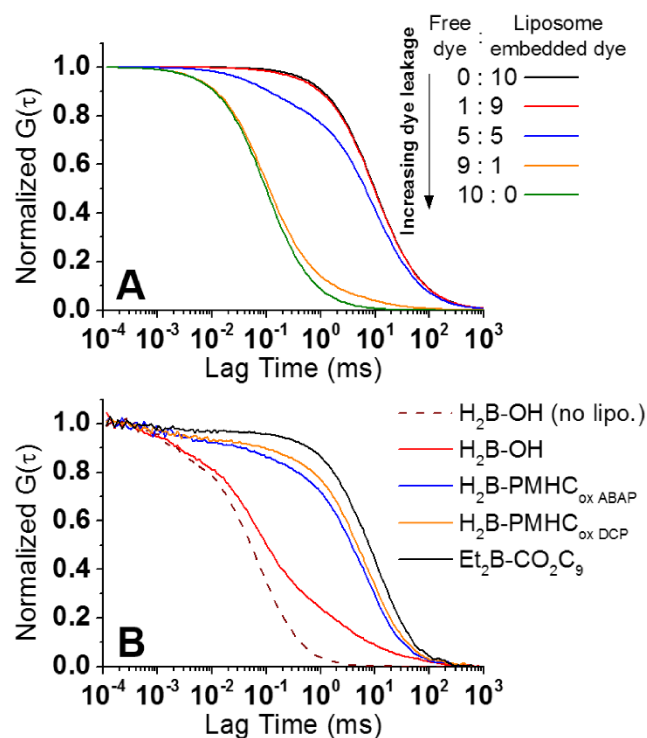


Figure 3.5. Panel A shows simulated normalized correlation function traces. Two components were simulated with different diffusion times (0.1 ms for free dye, 10 ms for liposome-embedded dye) and their ratios were varied according to the legend. Panel B shows normalized autocorrelation traces of selected samples acquired as quickly as possible after dilution ($t = 0$ h) (see Figure 3.10 for all traces). Dye only samples are depicted by a dashed line while dye/liposome samples are shown as full lines. Traces were obtained following a continuous 10-minute acquisition with the 70 kW/cm² output of a 514 nm laser.

The experimental FCS curve obtained with $Et_2B-CO_2C_9$ embedded in liposomes shows a single slow diffusive process with a diffusion time of 7.9 ms. The diffusion time is consistent with that measured for the DiI lipid stain (12.9 ms) and much longer than that recorded for rhodamine B in buffer solution (0.096 ms). This is in line with $Et_2B-CO_2C_9$ being embedded within the membrane. The addition of a second diffusion term did not improve the fitting, implying that no freely diffusing dye is present in the sample and supports the assumption that $Et_2B-CO_2C_9$ remained quantitatively embedded in the liposomal membrane. The mole fraction of dyes associated with the lipid membrane (x_m) was accordingly estimated to be ~ 1 .

Samples of H₂B–OH with and without liposomes were prepared to compare dye behavior in the presence or absence of liposomes. A satisfactory fitting of the H₂B–OH FCS trace acquired without liposomes is obtained from a model with a single fast diffusion term, consistent with freely diffusing dyes. On the other hand, to fit the H₂B–OH FCS trace acquired with liposomes, the addition of a slow diffusion term was necessary, pointing to dye–liposome interactions. Quantitative analysis of the FCS results yielded a value for x_m of 0.13 based on the fitted amplitudes of free dyes and liposome-associated dyes. FCS experiments thus show that 13% of the H₂B–OH dyes are associated with lipid membrane. In contrast, the ensemble quenching experiment estimated ~60% association with the membrane, and the dialysis experiment showed 92% dye retention in the membrane, while the confocal imaging returned ~0%. The results show the decreasing lipid fraction encountered in going from ensemble experiments (1 mM lipids) to FCS (75 μ M lipids) to confocal imaging (immobilized liposomes with ca. 75 nM lipids).

Oxidation of the H₂B–PMHC probe by DCP- or ABAP-generated ROS had an effect consistent with the confocal imaging observations: a higher amplitude of the slow diffusion component was seen with DCP, in line with the formation of more lipophilic products. Fitting for both H₂B–PMHC_{ox} samples required diffusion terms for both free dyes and membrane-associated dyes. This is in accordance with the confocal imaging experiments, where less than 100% of the dyes were found to remain in the lipid membrane, indicating that the presence of freely diffusing fluorophores is expected. The quantitative FCS analysis yielded $x_m = 0.69$ for H₂B–PMHC_{ox} DCP and $x_m = 0.59$ for H₂B–PMHC_{ox} ABAP.

The ethyl-substituted fluorogenic probe (Et₂B–TOH) showed an even stronger differentiation in its FCS profile upon oxidation by either ABAP or DCP initiators. Et₂B–TOH_{ox} DCP was found to be quantitatively associated with the liposomes, while for Et₂B–

TOH_{ox} ABAP, only 36% was calculated to be interacting with lipid membrane. We can appreciate that the influence of the oxidizing species does not only have an effect for H₂B–PMHC but is most likely a general phenomenon.

3.5.5. Summary

Single-molecule spectroscopy holds great strength in the fact that experiments can be carried out with infinitesimal amounts of probe, minimizing the potential impact on membrane structures and biological processes.¹² However, care should be taken when choosing lipophilic fluorophores for single-molecule studies. We have demonstrated that ensemble techniques do not portray an accurate picture of the tendency of lipophilic dyes and probes to remain embedded in a lipid membrane under dilute single-molecule conditions. FCS was not only successful in differentiating dye retention tendencies for different BODIPY dyes in lipid membranes but could also discriminate between the lipophilic and hydrophilic reaction products of fluorogenic probes following their activation.

The observed differences of the retention of dyes in lipid membranes are due to decreasing lipid concentrations when moving to single-molecule conditions (see Figure 3.6). At typical ensemble total lipid concentrations (>100 μ M), discrimination of the mole fraction of membrane-bound dyes for lipid/water partition constants (K_m) of 10^7 or greater is difficult ($x_m > 0.99$). However, under single-molecule conditions where the total lipid concentration is usually under 1 μ M, very little membrane-associated dye will be observed for systems with partition constants of $<10^7$. At these lipid concentrations, in contrast to ensemble conditions, partition coefficients between 10^7 and 10^9 will show large differences in x_m . Dyes and membrane solutes in general, which show very high membrane retention when studied by ensemble techniques, may not nearly display such a large proportion of probe within the lipid membrane when diluted to single-

molecule conditions. Dilution of the lipids should be taken into account to conclusively say if a chosen probe will be retained in the membrane.

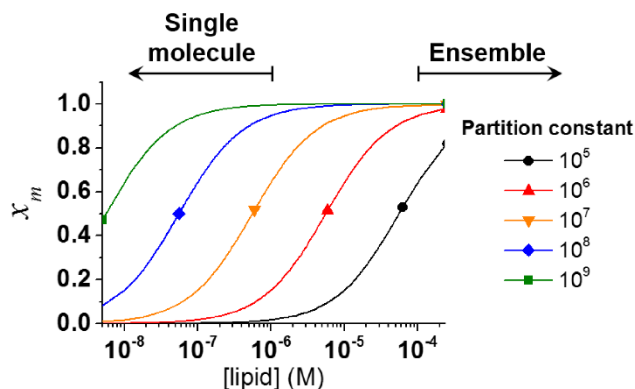


Figure 3.6. Dependence of the mole fraction of membrane-bound dye on the total lipid concentration and the partition constant of the system calculated from $x_m = K_m[\text{lipid}]/([\text{H}_2\text{O}] + K_m[\text{lipid}])$.³⁵

We also note that the vanishingly small rate of collision between liposomes and free dyes under single-molecule conditions may prevent the system from reaching thermodynamic equilibrium, where the rate of dye embedding into the lipid membrane (rate_{in}) must be equal to the rate of dye dissociating from the lipid membrane (rate_{out}). The rate at which dye embeds into the lipid membrane will be limited by the rate of collision between free dyes and liposomes ($\text{rate}_{\text{in max}} = k_d[\text{dye}]_{\text{free}}[\text{liposome}]_{\text{total}} < 1 \times 10^{-13} \text{ M s}^{-1}$).³⁶ Typical single-molecule imaging conditions also involve a constant flow of imaging buffer. Therefore, once a solute is released from the membrane into the bulk aqueous phase, it will tend to be washed away by the flow of imaging buffer before colliding with a liposome, making rate_{in} tend toward 0. On the other hand, the rate of dye dissociating from the lipid membrane ($\text{rate}_{\text{out}} = k_{\text{out}}n_{\text{dye/liposome}}[\text{dye-liposome}]$) will always be larger than 0 as long as some dye-liposome complex is present. Under these conditions ($\text{rate}_{\text{out}} > 0$, and $\text{rate}_{\text{in}} \sim 0$), the intrinsic rate of dissociation from the lipid membrane k_{out} may play a more dominant

role than the partition constant in determining if a particular solute will be retained during a single-molecule imaging experiment.

3.6. Conclusion

When designing single-molecule experiments to study lipid membranes, one naturally looks for a probe that will have a high affinity for the lipid milieu. Although many ensemble techniques have been developed to determine the partition constant of a solute within lipid membranes,³⁷⁻³⁸ the relatively high lipid concentrations typically used for these experiments make it difficult to distinguish between probes with very high values of partition constants. We have demonstrated that care should be exercised in predicting dye retention in lipid membranes under single-molecule imaging conditions based on ensemble measurements. Considering that control experiments involving a single-molecule imaging setup can be tedious, we propose FCS experiments as a simple, rapid technique to determine if a dye will be retained in lipid membranes under the extremely dilute conditions encountered in single-molecule spectroscopy. Our combined single-particle spectroscopy and FCS experiments lead us to recommend an x_m value, calculated from FCS experiments, of 0.5 or higher for adequate dye retention in membranes under the low lipid content characterizing single-molecule measurement conditions. Additionally, our investigations of dye retention in membranes enabled us to discover the dramatic effect that chemical modifications can have on probe localization. Changing the source of ROS from DCP (yielding the relatively lipophilic cumyloxyl radical) to ABAP (yielding a hydrophilic amidinium peroxy radical) led to a ~6-fold decrease in the amount of H₂B-PMHC_{ox} retained in the membrane as observed from individual liposome imaging. Similar changes of lipophilicity and retention in lipid membranes are expected in conditions, such as cellular environments, where many parallel chemical transformations of a probe may take place.

3.7. Acknowledgments

Gonzalo Cosa is grateful to the Natural Sciences and Engineering Research Council of Canada (NSERC) and Canadian Foundation for Innovation (CFI) for funding. Robert Godin is thankful to NSERC for a postgraduate scholarship. Hsiao-Wei Liu is thankful to the Drug Discovery and Training Program (CIHR) and GRASP (FRQS) for postdoctoral scholarships. Laura Smith is thankful to NSERC for an undergraduate scholarship. The authors also thank Dr. Katerina Krumova for the synthesis of Et₂B–CO₂C₉.

3.8. Supporting Information

The Supporting Information contains detailed experimental of individual liposome imaging and FCS setup, FCS results and development of the model used to extract the mole fraction of membrane-associated dye, BODIPY spectral characteristics and calculation of relative brightnesses in the BODIPY channel used for imaging, and synthesis of Et₂B–CO₂C₉.

3.8.1. Single Particle Confocal Microscopy Imaging of Individual Liposomes

Confocal imaging was performed on an Olympus IX-71 inverted microscope equipped with a closed-loop nanopositioning stage (Nano LP100, Mad City Labs, Madison, WI) used for imaging and sample positioning. Samples were excited by CW laser emission from either the 514 nm line from an Ar⁺ laser (43 series, Melles Griot) or the 633 nm output from a HeNe laser (Melles Griot). The linearly polarized laser beams introduced *via* a singlemode polarization maintaining fibre optic were circularly polarized by an achromatic quarter-wave plate and directed by a dichroic beamsplitter (z514-633rdc, Chroma, Rockingham, VT) to the sample through a high numerical aperture oil immersion objective (UPLSAPO 100XO, N.A. = 1.40, Olympus). Fluorescence emission was collected through the same objective, split into two channels by a second dichroic beam splitter (640DCXR, Chroma) and directed to two avalanche photodiode detectors (Perkin

Elmer Optoelectronics SPCM-AQR-14, Vaudreuil, QC). A 514 nm or 633 nm holographic Raman notch filter (Kaiser Optical Systems, Ann Arbor, MI) was placed in the emission beam path to block residual laser excitation. The BODIPY channel emission ($\lambda < 640$ nm) was cleaned by a 565/70m bandpass filter (Chroma) while the DiD channel emission ($\lambda > 640$ nm) was cleaned by a 685/80m bandpass filter (Chroma, Rockingham, VT). Images were acquired under ambient conditions with a constant flow (2 μ L/min) of HEPES buffer. Images of the BODIPY and DiD channels were acquired simultaneously with two independent detectors following excitation with a single laser line (514 nm). The 633 nm output was used to verify that the DiD-containing liposomes (strongly absorbing the 633 nm light) were the same liposomes imaged when DiD was excited by the 514 nm laser line.

3.8.2. Fluorescence Correlation Spectroscopy (FCS) Experiments

FCS acquisitions were performed on an Olympus IX-71 inverted microscope fitted with an optical-axis piezo-Z positioner (P-721 PIFOC[®], Physik Instrumente, Germany) and a nanopositioner (P-733, Physik Instrumente) controlled by a digital piezo controller (E-710, Physik Instrumente). The output of a CW HeNe laser was coupled to a singlemode, polarization maintaining fiber optic and launched into the microscope with a beam diameter of approx. 3 mm. The back aperture of a water immersion objective (UPLSAPO 60XW, N.A. = 1.20, Olympus) was underfilled in order to provide a better approximate Gaussian observation volume.²⁸ The laser excitation (70 kW/cm²) was focused 50 μ m above the glass-water interface and emission of dye-embedded liposomes and/or free dye molecules was collected through the objective. A Hanbury Brown and Twiss (HBT) intensity correlation setup was used to calculate the correlation traces where the emission output (cleaned by a 565/70m, Chroma) was split by a 50/50 non-polarising cube beam splitter (05BC17MB.1, Newport Corporation, Irvine, CA), focused onto two avalanche

photodiode detectors (SPCM-AQR-14, Perkin Elmer) in pseudo-cross-correlation format.²⁴ This setup was used to observe μ s dynamics that are obscured by detector afterpulsing in a single detector setup. Acquisition were performed using PicoHarp 300 electronics and autocorrelation functions calculated from SymPhoTime software (PicoQuant, Germany). A typical FCS trace was calculated from a 10 minute-long time point acquisition. The fitting was performed using the open source FCS fitting software PyCorrFit (v. 0.8.2). The concentration of liposomes was 1 nM, while free dye samples contained either 10 nM Rhodamine B or 1 nM H₂B-OH.

3.8.3. FCS Theory and Analysis

FCS relies on the principle that fluctuations in the observed fluorescence intensity is, in part, due to the diffusion of fluorophores through the observation volume of a confocal microscope. By measuring the fluorescence intensity over time one can construct the correlation function of fluorescence fluctuations:

$$G(\tau) = \frac{\langle \delta F(0) \delta F(\tau) \rangle}{\langle F \rangle^2} \quad (3.3)$$

where τ is the delay time, F denotes fluorescence intensity and δF denotes the difference from the mean fluorescence intensity. The correlation function is constructed from the acquired time-intensity trajectory and then fit to a model in order to extract parameters. We will now describe how we developed our chosen model and how we extracted the mole fraction of dyes associated with liposome membranes.

In a typical confocal microscope setup, a Gaussian laser excitation profile excites the fluorophores in the sample and a pinhole rejects out-of-focus light. The observation geometry can be approximated by a three-dimensional Gaussian. Solving for this geometry, we eventually obtain an expression for the correlation function for 3-dimensional diffusion:

$$G(\tau) = G(0) \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{\kappa^2 \tau_D}\right)^{-1/2} \quad (3.4)$$

with the structure parameter (κ) defined as the ratio of the long axis (ω_z) to the short axis (ω_{xy}) of the observation volume: $\kappa = \omega_z / \omega_{xy}$. The translational diffusional time of the fluorophore, τ_D , is further defined as $\tau_D = \omega_{xy}^2 / 4D$, where D is the diffusion constant of the diffusing fluorophore. Furthermore, the correlation function value at $\tau = 0$ yields the inverse of the average number of molecules in the observation volume ($G(0) = 1 / N_{eff}$). This now gives:

$$G(\tau) = \frac{1}{N_{eff}} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{\kappa^2 \tau_D}\right)^{-1/2} = \frac{1}{N_{eff}} G^D(\tau) \quad (3.5)$$

where the diffusional terms have been grouped as $G^D(\tau)$ for simplicity.

Multiple diffusion species can be distinguished in FCS only if their diffusion coefficients are sufficiently different. The minimum distinguishable ratio of diffusion times is dictated by the relative fraction and brightness of each type of species, and typically lies between ~ 2 and 10 .³⁴ When multiple species are present, the correlation function becomes:

$$G(\tau) = \frac{\sum N_i B_i^2 G_i^D(\tau)}{(\sum N_i B_i)^2} \quad (3.6)$$

The brightness of each species, B_i , will affect the relative contribution of each species to the correlation. In fact, the weighting factor is B_i^2 meaning that a species which is twice as fluorescent as another, but found in equal proportions, will contribute 4 times as much amplitude to the correlation trace. The interpretation of $G(0)$ is not as straightforward as in the single component case, but is nonetheless still defined as:²³

$$G(0) = \frac{1}{N_{app}} = \frac{\sum N_i B_i^2}{(\sum N_i B_i)^2} \quad (3.7)$$

Combining with the expression above (equation (3.6)), we obtain:

$$G(\tau) = \frac{1}{N_{app}} \cdot \frac{(\sum N_i B_i)^2}{\sum N_i B_i^2} \cdot \frac{\sum N_i B_i^2 G_i^D(\tau)}{(\sum N_i B_i)^2} = \frac{1}{N_{app}} \cdot \sum \frac{N_i B_i^2 G_i^D(\tau)}{\sum N_i B_i^2} \quad (3.8)$$

In our case, we assume that only two different species contribute to the correlation trace: free fluorophores (index *free*) and fluorophores associated with liposomes (index *lipo*). It is also helpful to express the relative brightness of components (q) as a ratio to a defined standard. By choosing the brightness of the freely diffusing fluorophores as our standard ($q_{free} \equiv 1$), the relative brightness of the liposomes (q_{lipo}) is equal to the number fluorophores that are associated with them, under the assumption that association with the lipid membrane doesn't affect the quantum yield of the fluorophore. For our specific case, we expand equation (3.8) replacing B_i with q_i to give

$$G(\tau) = \frac{1}{N_{app}} \cdot \left(\frac{N_{free}}{N_{free} + N_{lipo} q_{lipo}^2} G_{free}^D(\tau) + \frac{N_{lipo} q_{lipo}^2}{N_{free} + N_{lipo} q_{lipo}^2} G_{lipo}^D(\tau) \right) \quad (3.9)$$

where N_{free} and N_{lipo} are the average number of freely diffusing dye and freely diffusing liposomes, respectively, found in the observation volume.

Coutinho *et al.* has shown that the Poissonian distribution of fluorophores that associate with liposomes must be taken into account to correctly interpret FCS experiments as the brightness parameter will depend on the number of dyes per liposome particle.³³ The probability of finding a liposome with k embedded dyes, equivalent to the probability of finding a liposome with relative brightness equal to $k \cdot q_{free}$, will be:

$$P(k, \langle k \rangle) = \frac{\langle k \rangle^k \exp(-\langle k \rangle)}{k!} \quad (3.10)$$

where $\langle k \rangle$ is the average number of embedded fluorophore per liposome in the sample. It follows that the relative brightness, and the square of its value, of the liposome population become:

$$q_{lipo} = \sum_{k=1}^{\infty} k \cdot P(k, \langle k \rangle) = \langle k \rangle \quad (3.11)$$

$$q_{lipo}^2 = \sum_{k=1}^{\infty} k^2 \cdot P(k, \langle k \rangle) = \langle k \rangle (1 + \langle k \rangle) \quad (3.12)$$

Substituting in the description of the correlation curve (equation (3.8)), we obtain

$$G(\tau) = \frac{1}{N_{app}} \cdot \left(\frac{N_{free}}{N_{free} + N_{lipo} \langle k \rangle (1 + \langle k \rangle)} G_{free}^D(\tau) + \frac{N_{lipo} \langle k \rangle (1 + \langle k \rangle)}{N_{free} + N_{lipo} \langle k \rangle (1 + \langle k \rangle)} G_{lipo}^D(\tau) \right)$$

$$G(\tau) = \frac{1}{N_{app}} \cdot (A_{free} G_{free}^D(\tau) + A_{lipo} G_{lipo}^D(\tau)) \quad (3.13)$$

by defining the amplitudes $A_{free} = \frac{N_{free}}{N_{free} + N_{lipo} \langle k \rangle (1 + \langle k \rangle)}$ and $A_{lipo} = \frac{N_{lipo} \langle k \rangle (1 + \langle k \rangle)}{N_{free} + N_{lipo} \langle k \rangle (1 + \langle k \rangle)}$.

Exited state dynamics, such as population of the triplet state, also affect the correlation trace and an additional term needs to be added to describe the correlation function:

$$G(\tau) = \frac{1}{N_{app}} G^D(\tau) G^T(\tau) \quad (3.14)$$

where $G^T(\tau) = \left[1 + \frac{T}{1-T} \exp(-\tau/\tau_T) \right]$

Here T is the triplet fraction and τ_T is the characteristic triplet time. Experimentally, we determined the value of N_{app} from the fitted T value and experimental $G(0)$ value. Typically, triplet state parameters recovered from fitting were $\sim 10\%$ for T and $\sim 1.5 \mu s$ for τ_T .

Finally, taking diffusion and triplet dynamic terms into account, we obtain a model to describe the experimental autocorrelation curves (equation (3.15), below). The parameters A_{free} and A_{lipo} were determined based on finding the best fit to:

$$G(\tau) = \frac{1}{N_{app}} \cdot (A_{free} G_{free}^D(\tau) + A_{lipo} G_{lipo}^D(\tau)) G^T(\tau) \quad (3.15)$$

The open source software PyCorrFit (available at <http://paulmueller.github.io/PyCorrFit/>) was used to perform the fitting. Goodness of fits were evaluated by the reduced sum of squares, which ranged from 1.2×10^{-3} to 1.8×10^{-6} . In some cases, best fits were found for a model which included only a single diffusing species, which corresponded to either liposome with embedded dyes or freely diffusing dyes. The diffusion time of the liposome component varied from ~ 3 to 13 ms. We attribute these differences to variations in rates of photobleaching in the dye series. Certainly diffusion/photobleaching may not be deconvoluted in this case. For the free dyes, the diffusion times ranged from 20 μs to 78 μs . Variations in free dye diffusion times could be due to the different dye structures. Nonetheless, setting the diffusion time to 62 μs , that of free H₂B-OH in buffer, does not affect the experimental amplitudes (A_{free} and A_{lipo}) extracted from the fits nor does it affect the calculated results of the membrane interacting dye fraction.

The mole fraction of dyes that interact with the liposomal membrane can be determined from the correlation function amplitude corresponding to the liposome-associated component (A_{lipo}). The definition of x_m , which is as the number of liposome-embedded dyes divided by the total number of dye in solution. In terms of N_{free} , N_{lipo} and $\langle k \rangle$, can mathematically be written as:

$$x_m = \frac{N_{lipo} \langle k \rangle}{N_{free} + N_{lipo} \langle k \rangle} \quad (3.16)$$

Using this definition, we can rewrite A_{lipo} as:

$$A_{lipo} = \frac{N_{lipo} \langle k \rangle (1 + \langle k \rangle)}{N_{free} + N_{lipo} \langle k \rangle (1 + \langle k \rangle)} = \frac{1 + \langle k \rangle}{\frac{N_{free} + N_{lipo} \langle k \rangle}{N_{lipo} \langle k \rangle} + \frac{N_{lipo} \langle k \rangle^2}{N_{lipo} \langle k \rangle}} = \frac{1 + \langle k \rangle}{\frac{1}{x_m} + \langle k \rangle} = \frac{(1 + \langle k \rangle) x_m}{1 + \langle k \rangle x_m} \quad (3.17)$$

Since we controlled the liposome preparation, the initial amount of dye per liposome was set to 10, and accordingly $\langle k \rangle_0 = 10$. The remaining fraction of membrane interacting dyes can be deduced from:

$$x_m = \frac{\langle k \rangle}{\langle k \rangle_0} = \frac{\langle k \rangle}{10} \quad (3.18)$$

By substituting out $\langle k \rangle$ from equation (3.17), equation (3.18) finally leads us to:

$$A_{lipo} = \frac{(1 + \langle k \rangle)x_m}{1 + \langle k \rangle x_m} = \frac{(1 + x_m \langle k \rangle_0)x_m}{1 + \langle k \rangle_0 x_m^2} \quad (3.19)$$

We calculated the mole fraction of liposome-associated dyes by solving the quadratic equation using the amplitude A_{lipo} returned from FCS fitting and knowledge of $\langle k \rangle_0$. We mention that the choice of $\langle k \rangle_0$ will impact the sensitivity of x_m to changes in A_{lipo} , especially for high values of x_m . This is illustrated in Figure 3.7 which shows plots of x_m vs. A_{lipo} for different values of $\langle k \rangle_0$.

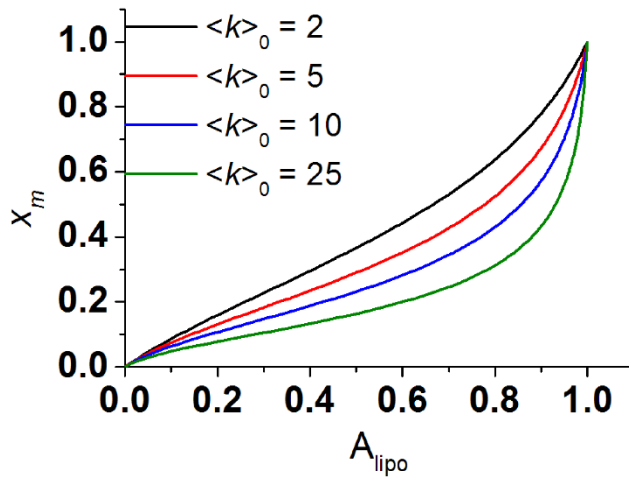


Figure 3.7. Dependence of x_m and A_{lipo} relationship on changes to then initial number of dyes per liposome ($\langle k \rangle_0$). Lower $\langle k \rangle_0$ gives better sensitivity for values of x_m closer to 1, while higher $\langle k \rangle_0$ gives better sensitivity for lower values of x_m .

3.8.4. *Synthesis of 8-(Decanoyloxy)-methyl-2,6-diethyl-1,3,5,7-tetramethyl
Pyrromethene Fluoroborate (Et₂B-CO₂C₉)*

8-Hydroxymethyl-2,6-diethyl-1,3,5,7-tetramethyl pyrromethene fluoroborate (Et₂B-OH, prepared as described in the literature^{13, 39} (0.05 g, 0.15 mmol, 1 eq) was dissolved in 5 mL dry dichloromethane at room temperature under argon. Diisopropylethylamine (183 μ L, 1.05 mmol, 7 eq) was added to the solution. The reaction mixture was cooled to 0 °C and decanoyl chloride (0.75 mmol, 155 μ L, 5 eq) was added drop-wise. The reaction mixture was left stirring at room temperature for 4 h or until all the Et₂B-OH was consumed. The solution was diluted with dichloromethane and washed twice with saturated aqueous NaHCO₃ and once with brine. The organic layer was dried with MgSO₄ and the solvent was next evaporated under reduced pressure. The solid residue was loaded onto a silica gel flash column and eluted with hexanes/ethyl acetate 4/1 and the product was obtained as red oil (0.069 g, 0.14 mmol, 95 % yield).

3.8.5. BODIPY and DiD Spectral Discrimination

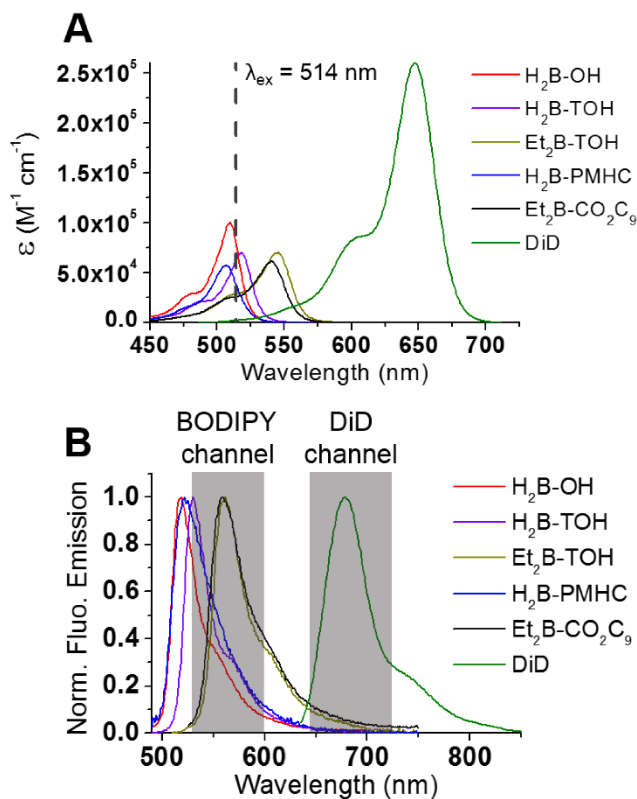
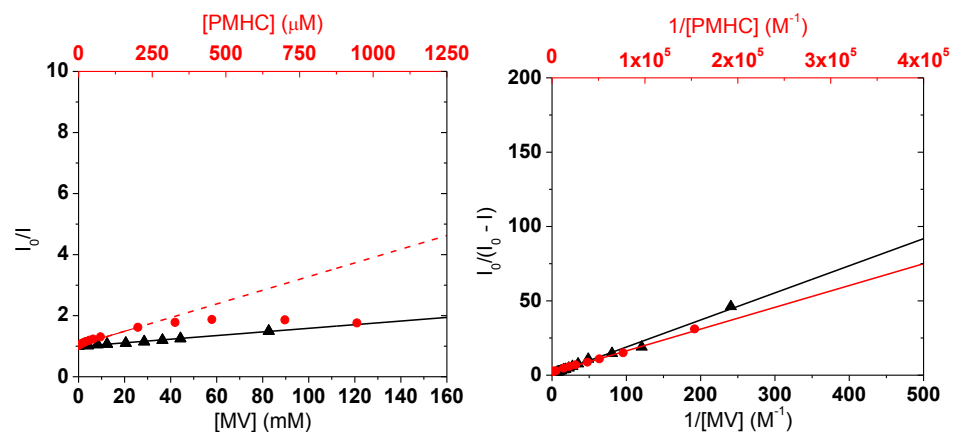
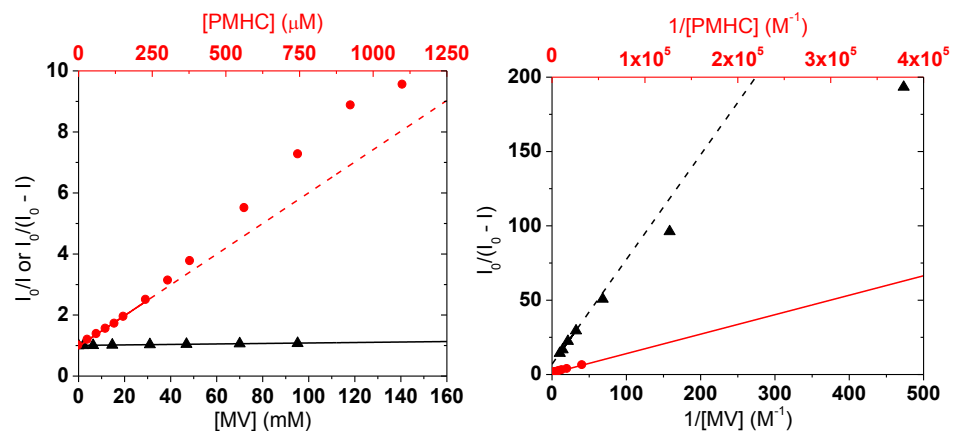
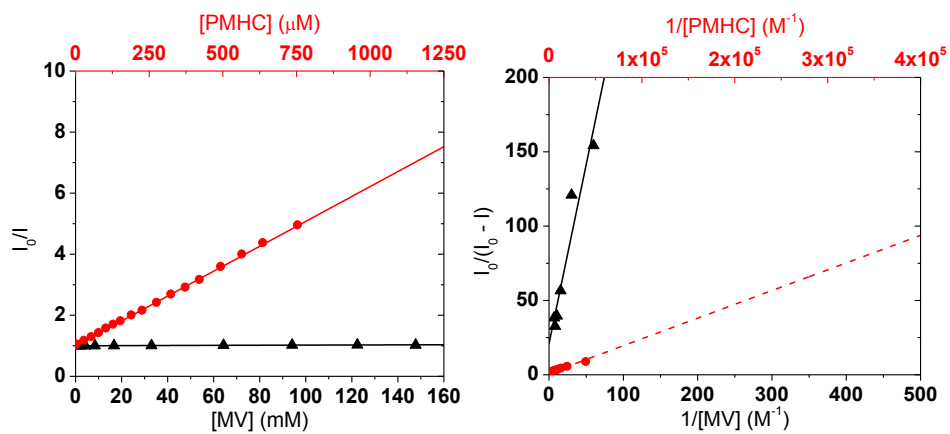
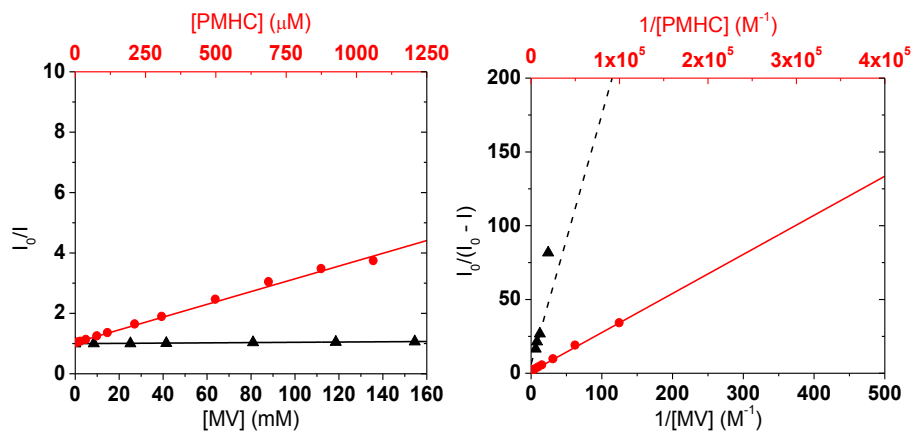


Figure 3.8. Absorbance (A, top) and fluorescence (B, bottom) spectra of BODIPY dyes and probes and DiD. BODIPYs and DiD show good spectral separation and their emission can be split and detected by two separate channels (BODIPY channel 530 – 600 nm; DiD channel 645 – 725 nm). The 514 nm output of an Ar^+ laser was used to excite the BODIPYs and also DiD at the blue edge of its absorbance spectrum. Crosstalk from BODIPYs to the DiD channel was 7% for $H_2B-PMHC$, 10% for $Et_2B-CO_2C_9$ and 3% for H_2B-OH . On the other hand, negligible DiD to BODIPY channel crosstalk was observed with our setup.

3.8.6. Ensemble Quenching Results

H₂B-OH**H₂B-TOH_{ox} DCP**

Et₂B-TOH_{ox} DCP**H₂B-PMHC_{ox} ABAP**

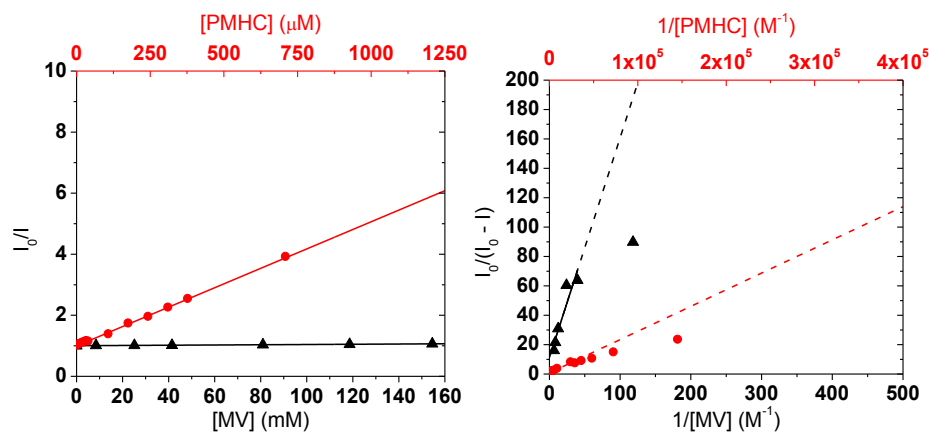
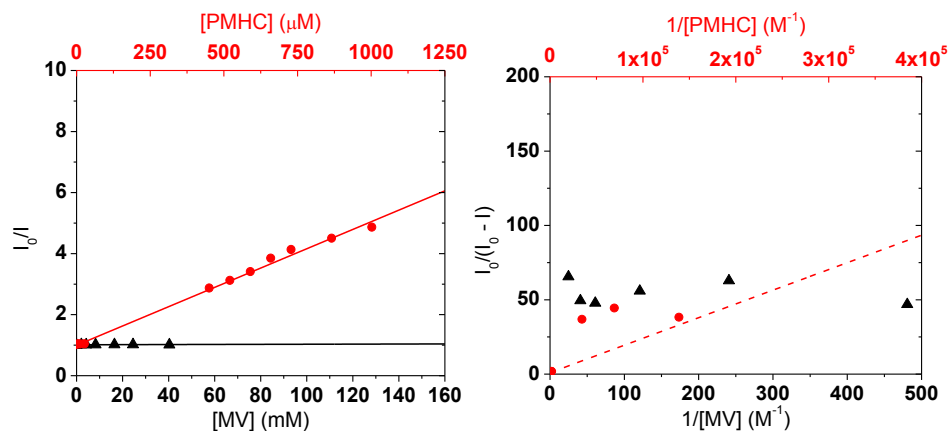
H₂B-PMHC_{ox} DPC**Et₂B-CO₂C₉**

Figure 3.9. Ensemble fluorescence quenching of liposome preparations containing BODIPY fluorophores. Standard Stern-Volmer (left) and modified Stern-Volmer (right) analyses and corresponding linear fits are shown for MV (black triangles) and PMHC (red circles) quenchers. Full lines extend over the selected fitting range, while dashed lines prolong the fits as a visual guide.

3.8.7. Calculation of % Retention in Lipid Membrane from Confocal Single Particle Imaging

In order to extract the % of dyes that are associated with liposome at each imaging time point, we relied on the assumption that the highly lipophilic Et₂B-CO₂C₉ was quantitatively embedded in the membrane after sample preparation and dilution. Samples containing 10 BODIPY/liposome were prepared and their fluorescence intensity measured in a fluorometer between 530 – 600 nm (corresponding to the BODIPY channel used in single particle imaging). The relative emission intensity of each BODIPY to Et₂B-CO₂C₉ was determined by integration of the fluorescence intensity in this spectral window. Finally, we converted the observed BODIPY/DiD fluorescence intensity ratios to % dyes retained in the membrane with the information about the relative brightness of BODIPYs following this relation:

$$\% retention_{dye} = \frac{\langle I_{BODIPY}/I_{DiD} \rangle_{dye}}{\langle I_{BODIPY}/I_{DiD} \rangle_{Et_2B-CO_2C_9} \times relative\ brightness_{dye}}$$

Table 3.2. Parameter used to determine % retention in membrane from confocal images.

Dye	Exp. $\langle I_{BODIPY}/I_{DiD} \rangle$ after 4 h	Relative brightness in BODIPY spectral channel (530 – 600 nm)	% retention after 4 hours
Et ₂ B-CO ₂ C ₉	1.95	1	~ 100
H ₂ B-OH	- 0.02	2.73	~ 0
H ₂ B- PMHC _{ox} ABAP	0.13	1.40	5
H ₂ B- PMHC _{ox} DCP	0.87	1.40	32

3.8.8. FCS Autocorrelation Traces

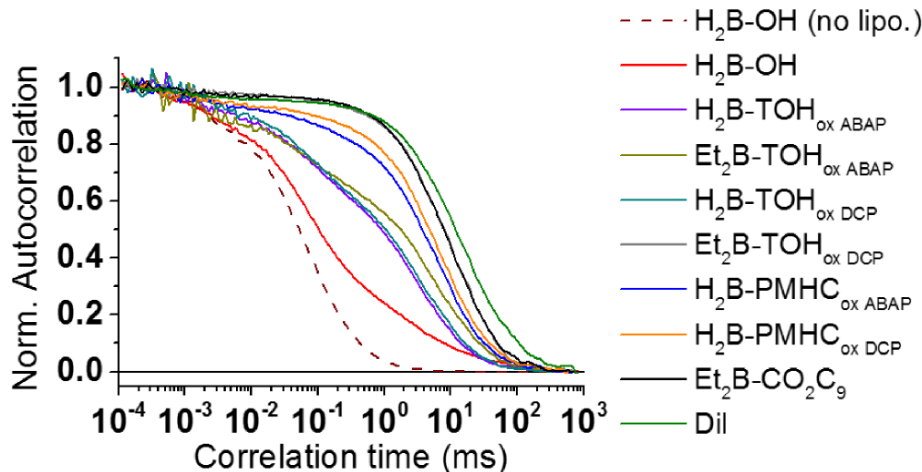


Figure 3.10. Normalized autocorrelation traces of samples acquired as quickly as possible after dilution ($t = 0$ h). H_2B-OH dye only sample is depicted by a dashed line while dye/liposome samples are shown as full lines. Traces were calculated from a continuous 10-minute acquisition with 70 kW/cm^2 514 nm laser excitation.

3.9. References

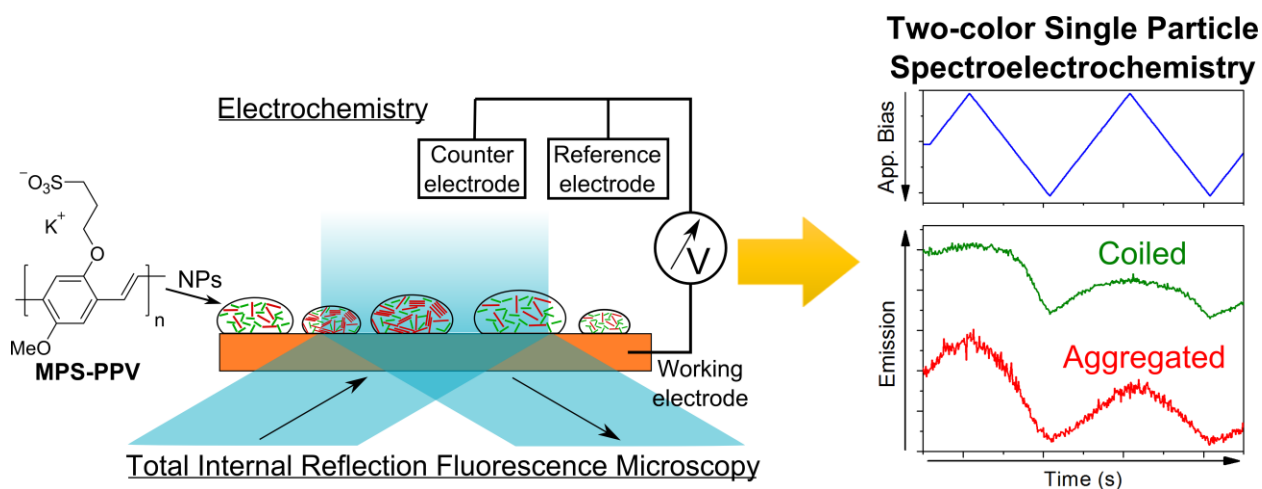
- (1) Phillips, R.; Ursell, T.; Wiggins, P.; Sens, P., Emerging Roles for Lipids in Shaping Membrane-Protein Function. *Nature* **2009**, *459*, 379-385.
- (2) Mouritsen, O. G., *Life - as a Matter of Fat. The Emerging Science of Lipidomics*; Springer Berlin Heidelberg 2005.
- (3) Allen, T. M.; Cullis, P. R., Drug Delivery Systems: Entering the Mainstream. *Science* **2004**, *303*, 1818-1822.
- (4) Vriezema, D. M.; Comellas Aragonès, M.; Elemans, J. A. A. W.; Cornelissen, J. J. L. M.; Rowan, A. E.; Nolte, R. J. M., Self-Assembled Nanoreactors. *Chem. Rev.* **2005**, *105*, 1445-1490.
- (5) Chen, C.; Zhu, S.; Huang, T.; Wang, S.; Yan, X., Analytical Techniques for Single-Liposome Characterization. *Anal. Methods* **2013**, *5*, 2150-2157.
- (6) Korlach, J.; Schwille, P.; Webb, W. W.; Feigensohn, G. W., Characterization of Lipid Bilayer Phases by Confocal Microscopy and Fluorescence Correlation Spectroscopy. *P. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 8461-8466.
- (7) Larsen, J.; Hatzakis, N. S.; Stamou, D., Observation of Inhomogeneity in the Lipid Composition of Individual Nanoscale Liposomes. *J. Am. Chem. Soc.* **2011**, *133*, 10685-10687.
- (8) Elizondo, E.; Larsen, J.; Hatzakis, N. S.; Cabrera, I.; Bjørnholm, T.; Veciana, J.; Stamou, D.; Ventosa, N., Influence of the Preparation Route on the Supramolecular Organization of Lipids in a Vesicular System. *J. Am. Chem. Soc.* **2012**, *134*, 1918-1921.
- (9) Chan, J.; Dodani, S. C.; Chang, C. J., Reaction-Based Small-Molecule Fluorescent Probes for Chemoselective Bioimaging. *Nat Chem.* **2012**, *4*, 973-984.

- (10) Krumova, K.; Cosa, G., Fluorogenic Probes for Imaging Reactive Oxygen Species. In *Photochemistry: Volume 41*, The Royal Society of Chemistry: 2013; Vol. 41, pp 279-301.
- (11) Terai, T.; Nagano, T., Fluorescent Probes for Bioimaging Applications. *Curr. Opin. Chem. Biol.* **2008**, *12*, 515-521.
- (12) Loura, L. S.; Prates Ramalho, J. P., Fluorescent Membrane Probes' Behavior in Lipid Bilayers: Insights from Molecular Dynamics Simulations. *Biophys. Rev.* **2009**, *1*, 141-148.
- (13) Oleynik, P.; Ishihara, Y.; Cosa, G., Design and Synthesis of a Bodipy- α -Tocopherol Adduct for Use as an Off/on Fluorescent Antioxidant Indicator. *J. Am. Chem. Soc.* **2007**, *129*, 1842-1843.
- (14) Krumova, K.; Oleynik, P.; Karam, P.; Cosa, G., Phenol-Based Lipophilic Fluorescent Antioxidant Indicators: A Rational Approach. *J. Org. Chem.* **2009**, *74*, 3641-3651.
- (15) Khatchadourian, A.; Krumova, K.; Boridy, S.; Ngo, A. T.; Maysinger, D.; Cosa, G., Molecular Imaging of Lipid Peroxyl Radicals in Living Cells with a Bodipy- α -Tocopherol Adduct. *Biochemistry* **2009**, *48*, 5658-5668.
- (16) Krumova, K.; Cosa, G., Bodipy Dyes with Tunable Redox Potentials and Functional Groups for Further Tethering: Preparation, Electrochemical, and Spectroscopic Characterization. *J. Am. Chem. Soc.* **2010**, *132*, 17560-17569.
- (17) Krumova, K.; Friedland, S.; Cosa, G., How Lipid Unsaturation, Peroxyl Radical Partitioning, and Chromanol Lipophilic Tail Affect the Antioxidant Activity of α -Tocopherol: Direct Visualization Via High-Throughput Fluorescence Studies Conducted with Fluorogenic α -Tocopherol Analogues. *J. Am. Chem. Soc.* **2012**, *134*, 10102-10113.
- (18) Krumova, K.; Greene, L. E.; Cosa, G., Fluorogenic α -Tocopherol Analogue for Monitoring the Antioxidant Status within the Inner Mitochondrial Membrane of Live Cells. *J. Am. Chem. Soc.* **2013**, *135*, 17135-17143.
- (19) Godin, R.; Liu, H.-W.; Cosa, G., Ambient Condition Oxidation in Individual Liposomes Observed at the Single Molecule Level. *Chem. Sci.* **2014**, *5*, 2525-2529.
- (20) Burton, G. W.; Ingold, K. U., Autoxidation of Biological Molecules. 1. Antioxidant Activity of Vitamin E and Related Chain-Breaking Phenolic Antioxidants in Vitro. *J. Am. Chem. Soc.* **1981**, *103*, 6472-6477.
- (21) Niki, E.; Saito, T.; Kawakami, A.; Kamiya, Y., Inhibition of Oxidation of Methyl Linoleate in Solution by Vitamin E and Vitamin C. *J. Biol. Chem.* **1984**, *259*, 4177-4182.
- (22) Torchilin, V. P.; Weissig, V., *Liposomes*. 2nd ed.; Oxford University Press Inc. : NY, 2003; Vol. 264.
- (23) Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*. 3rd ed.; Springer US: 2006.
- (24) Yu, J.; Lammi, R.; Gesquiere, A. J.; Barbara, P. F., Singlet-Triplet and Triplet-Triplet Interactions in Conjugated Polymer Single Molecules. *J. Phys. Chem. B* **2005**, *109*, 10025-10034.
- (25) Honig, M. G.; Hume, R. I., Fluorescent Carbocyanine Dyes Allow Living Neurons of Identified Origin to Be Studied in Long-Term Cultures. *J. Cell Biol.* **1986**, *103*, 171-87.
- (26) Alessi, M.; Paul, T.; Scaiano, J. C.; Ingold, K. U., The Contrasting Kinetics of Peroxidation of Vitamin E-Containing Phospholipid Unilamellar Vesicles and Human Low-Density Lipoprotein. *J. Am. Chem. Soc.* **2002**, *124*, 6957-6965.
- (27) Gelinck, G. H.; Staring, E. G. J.; Hwang, D.-H.; Spencer, G. C. W.; Holmes, A. B.; Warman, J. M., The Effect of Broken Conjugation and Aggregation on Photo-Induced Charge Separation on Polyphenylenevinylene Chains. *Synth. Met.* **1997**, *84*, 595-596.
- (28) Hess, S. T.; Huang, S.; Heikal, A. A.; Webb, W. W., Biological and Chemical Applications of Fluorescence Correlation Spectroscopy: A Review. *Biochemistry* **2002**, *41*, 697-705.

- (29) Krichevsky, O.; Bonnet, G., Fluorescence Correlation Spectroscopy: The Technique and Its Applications. *Rep. Prog. Phys.* **2002**, 65, 251.
- (30) Haustein, E.; Schwille, P., Fluorescence Correlation Spectroscopy: Novel Variations of an Established Technique. *Annu. Rev. Biophys. Biomol. Struct.* **2007**, 36, 151-169.
- (31) Rusu, L.; Gambhir, A.; McLaughlin, S.; Rädler, J., Fluorescence Correlation Spectroscopy Studies of Peptide and Protein Binding to Phospholipid Vesicles. *Biophys. J.* **2004**, 87, 1044-1053.
- (32) Posokhov, Y. O.; Rodnin, M. V.; Lu, L.; Ladokhin, A. S., Membrane Insertion Pathway of Annexin B12: Thermodynamic and Kinetic Characterization by Fluorescence Correlation Spectroscopy and Fluorescence Quenching. *Biochemistry* **2008**, 47, 5078-5087.
- (33) Melo, A. M.; Prieto, M.; Coutinho, A., The Effect of Variable Liposome Brightness on Quantifying Lipid-Protein Interactions Using Fluorescence Correlation Spectroscopy. *Biochim. Biophys. Acta, Biomembr.* **2011**, 1808, 2559-2568.
- (34) Meseth, U.; Wohland, T.; Rigler, R.; Vogel, H., Resolution of Fluorescence Correlation Measurements. *Biophys. J.* **1999**, 76, 1619-1631.
- (35) White, S. H.; Wimley, W. C.; Ladokhin, A. S.; Hristova, K., [4] Protein Folding in Membranes: Determining Energetics of Peptide-Bilayer Interactions. In *Methods Enzymol.*, Gary K. Ackers, M. L. J., Ed. Academic Press: 1998; Vol. Volume 295, pp 62-87.
- (36) Günes, S.; Neugebauer, H.; Sariciftci, N. S., Conjugated Polymer-Based Organic Solar Cells. *Chem. Rev.* **2007**, 107, 1324-1338.
- (37) Santos, N. C.; Prieto, M.; Castanho, M. A. R. B., Quantifying Molecular Partition into Model Systems of Biomembranes: An Emphasis on Optical Spectroscopic Methods. *Biochim. Biophys. Acta, Biomembr.* **2003**, 1612, 123-135.
- (38) Giaginis, C.; Tsantili-Kakoulidou, A., Alternative Measures of Lipophilicity: From Octanol-Water Partitioning to Iam Retention. *J. Pharm. Sci.* **2008**, 97, 2984-3004.
- (39) Amat-Guerri, F.; Liras, M.; Carrascoso, M. L.; Sastre, R., Methacrylate-Tethered Analogs of the Laser Dye PM567—Synthesis, Copolymerization with Methyl Methacrylate and Photostability of the Copolymers¶. *Photochem. Photobiol.* **2003**, 77, 577-584.

4. Heterogeneous Charge Mobility in Individual Conjugated Polyelectrolyte Nanoparticles Revealed by Two-Color Single Particle Spectroelectrochemistry Studies

Adapted with permission from Robert Godin, Rodrigo Palacios, and Gonzalo Cosa, *Journal of Physical Chemistry C*, **2015**, 119, 12875–12886. Copyright 2015 American Chemical Society.



4.1. Preface

We now explore systems of relevance to the optoelectronic materials community where we exploit the technique of SMS-EC. In this Chapter, we demonstrate the capabilities of SMS-EC to provide structural insights on the optoelectronic behaviour of conjugated polymers. We first describe the facile preparation of conjugated polyelectrolyte nanoparticles (CPE NPs). The chain conformation of the CPE is tuned by the solvent composition and affects the luminescence properties of the NPs. These NPs further provide us with a nanoconfined environment where we can study energy and charge transfer processes in individual particles. We reasoned that SMS-EC would be an ideal method to probe the heterogeneity of the CPE NPs environment and allow us to understand the interplay between optical and electronic effects at the nanoscale. Applying an oxidative potential resulted in the injection of positively charged holes in the polymer backbone that quenched the fluorescence of the CPE. We were also able to spectroscopically distinguish between deaggregated and aggregated CPE segments within individual NPs with a novel SMS-EC two-color setup. We could then conclude that extended and aggregated segments are more easily oxidized compared to deaggregated sites. We also investigated the effect of reductive conditions, and found that the fluorescence could be increased upon electron injection into the polymer backbone. We further observed that the interfacial charge transfer dynamics between the CPE NP and the transparent indium tin oxide (ITO) electrode could be modulated by an applied potential in the range where no ground state redox processes occur. Throughout these investigations, two populations of NPs could be identified based on their fluorescence response to applied electrochemical potentials. Comprehensive consideration of the effects observed led us to conclude that the two populations differed in their ordering of polymer segments. We established

that external ion mobility through the NPs, more difficult for highly ordered polymer chains, plays a role in dictating the optoelectronic properties of CPE NPs.

4.2. Abstract

The optoelectronic properties of conjugated polymers and conjugated polyelectrolytes (CPEs) depend on their chain conformation and packing. Correlations between emission color, charge mobility and extent of aggregation in these materials have been previously established from bulk studies. Here we describe the preparation of stable nanoparticle suspensions of the CPE poly[5-methoxy-2-(3-sulfopropoxy)-1,4-phenylenevinylene (MPS-PPV) where changes in the solvent composition enable tuning their emission spectra and quantum yield. Employing a newly developed color-sensitive single-molecule spectroelectrochemistry (SMS-EC) technique, the effect of chain conformation on the optoelectronic properties of MPS-PPV nanoparticles is monitored at the single particle level. Within a single particle the photoluminescence and redox response is chromatically-correlated reflecting on the differing contributions that coiled and deaggregated vs. extended and packed segments have on their optoelectronic properties. We also observe a heterogeneous response among nanoparticles to externally applied electrochemical potentials which further correlates with their emission color (chain packing). We rationalize our observations on differential charge injection, energy and charge transport and ion migration as a consequence of chain conformation, packing effects, and the presence of electrochemically reducible quenching sites. Our work provides a way to unravel the intrinsic heterogeneity of CPE materials to better understand the relationship between chain conformation and optoelectronic properties.

4.3. Introduction

Conjugated polymers and conjugated polyelectrolytes (CPEs)¹ have become customary building blocks for optoelectronic devices such as LEDs, thin-film transistors, chemical sensors, and photovoltaics.²⁻⁸ Their increasing importance in the manufacturing industry arises both from

their ease of processability and tunable electronic and optical properties. These properties are intimately related to the conformation of individual chains along the polymer backbone and to the distance between chains.⁹⁻¹⁴ In this context, increased charge carrier mobilities, reduced emission quantum yield, red-shifted emission spectrum, and increased exciton transport properties have been observed experimentally as a result of the increase of conjugation length caused by extension of the polymer backbone as well as by the formation of interchain species.¹⁵⁻²¹

Given the amphiphilic nature of CPEs (hydrophobic backbone with π -delocalized electronic structure and repeat units that bear pendant substituents with ionic functionalities)¹ their chain conformation and extent of aggregation in solution may be tuned upon careful solvent choice.²²⁻²⁵ CPEs provide in this regard unique systems to explore how the interplay of chain conformation and aggregation affect electronic and optical properties at the nanoscale.

Here we show that adjusting the water content in water/tetrahydrofuran (H₂O/THF) and water/acetonitrile (H₂O/MeCN) mixtures enables controlling the chain conformation and aggregation of the conjugated polyelectrolyte poly[5-methoxy-2-(3-sulfopropoxy)-1,4-phenylenevinylene] (MPS-PPV, see Figure 4.1). Conjugated polyelectrolyte nanoparticles (NPs) with emission spectra peaking in the range from ca. 620 nm to 545 nm and with up to 46-fold increase in relative emission intensity are readily obtained upon careful selection of the solution conditions.

We also show that the effect of chain conformation on the optical and electronic properties of MPS-PPV can be monitored at the single nanoparticle level with the aid of a novel color-sensitive single-molecule spectroelectrochemistry (SMS-EC) technique. This technique enabled recording the distribution of the potentials required to inject charges (both from the ground state and photoexcited states) for individual CPE NPs, offering new information on the heterogeneity

of the redox process of CPEs at the molecular level. The simultaneous monitoring of the red (extended/aggregated polymer backbone) and green (coiled and deaggregated backbone) emission components as a function of applied bias in an electrochemical cell configuration allowed us to unearth the interplay between charge injection (thermal and photoinduced), energy transfer and photoluminescence in individual MPS-PPV NPs and correlate them to CPE conformation.²⁶ We demonstrate that NPs of MPS-PPV display differing emissive behavior when subjected to an externally applied electrochemical bias. This is manifested both in the response speed and modulation amplitude exhibited between different nanoparticles. Within a single particle the photoluminescence response is also chromatically-correlated reflecting on the differing contributions that coiled and deaggregated vs. extended and packed segments have on the optoelectronic properties.

Our studies provide a method that distinguishes between individual MPS-PPV NPs with lower or higher charge mobility, caused by chain packing effects for example, and thus make it possible to evaluate the effectiveness of various approaches aimed at modifying chain conformation for specific applications. Our work provides a way to unravel the intrinsic heterogeneity of CPE materials to better understand the relationship between chain conformation and optoelectronic properties.

4.4. Experimental Section

4.4.1. Preparation of MPS-PPV solution in H₂O/organic solvent for ensemble measurements.

An initial MPS-PPV stock solution (Sigma-Aldrich, MW ~ 1 MDa; 0.25% w/w in H₂O, 9.5 mM in polymer repeat units) was centrifuged for 5 min at 5000 rpm on a benchtop centrifuge (accuSPINTM micro, Fisher Scientific) to precipitate large aggregates. In order to prepare the

various H₂O/organic solvent dispersions, 10 μ L aliquots of the supernatant were brought to a final desired H₂O volume before adding to the organic solvent in order to complete 2 mL of H₂O:organic solution with the desired % v/v H₂O content. To ensure reproducibility, the aqueous MPS-PPV solution thus obtained was diluted at most 10x in the water-miscible solvents THF or MeCN by rapid injection, and next the resulting solution was added to the remaining organic solvent to complete 2 mL. For H₂O volumes > 200 μ L, a single injection was performed into the organic solvent to yield a final volume of 2 mL. The MPS-PPV stock was thus diluted 200-fold in all ensemble experiments yielding a final polymer repeat unit concentration of 48 μ M.

4.4.2. Ensemble spectroscopy

Fluorescence measurements were performed on a PTI Quantamaster 40 fluorimeter at 25 ° C with excitation at 450 nm. UV-VIS spectra were obtained at room temperature (23 ± 1 ° C) on a Hitachi U-2800 spectrophotometer. DLS measurements were performed on a Brookhaven Instruments NanoBrook 90Plus at 90° detection angle, 25°C with nominally 640 nm laser light.

4.4.3. SEM imaging

Coverslips were cleaned immersing them for 1h in a piranha solution (30% H₂O₂ and H₂SO₄ 1:3 v:v). They were next thoroughly rinsed with H₂O and EtOH. MPS-PPV NPs (0.5% v/v H₂O content) were spin-coated by dropping 40 μ L of solution on the clean coverslips rotating at 3000 rpm. The spinning was then slowed to 2000 rpm and kept for 50 s afterwards. A thin gold coating (~ 10 nm) was then argon-sputtered in a vacuum environment to give sufficient sample conductivity. The SEM images were acquired on a FEI Inspect F-50 FEG-SEM operating at 10-20 kV in backscatter detection mode.

4.4.4. Single molecule spectroelectrochemistry

MPS-PPV NPs (0.5% v/v H₂O content in THF) were spin-coated by dropping 40 μ L of solution on a clean ITO-coated coverslip (Evaporated Coatings Inc., 50 $\Omega/\square$; cleaned by 20 minutes sequential sonication in acetone, trichloroethylene and methanol) rotating at 3000 rpm. The spinning was then slowed to 2000 rpm and kept for 50 s. A glass tube was affixed on top of the coverslip with the help of epoxy glue (Hardman®, McMaster-Carr). The epoxy was cured in an oven (110° C) for ca. 1 hour, which also dried the sample. The solvent utilized was MeCN and the supporting electrolyte was Bu₄NClO₄ (0.1 M). The electrochemical cell was completed by sealing with an inverted septum, which was pierced by a non-aqueous Ag/Ag⁺ reference electrode and Pt wire counter electrode. The Ag/Ag⁺ reference electrode was internally calibrated to the Fc⁺/Fc redox couple ($E^0 = + 0.64$ V vs. NHE). The ITO surface served as the working electrode and four ITO sectors on the coverslip could be addressed independently. The cell was degassed for at least 1 hour with Ar before measurements. An argon-filled balloon was affixed to the sample at all times to ensure an Ar positive pressure within the electrochemical cell. Individual MPS-PPV NPs were imaged using a wide-field objective-based total internal reflection fluorescence (TIRF) microscopy setup consisting of an inverted microscope (IX71, Olympus) equipped with a laser-based TIRF illumination module (IX2-RFAEVA-2, Olympus). Samples were excited with the evanescent wave of a 488 nm Ar⁺ laser output (Melles Griot), obtained by focusing the collimated laser beam at back focal plane of a high numerical aperture (N.A. = 1.45) oil-immersion objective (Olympus PLAN APO 60X) and launching the excitation past the critical angle. The laser power measured at the exit of the objective was 970 μ W. Emission was collected through the same objective and images were magnified 2-fold *via* a lens system and then captured on a back illuminated electron multiplying charge coupled device (EM-CCD) camera (Cascade II:512B,

Roper Scientific). Images were spectrally separated by a 640dcxr dichroic mirror to give two distinct emission channels, a green channel with $\lambda_{\text{em}} < 640$ nm, and a red channel with $\lambda_{\text{em}} > 640$ nm. A total area of about $66 \times 33 \mu\text{m}^2$ was imaged at any given time. The images were analyzed with custom Matlab and IDL scripts that identified diffraction-limited fluorescence spots, registered them in both spectral channels and calculated the background-corrected integrated fluorescence intensity for each individual NP in either the green or red channel. Electrochemical potential was controlled by a CHI600D electrochemical analyzer. The applied bias (E_{app}) and acquired movies were temporally synchronized. A cyclic voltammetry potential waveform was applied to the sample, the E_{app} were initiated at + 0.53 V vs. NHE and were first swept to the minimum peak potential (either + 0.03 V (Figure 4.2) or – 0.97 V (Figure 4.5)) and then to the maximum peak potential of + 1.03 V. Potentials were scanned at a rate of either 50 or 100 mV/s while frames were acquired at a rate of 10 Hz.

4.5. Results and Discussion

4.5.1. Effect of water content in $\text{H}_2\text{O}/\text{THF}$ and $\text{H}_2\text{O}/\text{MeCN}$ mixtures

We prepared CPE NPs by rapidly injecting a CPE aqueous solution into the water-miscible organic solvent, either THF or MeCN. During injection, individual MPS-PPV chains aggregate or collapse to form CPE NPs.²⁷ This nano-precipitation process is analogous (albeit with opposite polymer and solvent polarities) to the method reported to produce neutral conjugated polymer NPs following injection of their THF solutions into H_2O .²⁸ This reprecipitation method has been extensively used to form conjugated polymer NPs, but is far less common for the case of conjugated polyelectrolytes.²⁹⁻³¹ Nanoparticle formation was monitored via dynamic light scattering (DLS) analysis of dispersions of MPS-PPV in various $\text{H}_2\text{O}/\text{THF}$ and $\text{H}_2\text{O}/\text{MeCN}$ mixtures, ranging from 0.5% to 100% v/v in water (see Table 4.1 and

Table 4.2). Nanoparticles of MPS-PPV 212 nm in diameter (by DLS) were recorded at 0.5% H₂O/THF v/v. These NPs were also observed in scanning electron microscope (SEM) micrographs, although we recorded a smaller average size of 124 nm in diameter (Figure 4.1 and Figure 4.7). The difference in size presumably arises due to swelling of the NPs in solution for the DLS experiments. Increasing the water content above 0.5% v/v led to a reduction of the recorded size of the NPs in our DLS studies as better solubilization of the CPEs takes place. At water contents above 15% v/v, the scattering counts were below the detection limit of our setup.

The chain conformation of CPEs was extremely sensitive to nanoparticle preparation conditions. We observed a broad, red-shifted fluorescence at very low water content in water/THF solutions, in the range of 0.5-2.5% H₂O v/v, see Table 4.1 and Figure 4.1. At higher water contents (5-50% H₂O v/v) the fluorescence intensity was significantly enhanced (up to 10-fold increase) and the emission spectrum, while still broad, was markedly blue-shifted (up to 80 nm). Similar trends were observed for MPS-PPV dispersed in H₂O/MeCN solutions, however the fluorescence enhancements were not nearly as pronounced as those in THF presumably because MeCN is a poor solvent for the PPV backbone (see Figure 4.8). Variations in absorption spectra with increasing H₂O content were also consistent with conformational changes (*vide infra*). A decreasing proportion of red-absorbing sites were recorded in going from 0.5 to 50% H₂O by volume in THF (see Figure 4.9). At the lowest water content, broadening of the absorption peak, indicative of a large distribution of conjugation lengths and/or chain conformations, was observed. Also recorded was a red-absorbing tail which extended up to ~ 600 nm.

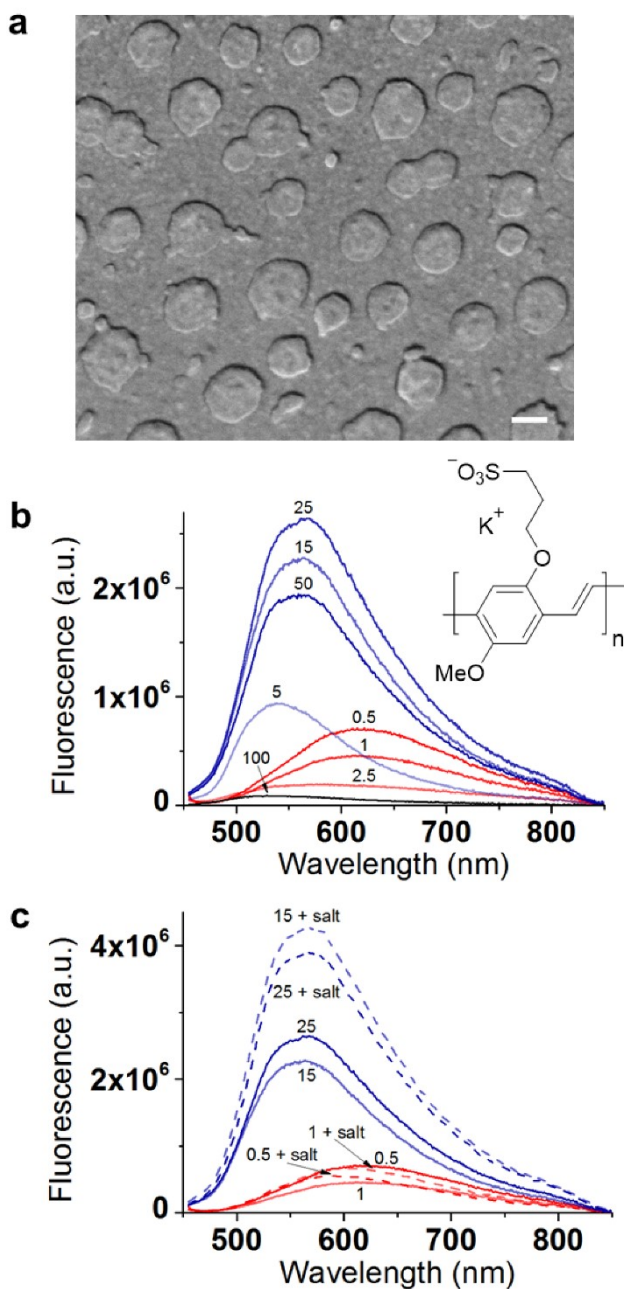


Figure 4.1. a) SEM image of MPS-PPV NPs (formed in 0.5% H_2O v/v in THF solution) dispersed on a glass coverslip. See sample preparation details in the text. Scale bar is 100 nm. Fluorescence emission properties of MPS-PPV dispersed in various H_2O /THF solutions (b) before (solid lines) and (c) after (dashed lines) addition of 100 mM Bu_4NClO_4 as a powder, $\lambda_{\text{ex}} = 450$ nm in both cases. Panel (c) also shows spectra of two samples before addition of Bu_4NClO_4 (solid lines) to allow for direct comparison. The water content (in % v/v) is indicated near the fluorescence maximum in each trace. The low water regime ($\leq 2.5\%$ H_2O by volume) is shown in red while the high water regime ($\geq 5\%$ H_2O by volume) is shown in blue. Fluorescence in water-only solution is shown in black. Shown in the inset is the chemical structure of MPS-PPV.

Table 4.1. Spectroscopic and DLS characterization of MPS-PPV dispersions in H₂O/THF solutions.

		MPS-PPV + H ₂ O + THF			MPS-PPV + H ₂ O + THF + Bu ₄ NClO ₄		
H ₂ O ^a (%v/v)	n _{H₂O} /n _T HF	λ_{em}^b (nm)	Diam. (nm) ^c	Relative emission ^b	λ_{em}^b (nm)	Diam. (nm) ^c	Relative emission ^b
0.5	0.02	622	212	9	588	1900	7
1.0	0.05	613	146	6	605	3300	8
2.5	0.12	583	110	3	544	1360	18
5.0	0.24	543	82	9	566	1494	36
15	0.79	557	N/D	24	570	N/D	46
25	1.50	568	N/D	28	571	N/D	42
50	4.50	559	N/D	21	N/A	N/A	N/A
100	-	518	N/D	1	554	N/D	4 ^d

^aIn THF. ^bWavelength corresponding to the maximum of the emission spectrum. The excitation wavelength utilized was 450 nm, and absorption at this wavelength did not vary more than ~ 10%. Emissions intensities are relative to the emission of MPS-PPV in pure water. Absorption and emission intensities of solutions containing particles larger than ~ 300 nm are likely to be affected by the penetration depth of light into the CPE.³² ^cAs measured by DLS. ^dSaturated solution of Bu₄NClO₄ in H₂O (concentration << 100 mM). N/D = not detectable.

Although it is difficult to assign the red fluorescence to a specific emitting species,³³ the fact that red-absorbing sites are formed in our system (see Figure 4.9) indicates an increase in the average conjugation length and/or the formation of aggregates. While more specific assignment of the red-emitting species is beyond the scope of the present study, it is important to recall that the origin of red-shifted fluorescence in conjugated polymers has been previously attributed either to

an increase in the conjugation length and/or to the formation of interchain species.³⁴⁻³⁹ Here ‘interchain species’ denotes a group of non-neighboring chromophore segments in the polymer backbone, regardless if these segments are part of the same molecule or not, that are in contact.¹⁸
⁴⁰ Following the nomenclature defined by Schwartz,¹⁸ the term aggregates refer to interchain species that interact in the ground state. Excimers in turn refers to interchain species that interact exclusively in the excited state, resulting in changes in the fluorescence, but not the absorption spectra.⁴¹

Insight on the CPE chain conformation can be gained by comparing the observed spectral and NP size trends (Table 4.1). Increasing the water content from 0.5% to 15% v/v leads to smaller NPs with blue-shifted fluorescence. With increasing water content the CPE becomes relatively well solvated and interchain interactions between MPS-PPV chains are less prominent, leading to a blue-shift in the absorption and fluorescence spectra and a marked increase in emission intensity. Similar observations have been made with MPS-PPV dispersed in mixtures of H₂O and DMSO (a good solvent for MPS-PPV chains), where a large fluorescence enhancement in pure DMSO were recorded and assigned to the absence of exciton-exciton annihilation within the PPV backbone.⁴² We postulate that, at very low water content, MPS-PPV chain segments are straightened out and bury the charged side groups within water rich pools, effectively increasing the average conjugation length of the chromophores.¹⁸ In turn, the extended conformation allows for better π - π stacking between chromophores promoting the formation of interchain species (aggregates and excimers) and NPs. Increased interchain interactions lead to red-shifted (and weak) fluorescence as low-energy emissive sites (and non-emissive sites) are eventually populated upon extensive energy transfer.⁴³⁻⁴⁵

We next explored the role that the large hydrophobic counterion tetrabutylammonium perchlorate (Bu_4NClO_4) has on the spectroscopic properties of MPS-PPV (Figure 4.1c). The information is relevant to our single molecule/particle spectroelectrochemistry studies where Bu_4NClO_4 was used as the supporting electrolyte (*vide infra*). In the presence of Bu_4NClO_4 , we observed a significant increase in fluorescence when MPS-PPV was dispersed in 15% H_2O v/v in THF. A maximal 46-fold fluorescence enhancement was observed compared to MPS-PPV dispersed in neat H_2O with no Bu_4NClO_4 . These observed changes in luminescence, also recorded in $\text{H}_2\text{O}/\text{MeCN}$ solutions, point to better solvation of MPS-PPV polymer chains when an organic cation is added to a $\text{H}_2\text{O}/\text{organic}$ solvent mixture rich in the organic fraction. The alkyl groups in the large counterion act as spacers that effectively separate polymer chains and inhibit their contact.⁴⁶ The greasy counterions may additionally minimize the tendency of CPEs towards packing with each other to reduce contact with water, exerting a surfactant effect. At water contents of 1% or lower, the emission enhancement of MPS-PPV was significantly smaller, indicating that Bu_4NClO_4 did not significantly alter the NPs structure under these conditions. A similar effect is expected under the anhydrous conditions we employed in our single molecule measurements described below.

4.5.2. *Single Molecule Spectroelectrochemistry of MPS-PPV particles*

In order to unravel the role that chain conformation plays in the differential optoelectronic behavior of individual MPS-PPV NPs we developed a two-color single-molecule spectroelectrochemistry (SMS-EC) technique to correlate changes in either green (coiled/deaggregated) or red (extended/aggregated) emission with applied electrochemical bias (E_{app}).⁴⁷ Specifically, NPs were spin cast onto an ITO coated glass coverslip (working electrode) and an air tight electrochemical cell was assembled with reference and counter electrodes (Figure

4.2a). Samples were next mounted on a total internal reflection fluorescence microscope (TIRFM). Images were recorded at 10Hz rate in an electron multiplied CCD camera (EMCCD) while sweeping the external potential E_{app} in the electrochemical cell. Tens of surface immobilized NPs could thus be followed over time. Intensity vs. time trajectories for each individual particle in both color channels were constructed from the stack of images (Figure 4.2b). We studied NPs formed in 0.5% v/v H₂O in THF as the solution properties of these NPs, characterized by a high level of interchain interactions, are expected to be representative of those corresponding to NPs deposited on a surface and monitored in dry MeCN solutions.

In the following we first describe our results for a ‘narrow’ E_{app} range (+ 0.03 to + 1.03 V vs. NHE, Figure 4.2) and discuss the phenomena occurring in this E_{app} range (thermal electrochemical oxidation and photoinduced electron transfer). Results obtained for a wider E_{app} range, keeping the maximum at + 1.03 V but extending on the reductive side to – 0.97 V (Figure 4.5), are next presented and the new processes occurring (asymmetric fluorescence enhancement and differences in hole filling) are discussed in the context of polymer morphology.

4.5.2.1. Results for $+1.03\text{ V} \geq E_{app} \geq +0.03\text{ V}$

Shown in Figure 4.2 are representative normalized fluorescence intensity trajectories for a single particle and subensembles (average of selected single particle trajectories), recorded in both the green and red channel upon scanning the E_{app} linearly over time between + 0.03 V and + 1.03 V vs. NHE (see Figure 4.10 for non-normalized intensities). Starting with an open circuit, applying a potential of + 0.53 V (at time = 4 s) resulted in a slight reduction of fluorescence intensity (I_f) in the green channel, yet a marked one in the red channel. As E_{app} was swept towards 0 V (up to time = 11 s), all particles showed a reversible I_f enhancement in the red channel. For the green channel a subset of particles (ca. 50%) showed I_f enhancements, albeit smaller in relative magnitude to

those observed in the red channel (Figure 4.2d). The changes in I_f are reversed as the bias is swept linearly over time from + 0.03 V back to + 0.53 V (from 11 s to 16 s). In contrast, the remaining particles showed negligible changes in I_f (Figure 4.2e).

At the onset E_{app} of $\sim + 0.54$ V and increasing until + 1.03 V a sharp quenching of the fluorescence intensity was observed followed by a steady recovery upon scanning E_{app} back from + 1.03 V to + 0.54 V (gray regions in Figure 4.2, corresponding to potentials $\geq + 0.54$ V). The quenching and recovery in this bias range were equally prominent in both the green and red channels. However, for both channels, the intensity did not fully recover at the end of the first cycle. This is probably the result of either permanent photodamage to the polymer or irreversible reactions taking place after extensive electrochemical oxidation of MPS-PPV.⁴⁸ Similar conclusions applied to I_f trajectories recoded during a second potential applied immediately following the first cycle (see Figure 4.11 and Figure 4.12).

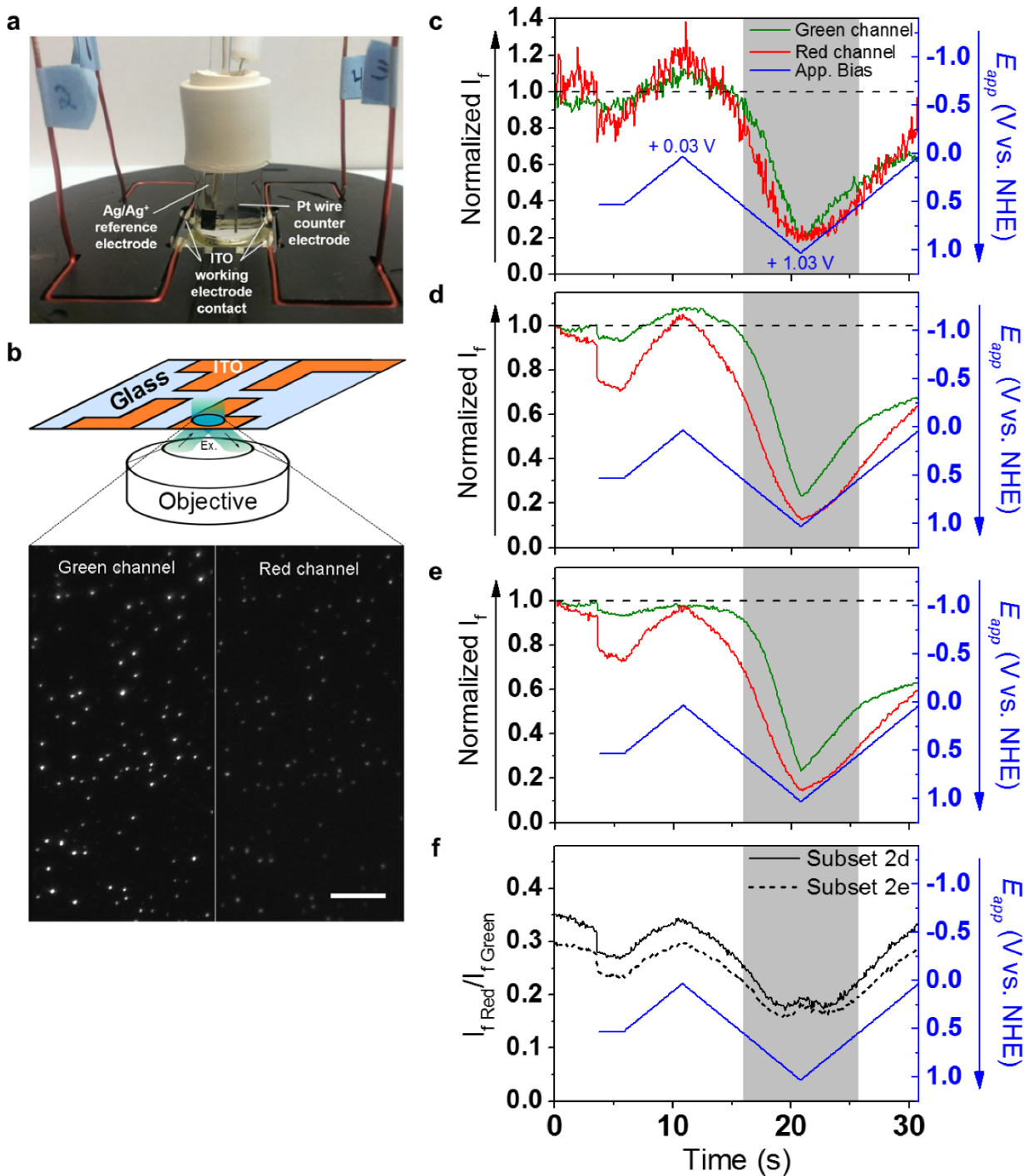


Figure 4.2. Effect of applied bias on MPS-PPV NP fluorescence as measured by TIRFM based color-sensitive SMS-EC. a) Photograph of the SMS-EC cell. b) Cartoon illustrating the coverslip with four independent ITO working electrodes and the evanescent optical excitation. The resulting emission from individual nanoparticles is collected by the objective and relayed to the chip of an EM CCD camera for image capture. One acquired frame is shown below depicting real data in the green and red channels. The scale bar is 10 μm . c) Chromatically-resolved (green and red) SMS-

EC single particle fluorescence intensity trajectories normalized to the initial fluorescence intensities. Panels d) (47% of the total) and e) (53% of the total) show normalized ensemble fluorescence intensity trajectories for two subsets of NPs showing differing enhancement behavior in the green channel under E_{app} in the region between + 0.03 V and + 0.54 V. Dashed lines corresponding to the initial intensities are visual guides to emphasize the changes in intensity. f) Non-normalized red-to-green fluorescence intensity ratios for NP subsets presented in d) and e). Peaks seen at E_{app} near + 1 V are artifacts resulting from the fluorescence intensity in the red channel dropping near background levels. A total of 68 NPs were analyzed. Time intervals where E_{app} was > + 0.54 V are highlighted in grey.

The marked I_f quenching observed as E_{app} is swept above the + 0.54 V onset potential and the recovery recorded in the return sweep are consistent with the reversible electrochemical oxidation of the ground state of MPS-PPV. Exciton quenching has been observed in similar studies conducted on MEH-PPV, the non-ionic counterpart of MPS-PPV, upon hole injection and formation of a radical cation.⁴⁹ The onset p-doping potentials of other 2,5-dialkoxy-substituted PPVs have been reported to be in the range of + 0.85 to + 0.91 V vs. NHE,⁵⁰⁻⁵¹ consistent with our assignment of oxidation of MPS-PPV NPs at potentials more positive than + 0.54 V (see discussion below for $E_{1/2}$ values) and indicative of the high sensitivity of photoluminescence intensity to the presence of holes (radical cation species) in this CPE.

Importantly, the distribution of potentials at which I_f is quenched by half relative to the initial I_f ($E_{1/2}$) and the I_f modulation depth we recorded were very similar for all particles in both color channels. The $E_{1/2}$ for each particle and for both color channels was very narrow, ca. ± 0.03 V. The mean $E_{1/2}$ recorded in the red channel, $\sim + 0.8$ V, was slightly smaller than that recorded for the green one, $\sim + 0.9$ V, see Figure 4.3. The smaller value of $E_{1/2}$ recorded in the red vs. green channel while initially surprising is readily rationalized considering that segments with an average longer conjugation (red-emitting sites) are easier to oxidize than those with shorter conjugation segments (green-emitting sites). The I_f modulation depth we recorded was ca. 80% (75% in the green channel, 85% in the red channel) relative to the initial I_f . The narrow distribution of $E_{1/2}$

along with the observation that all particles showed high I_f modulation depth indicate that all the studied particles were in good ohmic contact with the ITO working electrode.^{26, 49} The observation that the quenching modulation depth was $\sim 80\%$ within a relatively narrow 0.5 V bias range above the quenching onset indicates that holes injected in these NPs were significantly electrostatically screened from each other, presumably by the sulfonate side groups along the polymer backbone. This screening permitted the efficient injection of multiple holes necessary to account for the large modulation depth in a ca. 100 nm diameter nanoparticle, within the short bias range experimentally recorded. The large fluorescence intensity modulation in the short bias range compares to the results recorded with small (25nm diameter) NPs of MEH-PPV, where 98% fluorescence quenching was achieved within a 0.3 V range.⁴⁹ Solvent penetration and external ion screening were called upon to justify the narrow range and large modulation with MEH-PPV small particles. These factors would also be operational in the ionic MPS-PPV.

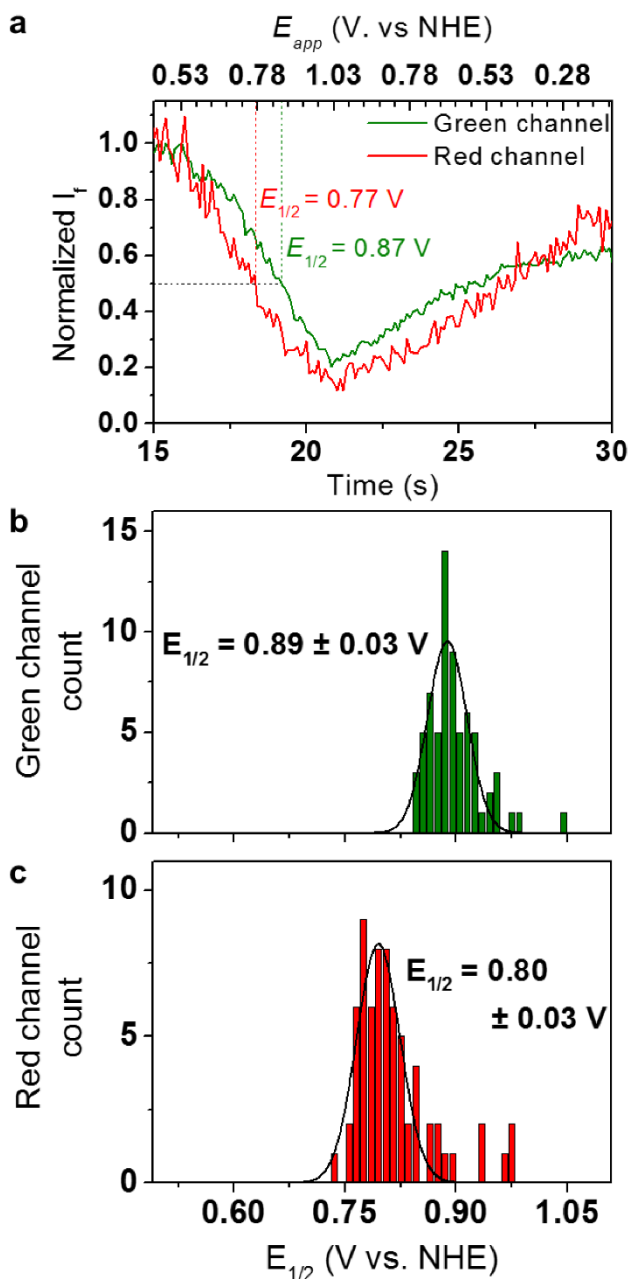


Figure 4.3. Distributions of $E_{1/2}$ values determined from single particle intensity-time trajectories. a) Representative chromatically-resolved (green and red) single particle fluorescence intensity trajectories recorded in our two-color SMS-EC setup. The I_f recorded at + 0.54 V, before the first oxidative quenching event, was taken to be 1. The $E_{1/2}$ is the applied bias where the intensity dropped by half. The center and width of the Gaussian distributions were taken to represent the mean and the standard deviation of $E_{1/2}$ values. Panels b) and c) display data extracted from the green and red channels for both NP subsets shown in Figure 4.2d and Figure 4.2e.

Having unraveled the origin of the fluorescence intensity quenching at $E_{app} \geq +0.54\text{V}$ as thermal (i.e. not photoinduced) hole injection we next discuss the observed modulation in I_f for E_{app} between $+0.54\text{ V}$ and $+0.03\text{ V}$ (white regions of Figure 4.2c-f). Specifically, we address first the origin of the I_f enhancement with reduction in E_{app} , and next the particle-to-particle heterogeneous behavior observed for the green channel and the differential relative amplitude of I_f for the two channels (green vs red).

Excited state electrochemical oxidation and reduction processes (photoinduced electron transfer (PeT) from (to) photoexcited MPS-PPV to (from) ITO) are both plausible in the potential range between $+0.54\text{ V}$ and $+0.03\text{ V}$, resulting in the quenching of polymer emission. In turn, no ground state redox process is known to occur for MPS-PPV in this bias range. We thus assigned the observed voltage-induced intensity modulation in the above potential range to changes in the rate of photoinduced redox processes from photoexcited MPS-PPV (see Figure 4.4). Lowering E_{app} (raising the Fermi level (E_F) of ITO) should decrease the rate constant for photoinduced oxidation (k_{ox}^*) concomitant with a reduction in the driving force for the oxidation of MPS-PPV. In turn, the rate constant for photoinduced reduction (k_{red}^*) should increase as E_{app} is lowered. Considering the initial segment of the intensity-time trajectories, applying a bias of $+0.53\text{ V}$ (Figure 4.2c-e, time = 4 s) leads to a drop in the I_f of the nanoparticles compared to the open circuit case. This is consistent with an overall increase in the PeT rate (sum of k_{ox}^* and k_{red}^* , Figure 4.4). Subsequent lowering of the bias enhanced the I_f , in line with a relatively smaller increase of k_{red}^* compared to the decrease of k_{ox}^* under these conditions. The observation that a nanoparticle I_f recorded under applied bias may surpass the I_f recorded at open circuit further signals that PeT was already operational at open circuit, resulting in static quenching of the polymer emission.⁴⁹

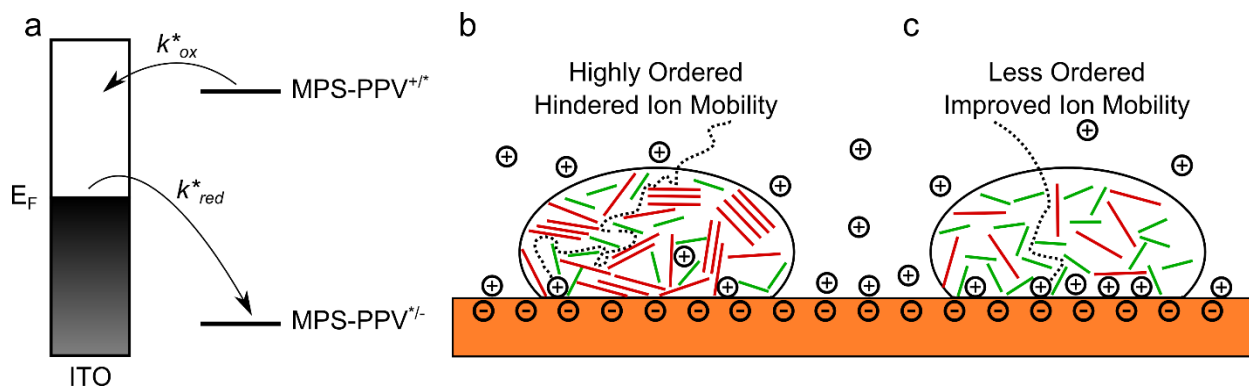


Figure 4.4. Cartoons illustrating: (a) state energy diagram for the MPS-PPV/ITO system. In SMS-EC experiments the energy of the ITO Fermi level (E_F) is modulated by an external bias. (b,c) cartoon rendering the hypothetical ordering of MPS-PPV NPs on an ITO electrode, where the polymer backbone is either (b) extended and aggregated or (c) coiled and deaggregated.

It is interesting to note that different results were obtained for the modulation of I_f with E_{app} for charged vs. uncharged conjugated polyphenylene vinylene (PPV) polymers in the bias range between 0 V and + 0.54 V. These discrepancies underscore the role of side groups in tuning the photophysical properties of the PPV parent backbone. Specifically, previous SMS-EC studies conducted on MEH-PPV NPs showed a linear modulation of I_f with E_{app} under mild electrochemical potentials.⁴⁹ The results however showed a drop in intensity with decreasing E_{app} , contrary to our results with MPS-PPV. With MEH-PPV it was concluded that excited state reduction of the polymer chains was the dominant pathway, i.e., with decreasing bias (raising the E_F of ITO) the increase in k_{red}^* and ensuing quenching is more important than the drop in k_{ox}^* that would lead to enhanced I_f . Our studies with MPS-PPV are in line with excited state oxidation being the dominant process at play in this bias range. The change of regime from overall photoinduced reduction (MEH-PPV) to overall photoinduced oxidation (MPS-PPV) is possibly related to the lowering of the barrier to electron injection into ITO due to the dipole created at the ITO surface from negatively charged side groups on MPS-PPV.⁵²⁻⁵⁴

Following our discussion on thermal and photoinduced redox processes accounting for the modulation of I_f in the bias range between 0 V and + 1 V, we next address the particle-to-particle heterogeneous behavior observed for the green channel and the differential relative amplitude of I_f for the two channels (green vs red) (see Figure 4.2). These experimental observations may be rationalized by considering the effects of ion motion through the MPS-PPV NPs and the extent of aggregation and energy transfer characterizing green and red emissive sites as it is described in detail below. Briefly, the rearrangement of ions that occurs when a potential is applied results in the formation of a double layer and to changes of the electric field in the vicinity of the electrode.⁵⁵ The resulting electric field affects the kinetics of photoinduced redox processes between ITO and MPS-PPV.

We postulate that the ease of external ion migration through the NPs will dictate the sensitivity of I_f to E_{app} in a potential window where the only mechanism that modulates I_f is PeT. Higher external ion mobility and easier chain reorganization should lead to better screening of the electric field generated at the working electrode and a lower sensitivity of I_f (PeT) to changes in potential. We note that the indistinguishable ground state oxidative quenching behavior recorded for the subset of NPs in Figure 4.2d and e (see also Figure 4.15b) is consistent with similar polymer chain interactions in both systems, including charge transport characteristics. In this regard, the differential behavior between subsets in the green color channel should thus be dominated by external factors such as ion percolation/mobility. We therefore postulate that NPs with a marked green channel I_f modulation in the + 0.03 V to + 0.54 V range (subset 2d; also see Figure Figure 4.13) are characterized by a lower external ion mobility (larger sensitivity to E_{app}) than those that show low modulation within this potential range (subset 2e).

The differential relative amplitude of I_f for the two channels (green vs. red) may be rationalized considering the extent of aggregation and energy transfer characterizing green and red emissive sites. Compared to green-emitting sites, red-emitting sites are thought to be better packed and have stronger interchain interactions,⁵⁶⁻⁵⁷ resulting in better charge transport²² and faster energy transfer.^{19, 58} Note that in this context *charge transport* refers to the movement of charge (radical anions or cations) within the conjugated polymer material by means of a hopping mechanism which takes place among nearby chain segments (charge carriers) belonging to the same chain or different chains.⁵⁹ Under applied electrochemical potentials, we postulate that external ion migration through open spaces in a structure dominated by red-emitting sites is difficult because the corresponding chains are closely packed and have less freedom of motion (Figure 4.4c).⁶⁰ The electric field generated at the working electrode is thus poorly screened for red-emissive sites resulting in a high sensitivity of the associated I_f to changes in E_{app} for a potential window where the only active mechanism is PeT (i.e. marked I_f modulation with E_{app} in the + 0.03 V to + 0.54 V range, Figure 4.2 and Figure 4.5). We further note that the better light harvesting ability of red-emitting over green-emitting sites, due to their intrinsically higher interchain energy transfer efficiency, would lead to a strong amplification of the I_f quenching via excited state redox processes. In other words, the intrinsic longer exciton diffusion length of red-emitting sites results in a higher fraction of excitons reaching the electrode for PeT relative to green-emitting sites. The combined action of poorer external ion mobility (lesser screening) and better energy transfer results in a stronger relative I_f modulation for red emissive vs. green emissive sites (Figure 4.2f, Figure 4.5d), and is in line with the higher modulation depth recorded for red-emitting sites as previously described (Figure 4.3). In line with better energy transport, red-emitting sites are more prone to irreversible photodamage following prolonged imaging, as can be observed from the

larger relative drop in I_f for red-emitting sites vs. green-emitting sites before engaging the applied bias and after disengaging the applied bias (see Figure 4.11 and Figure 4.12).

Altogether, the above observations reveal that ion mobility, in addition to PPV backbone aggregation, play a crucial role in determining the interfacial (ITO/MPS-PPV) charge transfer dynamics of the system.

4.5.2.2. Results for $+1.03\text{ V} \geq E_{app} \geq -0.97\text{ V}$

Larger I_f enhancements were recorded, in both red and green channels when E_{app} was scanned to negative peak values of -0.97 V (Figure 4.5). Two differing behaviors were observed at these negative biases. One subset of NPs (~50% of the total of 76 NPs monitored) displayed a linear response in I_f with E_{app} in the range of $+0.54\text{ V}$ to -0.97 V (Figure 4.5a) while a second subset showed asymmetric I_f response for E_{app} lower than -0.68 V (regions in purple in Figure 4.5), particularly evident in the green channel (Figure 4.5b). We draw comparisons between NP subsets 2d and 5a (2e and 5b) that show a higher (lower) sensitivity of I_f to E_{app} . When slowing the potential scan rate by half, to 50 mV/s , all particles showed a single behavior (Figure 4.5c): specifically, a small change of I_f for potentials between -0.68 V and $+0.54\text{ V}$ and a sharp I_f enhancement for $E_{app} < -0.68\text{ V}$. This is the same behavior observed for the subset of particles presented in Figure 4.5b.

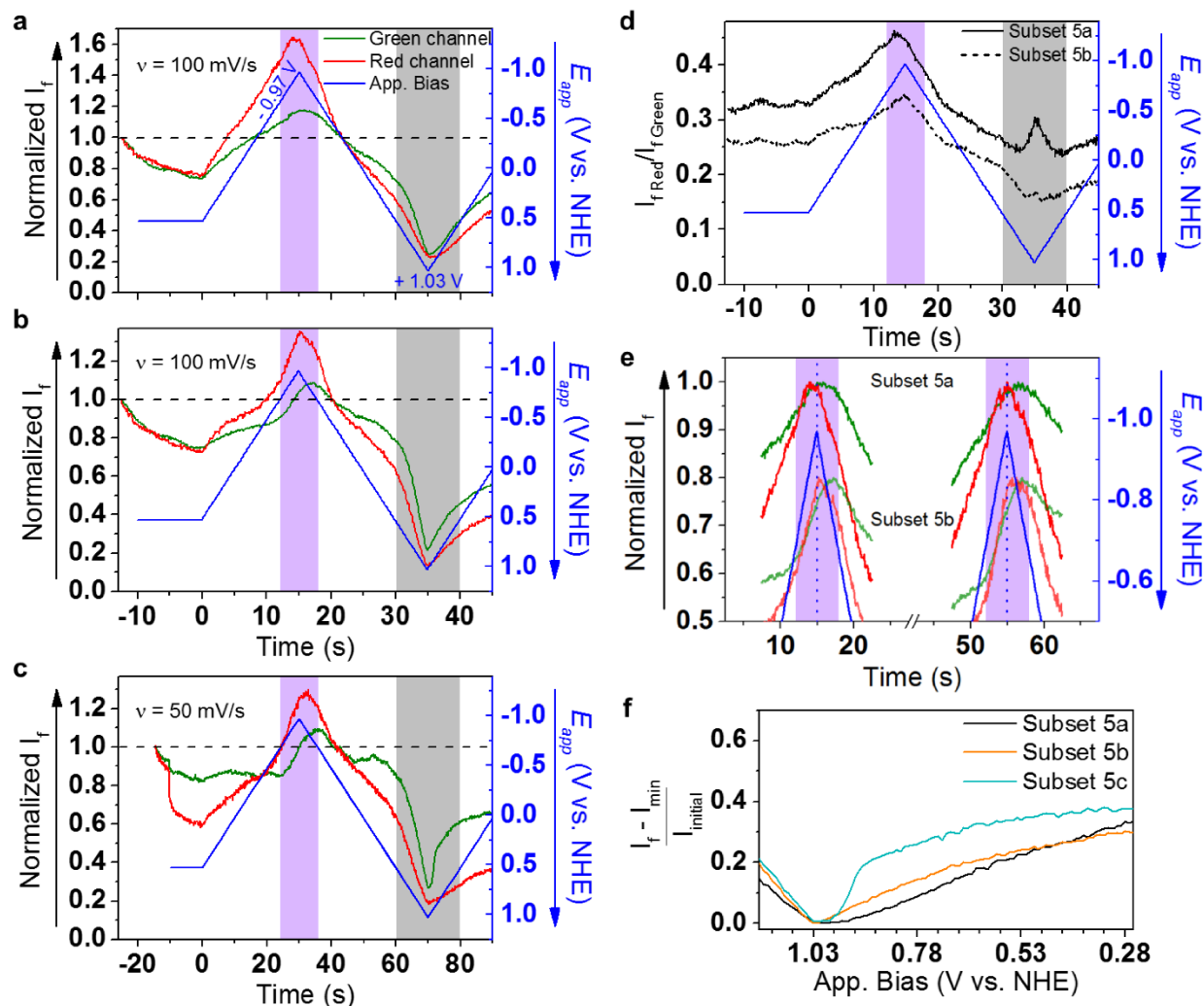


Figure 4.5. MPS-PPV NP fluorescence as measured by two-color SMS-EC showing the effect of negative applied biases ($-0.97 \leq E_{app} \leq +1.03$ V). (a and b) Average time trajectories normalized to the initial intensities of two subsets of NPs imaged in the same experiment and showing differing behavior at a potential scan rate of 100 mV/s. a) NPs where I_f is highly sensitive to changes in E_{app} . b) NPs that show an asymmetric I_f enhancement at an onset of ~ -0.68 V. c) Results for MPS-PPV NPs when the potential scan rate is reduced to 50 mV/s, normalized to the initial intensities. Only one type of NP behavior is seen ($n = 37$ particles). d) Non-normalized red-to-green fluorescence intensity ratios for NP subsets presented in a) and b). Peaks seen at E_{app} near $+1$ V are artifacts resulting from the fluorescence intensity in the red channel dropping near background levels. e) Changes in normalized fluorescence intensity relative to the maximum I_f for the NP subsets shown in a) and b). The traces of subset 5b are offset by 0.2 units to increase clarity. e) Changes in normalized fluorescence relative to the minimum I_f recorded in the green channels at the first positive peak potential for NP subsets shown in a), b) and c). Data shown corresponds to time = $25 - 45$ s for subsets 5a and 5b, and time = $50 - 70$ s for subset 5c. Time intervals where E_{app} is $> +0.54$ V are highlighted in grey, while those where E_{app} is < -0.68 V are highlighted in purple.

The observed asymmetric behavior at negative bias (purple region in Figure 4.5) is consistent with the thermal reduction of otherwise non-emissive quenched sites. The reduction potential onset of MPS-PPV is expected to be in the range of - 1.3 V to - 1.55 V vs. NHE by comparison to other 2,5-dialkoxy-substituted PPVs.⁵⁰⁻⁵¹ Considering this redox potential and also that the redox process we observed at $E_{app} < - 0.68$ V led to an increase in I_f , as opposed to intensity quenching expected from the formation of MPS-PPV radical anions, we conclude that a species different to pristine MPS-PPV is undergoing reduction at E_{app} more negative than - 0.68 V.

We postulate that non-emitting, photooxidized sites along the MPS-PPV backbone were transiently modified under the reductive potentials ($< - 0.68$ V) to non-quenching sites, giving rise to the sharp I_f increase. It is well known that PPV polymers photooxidize easily resulting in reduced photoluminescence. It is likely that electrochemical reduction of such quenching sites would restore the I_f of the MPS-PPV polymer.⁶¹ Importantly, fluorescence studies on individual MEH-PPV chains suggested that reversible fluorescence restoration is possible at reductive potentials, but only for photooxidized polymer.⁶²⁻⁶³ It has been suggested that the photoproducts include species such as dioxetanes or carbonyl defects.⁶⁴⁻⁶⁸ Carboxylic acids are also thought to be formed during PPV photooxidation.⁶⁹ Reversibility of the fluorescence enhancement seen previously,⁶² and in this study (in particular Figure 4.5c, time window 40 – 60 s) are inconsistent with the irreversible nature of the dissociative reduction of aromatic dioxetanes.⁶⁵ Based on the onset potential for the fluorescence enhancement (- 0.68 V), aldehyde reduction is unlikely to be the process at play since the reduction potential of an aldehyde-terminated oligo PV was reported to be - 2.07 V.⁷⁰ On the other hand, the reduction potential of benzoic acid at $\sim - 0.7$ V vs. NHE⁷¹ is in excellent agreement with our observations. It is thus tempting to conclude that carboxylic acid sites are present in the polymer backbone quenching MPS-PPV fluorescence and that they are

reversibly reduced at electrode potentials more negative than -0.68 V, restoring the intrinsic polymer fluorescence. A mechanistic overview of the proposed process is detailed in the Supporting Information. Further studies exploring the effect of negative applied biases on the photophysics of (photooxidized) MPS-PPV are warranted to elucidate the nature of quenching sites.

Differential ion motion through MPS-PPV NPs, previously invoked to explain the distinct response of the green and red channels towards PeT (*vide supra*), may also account for the occurrence of electrochemical reduction of a quenching site in only a single subset of NPs at a scan rate of 100 mV/s. Rapid ion motion in response to lowering of the potential (Figure 4.5b and c) leads to an accumulation of cations at the electrode surface. These cations will stabilize radical anions formed by photoinduced reduction of MPS-PPV and retard the thermodynamically favorable back electron transfer to the ITO (Figure 4.6). The injected electron will consequently remain longer in MPS-PPV, increasing the probability of the electron to be transported away from the electrode through self-exchange reactions (i.e. hopping mechanism), and reduce a quenching site. On the other hand, if cations cannot accumulate to stabilize the initial radical anion formed near the electrode, only a few sites in the NP will be sampled before fast back electron transfer occurs thus lowering the probability of reductively repairing a quenching site. Our view that the differential characteristics between NPs are due to the relative ease of external ion diffusion is further supported by the observation of a higher proportion of aggregated and ordered red-emitting sites, which impede ion diffusion, in the NP subsets associated with slower ion motion (Subsets 2d, 5a; see Figure 4.2f, Figure 4.5d).

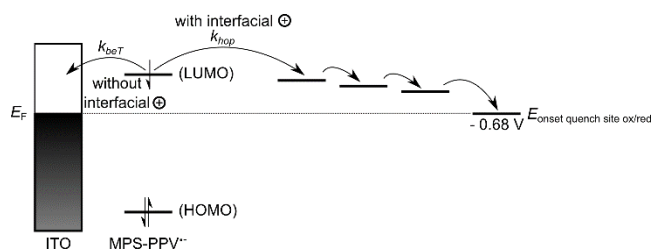


Figure 4.6. Proposed effect of MPS-PPV/ITO interfacial ions on the electron transport in MPS-PPV NPs. k_{beT} is the rate of back electron transfer from a MPS-PPV radical anion segment (formed upon PeT from ITO to MPS-PPV) to the ITO electrode. k_{hop} is the self-exchange electron transfer rate between MPS-PPV segments, leading to electron mobility in the CPE. The onset energy for reduction of quenching sites is indicated at -0.68 V ($E_{\text{onset quench site ox/red}}$) in accordance to experimental observations. Redox potentials for the MPS-PPV $^{\cdot-}$ segment can be roughly approximated to the energy levels of the highest occupied and lowest unoccupied molecular orbitals (HOMO, LUMO) of ground state MPS-PPV.

Focusing next our attention on the recovery of I_f recorded as E_{app} is swept from its peak positive value of $+1.03\text{ V}$ towards lower values (e.g. Figure 4.5a, times 35 s and later), we note that I_f is both dependent on the scan rate and the subset of NPs analyzed (see Figure 4.5f). Previous studies on MEH-PPV NPs have revealed the presence of deep holes (hole traps) upon observation of a slow recovery of I_f following extensive hole injection, similar to our own observations.^{26, 49, 72} As E_{app} decreases from the positive potential peak ($+1.03\text{ V}$), the MPS-PPV fluorescence is restored by filling (electrochemically reducing) holes that were injected in the PPV backbone. We observe a faster recovery of I_f , in other words a faster filling of injected holes, for the subset of NPs depicted in Figure 4.5b and previously associated with fast external ion migration. Slowing the potential scan rate (subset 5c) results in a larger yield of filled holes at a given bias (i.e. a larger relative intensity restoration at a given bias) consistent with better responsiveness of external ions to changes in E_{app} . There is a positive correlation between the external ion mobility and the charge mobility in the CPE, as has been previously reported.⁴⁶

Surprisingly, neither the differential rate of recovery of I_f nor the I_f peak response time at $E_{app} = +0.03\text{ V}$ were observed when the data was collected in the range between $+1.03\text{ V}$ and $+$

0.03 V as shown in Figure 4.2 (see Figure 4.15). We attribute the discrepancy when compared to the data with E_{app} reaching -0.97 V to the limiting conditions experienced by the polymer environment; in the latter case, substantial external ion reorganization is expected in response to the relatively large negative bias. The larger changes in E_{app} used for the experiment illustrated in Figure 4.5 and concomitant ion reorganization directly translate to the optoelectronic properties of MPS-PPV.

4.6. Conclusions

In summary, we report a simple method for the formation of NPs of the conjugated polyelectrolyte MPS-PPV that exhibit dim and red-shifted fluorescence. The chain conformation of MPS-PPV is greatly influenced by water content in H₂O/organic solvent mixtures. Tuning the solvent composition enables controlling the emission intensity and color. Given the amphiphilic nature of CPEs, these observations are fully applicable to the preparation of NPs of CPEs in general, providing a simple means to control their photophysical properties.

Newly prepared CPE NPs revealed heterogeneous fluorescence response to an externally applied potential. The two-color SMS-EC setup used allowed us to spectrally discriminate between deaggregated and aggregated emission sites. The deaggregated coiled MPS-PPV sites, where ion rearrangement is less hindered, were less sensitive to changes in applied bias as a result of, we postulate, better shielding of the electric field generated at the working electrode due to higher electrolyte mobility. Quenching sites are present in the CPE, most probably products of photooxidation of polymer segments, and are electrochemically reducible in a reversible fashion which restores the fluorescence of the polymer. When migration of ions through the NP is hindered, charge mobility between CPE segments is slowed down leading to reduced electronic

communication between chromophores in the NP and the working electrode and affecting the repair of quenching sites.

Methods that influence CPE chain conformation are highly sought after to tune and improve device applications, where a choice between either high charge mobility or high emission yield is often necessary.²² Our two-color SMS-EC method herein described provides a tool to explore at the single NP level the interplay between CPE conformation and its optoelectronic properties, distinguishing between poorly and well-packed segments within single MPS-PPV NPs. Our method thus enables evaluating the effectiveness of protocols that modify polymer chain conformation either in solution or after deposition on a surface.

4.7. Acknowledgements

This work has been supported in part by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina (PICT 140/08, 2213/07, 2691/11, and PRH23 PME01); the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina (PIP 11220090100839/10, 11220100100284/11, and CIAM/09); the Secretaría de Ciencia y Técnica, UNRC Argentina; the Ministerio de Ciencia y Tecnología Córdoba, Argentina (PID 033/2010); the National Science and Engineering Research Council (Canada) and a Tomlinson Award, McGill University. R.G. thanks the NSERC of Canada for a postgraduate scholarship. R.E.P. is permanent research staff of CONICET.

4.8. Supporting Information

The Supporting Information contains particle size distribution from SEM micrograph, spectroscopic data for MPS-PPV NPs dispersed in H₂O/MeCN solutions, additional SMS-EC data and representations and detailed mechanism of fluorescence enhancement under negative applied biases.

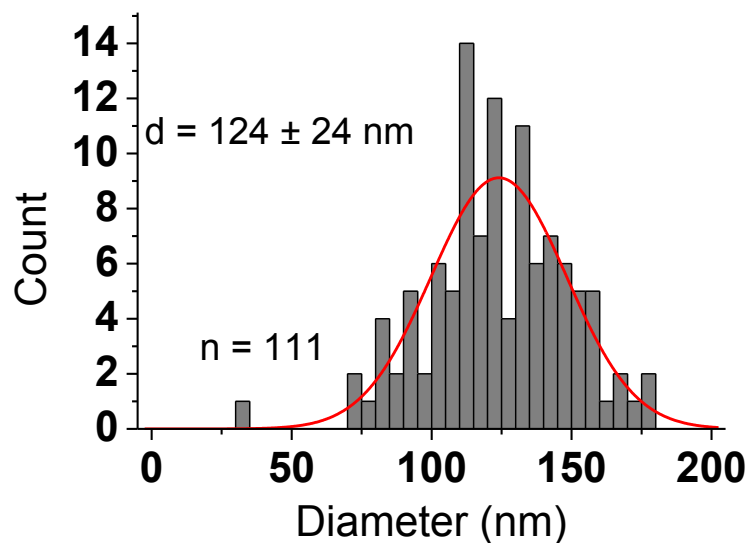
4.8.1. Particle Size Distribution from SEM Micrograph

Figure 4.7. MPS-PPV particle size distribution obtained from SEM micrographs, particles were formed in 0.5% H₂O v/v in THF solution, deposited over a clean coverslip and gold coated, see main text's experimental section for details. A Gaussian distribution was used to extract the mean diameter and width of the distribution.

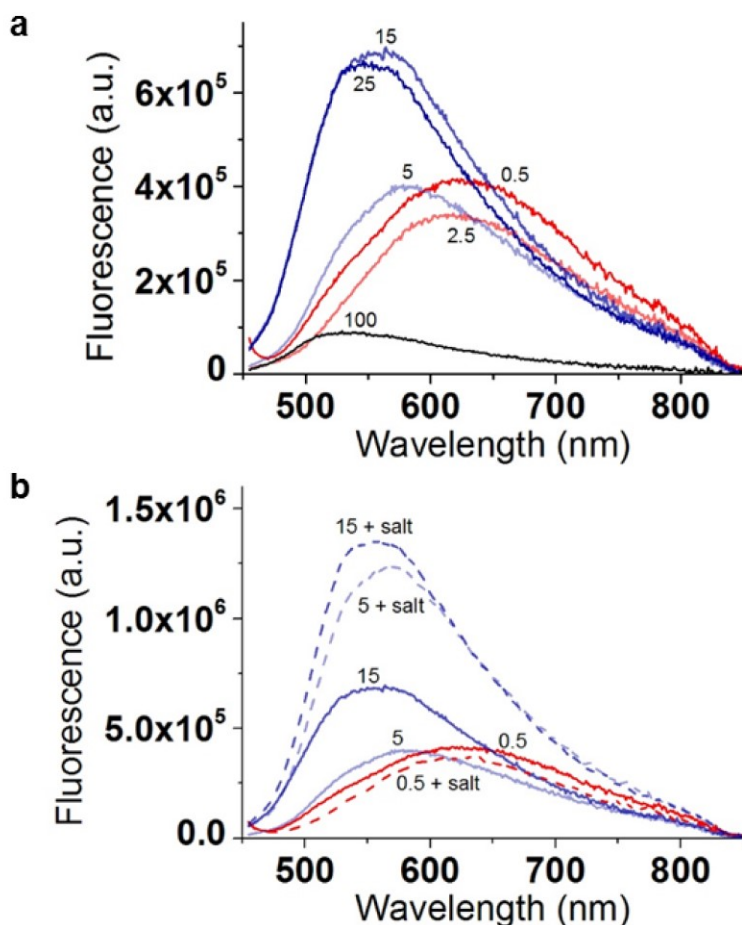
4.8.2. Ensemble Spectroscopy of MPS-PPV in either H_2O/THF or $H_2O/MeCN$ Mixtures

Figure 4.8. Fluorescence emission spectra of MPS-PPV ($12.5 \mu\text{g/mL}$, $48 \mu\text{M}$ in polymer repeat units) dispersed in various $H_2O/MeCN$ solutions (a) before (solid lines) and (b) after (dashed lines) addition of $100 \text{ mM } Bu_4NClO_4$ as a powder, $\lambda_{\text{ex}} = 450 \text{ nm}$ in both cases. Panel (b) also shows spectra of two samples before addition of Bu_4NClO_4 (solid lines) to allow for direct comparison. The water content (in % v/v) is indicated near the fluorescence maxima in each trace. The low water regime ($\leq 2.5\% H_2O$ by volume) is indicated in red while the high water regime ($\geq 5\% H_2O$ by volume) is indicated in blue. Fluorescence in water-only solution is shown in black.

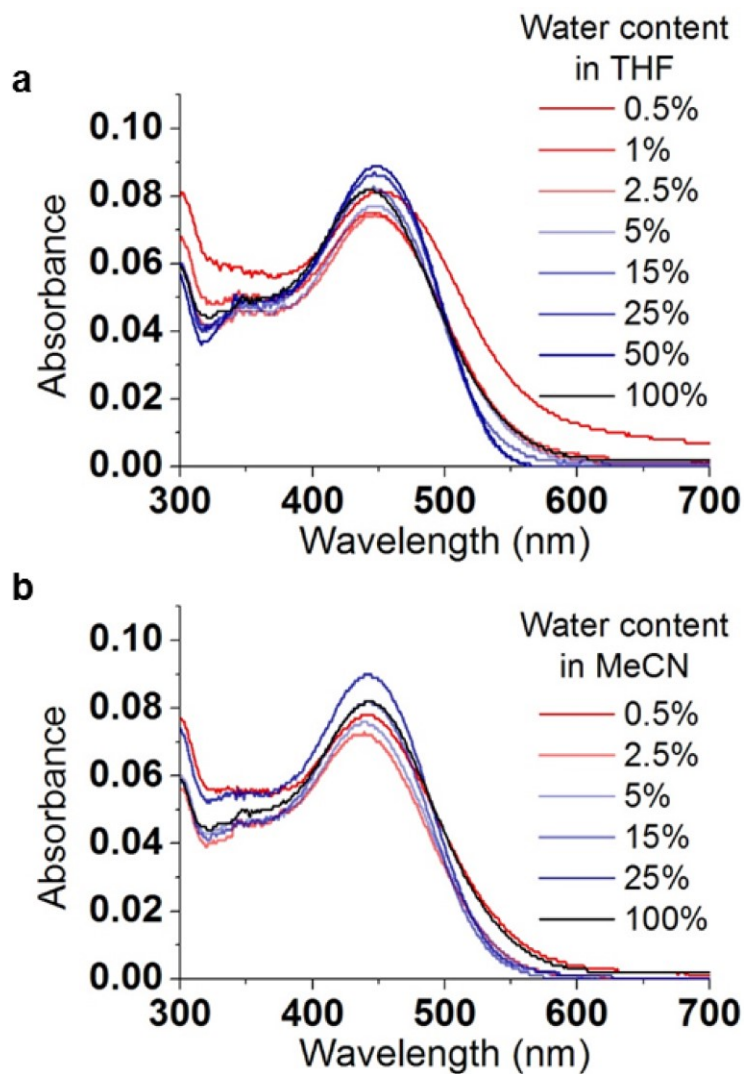


Figure 4.9. Absorption spectra of MPS-PPV dispersions (12.5 $\mu\text{g/mL}$, 48 μM in polymer repeat unit) in a) $\text{H}_2\text{O}/\text{THF}$ and b) $\text{H}_2\text{O}/\text{MeCN}$ solutions.

Table 4.2. Spectroscopic and DLS characterization of MPS-PPV dispersions in H₂O/MeCN solutions.

		MPS-PPV + H ₂ O + MeCN			MPS-PPV + H ₂ O + MeCN + Bu ₄ NClO ₄		
H ₂ O ^a (% v/v)	n _{H₂O} /n _{MeCN}	λ _{em} (nm) ^b	Diam. (nm) ^c	Relative emission ^b	λ _{em} (nm) ^b	Diam. (nm) ^c	Relative emission ^b
0.5	0.01	621	155	5.8	622	1723	4.8
2.5	0.07	627	N/D	4.5	N/A	N/A	N/A
5	0.15	578	N/D	5.0	570	N/D	14.1
15	0.51	564	N/D	8.2	568	N/D	15.1
25	0.97	546	N/D	7.6	N/A	N/A	N/A
100	-	518	N/D	1	554	N/D	4 ^d

^aIn MeCN. ^bWavelength corresponding to the maximum of the emission spectrum. The excitation wavelength utilized was 450 nm, and absorption at this wavelength did not vary more than ~ 10%. Emissions intensities are relative to the emission of MPS-PPV in pure water. Absorption and emission intensities of solutions containing particles larger than ~ 300 nm are likely to be affected by the penetration depth of light into the CPE.³² ^cAs measured by DLS. ^dSaturated solution of Bu₄NClO₄ in H₂O (concentration << 100 mM). N/D = not detectable.

4.8.3. Examples of Non-normalized SMS-EC Fluorescence Intensity Trajectories

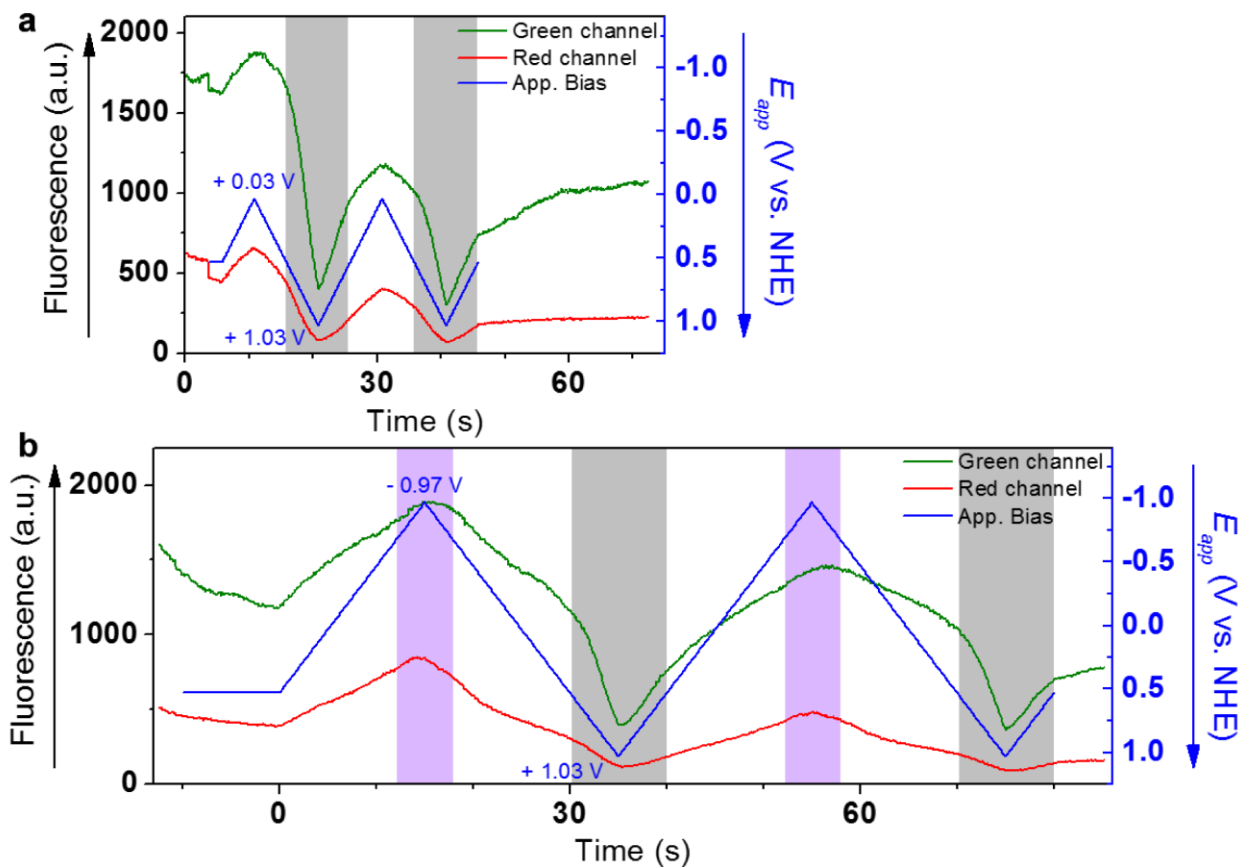


Figure 4.10. Non-normalized MPS-PPV NP fluorescence for NP subsets a) 2d and b) 5a of the main text. Time intervals where the applied bias is $> +0.54$ V are highlighted in grey, while those where the applied bias is < -0.68 V are highlighted in purple.

4.8.4. Full Two Potential Cycles of SMS-EC Experiments

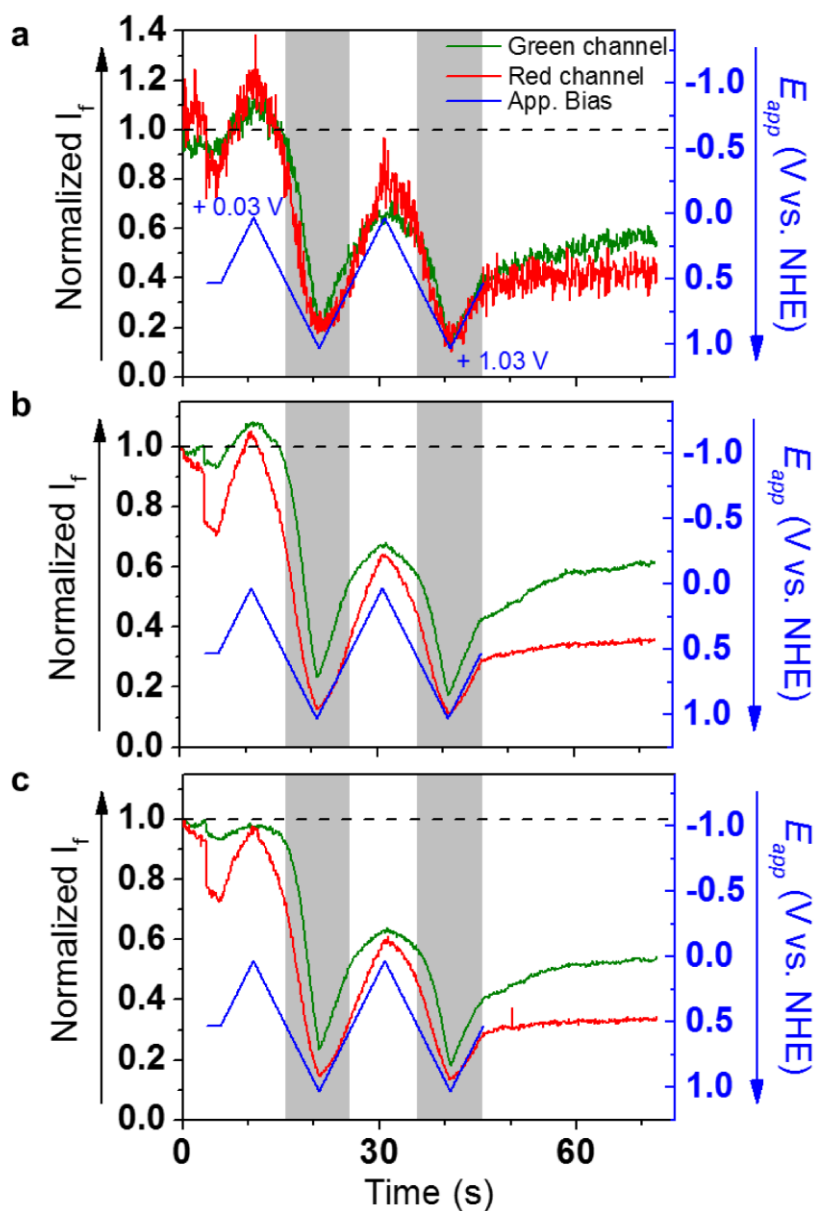


Figure 4.11. MPS-PPV NP normalized fluorescence as measured by two-color SMS-EC showing the effect of both narrow applied biases ($+0.03 \leq E_{app} \leq +1.03$ V) cycles. First cycles were shown in Figure 4.2 of the main text: Panel a) corresponds to Figure 4.2c, b) to Figure 4.2d and c) to Figure 4.2e. Time intervals where the applied bias is $> +0.54$ V are highlighted in grey.

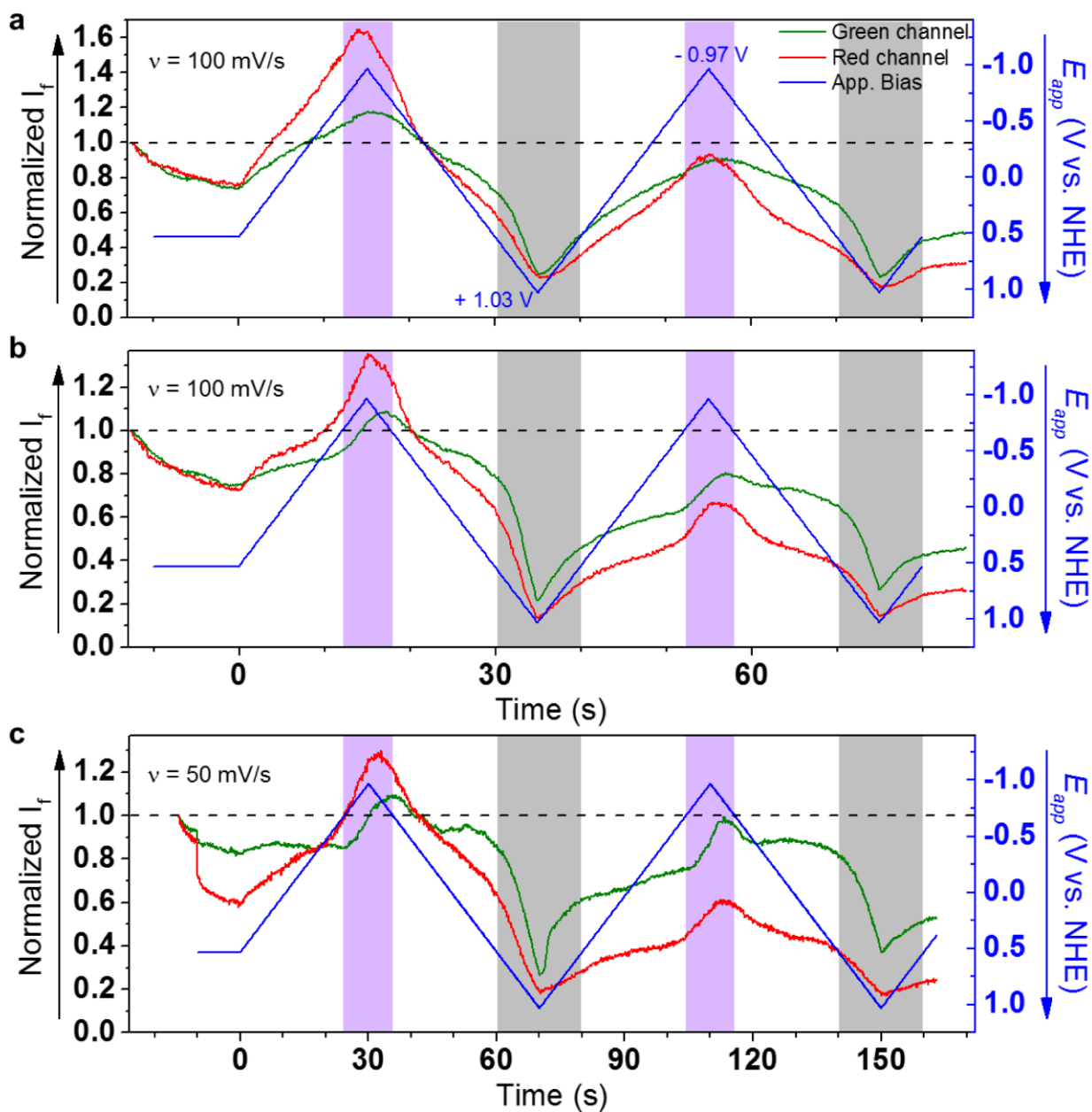


Figure 4.12. MPS-PPV NP normalized fluorescence as measured by two-color SMS-EC showing the effect of both negative applied biases ($-0.97 \leq E_{app} \leq +1.03$ V) cycles. First cycles were shown in Figure 4.5 of the main text: Panel a) corresponds to Figure 4.5a, b) to Figure 4.5b and c) to Figure 4.5c. Time intervals where the applied bias is $> +0.54$ V are highlighted in grey, while those where the applied bias is < -0.68 V are highlighted in purple.

4.8.5. Comparing the Effect of External Bias for the Different NP Subsets

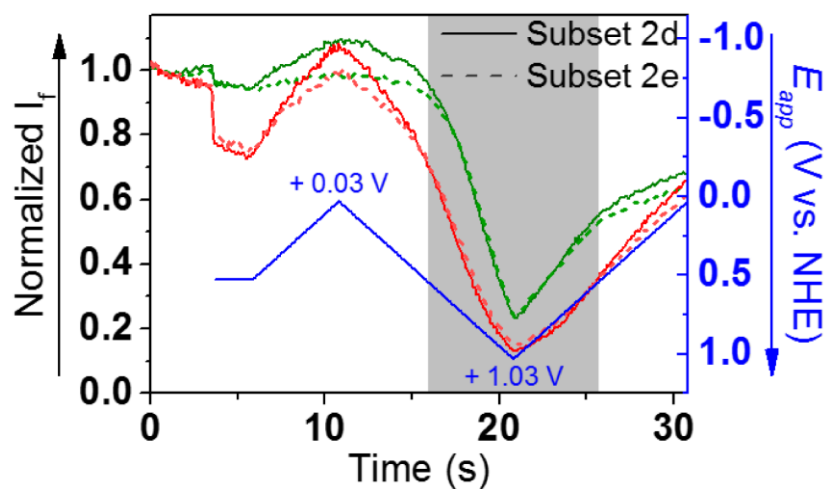


Figure 4.13. Subensemble fluorescence intensity trajectories normalized to the initial intensities under open circuit. Green and red channel fluorescence intensities are shown for the same subsets of NPs as in Figure 4.2d (solid line) and 4.2e (dashed line) of the main text. Time intervals where the applied bias is $> +0.54$ V are highlighted in grey.

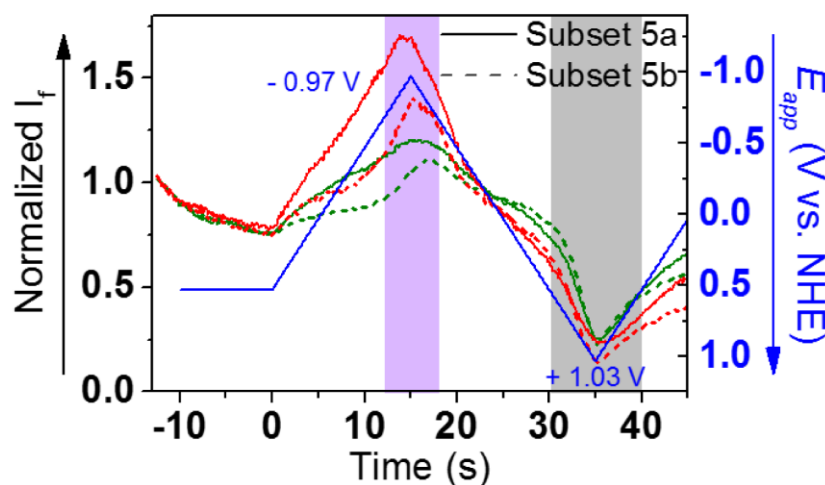


Figure 4.14. Subensemble fluorescence intensity trajectories normalized to the initial intensities under open circuit. Green and red channel fluorescence intensities are shown for the same subsets of NPs as in Figure 4.5a (solid line) and 4.5b (dashed line) of the main text. Time intervals where the applied bias is $> +0.54$ V are highlighted in grey, while those where the applied bias is < -0.68 V are highlighted in purple.

4.8.6. Comparing NP Subsets for Applied Potentials from + 0.03 to + 1.03 V

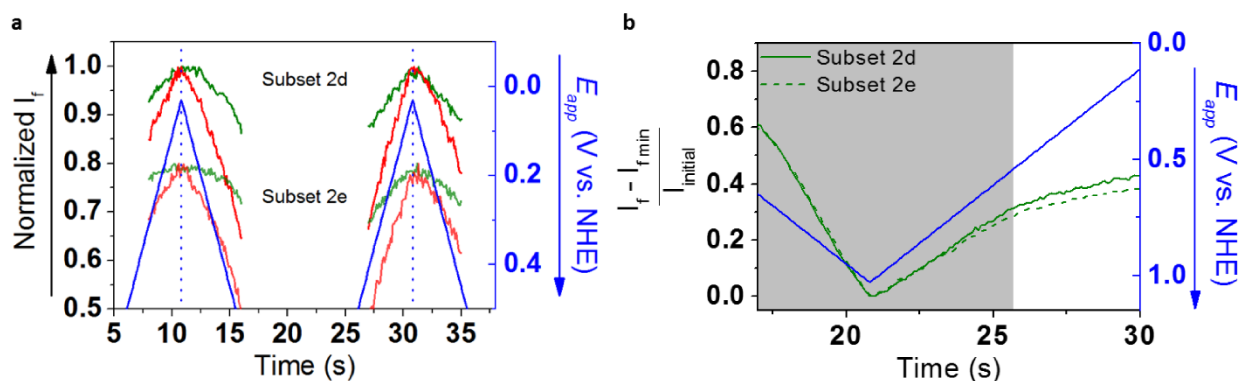


Figure 4.15. MPS-PPV NP fluorescence as measured by SMS-EC showing the effect at a ‘narrow’ applied biases range ($+ 0.03 \text{ V} \leq E_{\text{app}} \leq + 1.03 \text{ V}$). a) Changes in normalized fluorescence intensity relative to the maximum I_f for the NP subset shown in Figure 4.2d and 4.2e of the main text. The traces for subset 2e is offset by 0.2 units for clarity. The peak maximum potentials are indicated by the vertical blue dotted lines. b) Change in normalized fluorescence relative to the minimum I_f recorded in the green channels at the first maximal positive potential peak of both NP subsets shown in Figure 4.2d and 4.2e. Time intervals where the applied bias is $> + 0.54 \text{ V}$ are highlighted in grey.

4.8.7. Mechanism of Fluorescence Enhancement under Negative Applied Biases

We postulate that the sharp asymmetric fluorescence enhancement is due to the following mechanism: a) injection of electrons from ITO to photo-excited MPS-PPV segments generating negative charge carriers (radical anions), b) an injected electron can move through the MPS-PPV NP by sequential electron transfer steps between chain segments within tunnelling distance. The electron movement in the polymer material is mainly dominated by the effective electric field, which has contributions from the charged sulfonate groups, salt ions and the biased ITO electrode, and by the electronic coupling among chain segments, c) an injected electron reaches a non-emitting photooxidized quenching segment transforming it into a non-quenching site upon reversible reduction. The applied reducing potentials (down to $- 0.97 \text{ V}$) are not negative enough to inject an electron into MPS-PPV chains ($E_{\text{MPS-PPV }-/0} \sim - 1.3 \text{ to } - 1.55 \text{ V}$) and an insulating barrier is expected. However, an electron can be injected into photoexcited MPS-PPV segments ($E_{\text{MPS-PPV }-/ * } \sim 1.1 \text{ V}$) at less negative applied potentials than those necessary to reduce it in its ground state. Once a radical anion is formed, we envision that diffusion through self-exchange reactions (or hopping) between segments will occur until it reaches a site with a favorable reduction potential,

such as a photooxidized quenching site. Upon returning to potentials less negative than -0.68 V, the reduced quenching sites will energetically be favored to return the electron to the ITO electrode through photo-assisted charge hopping. This process could be impeded by a reversible chemical transformation that followed the electron injection. The hysteresis observed in the fluorescence modulation at very negative potentials in Figures 4.5b and 4.5c, as deduced from the slow decrease in I_f as the potentials are swept higher than -0.68 V (especially for the green channel), is consistent with the proposed mechanism and the relatively low electron mobility in PPVs polymer materials.

4.9. References

- (1) Pinto, M. R.; Schanze, K. S., Conjugated Polyelectrolytes: Synthesis and Applications. *Synthesis* **2002**, 2002, 1293-1309.
- (2) Burroughes, J. H.; Bradley, D. D. C.; Brown, A. R.; Marks, R. N.; Mackay, K.; Friend, R. H.; Burns, P. L.; Holmes, A. B., Light-Emitting Diodes Based on Conjugated Polymers. *Nature* **1990**, 347, 539-541.
- (3) Günes, S.; Neugebauer, H.; Sariciftci, N. S., Conjugated Polymer-Based Organic Solar Cells. *Chem. Rev.* **2007**, 107, 1324-1338.
- (4) Hoeben, F. J. M.; Jonkheijm, P.; Meijer, E. W.; Schenning, A. P. H. J., About Supramolecular Assemblies of Π -Conjugated Systems. *Chem. Rev.* **2005**, 105, 1491-1546.
- (5) Duarte, A.; Pu, K.-Y.; Liu, B.; Bazan, G. C., Recent Advances in Conjugated Polyelectrolytes for Emerging Optoelectronic Applications. *Chem. Mater.* **2010**, 23, 501-515.
- (6) Chen, H.-Y.; Hou, J.; Zhang, S.; Liang, Y.; Yang, G.; Yang, Y.; Yu, L.; Wu, Y.; Li, G., Polymer Solar Cells with Enhanced Open-Circuit Voltage and Efficiency. *Nat Photon* **2009**, 3, 649-653.
- (7) Esfandiari, N. M.; Wang, Y.; Bass, J. Y.; Blum, S. A., Deconvoluting Subensemble Chemical Reaction Kinetics of Platinum–Sulfur Ligand Exchange Detected with Single-Molecule Fluorescence Microscopy. *Inorg. Chem.* **2011**, 50, 9201-9203.
- (8) Thomas, S. W.; Joly, G. D.; Swager, T. M., Chemical Sensors Based on Amplifying Fluorescent Conjugated Polymers. *Chem. Rev.* **2007**, 107, 1339-1386.
- (9) Hu, D.; Yu, J.; Wong, K.; Bagchi, B.; Rossky, P. J.; Barbara, P. F., Collapse of Stiff Conjugated Polymers with Chemical Defects into Ordered, Cylindrical Conformations. *Nature* **2000**, 405, 1030-1033.
- (10) Kim, J.; Swager, T. M., Control of Conformational and Interpolymer Effects in Conjugated Polymers. *Nature* **2001**, 411, 1030-1034.
- (11) Sarzi Sartori, S.; De Feyter, S.; Hofkens, J.; Van der Auweraer, M.; De Schryver, F.; Brunner, K.; Hofstraat, J. W., Host Matrix Dependence on the Photophysical Properties of Individual Conjugated Polymer Chains. *Macromolecules* **2003**, 36, 500-507.
- (12) Ebihara, Y.; Vacha, M., Relating Conformation and Photophysics in Single MEH-PPV Chains. *J. Phys. Chem. B* **2008**, 112, 12575-12578.
- (13) Lupton, J. M., Chromophores in Conjugated Polymers—All Straight? *ChemPhysChem* **2012**, 13, 901-907.

- (14) Botiz, I.; Stingelin, N., Influence of Molecular Conformations and Microstructure on the Optoelectronic Properties of Conjugated Polymers. *Materials* **2014**, *7*, 2273-2300.
- (15) Nguyen, T.-Q.; Martini, I. B.; Liu, J.; Schwartz, B. J., Controlling Interchain Interactions in Conjugated Polymers: The Effects of Chain Morphology on Exciton–Exciton Annihilation and Aggregation in MEH-PPV Films. *J. Phys. Chem. B* **2000**, *104*, 237-255.
- (16) Kobayashi, H.; Hirata, S.; Vacha, M., Mechanical Manipulation of Photophysical Properties of Single Conjugated Polymer Nanoparticles. *J. Phys. Chem. Lett.* **2013**, *4*, 2591-2596.
- (17) Woll, D.; Braeken, E.; Deres, A.; De Schryver, F. C.; Uji-i, H.; Hofkens, J., Polymers and Single Molecule Fluorescence Spectroscopy, What Can We Learn? *Chem. Soc. Rev.* **2009**, *38*, 313-328.
- (18) Schwartz, B. J., Conjugated Polymers as Molecular Materials: How Chain Conformation and Film Morphology Influence Energy Transfer and Interchain Interactions. *Annu. Rev. Phys. Chem.* **2003**, *54*, 141-172.
- (19) Nguyen, T.-Q.; Wu, J.; Doan, V.; Schwartz, B. J.; Tolbert, S. H., Control of Energy Transfer in Oriented Conjugated Polymer-Mesoporous Silica Composites. *Science* **2000**, *288*, 652-656.
- (20) Hu, Z.; Adachi, T.; Haws, R.; Shuang, B.; Ono, R. J.; Bielawski, C. W.; Landes, C. F.; Rossky, P. J.; Vanden Bout, D. A., Excitonic Energy Migration in Conjugated Polymers: The Critical Role of Interchain Morphology. *J. Am. Chem. Soc.* **2014**.
- (21) Hwang, I.; Scholes, G. D., Electronic Energy Transfer and Quantum-Coherence in Π -Conjugated Polymers. *Chem. Mater.* **2011**, *23*, 610-620.
- (22) Garcia, A.; Nguyen, T.-Q., Effect of Aggregation on the Optical and Charge Transport Properties of an Anionic Conjugated Polyelectrolyte. *J. Phys. Chem. C* **2008**, *112*, 7054-7061.
- (23) Wang, D.; Lal, J.; Moses, D.; Bazan, G. C.; Heeger, A. J., Small Angle Neutron Scattering (SANS) Studies of a Conjugated Polyelectrolyte in Aqueous Solution. *Chem. Phys. Lett.* **2001**, *348*, 411-415.
- (24) Gao, Y.; Wang, C.-C.; Wang, L.; Wang, H.-L., Conjugated Polyelectrolytes with pH-Dependent Conformations and Optical Properties. *Langmuir* **2007**, *23*, 7760-7767.
- (25) Schnablegger, H.; Antonietti, M.; Göltner, C.; Hartmann, J.; Cölfen, H.; Samori, P.; Rabe, J. P.; Häger, H.; Heitz, W., Morphological Characterization of the Molecular Superstructure of Polyphenylene Ethynylene Derivatives. *J. Colloid Interface Sci.* **1999**, *212*, 24-32.
- (26) Palacios, R. E.; Fan, F.-R. F.; Grey, J. K.; Suk, J.; Bard, A. J.; Barbara, P. F., Charging and Discharging of Single Conjugated-Polymer Nanoparticles. *Nat. Mater.* **2007**, *6*, 680-685.
- (27) Liu, J.; Feng, G.; Liu, R.; Tomczak, N.; Ma, L.; Gurzadyan, G. G.; Liu, B., Bright Quantum-Dot-Sized Single-Chain Conjugated Polyelectrolyte Nanoparticles: Synthesis, Characterization and Application for Specific Extracellular Labeling and Imaging. *Small* **2014**, *10*, 3110-3118.
- (28) Szymanski, C.; Wu, C.; Hooper, J.; Salazar, M. A.; Perdomo, A.; Dukes, A.; McNeill, J., Single Molecule Nanoparticles of the Conjugated Polymer MeH-PPV, Preparation and Characterization by near-Field Scanning Optical Microscopy. *J. Phys. Chem. B* **2005**, *109*, 8543-8546.
- (29) Tuncel, D.; Demir, H. V., Conjugated Polymer Nanoparticles. *Nanoscale* **2010**, *2*, 484-494.
- (30) Pecher, J.; Mecking, S., Nanoparticles of Conjugated Polymers. *Chem. Rev.* **2010**, *110*, 6260-6279.

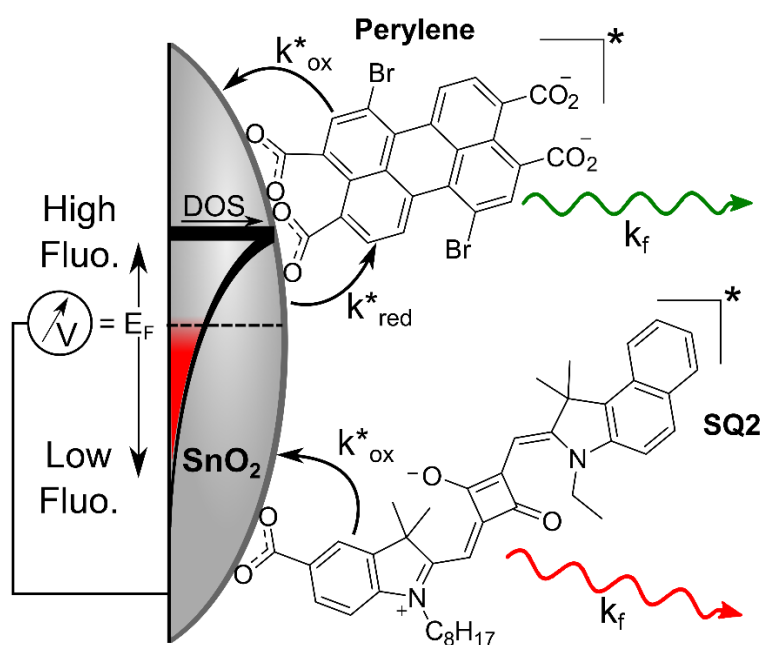
- (31) Wu, C.; Chiu, D. T., Highly Fluorescent Semiconducting Polymer Dots for Biology and Medicine. *Angew. Chem. Int. Ed.* **2013**, *52*, 3086-3109.
- (32) Bustamante, C.; Maestre, M. F., Statistical Effects in the Absorption and Optical Activity of Particulate Suspensions. *Proc. Natl. Acad. Sci. U. S. A.* **1988**, *85*, 8482-8486.
- (33) Choi, J.; Ruiz, C. R.; Nesterov, E. E., Temperature-Induced Control of Conformation and Conjugation Length in Water-Soluble Fluorescent Polythiophenes. *Macromolecules* **2010**, *43*, 1964-1974.
- (34) Tretiak, S.; Saxena, A.; Martin, R. L.; Bishop, A. R., Interchain Electronic Excitations in Poly(phenylenevinylene) (PPV) Aggregates. *J. Phys. Chem. B* **2000**, *104*, 7029-7037.
- (35) Pinto, M. R.; Kristal, B. M.; Schanze, K. S., A Water-Soluble Poly(Phenylene Ethynylene) with Pendant Phosphonate Groups. Synthesis, Photophysics, and Layer-by-Layer Self-Assembled Films. *Langmuir* **2003**, *19*, 6523-6533.
- (36) Traiphol, R.; Charoenthai, N.; Sriksirin, T.; Kerdcharoen, T.; Osotchan, T.; Maturos, T., Chain Organization and Photophysics of Conjugated Polymer in Poor Solvents: Aggregates, Agglomerates and Collapsed Coils. *Polymer* **2007**, *48*, 813-826.
- (37) Wang, M.; Zou, S.; Guerin, G.; Shen, L.; Deng, K.; Jones, M.; Walker, G. C.; Scholes, G. D.; Winnik, M. A., A Water-Soluble Ph-Responsive Molecular Brush of Poly(N,N-Dimethylaminoethyl Methacrylate) Grafted Polythiophene. *Macromolecules* **2008**, *41*, 6993-7002.
- (38) Feist, F. A.; Zickler, M. F.; Basché, T., Origin of the Red Sites and Energy Transfer Rates in Single MEH-PPV Chains at Low Temperature. *ChemPhysChem* **2011**, *12*, 1499-1508.
- (39) Potai, R.; Traiphol, R., Controlling Chain Organization and Photophysical Properties of Conjugated Polymer Nanoparticles Prepared by Reprecipitation Method: The Effect of Initial Solvent. *J. Colloid Interface Sci.* **2013**, *403*, 58-66.
- (40) Lemmer, U.; Heun, S.; Mahrt, R. F.; Scherf, U.; Hopmeier, M.; Siegner, U.; Göbel, E. O.; Müllen, K.; Bässler, H., Aggregate Fluorescence in Conjugated Polymers. *Chem. Phys. Lett.* **1995**, *240*, 373-378.
- (41) Aryanpour, K.; Sheng, C. X.; Olejnik, E.; Pandit, B.; Psiachos, D.; Mazumdar, S.; Vardeny, Z. V., Evidence for Excimer Photoexcitations in an Ordered Π -Conjugated Polymer Film. *Phys. Rev. B* **2011**, *83*, 155124.
- (42) Smith, A.; Shen, C.-F.; Roberts, S.; Helgeson, R.; Schwartz, B., Ionic Strength and Solvent Control over the Physical Structure, Electronic Properties and Superquenching of Conjugated Polyelectrolytes. *Res. Chem. Intermed.* **2007**, *33*, 125-142.
- (43) Jakubiak, R.; Collison, C. J.; Wan, W. C.; Rothberg, L. J.; Hsieh, B. R., Aggregation Quenching of Luminescence in Electroluminescent Conjugated Polymers. *J. Phys. Chem. A* **1999**, *103*, 2394-2398.
- (44) Collini, E.; Scholes, G. D., Coherent Intrachain Energy Migration in a Conjugated Polymer at Room Temperature. *Science* **2009**, *323*, 369-373.
- (45) Hoven, C. V.; Garcia, A.; Bazan, G. C.; Nguyen, T.-Q., Recent Applications of Conjugated Polyelectrolytes in Optoelectronic Devices. *Adv. Mater.* **2008**, *20*, 3793-3810.
- (46) Yang, R.; Garcia, A.; Korystov, D.; Mikhailovsky, A.; Bazan, G. C.; Nguyen, T.-Q., Control of Interchain Contacts, Solid-State Fluorescence Quantum Yield, and Charge Transport of Cationic Conjugated Polyelectrolytes by Choice of Anion. *J. Am. Chem. Soc.* **2006**, *128*, 16532-16539.
- (47) Palacios, R. E.; Fan, F.-R. F.; Bard, A. J.; Barbara, P. F., Single-Molecule Spectroelectrochemistry (SMS-EC). *J. Am. Chem. Soc.* **2006**, *128*, 9028-9029.

- (48) Bard, A. J.; Faulkner, L. R., *Electrochemical Methods: Fundamentals and Applications*. 2nd ed.; Wiley: Hoboken, 2001.
- (49) Palacios, R. E.; Chang, W.-S.; Grey, J. K.; Chang, Y.-L.; Miller, W. L.; Lu, C.-Y.; Henkelman, G.; Zepeda, D.; Ferraris, J.; Barbara, P. F., Detailed Single-Molecule Spectroelectrochemical Studies of the Oxidation of Conjugated Polymers. *J. Phys. Chem. B* **2009**, *113*, 14619-14628.
- (50) Li, Y.; Cao, Y.; Gao, J.; Wang, D.; Yu, G.; Heeger, A. J., Electrochemical Properties of Luminescent Polymers and Polymer Light-Emitting Electrochemical Cells. *Synth. Met.* **1999**, *99*, 243-248.
- (51) Holt, A. L.; Leger, J. M.; Carter, S. A., Electrochemical and Optical Characterization of P- and N-Doped Poly[2-Methoxy-5-(2-Ethylhexyloxy)-1,4-Phenylenevinylene]. *J. Chem. Phys.* **2005**, *123*, 044704 1-7.
- (52) Wu, H.; Huang, F.; Mo, Y.; Yang, W.; Wang, D.; Peng, J.; Cao, Y., Efficient Electron Injection from a Bilayer Cathode Consisting of Aluminum and Alcohol-/Water-Soluble Conjugated Polymers. *Adv. Mater.* **2004**, *16*, 1826-1830.
- (53) Wu, H.; Huang, F.; Peng, J.; Cao, Y., High-Efficiency Electron Injection Cathode of Au for Polymer Light-Emitting Devices. *Org. Electron.* **2005**, *6*, 118-128.
- (54) Ishii, H.; Sugiyama, K.; Ito, E.; Seki, K., Energy Level Alignment and Interfacial Electronic Structures at Organic/Metal and Organic/Organic Interfaces. *Adv. Mater.* **1999**, *11*, 605-625.
- (55) Hoven, C. V.; Peet, J.; Mikhailovsky, A.; Nguyen, T.-Q., Direct Measurement of Electric Field Screening in Light Emitting Diodes with Conjugated Polyelectrolyte Electron Injecting/Transport Layers. *Appl. Phys. Lett.* **2009**, *94*, 033301 1-3.
- (56) Nguyen, T.-Q.; Schwartz, B. J.; Schaller, R. D.; Johnson, J. C.; Lee, L. F.; Haber, L. H.; Saykally, R. J., Near-Field Scanning Optical Microscopy (NSOM) Studies of the Relationship between Interchain Interactions, Morphology, Photodamage, and Energy Transport in Conjugated Polymer Films. *J. Phys. Chem. B* **2001**, *105*, 5153-5160.
- (57) Schaller, R. D.; Snee, P. T.; Johnson, J. C.; Lee, L. F.; Wilson, K. R.; Haber, L. H.; Saykally, R. J.; Nguyen, T.-Q.; Schwartz, B. J., Nanoscopic Interchain Aggregate Domain Formation in Conjugated Polymer Films Studied by Third Harmonic Generation near-Field Scanning Optical Microscopy. *J. Chem. Phys.* **2002**, *117*, 6688-6698.
- (58) Nguyen, T. Q.; Wu, J.; Tolbert, S. H.; Schwartz, B. J., Control of Energy Transport in Conjugated Polymers Using an Ordered Mesoporous Silica Matrix. *Adv. Mater.* **2001**, *13*, 609-611.
- (59) Heeger, A. J.; Kivelson, S.; Schrieffer, J. R.; Su, W. P., Solitons in Conducting Polymers. *Rev. Mod. Phys.* **1988**, *60*, 781-850.
- (60) Garcia, A.; Bakus Ii, R. C.; Zalar, P.; Hoven, C. V.; Brzezinski, J. Z.; Nguyen, T.-Q., Controlling Ion Motion in Polymer Light-Emitting Diodes Containing Conjugated Polyelectrolyte Electron Injection Layers. *J. Am. Chem. Soc.* **2011**, *133*, 2492-2498.
- (61) Liu, H.-W.; Ngo, A. T.; Cosa, G., Enhancing the Emissive Properties of Poly(P-Phenylenevinylene)-Conjugated Polyelectrolyte-Coated SiO₂ Nanoparticles. *J. Am. Chem. Soc.* **2011**, *134*, 1648-1652.
- (62) Park, S.-J.; Gesquiere, A. J.; Yu, J.; Barbara, P. F., Charge Injection and Photooxidation of Single Conjugated Polymer Molecules. *J. Am. Chem. Soc.* **2004**, *126*, 4116-4117.

- (63) Gesquiere, A. J.; Park, S.-J.; Barbara, P. F., F–V/SMS: A New Technique for Studying the Structure and Dynamics of Single Molecules and Nanoparticles. *J. Phys. Chem. B* **2004**, *108*, 10301-10308.
- (64) Cornil, J.; dos Santos, D. A.; Crispin, X.; Silbey, R.; Brédas, J. L., Influence of Interchain Interactions on the Absorption and Luminescence of Conjugated Oligomers and Polymers: A Quantum-Chemical Characterization. *J. Am. Chem. Soc.* **1998**, *120*, 1289-1299.
- (65) Stringle, D. L. B.; Nevin Campbell, R.; Workentin, M. S., Radical Anion Chain Process Initiated by a Dissociative Electron Transfer to a Monocyclic Endoperoxide. *Chem. Commun.* **2003**, 1246-1247.
- (66) Scurlock, R. D.; Wang, B.; Ogilby, P. R.; Sheats, J. R.; Clough, R. L., Singlet Oxygen as a Reactive Intermediate in the Photodegradation of an Electroluminescent Polymer. *J. Am. Chem. Soc.* **1995**, *117*, 10194-10202.
- (67) Dam, N.; Scurlock, R. D.; Wang, B.; Ma, L.; Sundahl, M.; Ogilby, P. R., Singlet Oxygen as a Reactive Intermediate in the Photodegradation of Phenylenevinylene Oligomers. *Chem. Mater.* **1999**, *11*, 1302-1305.
- (68) Burrows, H. D.; Narwark, O.; Peetz, R.; Thorn-Csanyi, E.; Monkman, A. P.; Hamblett, I.; Navaratnam, S., Mechanistic Studies on the Photodegradation of 2,5-Dialkyloxy-Substituted Para-Phenylenevinylene Oligomers by Singlet Oxygen. *Photochem. Photobio. Sci.* **2010**, *9*, 942-948.
- (69) Chambon, S.; Rivaton, A.; Gardette, J.-L.; Firon, M., Photo- and Thermo-Oxidation of Poly(p-Phenylene-Vinylene) and Phenylene-Vinylene Oligomer. *Polym. Degrad. Stab.* **2011**, *96*, 1149-1158.
- (70) Guerlin, A.; Dumur, F.; Dumas, E.; Miomandre, F.; Wantz, G.; Mayer, C. R., Tunable Optical Properties of Chromophores Derived from Oligo(p-Phenylene Vinylene). *Org. Lett.* **2010**, *12*, 2382-2385.
- (71) Silvester, D. S.; He, W.; Aldous, L.; Hardacre, C.; Compton, R. G., Electrochemical Reduction of Benzoic Acid and Substituted Benzoic Acids in Some Room Temperature Ionic Liquids. *J. Phys. Chem. C* **2008**, *112*, 12966-12973.
- (72) Bolinger, J. C.; Fradkin, L.; Lee, K.-J.; Palacios, R. E.; Barbara, P. F., Light-Assisted Deep-Trapping of Holes in Conjugated Polymers. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 1342-1346.

5. Fluorescence-Mediated Visualization of Charge Transfer Dynamics in Dye-Sensitized SnO₂ Electrodes under Applied Biases

Adapted with permission from Robert Godin, Benjamin D. Sherman, Jesse J. Bergkamp, Carlos A. Chesta, Ana L. Moore, Thomas A. Moore, Rodrigo Palacios, and Gonzalo Cosa, *Journal of Physical Chemistry Letters*, **2015**, 6, 2688–2693. Copyright 2015 American Chemical Society.



5.1. Preface

The interfacial charge transfer (ICT) dynamics that occur in dye-sensitized solar cells (DSSCs) are highly complex in nature. Despite ICT being at the core of the energy conversion process, the effects of electron density in the semiconductor on the ICT dynamics is still not fully understood. In this Chapter, we draw on SMS-EC to probe the ICT dynamics between organic dye sensitizers and SnO₂ semiconductor. Electrochemical control of the applied bias allowed us to control the Fermi level and electron density of the semiconductor. SMS-EC is then used to visualize and monitor the fluorescence emitted from dye sensitizers under applied biases. Since fluorescence competes with ICT to deactivate the excited state, we can infer changes to ICT through the fluorescence intensity modulation. Although we have not yet successfully studied individual molecules in this system, the SMS-EC setup provided the high sensitivity necessary to study very thin SnO₂ layers, while total internal reflection (TIRF) microscopy spatially excluded contributions from dyes not bound to the SnO₂ surface. The photobleaching dependence on the applied bias of a perylene sensitizer proved to be insightful in probing the rate of dye photooxidation. The fluorescence intensity modulation that was observed at positive applied biases led us to conclude that a dye photoreduction pathway is also active, with implications for the design of solar cells. By studying two dye sensitizers with significantly different excited state redox properties, we deduced that electron occupancy of the density of state (DOS) of the semiconductor takes precedent over energetic effects. Previous reports have observed the dominance of either DOS or energetics effects. This unique set of data, useful to researchers working in dye-sensitized solar energy conversion devices, highlights our limited understanding of ICT dynamics at heterogeneous dye-semiconductor interfaces. It further serves as a motivation to realize our goal

of true SMS-EC studies of dye-sensitized electrodes to reveal the spatial and dynamical heterogeneity of ICT processes.

5.2. Abstract

The development of dye-sensitized solar cells requires in depth understanding of the interfacial charge transfer dynamics that take place between dye sensitizers and semiconductors. Here we describe a prototype system to probe these dynamics by monitoring in real-time the fluorescence of two organic sensitizers, a perylene and a squaraine, bound to a SnO_2 semiconductor thin film under control of the Fermi level. The two different sensitizer fluorophores characterized by vastly different redox potentials undergo similar fluorescence modulation with applied bias, an indication that the density of states of the semiconductor largely influences the charge transfer dynamics while energetics play a minimal role. We further show that the rate of photodegradation of the perylene sensitizer with applied bias provides a suitable marker to study the rate of charge injection and charge recombination. Taken together, our results demonstrate a suitable platform to visualize and study charge transfer dynamics on films and constitute a step forward towards achieving single molecule resolution in our quest to decipher the static and dynamic heterogeneity of charge transfer dynamics in dye-sensitized photoanodes.

5.3. Introduction

Dye-sensitized solar cells (DSSCs)¹⁻² have tremendous potential as a low-cost, easy to manufacture solution to the world's growing energy needs. The dynamics of interfacial charge transfers play a key role in determining how charges flow in the cell and ultimately the power conversion efficiency of DSSCs. Significant advances have been made towards maximizing efficiency of DSSCs by studying, via transient absorption spectroscopy, the influence of applied biases on rates of electron injection and charge recombination. By monitoring the absorption of the photooxidized sensitizer and that of the injected electron over time, it has been shown that when the Fermi level (E_F) of the semiconductor is raised by applying a negative potential,

electronic states are filled leading to a high density of electrons.³ The increase in electronic density in turn hampers device performance by both inhibiting electron injection into the semiconductor and by accelerating charge recombination with the photooxidized sensitizer.⁴⁻⁷ The applied bias dependence of charge transfer pathways are important considerations for the working conditions of DSSCs under sunlight irradiation.⁸

Here we report the use of fluorescence spectroscopy to probe interfacial charge transfer dynamics under applied bias. Originally introduced in seminal work by Kamat et al. using MLCT emission rather than fluorescence,⁹ the method enables monitoring charge transfer dynamics from the photoexcited sensitizer as it competes with radiative emission.¹⁰ Fluorescence provides an internal clock of relevant timescales for interfacial charge dynamics, that of the excited state lifetime dictated by the interplay of radiative decay (ps – ns) and charge transfer. Furthermore, fluorescence boasts unsurpassed sensitivity, reaching single molecule levels.¹¹

In this work we describe a prototype system where we monitor and visualize real-time the fluorescence of organic fluorescent sensitizers, either a perylene or squaraine (SQ2), bound to a SnO₂ semiconductor thin film under control of the electron density and E_F (Figure 5.1). Working with two different sensitizer fluorophores, characterized by different redox potentials, we show that the emission intensity is tuned by the applied bias. Similar emission enhancements with decreasing potential, a result of suppressed electron injection into the semiconductor in favor of emission, is recorded for both dyes. That emission from both dyes is equally enhanced indicates that the effect of sensitizer energetics on charge transfer rates is minimal but that the density of states in the semiconductor largely influences the charge transfer dynamics. Photodegradation (photobleaching) of the oxidized form of the perylene sensitizer, but not the squaraine dye, provides additional insight on the rate of charge injection, and also on the rate of charge

recombination. Analysis of the fluorescence intensity observed at high positive applied biases yield information on the critical role that photoreduction (electron injection from the semiconductor into the photoexcited sensitizer) may play in reducing overall performance in DSSCs. Taken together, our results provide a suitable platform to visualize and study charge transfer dynamics on films and constitute a step forward towards achieving single molecule resolution in our quest to decipher the charge transfer dynamics in a model dye-sensitized photoanode.

5.4. Results and Discussion

In order to study the interfacial charge transfer dynamics between organic dyes and metal oxide semiconductors, we resorted to a single molecule/particle spectroelectrochemistry (SMS-EC)¹² setup based on a total internal reflection fluorescence microscope (TIRFM), see Figure 5.1. The setup enabled photoexcitation at the dye-semiconductor interface with an evanescent field that only penetrates a few hundred nanometers in the solution. We therefore spatially exclude fluorescence contributions from dyes that potentially desorb from the semiconductor surface under negative applied bias.¹³ We separately monitored the fluorescence of two different organic sensitizers, either a perylene or a commercial squaraine (SQ2), bound to a SnO₂ semiconductor thin film under electrochemical control of the electron density and E_F . These two sensitizers were chosen since their redox potentials are significantly different (Figure 5.1) allowing for the investigation of the effects of the sensitizer energetics. The fluorescence emitted from the sensitizers was collected by the microscope objective and recorded by an electron multiplied CCD camera (EMCCD). The camera was synchronized with a dedicated potentiostat applying an external bias (E_{app}) to control the electron density and E_F of the SnO₂ semiconductor.

Microscope coverslips were prepared by spin-casting a ~ 35 nm thick SnO₂ film on top of ITO-coated coverslips (Figure Figure 5.5). From the AFM experiments, small areas of ITO were

exposed and not covered by the SnO₂ film (Figure 5.5B). The fluorescence observed and results discussed are still expected to originate from sensitizers bound to SnO₂ as control experiments on bare ITO with no SnO₂ present showed negligible fluorescence intensity (Figure 5.6). The sensitized SnO₂ was prepared by soaking selected areas of the electrode overnight in baths of the appropriate dye, followed by thorough rinsing. An electrochemical cell was then built on top of the dye-functionalized SnO₂-coated coverslip to finalize the sample.

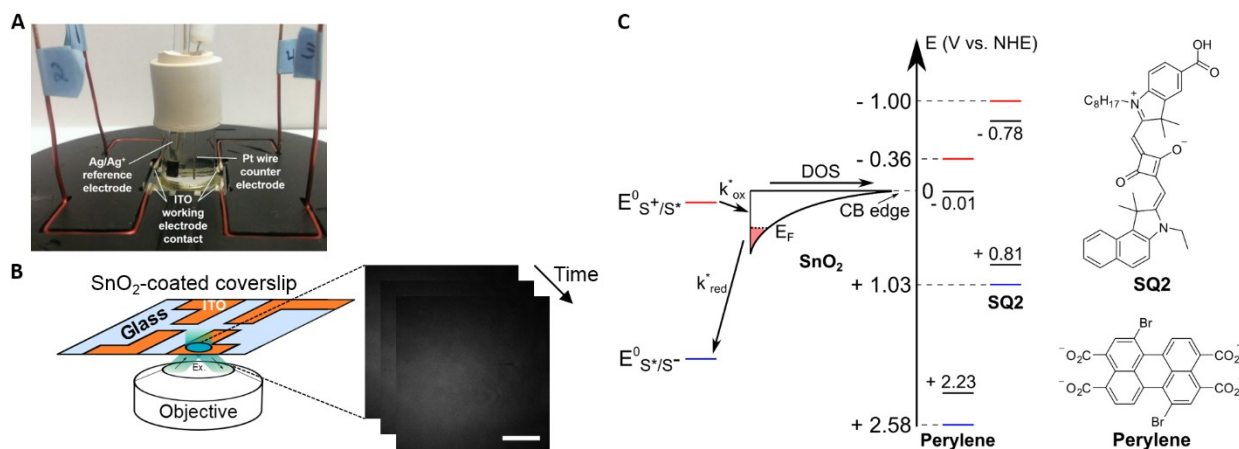


Figure 5.1. SMS-EC microscope setup and configuration of the samples studied. A) Photograph of the SMS-EC cell illustrating the electrodes and leads for the electrochemical chamber. B) Cartoon showing the coverslip with 4 independent L-shaped ITO electrodes and the evanescent optical excitation. The fluorescent emission from dyes bound to the SnO₂ film passes through the objective and is relayed to an EM-CCD camera for image capture. Shown to the right is a series of frames from a movie acquired on a perylene-sensitized region of the SnO₂ film under an applied bias of 0.83 V vs. NHE. The scale bar is 20 μ m. C) Energy diagram showing the relevant energy levels of the SnO₂ and dyes utilized in this work (see also the chemical structures of the dyes). Occupied electronic states of the SnO₂ are highlighted in red. The ground state redox potentials are drawn in black (closed form of the perylene). The excited state redox potentials (excited state oxidation in red, excited state reduction in blue) are calculated taking into account the E_{00} energies of the sensitizers (opened form for the perylene). Excited state oxidation potentials are drawn in red and excited state reduction potentials are in blue. To the left is an example sensitizer depicting dye photooxidation (k^*_{ox}) and photoreduction (k^*_{red}) pathways.

We initially compared the fluorescence from the two dyes when no external bias is applied. Images acquired in the microscopy setup show smooth fluorescence profiles indicating homogeneous surface coverage for both dyes. In the absence of external bias a resting potential of

~ 0.4 V (vs. NHE) was measured and we observed fast photobleaching of the perylene fluorescence; however, SQ2 emission was stable over time. Under control of E_{app} and thus of the E_{F} of SnO_2 , the fluorescence of the perylene dye decayed very rapidly upon illumination when biases of ~ 1 V were applied. Applying a positive potential decreases the electron density present in the semiconductor film. Qualitatively, the rate of perylene photobleaching decreased significantly with decreasing potential (Figure 5.2A). SQ2 on the other hand showed limited, bias-independent, photobleaching (Figure 5.2C). Control experiments with perylene bound to a SiO_2 redox-inactive support showed minimal photobleaching (Figure 5.7). The photobleaching of the perylene in the absence of charge transfer was thus insignificant. Similar results and conclusions were obtained with SQ2 bound to SiO_2 .

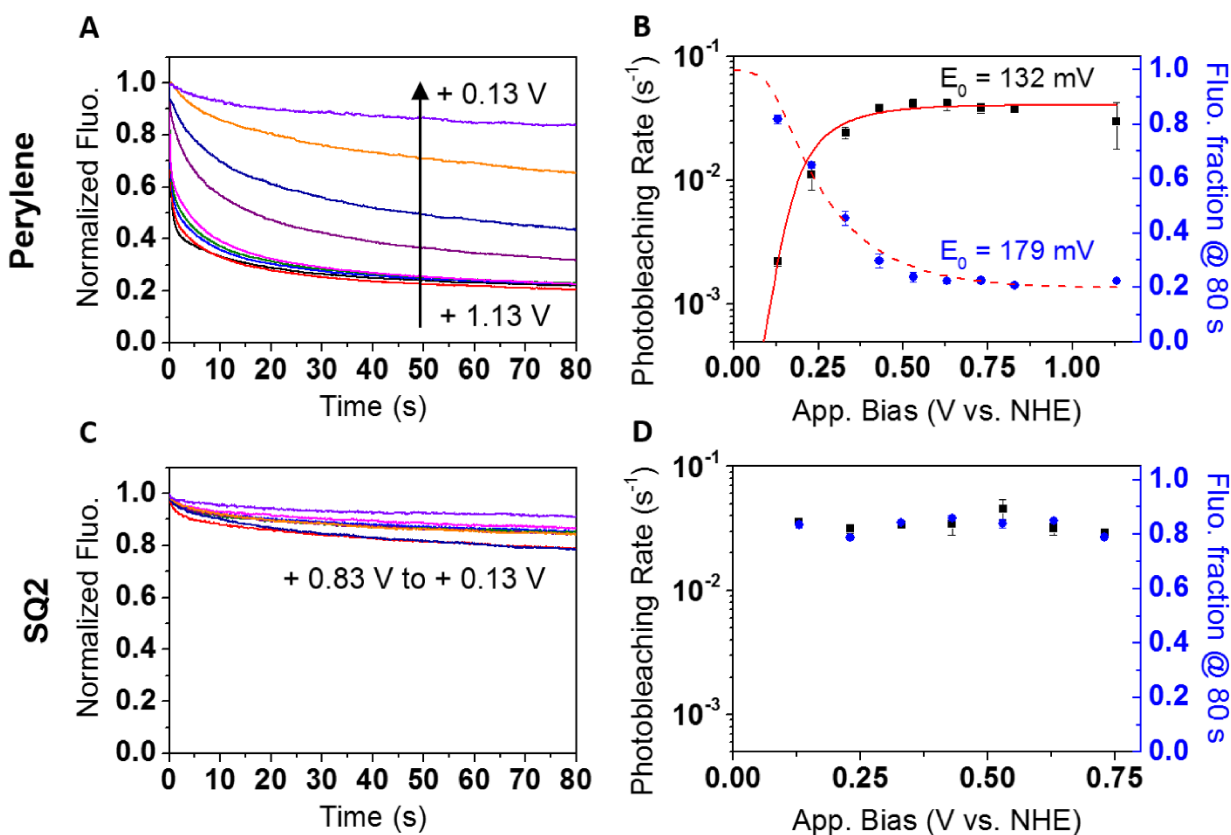
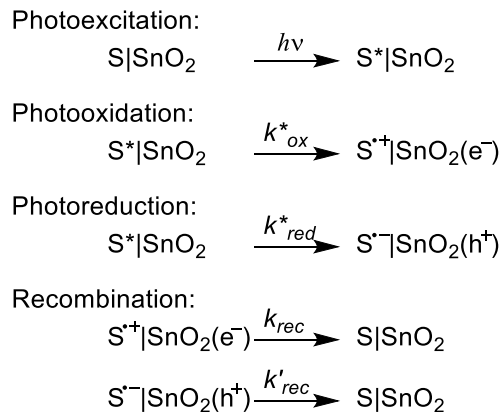


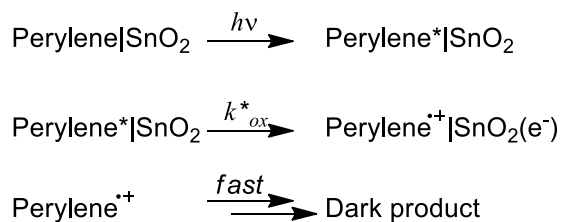
Figure 5.2. Influence of applied bias on the photobleaching of the fluorescent sensitizer dyes under study. Shown to the left are the normalized fluorescence intensity trajectories of the perylene (A) and SQ2 (C) dyes. See Experimental section for details on excitation and emission wavelengths. Shown to the right are the photobleaching rates obtained from stretched exponential fits to the data (Equations (5.1) and (5.2)) and residual fluorescence after 80 s of illumination for the perylene (B) and SQ2 (D) dyes. The red lines in B) represent fits to the sigmoidal expressions depicted in Equations (5.3) and (5.4) below. The best fit parameters for E_0 are noted in the plot.

These initial results highlight the rapid photodegradation of the perylene dye following formation of its radical cation (perylene^{•+}) subsequent to electron injection into the SnO₂ (photoinduced oxidation (k_{ox}^*), see also Figure 5.1 and Schemes 5.1 and 5.2). The perylene dye used in this work has been shown to inject electrons into TiO₂ nanoparticles (NPs).¹⁴ It is thus also expected to inject electrons into SnO₂, characterized by a conduction band edge (CBE $\sim$ 0 V vs. NHE)⁹ positively shifted by 0.5 V compared to TiO₂ (CBE $\sim$ - 0.5 V vs. NHE).¹⁵ The irreversible previously reported electrochemical oxidation¹⁴ of the perylene supports the rapid transformation of the newly formed perylene^{•+} to non-emissive dark products (Scheme 5.2). The rate limiting step of photobleaching being given by the photooxidation of the perylene (k_{ox}^*) by electron transfer to SnO₂. Given the excited state oxidation potentials of perylene and SQ2, and the fact that SQ2 is used in high performance TiO₂-based DSSCs,¹⁶ photoinduced oxidation should also readily occur for SQ2 on SnO₂ to yield the radical cation of SQ2 (SQ2^{•+}). The relatively minor and bias-independent photobleaching observed for SQ2 on SnO₂ suggests that SQ2^{•+} is stable under our experimental conditions. The proposed stability of SQ2^{•+} attached to SnO₂ is consistent with the results of electrochemical cyclic voltammetry (CV) experiments which show highly reversible oxidation waves for SQ2 in solution.¹⁶

Scheme 5.1. Excited state charge transfer pathways.



Scheme 5.2. Proposed degradation pathway for perylene fluorescent sensitizer.



We next extracted from the data in Figure 5.2 the rates of photobleaching for the perylene dye in order to correlate them to E_{app} and thus obtain the dependence of k_{ox}^* with E_{app} . To obtain these rates, we first fitted the fluorescence decay trajectories to the stretched exponential expression shown in Equation (5.1) (see Figure 5.8). Here $I_{(t)}$, and I_0 are the fluorescence intensities for the sample at time t and time $t = 0$, respectively. I_∞ corresponds to the residual intensity recorded once the sample photobleached. By comparing the well-defined values of I_∞ obtained with the perylene for $E_{app} > 0.5$ V (~ 2200 for non-normalized trajectories) to the background intensity observed on non-functionalized SnO₂ films (~ 330), we conclude that the residual fluorescence mostly stems from dyes that do not participate in interfacial charge transfer with the SnO₂. The parameter τ_{bl} is the characteristic time scale of the decay, and h is the heterogeneity parameter.

$$I_{(t)} = I_0 \times \exp \left[- \left(\frac{t}{\tau_{bl}} \right)^{1/h} \right] + I_\infty \quad (5.1)$$

The average rate of photobleaching was then determined from Equation (5.2), where Γ is the gamma function.

$$\langle \text{rate}_{bl} \rangle = \frac{1}{h \tau_{bl} \Gamma[h]} \quad (5.2)$$

We note that the I_∞ parameters were not fixed in the fitting procedure in order to ensure good fits of the initial data points. The values of I_∞ were larger for lower E_{app} , and may have led to an underrepresentation of slow photobleaching processes. This consideration, along with the relatively short time window of the fluorescence trajectories, set the photobleaching rates shown in Figure 5.2B as upper bounds. Correspondingly, the E_0 value obtained from fitting of the photobleaching rate ($E_0 = 132$ mV) may also be considered an upper bound.

Stretched exponential functions have been previously used to describe the charge recombination dynamics of sensitized metal oxide semiconductors, including SnO₂.^{3, 6} It was proposed that electron transport mediated by trapping in an exponential energetic distribution of trap states leads to the dispersive kinetics. In our case we suspect that the stretched exponential reflects a distribution of electron injection barriers¹⁷⁻¹⁸ that result in a distribution of rates of electron injection (k^*_{ox}). For the non-injecting dyes that give the residual intensity either the barrier is too high for charge transfer to compete with fluorescence or they are not in close proximity to an electron accepting site on the SnO₂.

For SQ2, the stretched exponentials traced well the fluorescence trajectories but led to large variations in the photobleaching rate, albeit all trajectories looked similar. This seems to be caused by difficulties in determining the background fluorescence (I_∞) parameter. We instead chose to

extract the photobleaching rate from single exponential functions as they were sufficient to fit the fluorescence traces. Neither the photobleaching rates nor the photobleached fraction after 80 s of irradiation (bleach less than 20% of the initial intensity) were affected by E_{app} .

The perylene photobleaching rates (proportional to k^*_{ox})¹⁹ obtained from the fitted average fluorescence decay rates according to Equation (5.2) above showed a sigmoidal dependence with E_{app} (Figure 5.2). A similar dependence with bias was observed when the fraction of fluorescence remaining after 80 s of illumination ($I_{80\text{ s}}$) at a constant E_{app} was used as the quantitative metric of photobleaching. Equations (5.3) and (5.4) below provided good fits to the dependence of the rate of photobleaching (and therefore the rate-limiting k^*_{ox} under the assumption made in Scheme 5.2) and $I_{80\text{ s}}$ with E_{app} , respectively.

$$k^*_{ox} \propto \text{rate}_{bl} = \text{rate}_{max} \times \int_{\infty}^{\text{Edge}} \left(1 - \frac{1}{1 + \exp\left(\frac{E - E_{app}}{E_0}\right)} \right) \exp\left(-\frac{E}{E_0}\right) dE \quad (5.3)$$

$$I_{80\text{ s}} = 1 - I_{bl, max} \times \int_{\infty}^{\text{Edge}} \left(1 - \frac{1}{1 + \exp\left(\frac{E - E_{app}}{E_0}\right)} \right) \exp\left(-\frac{E}{E_0}\right) dE \quad (5.4)$$

In order to interpret Equations (5.3) and (5.4), it is important to recall that the interfacial charge transfer rate between sensitizers and a semiconductor has been shown to depend on an electronic coupling term (V^2), a Fermi-Dirac term to account for the electronic occupancy, a density of state (DOS) term for the semiconductor and finally an energetic term described by the Marcus-Gerischer theory,²⁰⁻²² shown in going from left to right in Equation (5.5)/(5.6).²³ The DOS

of metal oxide semiconductors such as TiO₂ and SnO₂ are usually characterized by an exponential tail of states acting as trap sites (with energetic distribution of E_0) below the conduction band edge, accounting for the $\exp(-E/E_0)$ term.^{7, 23-25} We conducted chronoamperometry and cyclic voltammetry (CV) experiments in order to experimentally verify the shape of the DOS of the SnO₂ used in our studies. Our data confirmed the energetic exponential dependence of the semiconductor DOS (Figure 5.9).

$$k_{\text{ox}}^* = A \int V^2 \times \left(1 - \frac{1}{1 + \exp\left(\frac{E - E_F}{k_B T}\right)} \right) \times \exp\left(-\frac{E}{E_0}\right) \times \exp\left(-\frac{(E - E_{\text{ox}}^* + \lambda)^2}{4\lambda k_B T}\right) dE \quad (5.5)$$

$$k_{\text{red}}^* = A \int V^2 \times \frac{1}{1 + \exp\left(\frac{E - E_F}{k_B T}\right)} \times \exp\left(-\frac{E}{E_0}\right) \times \exp\left(-\frac{(E - E_{\text{red}}^* - \lambda)^2}{4\lambda k_B T}\right) dE \quad (5.6)$$

Equations (5.3) and (5.4) take into account an exponentially dependent DOS term, where E_0 represents the energetic distribution of the electronic states. Although energetic contributions (Marcus-type dependence of the electron transfer rate on the free energy change) cannot be conclusively ruled out without accurate knowledge of the reorganisation energy parameter, our data consistently show the DOS of the SnO₂ as the dominant factor that controls the changes in rate of interfacial charge transfers. The DOS E_0 s extracted from the photobleaching fits are ca. 130 – 180 mV, and similar values have been reported for the much more studied TiO₂.^{23, 26-28} More importantly, the fitting results are in line with the E_0 values of 90 – 130 mV obtained from electrochemical measurements of a thick SnO₂ film cast from a 15% wt. SnO₂ NP dispersion (Figure 5.9). The Edge parameter found in Equations (5.3) and (5.4) may be physically linked to the approximate upper energies of states that are both active in the charge transfers and whose occupancy is influenced by the changes in E_{app} . Edge values of 0.11 - 0.15 V are extracted from the fits shown in Figure 5.2B.

In order to determine the fate of electrons injected into the semiconductor we measured next the photocurrents produced upon photoexcitation of the sensitizers bound to SnO_2 films. Under a constant applied bias of 0.43 V, a 10-fold larger photocurrent was produced when exciting the perylene over SQ2 despite the lower photon flux used to excite the former vs. the latter (Figure 5.3). Here a significant fraction of the electrons photoinjected into SnO_2 may not recombine with perylene $^{*+}$, presumably due to its rapid degradation. These electrons thus diffuse away from the interface and are efficiently collected at the ITO back contact, giving rise to the observed photocurrent. In the case of SQ2, we attribute the lower measured photocurrent to an efficient electron-SQ2 $^{*+}$ recombination pathway. Considering the charge collected upon irradiation of the perylene and the size of the area illuminated, we estimate ~ 10 dyes/ nm^2 of geometrical area, in line with the expected coverage of a dye monolayer and the rough surface of the SnO_2 layer (Figure 5.5).

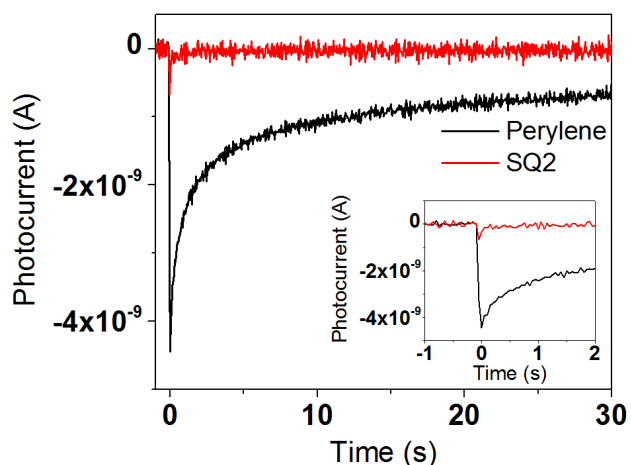


Figure 5.3. Comparison of the photocurrent measured upon excitation of perylene and SQ2 bound to SnO_2 . The laser excitation (488 nm, 14 μW for perylene; 647 nm, 396 μW for SQ2) was initiated at time $t = 0$ s. The applied bias was kept at 0.43 V before and during irradiation. Inset: Zoomed in view of the first 2 s of the photocurrent decay.

Importantly, a reduction in photocurrent is observed with perylene when the applied bias is more negative. Two possible scenarios both related to the higher electron density in SnO_2 may

account for this observation: i) the photooxidation rate of the dye (k_{ox}^*) becomes smaller with more negative bias^{9, 29-30} and/or ii) there is a faster recombination between electrons and perylene^{•+} effectively competing with the degradation of the dye.⁸

To distinguish between the two scenarios, we studied the effect of the applied bias on the initial fluorescence intensity (I_0) with our SMS-EC setup (Figure 5.4). By only taking into account the initial fluorescence intensity, we minimize the effect of bias-dependent photobleaching rates (*vide supra*). In agreement with the first hypothesis, our studies showed that for perylene (and also SQ2) fluorescence enhancements are recorded at negative biases, a result of suppressed electron injection into the semiconductor in favor of emission. The I_0 thus increased with a lowering of E_{app} , consistent with the reduction of k_{ox}^* at more negative biases. The I_0 analysis, however, is insensitive to changes in recombination rates between electrons and perylene^{•+} so we may not determine whether those processes are occurring in the first place, to what extent they are occurring, and how their rate may be affected by the applied potentials. Nonetheless, it is likely that the recombination rates mirror the observed retardation of photoreduction of the perylene with increasing applied bias (*vide infra*).

Further analysis of the changes on I_0 with E_{app} show a striking similarity in the data recorded for both the perylene and SQ2 compounds (Figure 5.4B). Given the large difference in the excited state redox potentials of both dyes (Figure 5.1), the observed trends indicate that it is highly unlikely that energetics are playing a significant role in dictating the *changes* in rates of charge transfer in our system. Rather, the occupancy of the electronic states of SnO₂ seems to be the dominant factor, in agreement with the above results from photobleaching studies of perylene. Fitting of the I_0 vs. applied bias experimental data to Equation (5.7) shown below, retrieved E_0 values between 50 and 90 mV. Equation (5.7) represents the dependence of emission quantum

yield Φ_f (and I_0) on k_{ox}^* and k_{red}^* that are defined in turn by Equations (5.8) and (5.9), respectively (sigmoidal expressions as stated for Equation (5.3)). Other parameters listed in Equation (5.7) include a proportionality constant (I_{max}) between intensity and Φ_f , the rate constant for radiative decay (k_r) and the rate constant for non-radiative decay (k_{nr}). The fitted E_0 values are similar to those obtained from the analysis of photobleaching and also in chronoamperometry measurements on the DOS of SnO_2 . The Edge values were 0.18 – 0.19 eV in the case of k_{ox}^* while a value of 0.75 eV was obtained for the k_{red}^* analysis of the perylene.

$$I_0 = I_{max} \times \Phi_f = I_{max} \times \frac{k_f}{k_f + k_{nr} + k_{ox}^*(E_{app}) + k_{red}^*(E_{app})} \quad (5.7)$$

$$k_{ox}^*(E_{app}) = k_{ox\ max}^* \times \int_{\infty}^{Edge} \left(1 - \frac{1}{1 + \exp\left(\frac{E - E_{app}}{E_0}\right)} \right) \exp\left(-\frac{E}{E_0}\right) dE \quad (5.8)$$

$$k_{red}^*(E_{app}) = k_{red\ max}^* \times \int_{\infty}^{Edge} \frac{1}{1 + \exp\left(\frac{E - E_{app}}{E_0}\right)} \exp\left(-\frac{E}{E_0}\right) dE \quad (5.9)$$

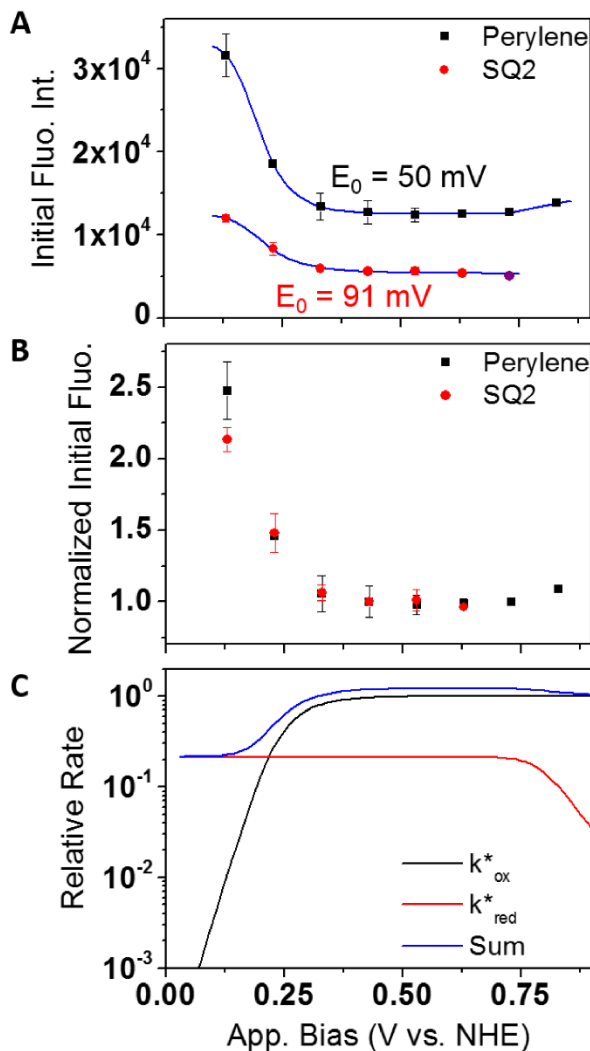


Figure 5.4. Influence of applied bias on the initial fluorescence intensity of the perylene and SQ2 dyes. A) The blue lines represent fits to the sigmoidal expression depicted in Equation (5.7). The k_{red}^* term is not observed in the case of SQ2 and was set to 0 for the fitting. The best fit E_0 parameters are noted in the plots. For the SQ2, the purple point at 0.73 V was excluded from the fitting since the fluorescence intensity decreases due to ground state oxidation at this E_{app} . B) Same data as in A) with normalized fluorescence intensity of both dyes, normalized to the values at 0.43 V. C) Relative charge transfer rates for the perylene as calculated from the fit parameters obtained in A).

Inspection of Figure 5.4 reveals that I_0 values increased for perylene in going from 0.63 V to 0.83 V [no data was available for SQ2 as thermal oxidation takes place at these biases ($E_{ox}^0 = 0.81$ V)].¹⁶ To account for this observation with perylene one must consider a decrease in k_{red}^* resulting from a depletion of electrons occupying the electronic states of SnO₂ at increasing bias.

On the contrary, k_{ox}^* would be expected to increase as a result of the decreasing electron density, which would result in the inverse trend, i.e. lowering of I_0 with increasing applied bias (Figure 5.4C).

The above result suggests that photoinduced reduction of a dye (electron transfer from the semiconductor to the photoexcited sensitizer) may take place in operating DSSCs. The efficiency of DSSCs has long been known to be hampered by recombination, the return of an electron injected into SnO_2 to an oxidized dye. Photoinduced reduction, however, has been frequently overlooked when studying the charge transfer dynamics of DSSCs. Our results point to a contribution arising from photoinduced reduction towards lower power conversion efficiency of a DSSC. The electron density (E_F) in SnO_2 is typically high under operating conditions ($E_F \sim -0.3$ V for $V_{\text{OC}} \sim 0.5$ V with I^-/I_3^- redox mediator), potentially exacerbating the non-productive k_{red}^* process under solar cell working conditions.

In closing, we may address the observed trends indicating that it is highly unlikely that energetics are playing a significant role in dictating the changes in rates of charge transfer in our system. In order to rationalize these observations, we propose the following molecular description. We note that the bias range explored herein covers only states of SnO_2 found in lower energies compared to the conduction band. States within the band gap, for TiO_2 , have been associated with surface defects such as under-coordinated Ti atoms or grain boundaries.³¹⁻³⁶ Considering the potentially well-defined structure of these states, it seems reasonable to consider that these states are individually fixed in terms of energy.¹⁷ Therefore, the driving force for electron transfer between the localized energy state of the semiconductor and the molecular orbital of the dye sensitizers will be for the most part independent of the E_F of the SnO_2 . Instead, the occupancy of the electronic state will dictate whether electron transfer is fast/possible (empty accepting state) or

slow/inhibited (filled accepting state), in line with single molecule studies that have shown stochastic fluctuations in electron transfer rates.^{18, 37-38} We envisage that single molecule spectroelectrochemical studies will provide novel insights in determining the nature of semiconductor energy states that participate in charge transfer.

5.5. Conclusions

In summary, we have investigated the interfacial charge transfer dynamics of dye-sensitized SnO₂ thin films using fluorescence spectroelectrochemistry in a SMS-EC setup. The instability of the radical cation of a perylene dye proved useful to monitor changes in rates of electron injection into SnO₂. We also studied the effect of applied biases on the initial fluorescence intensity of both the perylene dye and SQ2, a commercial sensitizer used in TiO₂ DSSCs. For both the photobleaching and the initial intensity analyses, the occupancy of the electronic states of the SnO₂ film dictated the dependence of charge transfer rates on the applied potential. Previous reports have shown the influence of both DOS^{7-8, 10} and energetics effects^{30, 39-41} (or lack of energetic effects⁴²⁻⁴⁴) on charge transfer pathways.⁴⁵ Our analyses indicate that the DOS of the SnO₂ film dominated over energetic Marcus-type effect in our system, underlining the complex nature of interfacial charge transfers and our limited understanding of the matter. We have also shown evidence of an active sensitizer photoreduction pathway that warrants consideration in the design of solar cells.

5.6. Experimental Methods

5.6.1. Materials

All materials were used as received without further purification. SnO₂ NPs roughly 10 nm in diameter were purchased from Alfa Aesar (Ward Hill, MA). Electrochemical grade Bu₄NClO₄ and anhydrous MeCN were acquired from Sigma Aldrich (St. Louis, MO). Custom patterned ITO-

coated coverslips with resistivity of $50 \Omega/\square$ were obtained from Evaporated Coatings Inc. (Willow Grove, PA). SQ2 dye was purchased from Solaronix (Aubonne, Switzerland). 1,7-Dibromoperylene-3,4,9,10-tetracarboxy dianhydride (perylene) was obtained following a literature procedure.⁴⁶

5.6.2. Sample Preparation

The samples were prepared by first spin-casting a thin layer of SnO_2 NPs (diluted to 2% w/w in 0.1 M KOH aqueous solution) onto clean ITO-coated microscope coverslips (sequential sonication in acetone, trichloroethylene and methanol, 10 minutes each). 40 μL of the SnO_2 NP solution was dropped while the coverslip rotates at 3000 rpm. The spinning was then slowed to 2000 rpm for an additional 50 s. The SnO_2 films were sintered at 450 $^\circ\text{C}$ for 1 hour, then kept at 110 $^\circ\text{C}$. The thickness of the SnO_2 layer was determined to be ~ 35 nm by AFM measurements. The organic dye sensitizers were then bound to the SnO_2 surface by exposing selected areas of the film to dye sensitizer solutions with a custom sample holder. Immediately after taking the coverslip out of the oven, the SnO_2 film was soaked overnight in filtered (PTFE, 0.2 μm) dye sensitizer solutions (ca. 50 - 100 μM in EtOH). The perylene was converted from the closed dianhydride form to the open opened tetracarboxylic species (most likely as a mixture of the acid and the ethyl ester corresponding to ethoxide addition) by dissolving the powder in a 1 mM KOH ethanoic solution. Unbound dyes were washed by EtOH and MeCN rinses and sonication. A glass tube was next affixed on top of the coverslip by an epoxy glue (Hardman®, McMaster-Carr). The sample was placed in a 110 $^\circ\text{C}$ oven for 1 hour to cure the epoxy and also dry the sample. The chamber was then filled with anhydrous MeCN containing the supporting electrolyte (0.1 M Bu_4NClO_4). The reference electrode (non-aqueous Ag/Ag^+) and counter electrode (Pt wire) were introduced to the electrochemical cell by piercing through the inverted septum that sealed the cell. The Ag/Ag^+

electrode (10 mM AgNO₃ with 0.1 M Bu₄NClO₄ in MeCN) was calibrated to the Fc/Fc⁺ redox couple ($E^0 = +0.64$ V vs. NHE).

5.6.3. *Single molecule/particle spectroelectrochemistry*

In the electrochemical cell, the working electrode lead could be connected to any of the 4 independent ITO sectors on the coverslip. The cell was degassed with argon for at least 1 hour before the spectroelectrochemistry measurements. During data acquisition, an argon-filled balloon was affixed to the sample to ensure an oxygen-free atmosphere. Time-lapse movies were acquired at a 20 Hz frame rate using a wide-field objective-based total internal reflection fluorescence (TIRF) microscopy setup consisting of an inverted microscope (Nikon Eclipse Ti) equipped with a Perfect Focus System (PSF) to minimize axial drifting. Samples were excited with the evanescent wave of either a 488 nm or 647 nm diode laser output of a laser combiner (Agilent Technologies, MLC-400B), obtained by focusing the collimated laser beam at the back focal plane of a high numerical aperture oil-immersion objective (Nikon CFI SR Apochromat TIRF 100x, NA = 1.49) and launching the beam past the critical angle. A dual band dichroic mirror (ZT488/640rpc-UF2, Chroma) directed the excitation beam to the sample while the emission was cleaned by a dual band pass filter (ZET488/640m, Chroma). The excitation laser powers measured out of the objective with the beam projecting normal to the objective lens surface were 14 μ W and 396 μ W for the 488 nm and 647 nm laser lines, respectively. Emission was collected through the same objective and captured on a back illuminated electron multiplying charge coupled device (EM-CCD) camera (Andor iXon Ultra DU-897). Images were analyzed using Fiji/ImageJ software⁴⁷ with GDSC plugins developed by the University of Sussex.⁴⁸ The electrochemical potential was controlled by a CHI600D (CH Instruments) electrochemical analyzer. The biases were applied for 5 – 10 minutes before imaging to allow the Fermi level of the SnO₂ film to stabilize.

5.7. Acknowledgements

We would like to thank Dr. Mohini Ramkaran from the CSACS SPM facility at McGill University for assistance with the AFM measurements.

5.8. Supporting Information

The Supporting Information contains AFM micrographs of the SnO₂ films, results from controls of the perylene and SQ2 dyes on ITO and SiO₂ surfaces, additional representation of Figure 5.2A, and electrochemical characterization of thick SnO₂ films.

5.8.1. Surface Characterization of the SnO₂ Films

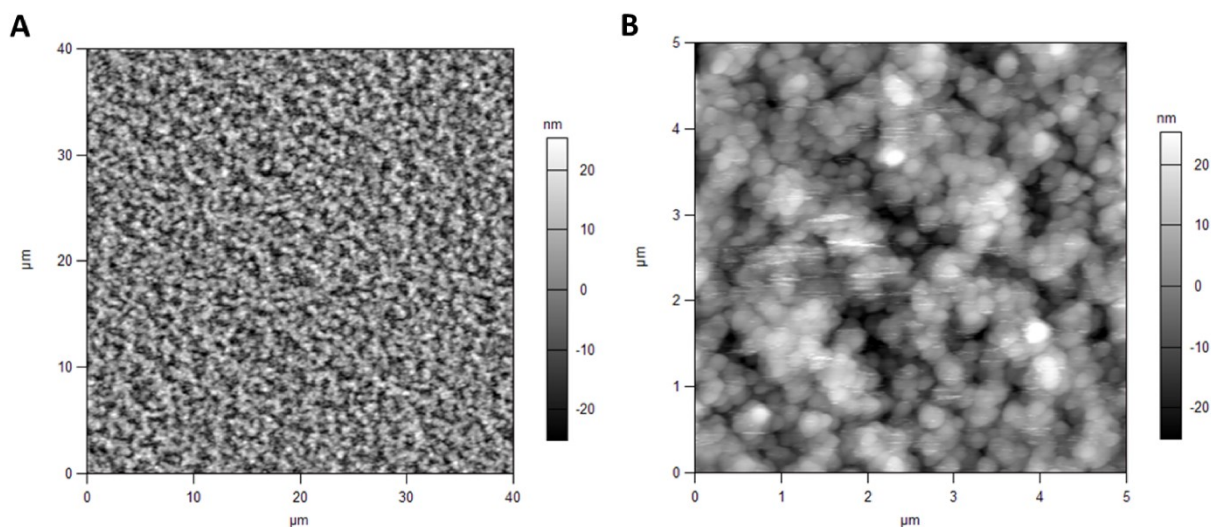


Figure 5.5. Micrographs of SnO₂ film prepared by spin coating a NP dispersion on ITO coverslips followed by sintering at 450 °C for 1 hour as obtained by atomic force microscopy (AFM) for (A) 40 x 40 μm² and (B) 5 x 5 μm² areas.

5.8.2. Control of Dyes Bound to ITO Surface

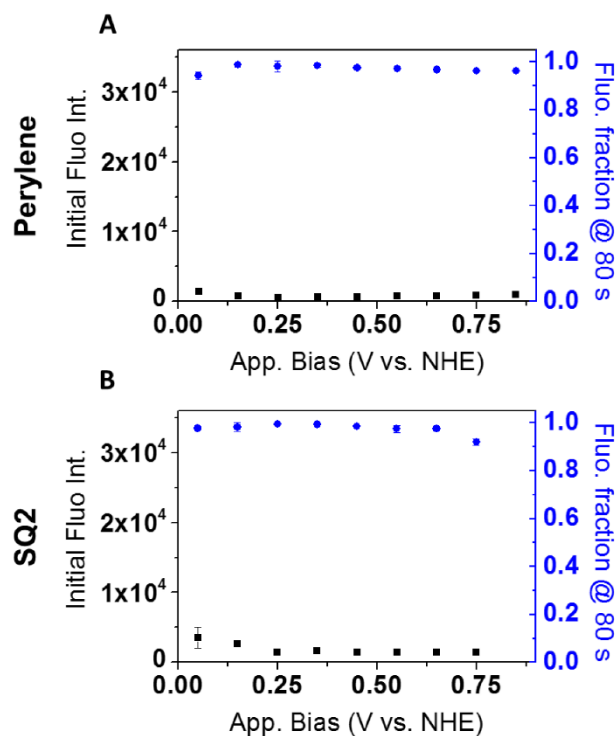


Figure 5.6. Results of control experiments of sensitized ITO surfaces. All conditions were the same as those used in the experiments of sensitized SnO₂ films presented in the main text. See Figure 5.4 for comparisons of the intensities of dyes on bare ITO surfaces (shown here) and on the SnO₂ surface.

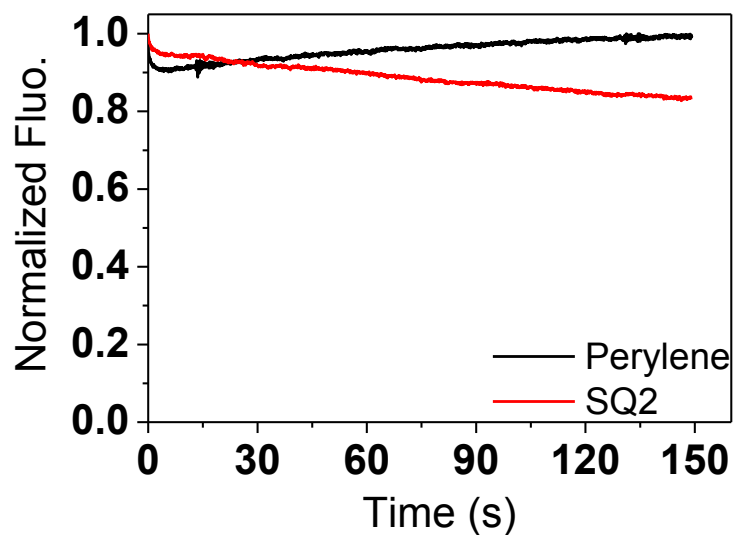
5.8.3. Photobleaching of Perylene and SQ2 Dyes Bound to Inert SiO₂ Film

Figure 5.7. Photobleaching of the perylene and SQ2 dyes bound to an insulating SiO₂ support. We attribute the surprising fluorescence intensity increase observed for the perylene over time to photodegradation of quenching sites caused by aggregated dyes (e.g. non-emissive dimers).

5.8.4. Additional Representations of Perylene Photobleaching Trajectories

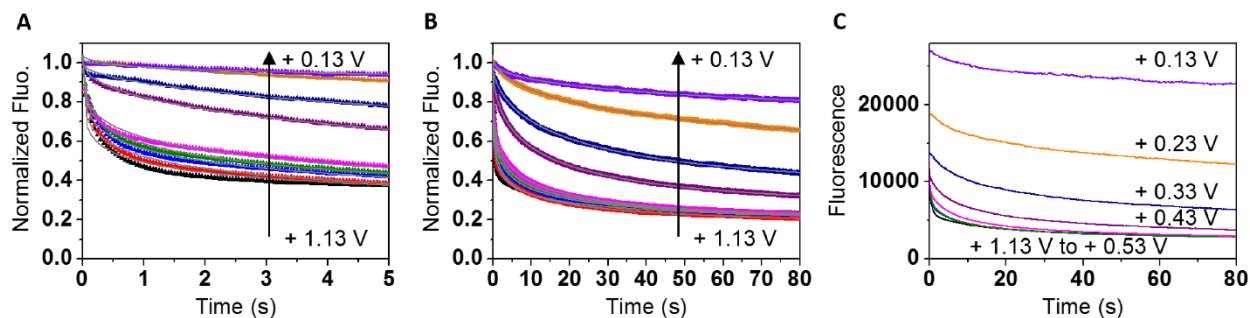


Figure 5.8. Additional representation of the photobleaching traces of the perylene dye bound to SnO₂ shown in Figure 5.2A of the main text. A and B) Normalized fluorescence intensity trajectories of the perylene at different timescales. Each data point is represented by a triangle. Stretched exponential fits (Equation (5.1)) are shown as gray lines overlaid on the fluorescence trajectories. C) Non-normalized fluorescence trajectory showing of the same data shown in B). The E_{app} under which the trajectories were acquired are noted in the plots.

5.8.5. Electrochemical Characterization of Thick SnO₂ Films

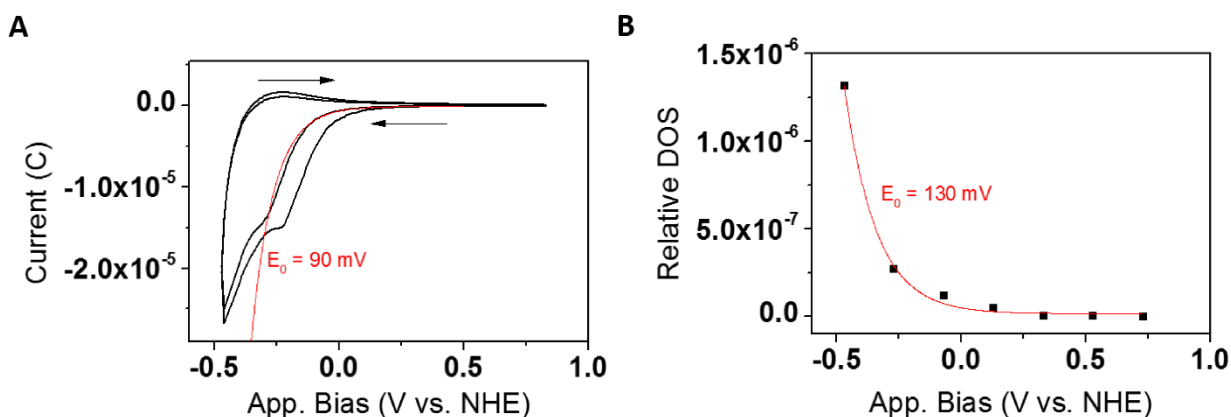


Figure 5.9 Comparison of the SnO₂ film DOS estimated by CV (A) or chronoamperometry (B) techniques. The film was cast from a 15% wt. SnO₂ NP solution. Electrochemical measurements were performed in argon-purged 0.1 M Bu₄NClO₄ acetonitrile solutions. The direction of the scans in the CV experiment (scan rate of 50 mV/s) is indicated by arrows. Exponential fits are shown in red with the extracted exponential constant indicated.

5.9. References

- (1) Oregan, B.; Gratzel, M., A Low-Cost, High-Efficiency Solar-Cell Based on Dye-Sensitized Colloidal TiO₂ Films. *Nature* **1991**, *353*, 737-740.
- (2) Yella, A.; Lee, H.-W.; Tsao, H. N.; Yi, C.; Chandiran, A. K.; Nazeeruddin, M. K.; Diau, E. W.-G.; Yeh, C.-Y.; Zakeeruddin, S. M.; Grätzel, M., Porphyrin-Sensitized Solar Cells with Cobalt (II/III)-Based Redox Electrolyte Exceed 12 Percent Efficiency. *Science* **2011**, *334*, 629-634.
- (3) Nelson, J.; Haque, S. A.; Klug, D. R.; Durrant, J. R., Trap-Limited Recombination in Dye-Sensitized Nanocrystalline Metal Oxide Electrodes. *Phys. Review. B* **2001**, *63*, 205321.
- (4) Willis, R. L.; Olson, C.; O'Regan, B.; Lutz, T.; Nelson, J.; Durrant, J. R., Electron Dynamics in Nanocrystalline ZnO and TiO₂ Films Probed by Potential Step Chronoamperometry and Transient Absorption Spectroscopy. *J. Phys. Chem. B* **2002**, *106*, 7605-7613.
- (5) Durrant, J. R., Modulating Interfacial Electron Transfer Dynamics in Dye Sensitized Nanocrystalline Metal Oxide Films. *J. Photochem. Photobiol. A: Chem.* **2002**, *148*, 5-10.
- (6) Green, A. N. M.; Palomares, E.; Haque, S. A.; Kroon, J. M.; Durrant, J. R., Charge Transport Versus Recombination in Dye-Sensitized Solar Cells Employing Nanocrystalline TiO₂ and SnO₂ Films. *J. Phys. Chem. B* **2005**, *109*, 12525-12533.
- (7) Knauf, R. R.; Brennaman, M. K.; Alibabaei, L.; Norris, M. R.; Dempsey, J. L., Revealing the Relationship between Semiconductor Electronic Structure and Electron Transfer Dynamics at Metal Oxide-Chromophore Interfaces. *J. Phys. Chem. C* **2013**.
- (8) Haque, S. A.; Tachibana, Y.; Willis, R. L.; Moser, J. E.; Grätzel, M.; Klug, D. R.; Durrant, J. R., Parameters Influencing Charge Recombination Kinetics in Dye-Sensitized Nanocrystalline Titanium Dioxide Films. *J. Phys. Chem. B* **2000**, *104*, 538-547.
- (9) Kamat, P. V.; Bedja, I.; Hotchandani, S.; Patterson, L. K., Photosensitization of Nanocrystalline Semiconductor Films. Modulation of Electron Transfer between Excited Ruthenium Complex and SnO₂ Nanocrystallites with an Externally Applied Bias. *J. Phys. Chem.* **1996**, *100*, 4900-4908.
- (10) McNeil, I. J.; Alibabaei, L.; Ashford, D. L.; Fecko, C. J., Investigation of Factors That Affect Excited-State Lifetime Distribution of Dye-Sensitized Nanoparticle Films. *J. Phys. Chem. C* **2013**, *117*, 17412-17420.
- (11) Hill, C. M.; Clayton, D. A.; Pan, S., Combined Optical and Electrochemical Methods for Studying Electrochemistry at the Single Molecule and Single Particle Level: Recent Progress and Perspectives. *PCCP* **2013**, *15*, 20797-20807.
- (12) Palacios, R. E.; Fan, F.-R. F.; Bard, A. J.; Barbara, P. F., Single-Molecule Spectroelectrochemistry (SMS-EC). *J. Am. Chem. Soc.* **2006**, *128*, 9028-9029.
- (13) Lemon, B. I.; Hupp, J. T., EQCM Investigations of Dye-Functionalized Nanocrystalline Titanium Dioxide Electrode/Solution Interfaces: Does Luminescence Report Directly on Interfacial Electron Transfer Kinetics? *J. Phys. Chem. B* **1999**, *103*, 3797-3799.
- (14) Hernández, L. I., et al., Spectral Characteristics and Photosensitization of TiO₂ Nanoparticles in Reverse Micelles by Perylenes. *J. Phys. Chem. B* **2012**, *117*, 4568-4581.
- (15) Asbury, J. B.; Hao, E.; Wang, Y.; Ghosh, H. N.; Lian, T., Ultrafast Electron Transfer Dynamics from Molecular Adsorbates to Semiconductor Nanocrystalline Thin Films. *J. Phys. Chem. B* **2001**, *105*, 4545-4557.
- (16) Geiger, T.; Kuster, S.; Yum, J.-H.; Moon, S.-J.; Nazeeruddin, M. K.; Grätzel, M.; Nüesch, F., Molecular Design of Unsymmetrical Squaraine Dyes for High Efficiency Conversion of Low

Energy Photons into Electrons Using TiO₂ Nanocrystalline Films. *Adv. Funct. Mater.* **2009**, *19*, 2720-2727.

(17) McNeil, I. J.; Ashford, D. L.; Luo, H.; Fecko, C. J., Power-Law Kinetics in the Photoluminescence of Dye-Sensitized Nanoparticle Films: Implications for Electron Injection and Charge Transport. *J. Phys. Chem. C* **2012**, *116*, 15888-15899.

(18) Wong, N. Z.; Ogata, A. F.; Wustholz, K. L., Dispersive Electron-Transfer Kinetics from Single Molecules on TiO₂ Nanoparticle Films. *J. Phys. Chem. C* **2013**.

(19) The observed rate of photobleaching is linked to the rate constant k^*_{ox} through the concentration of excited dye, itself dependent on the excitation rate, the quantum yield of photooxidation. Without access to these parameters, we limit ourselves to the changes in rates under varying E_{app} .

(20) Marcus, R. A., On the Theory of Electron-Transfer Reactions. VI. Unified Treatment for Homogeneous and Electrode Reactions. *J. Chem. Phys.* **1965**, *43*, 679-701.

(21) Gerischer, H., Electrochemical Techniques for the Study of Photosensitization. *Photochem. Photobiol.* **1972**, *16*, 243-260.

(22) Gerischer, H.; Willig, F., Reaction of Excited Dye Molecules at Electrodes. In *Physical and Chemical Applications of Dyestuffs*, Schäfer, F. P.; Gerischer, H.; Willig, F.; Meier, H.; Jahnke, H.; Schönborn, M.; Zimmermann, G., Eds. Springer Berlin Heidelberg: 1976; Vol. 61, pp 31-84.

(23) Listorti, A.; O'Regan, B.; Durrant, J. R., Electron Transfer Dynamics in Dye-Sensitized Solar Cells. *Chem. Mater.* **2011**, *23*, 3381-3399.

(24) Duffy, N. W.; Peter, L. M.; Rajapakse, R. M. G.; Wijayantha, K. G. U., A Novel Charge Extraction Method for the Study of Electron Transport and Interfacial Transfer in Dye Sensitized Nanocrystalline Solar Cells. *Electrochem. Commun.* **2000**, *2*, 658-662.

(25) Ardo, S.; Meyer, G. J., Photodriven Heterogeneous Charge Transfer with Transition-Metal Compounds Anchored to TiO₂ Semiconductor Surfaces. *Chem. Soc. Rev.* **2009**, *38*, 115-164.

(26) Bailes, M.; Cameron, P. J.; Lobato, K.; Peter, L. M., Determination of the Density and Energetic Distribution of Electron Traps in Dye-Sensitized Nanocrystalline Solar Cells. *J. Phys. Chem. B* **2005**, *109*, 15429-15435.

(27) O'Regan, B. C.; Durrant, J. R.; Sommeling, P. M.; Bakker, N. J., Influence of the TiCl₄ Treatment on Nanocrystalline TiO₂ Films in Dye-Sensitized Solar Cells. 2. Charge Density, Band Edge Shifts, and Quantification of Recombination Losses at Short Circuit. *J. Phys. Chem. C* **2007**, *111*, 14001-14010.

(28) Abayev, I.; Zaban, A.; Kytin, V.; Danilin, A.; Garcia-Belmonte, G.; Bisquert, J., Properties of the Electronic Density of States in TiO₂ Nanoparticles Surrounded with Aqueous Electrolyte. *J. Solid State Electrochem.* **2007**, *11*, 647-653.

(29) Iwai, S.; Hara, K.; Murata, S.; Katoh, R.; Sugihara, H.; Arakawa, H., Ultrafast Interfacial Charge Separation Processes from the Singlet and Triplet MLCT States of Ru(bpy)₂(dcbpy) Adsorbed on Nanocrystalline SnO₂ under Negative Applied Bias. *J. Chem. Phys.* **2000**, *113*, 3366-3373.

(30) Tachibana, Y.; Haque, S. A.; Mercer, I. P.; Moser, J. E.; Klug, D. R.; Durrant, J. R., Modulation of the Rate of Electron Injection in Dye-Sensitized Nanocrystalline TiO₂ Films by Externally Applied Bias. *J. Phys. Chem. B* **2001**, *105*, 7424-7431.

(31) Kopidakis, N.; Neale, N. R.; Zhu, K.; van de Lagemaat, J.; Frank, A. J., Spatial Location of Transport-Limiting Traps in TiO₂ Nanoparticle Films in Dye-Sensitized Solar Cells. *Appl. Phys. Lett.* **2005**, *87*, 202106.

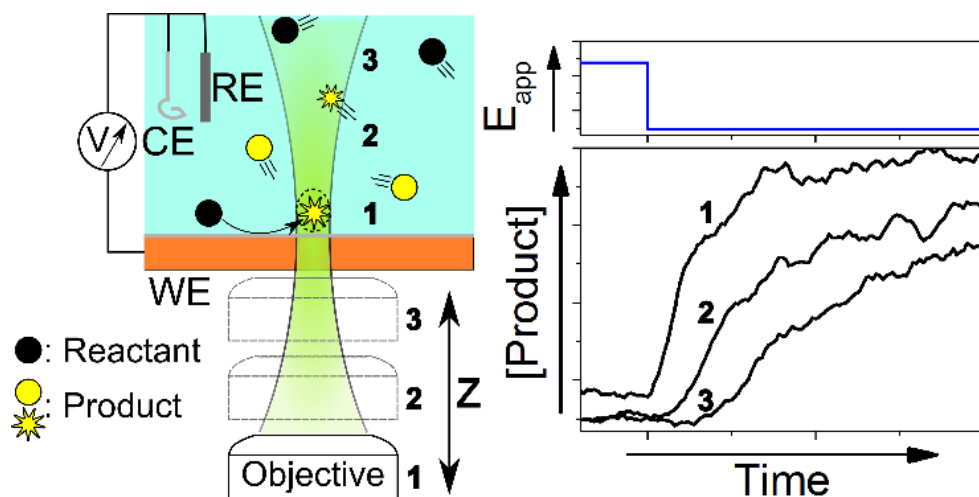
- (32) Zhu, K.; Kopidakis, N.; Neale, N. R.; van de Lagemaat, J.; Frank, A. J., Influence of Surface Area on Charge Transport and Recombination in Dye-Sensitized TiO₂ Solar Cells. *J. Phys. Chem. B* **2006**, *110*, 25174-25180.
- (33) Jankulovska, M.; Berger, T.; Wong, S. S.; Gómez, R.; Lana-Villarreal, T., Trap States in TiO₂ Films Made of Nanowires, Nanotubes or Nanoparticles: An Electrochemical Study. *ChemPhysChem* **2012**, *13*, 3008-3017.
- (34) Nunzi, F.; Mosconi, E.; Storchi, L.; Ronca, E.; Selloni, A.; Grätzel, M.; De Angelis, F., Inherent Electronic Trap States in TiO₂ Nanocrystals: Effect of Saturation and Sintering. *Energ. Env. Sci.* **2013**, *6*, 1221-1229.
- (35) Rex, R. E.; Knorr, F. J.; McHale, J. L., Surface Traps of TiO₂ Nanosheets and Nanoparticles as Illuminated by Spectroelectrochemical Photoluminescence. *J. Phys. Chem. C* **2014**, *118*, 16831-16841.
- (36) Rittmann-Frank, M. H.; Milne, C. J.; Rittmann, J.; Reinhard, M.; Penfold, T. J.; Chergui, M., Mapping of the Photoinduced Electron Traps in TiO₂ by Picosecond X-Ray Absorption Spectroscopy. *Angew. Chem. Int. Ed.* **2014**, *53*, 5858-5862.
- (37) Biju, V.; Micic, M.; Hu, D.; Lu, H. P., Intermittent Single-Molecule Interfacial Electron Transfer Dynamics. *J. Am. Chem. Soc.* **2004**, *126*, 9374-9381.
- (38) Rao, V. G.; Dhital, B.; He, Y.; Lu, H. P., Single-Molecule Interfacial Electron Transfer Dynamics of Porphyrin on TiO₂ Nanoparticles: Dissecting the Complex Electronic Coupling Dependent Dynamics. *J. Phys. Chem. C* **2014**, *118*, 20209-20221.
- (39) Yan, S. G.; Prieskorn, J. S.; Kim, Y.; Hupp, J. T., In Search of the Inverted Region: Chromophore-Based Driving Force Dependence of Interfacial Electron Transfer Reactivity at the Nanocrystalline Titanium Dioxide Semiconductor/Solution Interface. *J. Phys. Chem. B* **2000**, *104*, 10871-10877.
- (40) Kuciauskas, D.; Freund, M. S.; Gray, H. B.; Winkler, J. R.; Lewis, N. S., Electron Transfer Dynamics in Nanocrystalline Titanium Dioxide Solar Cells Sensitized with Ruthenium or Osmium Polypyridyl Complexes. *J. Phys. Chem. B* **2001**, *105*, 392-403.
- (41) Farnum, B. H.; Morseth, Z. A.; Brennaman, M. K.; Papanikolas, J. M.; Meyer, T. J., Driving Force Dependent, Photoinduced Electron Transfer at Degenerately Doped, Optically Transparent Semiconductor Nanoparticle Interfaces. *J. Am. Chem. Soc.* **2014**, *136*, 15869-15872.
- (42) Hasselmann, G. M.; Meyer, G. J., Diffusion-Limited Interfacial Electron Transfer with Large Apparent Driving Forces. *J. Phys. Chem. B* **1999**, *103*, 7671-7675.
- (43) Tachibana, Y.; Haque, S. A.; Mercer, I. P.; Durrant, J. R.; Klug, D. R., Electron Injection and Recombination in Dye Sensitized Nanocrystalline Titanium Dioxide Films: A Comparison of Ruthenium Bipyridyl and Porphyrin Sensitizer Dyes. *J. Phys. Chem. B* **2000**, *104*, 1198-1205.
- (44) Clifford, J. N.; Palomares, E.; Nazeeruddin, M. K.; Grätzel, M.; Nelson, J.; Li, X.; Long, N. J.; Durrant, J. R., Molecular Control of Recombination Dynamics in Dye-Sensitized Nanocrystalline TiO₂ Films: Free Energy Vs Distance Dependence. *J. Am. Chem. Soc.* **2004**, *126*, 5225-5233.
- (45) Hagfeldt, A.; Boschloo, G.; Sun, L.; Kloo, L.; Pettersson, H., Dye-Sensitized Solar Cells. *Chem. Rev.* **2010**, *110*, 6595-6663.
- (46) Shibano, Y.; Umeyama, T.; Matano, Y.; Imahori, H., Electron-Donating Perylene Tetracarboxylic Acids for Dye-Sensitized Solar Cells. *Org. Lett.* **2007**, *9*, 1971-1974.
- (47) Schindelin, J., et al., Fiji: An Open-Source Platform for Biological-Image Analysis. *Nat. Methods* **2012**, *9*, 676-682.

(48) Downloaded
http://www.sussex.ac.uk/gdsc/intranet/microscopy/imagej/gdsc_plugins.

from

6. Counting Single Redox Turnovers: Fluorogenic Antioxidant Conversion and Mass Transport Visualization via Single Molecule Spectroelectrochemistry

Robert Godin and Gonzalo Cosa, *Manuscript in preparation*



6.1. Preface

The final research Chapter of this thesis seeks to demonstrate the many opportunities enabled by SMS-EC for the study of heterogeneous electrochemical reactions at the SM level. The fluorogenic antioxidant H₂B-PMHC, shown in Chapters 2 and 3 to undergo dramatic fluorescence enhancements under lipid peroxidation conditions, was here utilized to monitor electrochemical oxidation processes. We observed fluorescence enhancements when the working electrode was poised to oxidize the chromanol moiety of H₂B-PMHC. Greater distinction between open circuit and oxidizing conditions could be made by monitoring the frequency of fluorescent bursts instead of the fluorescent intensity, a feat only possible through SM detection. Interestingly, we could also oxidize H₂B-PMHC under reductive electrochemical conditions in the presence of oxygen. Reduction of O₂ generates superoxide radical anion (O₂^{•-}) which deprotonates the chromanol segment of H₂B-PMCH to initiate chemical oxidation and production of fluorescent species. We also demonstrated that SMS-EC could experimentally measure the spatiotemporal concentration profile of electrochemically-generated products, significantly extending the scope of the technique. Furthermore, we conducted the first SMS-EC studies of small molecules in organic solvents, progressing the field of SMS in the monitoring of chemical reactions in organic solvents.

6.2. Abstract

The design of optimized mesoporous catalysts and detailed electroanalytical measurements require knowledge of the mass transport processes for various system geometries. However, it is experimentally challenging to measure the resulting concentration profile of products. Here we report the use of single molecule spectroelectrochemistry (SMS-EC) combined with the redox-sensitive fluorogenic probe H₂B-PMHC to optically detect electrochemically-generated individual fluorescent molecules and to measure the changes in their concentration over time and distance from a planar electrode. Fluorescent products were generated under both oxidative (direct oxidation of H₂B-PMHC) and reductive (produce O₂^{•-} to initiate chemical oxidation of H₂B-PMHC) conditions. Significant differences in the resulting concentration profile of fluorescent products reflect the distinct reaction pathways. These studies represent the first report of SMS-EC of small molecules conducted in organic solvents. Overall, our results significantly broaden the scope of electrochemical reactions that can be studied by SMS-EC and demonstrate novel applications in experimentally determining spatiotemporal concentration profiles.

6.3. Introduction

Mass transport is an important consideration in the optimization of catalytic processes,¹ device functioning such as photovoltaics,² and in electroanalytical methods.³⁻⁴ The combination of diffusion, migration and convection mass transport processes impact the measured current in electrochemical measurements.⁵ Although migration is insignificant under standard experimental conditions where an excess of supporting electrolyte is present, diffusion is always active and the extent to which convection plays a role in mass transport depends on geometrical factors.⁶⁻⁷ As such, detailed understanding of electrochemical processes occurring on intricate electrode

geometries, such as microarrays,⁸⁻⁹ or taking place in microfluidic devices¹⁰ require knowledge of mass transport processes and the concentration profiles surrounding the electrode.

In order to develop a general method to experimentally determine the concentration profile of electrochemically-generated species, we have resorted to single molecule spectroelectrochemistry (SMS-EC)¹¹ combined with the redox-sensitive fluorogenic probe H₂B-PMHC. SMS-EC is a powerful tool that may be used to elucidate the intricacies of a wide range of optoelectronic systems by monitoring the fluorescence of individual molecules under electrochemical control.¹²⁻¹⁹ The fluorescence-based detection of SMS-EC allows the monitoring of single electrochemical reaction events and allows for the study of highly dilute (~ pM) analytes. The use of a confocal microscope creates a hundreds of zeptoliter observation volume in solution that is arbitrarily positioned and spatially well-defined.

We chose the two-segment, reporter-trap, fluorogenic probe H₂B-PMHC developed in our group to perform the SMS-EC experiments.²⁰⁻²¹ H₂B-PMHC shows reactivity towards reactive oxygen species (ROS) analogous to that of the naturally-occurring antioxidant α -tocopherol.²¹ H₂B-PMHC has thus been used to study oxidation of lipids in heterogeneous media,²¹ including single molecule detection of oxidized probes in individual liposomes.²² The BODIPY fluorophore segment of this probe reports on the redox state of the chromanol trap segment. In the reduced form of the chromanol, photoinduced electron transfer (PeT) takes place from the chromanol donor to the photoexcited BODIPY acceptor resulting in a non-fluorescent state. Upon oxidation of the chromanol, the PeT pathway is deactivated and fluorescence enhancements of ~ 100-fold are observed. The large enhancement observed provides a most suitable probe, we reasoned, for monitoring electrochemically-induced reactions in the vicinity of an electrode surface.

6.4. Results and Discussion

We performed SMS-EC experiments on a confocal microscope setup (see Figure 6.1). Briefly, an electrochemical cell was custom-built by gluing a glass tube on top of a coverslip coated with optically-transparent platinized ITO (working electrode). The cell was filled with 2 mL of solution (0.5 nM analyte, 0.1 M Bu₄NClO₄ in dry acetonitrile) and was then sealed by a septum pierced with a Pt wire (counter electrode) and a AgCl-plated Ag wire (quasireference electrode). The cell was purged with argon to remove oxygen and then mounted on the microscope. The confocal observation volume was positioned above the platinized ITO electrode, the sample was excited by the 498.2 nm output of a supercontinuum broadband laser source (24 μ W measured out of the objective) and the emission was cleaned by an ET540/80 band pass filter and finally detected by avalanche photodiode detectors (APDs).

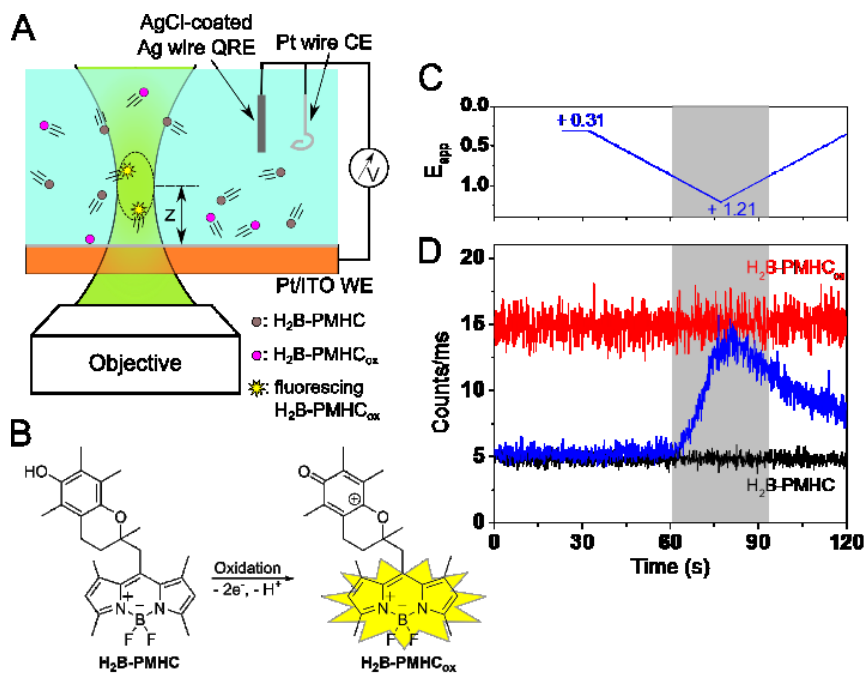


Figure 6.1. A) SMS-EC setup depicting H₂B-PMHC molecules that are oxidized at the surface of the Pt/ITO WE and then diffuse through the confocal observation volume (dotted oval) and emit fluorescence. The distance between the electrode surface and the observation volume was controlled by a piezo nanopositioner. B) Oxidation of H₂B-PMHC to yield fluorescent H₂B-

PMHC_{ox}. Addition of traces of H₂O may subsequently give rise to other oxidized species. C) Applied electrochemical potential. The potential scan rate was 20 mV/s. D) Fluorescence trajectories of chemically oxidized H₂B-PMHC (red), pristine H₂B-PMHC under open circuit conditions (black) and pristine H₂B-PMHC under influence of the bias shown in C). Times where $E_{app} \geq 0.88$ V are highlighted in gray. Trajectories were calculated using 100 ms time bins. The concentration of H₂B-PMHC was 0.5 nM in all cases. Trajectories were acquired with the observation volume 20 μ m above the electrode surface.

We first compared the fluorescence intensity of pristine H₂B-PMHC to that of H₂B-PMHC oxidized in a controlled chemical manner (H₂B-PMHC_{ox}). A 1 μ M solution of H₂B-PMHC in acetonitrile showed low fluorescence (see Figure 6.5). Addition of 2 eq. of the strong oxidant cerium(IV) ammonium nitrate resulted in a fluorescence enhancement of over 80-fold. Although we have not identified the final oxidation product(s), where possibilities include a formation of a phenoxonium cation²³ and various quinones,²⁴ the fluorescence enhancement is in line with deactivation of the PeT quenching mechanism.

We then diluted the pristine H₂B-PMHC sample and the chemically oxidized H₂B-PMHC (H₂B-PMHC_{ox}) to 0.5 nM and compared the signals obtained on our single molecule confocal setup. The fluorescence intensity of the H₂B-PMHC_{ox} (average 15.2 counts/ms) was only 3-fold larger than that of H₂B-PMHC (average 4.8 counts/ms), a smaller enhancement compared to the ensemble experiments (Figure 6.1). The highly dilute conditions combined with the diffraction-limited observation volume result, on average, in the presence of less than 1 H₂B-PMHC_(ox) molecule in the observation volume. In consequence, the fluorescence background and dark counts of the detectors mask the fluorescence enhancement. It is also possible that a minute amount H₂B-PMHC was oxidized during dilution by the presence of impurities in the solvent, contributing to the small increase in the fluorescence intensity compared to the background of a control sample containing only the 0.1 M Bu₄NClO₄ supporting electrolyte solution (average 3.4 counts/ms).

Correcting for the background fluorescence intensity yielded an improved, 8-fold, fluorescence enhancement.

Figure 6.1D shows the fluorescence enhancement of H₂B-PMHC under electrochemically oxidative conditions. The observed fluorescence trajectory is shown below the triangular potential waveform applied (E_{app}). We observe a rapid rise in fluorescence intensity with potentials above ~ 0.9 V. The onset potential for fluorescence enhancement is in line with the redox potential of α -tocopherol (1.11 V vs. NHE)²⁵ and further consistent with the oxidation reaction that occurs with cerium(IV) ammonium nitrate (1.61 V vs. NHE).²⁶ The maximal fluorescence intensity obtained upon electrochemical oxidation is comparable to that of the fully chemically oxidized H₂B-PMHC sample. We may thus conclude that H₂B-PMHC was practically completely converted to H₂B-PMHC_{ox} by electrochemical oxidation. As E_{app} is scanned back towards 0 V, at values less positive than 0.9 V where H₂B-PMHC_{ox} is no longer being generated, the fluorescence intensity does not trace the applied bias and decays slowly compared to the fluorescence increase. This is in line with the quasi-irreversible nature of the electrochemical oxidation of the chromanol moiety also seen in bulk cyclic voltammetry experiments (Figure 6.6). The decay of fluorescence is consequently attributed to diffusion-limited dispersion of the fluorescent H₂B-PMHC_{ox}.

We then investigated the fluorescence response of H₂B-PMHC under higher electrochemical oxidation potentials. When E_{app} was swept from 0.31 V to 1.81 V, we again observe a fluorescence enhancement with an onset of 0.88 V (Figure 6.2). Depletion of H₂B-PMHC_{ox} at $E_{app} > 1.34$ V was observed. We assign the depletion to oxidation of the BODIPY core to form BODIPY⁺⁺ at the electrode surface resulting in a non-fluorescent molecule. The onset potential of quenching of 1.34 V is in line with the oxidation potentials reported for analogous H-substituted BODIPYs.²⁷ To further support this assignment, we observe two anodic peaks in a

cyclic voltammetry (CV) experiment at 1.26 V and 1.61 V, which we attributed to the oxidation of the chromanol and the BODIPY segments, respectively (Figure 6.6). Note that the potentials reported for the anodic peaks for the CV experiment are indeed expected to be more positive than the onset potentials determined by SMS-EC. For example, the estimated onset potential for chromanol oxidation from the CV experiment is ~ 1.0 V.

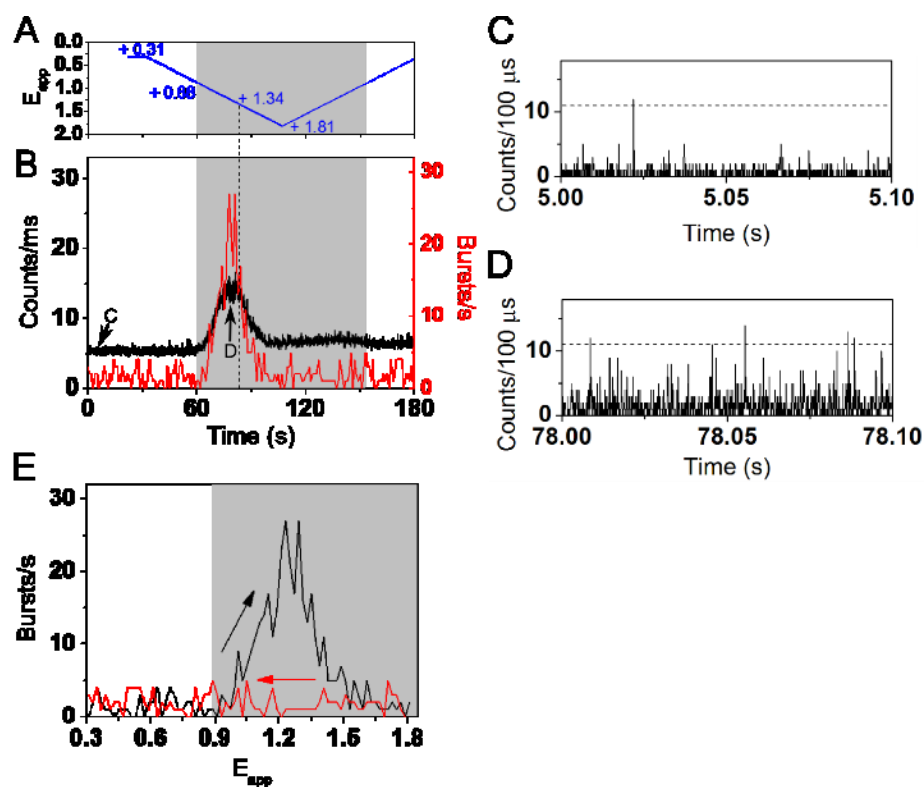


Figure 6.2. SMS-EC of $\text{H}_2\text{B-PMHC}$ under high positive E_{app} . A) Applied electrochemical potential. The potential scan rate was 20 mV/s. B) Fluorescence trajectory (black) and burst frequency (red) of $\text{H}_2\text{B-PMHC}$ under influence of the bias shown in A). Trajectories were calculated using 100 ms time bins. C) and D) 100 ms segments of the fluorescence trajectories indicated in B) by the arrows. Time bins of 100 μs were used, and high fluorescence bursts are counted for bins where counts/100 $\mu\text{s} \geq 11$. E) Burst frequency as in B) but plotted against E_{app} instead of time. The potential scan towards 1.81 V is traced in red while the scan back towards 0.31 V is traced in black. Direction of potential scans are further indicated by arrows. Times where $E_{\text{app}} \geq 0.88$ V are highlighted in gray. Trajectories acquired with the observation volume 20 μm above the electrode surface.

The single molecule detection of our setup allowed us to monitor the frequency of high fluorescence bursts. These bursts are associated with individual fluorescent molecules diffusing through the observation volume. As more $\text{H}_2\text{B-PMHC}_{\text{ox}}$ is produced by oxidation, a higher frequency of bursts is observed. Figure 6.2B compares the fluorescence intensity to the frequency of fluorescence bursts over time. The analysis of bursts shows a strikingly more marked distinction between low and high concentrations of $\text{H}_2\text{B-PMHC}_{\text{ox}}$ than does the fluorescence intensity. We observe a maximal 13-fold enhancement in the bursts frequency (from 1.6 bursts/s to ~ 20 bursts/s) versus a background-corrected 7-fold fluorescence intensity enhancement. Greater sensitivity to the production of fluorescence molecules in highly dilute solution is therefore possible by the discrimination of fluorescence bursts from individual molecules.

We experimentally determined the time-dependant concentration profile of electrochemically generated $\text{H}_2\text{B-PMHC}_{\text{ox}}$ by observing the changes in fluorescence intensity over time at varying distances from the electrode surface under a step potential function. We could easily move the distance between the confocal observation volume and the electrode surface with the aid of an-objective mounted z-axis nanopositioning piezoelectric actuator. We monitored the generation of $\text{H}_2\text{B-PMHC}_{\text{ox}}$ upon applying a potential step of 0.95 V at three different distances from the electrode surface: 20 μm , 50 μm and 80 μm . Figure 6.3 shows that the growth of the fluorescence signal is larger and faster when closer to the planar electrode surface.

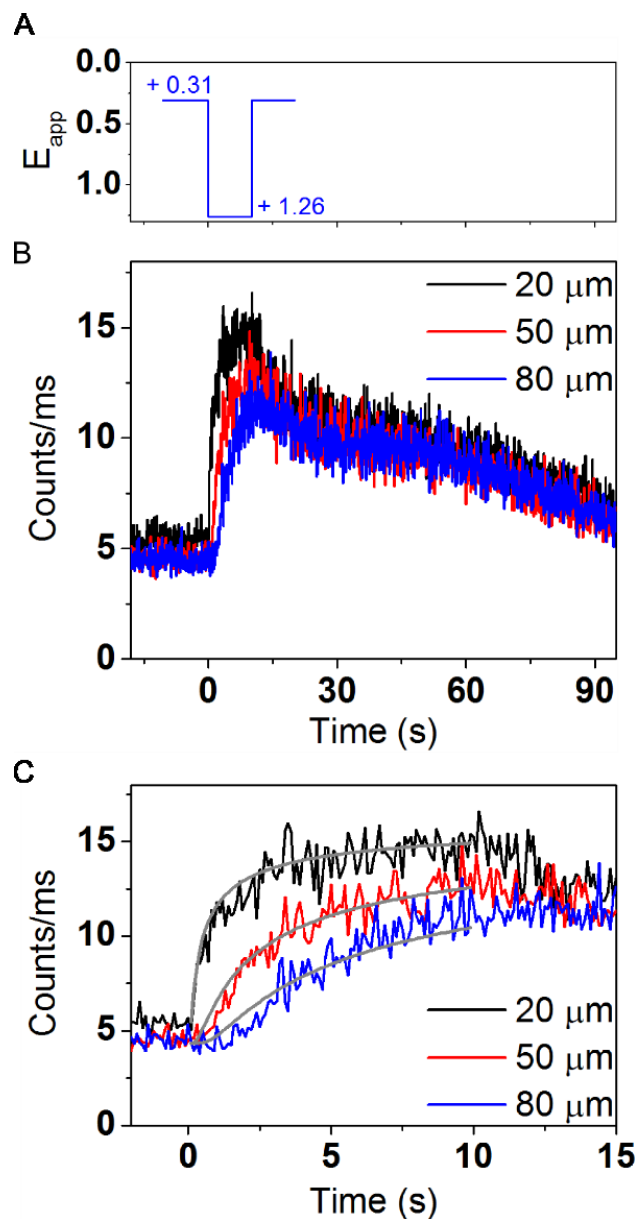


Figure 6.3. SMS-EC determination of the concentration profile of electrochemically-generated $\text{H}_2\text{B-PMHC}_{\text{ox}}$. A) Applied electrochemical potential. The pulses were 10 s in duration. B) Fluorescence trajectories of $\text{H}_2\text{B-PMHC}$ under influence of the bias shown in A) for confocal observation volumes located 20 μm (black), 50 μm (red) and 80 μm (blue) above the electrode surface. The time axis was set to 0 s at the start of the + 1.26 V pulse that lasted for 10 s. C) Segment of the fluorescence trajectories shown in B) emphasizing the $E_{app} = + 1.26$ V region. Gray lines represent the best fit result of the model described by equation (6.2).

The concentration profile of electrochemically generated species has been analytically solved for our experimental case of a planar electrode and semi-infinite linear diffusion.²⁸ We

globally fitted our experimental data to this model (eq. 22 of ref. 28) with modifications to relate concentration to the observed fluorescence, see Equations (6.1) and (6.2).

$$[H_2B-PMHC_{ox}](z,t) = [H_2B-PMHC_{ox}]_0 - \text{erfc}\left(\frac{z}{2\sqrt{Dt}}\right) \times ([H_2B-PMHC_{ox}]_0 - [H_2B-PMHC_{ox}]_{elec}) \quad (6.1)$$

$$I(z,t) = I_0 + I_{H_2B-PMHC_{ox}} \times [H_2B-PMHC_{ox}](z,t) \quad (6.2)$$

Here z stands for the distance from the electrode, t for time, $[H_2B-PMHC_{ox}](z,t)$ for the concentration profile of $H_2B-PMHC_{ox}$, erfc for the complementary error function, D for the diffusion coefficient of $H_2B-PMHC_{ox}$, $[H_2B-PMHC_{ox}]_0$ for the initial $H_2B-PMHC_{ox}$ concentration (assumed to be 0) and $[H_2B-PMHC_{ox}]_{elec}$ for the concentration of $H_2B-PMHC_{ox}$ at the electrode surface (assumed complete conversion, i.e. 0.5 nM), $I(z,t)$ for the observed fluorescence intensity, I_0 for the initial intensity, and $I_{H_2B-PMHC_{ox}}$ for the intensity associated with $H_2B-PMHC_{ox}$ at complete conversion. For the fitting procedure, the floating parameters were I_0 , $I_{H_2B-PMHC_{ox}}$ and D . Our data was well-described by equation (6.2) and the resulting fits are shown in Figure 6.3C. The diffusion coefficient extracted from this fit was $D = 6.0 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$. Of note, we can also independently estimate the diffusion coefficient of $H_2B-PMHC_{ox}$ from fluorescence correlation spectroscopy (FCS) analysis of the data acquired. Consistent with the results of the concentration profiles, FCS analysis yielded a diffusion coefficient of $1.5 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$. This value should be considered as a rough estimate as we relied on the concentration of $H_2B-PMHC_{ox}$ to calibrate the confocal observation volume.

We additionally demonstrate herein the ability of $H_2B-PMHC$ to indirectly probe electroreduction reactions via coupling with a chemical reaction.²⁹ Reductive conditions were applied to an air-equilibrated $H_2B-PMHC$ solution where $E_{app} = -0.89 \text{ V}$ is expected to reduce O_2

to yield superoxide radical anion, $O_2^{\bullet-}$ (O_2 reduction potential of -0.61 V vs. NHE in acetonitrile).³⁰ Taking advantage of the intrinsic antioxidant activity of H_2B -PMHC, $O_2^{\bullet-}$ in the presence of O_2 may then chemically oxidize H_2B -PMHC to form fluorescent products.³¹ Presumably, $O_2^{\bullet-}$ deprotonates the phenolic O-H group³² and O_2 subsequently adds to the anion. Redox reaction of both protonated and deprotonated H_2B -PMHC with the hydroperoxyl radical generated ($HO_2^{\bullet}$) is also feasible based on the redox potentials.³³ Applying a 10 s long reductive pulse of $E_{app} = -0.89$ V resulted in a small and sudden rise in the observed fluorescence intensity. More pronounced are the wave-like variations in the fluorescence intensity that persist long after E_{app} is disengaged (for over 270 s). The concentration profile of the fluorescence products generated is clearly distinct from that of H_2B -PMHC_{ox} formed directly by electrochemical oxidation of H_2B -PMHC at the electrode surface. The variations in fluorescence intensity are inconsistent with a diffusion-controlled process, and it is possible that the complex mass transport behaviour stems from the activation-controlled chemical reaction between H_2B -PMHC and $O_2^{\bullet-}$,³⁴ and the apparent necessity of at least one additional O_2 molecule to oxidize the chromanol segment.³¹ Convolution with the reduction of the BODIPY core is also a possibility (see Figure 6.6). In contrast, control experiments in the absence of O_2 did not show long-lasting variations in fluorescence intensity after E_{app} was swept down to -0.89 V (Figure 6.7).

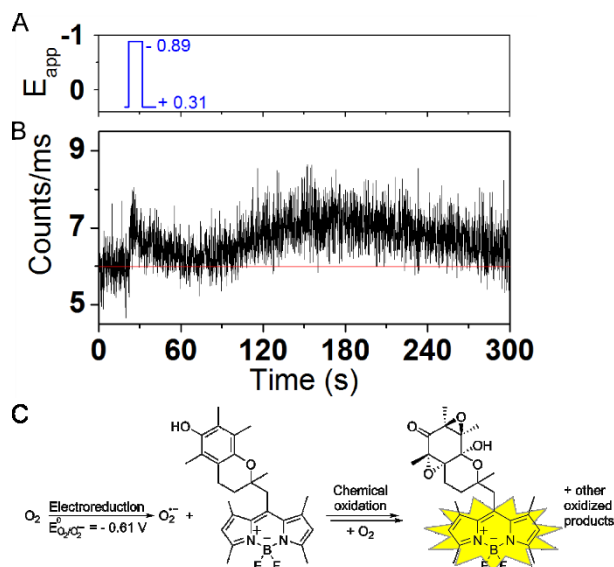


Figure 6.4. Indirect fluorescence monitoring of the electroreduction of O_2 . A) Applied electrochemical potential. B) Fluorescence trajectories of $H_2B-PMHC$ under influence of the bias shown in A) in air-equilibrated conditions. The red line represents the average fluorescence intensity prior to applying a bias. Trajectories were acquired with the observation volume $20\ \mu m$ above the electrode surface. C) Scheme illustrating the oxidation of $H_2B-PMHC$ initiated by electrochemically-generated $O_2^{\bullet -}$.

Previous SMS-EC studies of small molecules have only been reported for aqueous solutions.^{14, 16-17, 19} In contrast, the SMS-EC experiments reported herein were conducted in organic solvents, a significant advancement. The development of single molecule techniques applied to chemical reactions has been in part hampered by the relatively low number of studies conducted using organic solvents, typically limited by increased fluorescent background.³⁵ We show that SMS-EC is suitable to monitor electrochemically-generated fluorescent products in organic solvents, greatly expanding the available redox reactions that may be studied at the single molecule level.

6.5. Conclusions

In summary, we report the extension of SMS-EC to organic solvents using $H_2B-PMHC$ as the redox-active fluorescent probe. Electrochemical oxidation of $H_2B-PMHC$ was confirmed by

observation of higher fluorescence intensity and a higher frequency of fluorescence bursts arising from individual molecules. We also showed the apparent ambipolar reactivity of H₂B-PMHC, where fluorescent H₂B-PMHC_{ox} was produced via chemical reaction initiated by O₂^{•-} that was generated under reductive conditions. We further used SMS-EC methodology to experimentally determine the concentration profile of H₂B-PMHC_{ox} electrochemically-generated from a simple planar electrode. We thus demonstrate that SMS-EC introduces exciting opportunities to optically monitor the concentration profile of systems with complex electrode geometries or hampered diffusion in highly dilute solutions.

6.6. Materials and Methods

6.6.1. SMS-EC setup

The ITO coverslips (Evaporated Coatings Inc., 50 Ω/□; cleaned by 20 minutes sequential sonication in acetone, trichloroethylene and methanol) were platinized to improve the catalytic activity of the electrode. Platinum was deposited by spin coating a drop of ~ 20 μL of platinum precursor solution (Plastisol T, Solaronix). The coverslips were then sintered at 450 °C for 30 minutes. After cooling to allow for handling, a glass tube was glued with epoxy (Hardman®, McMaster-Carr) on top of the coverslip to form the sample chamber. The epoxy glue was cured by placing the samples in a 110 °C oven for ~ 30 minutes. The sample chamber was filled with the desired analyte solution and sealed by a septum pierced with the reference and counter electrodes. When oxygen-free conditions were required, argon was bubbled through the solution for 1 hour in order to deaerate the solution, and an argon-filled balloon was fixed on the sample during acquisition. The electrochemical cell was then mounted on the microscope (IX-71, Olympus). Electrochemical potential was controlled by a CHI600D electrochemical analyzer. The AgCl-

coated Ag wire quasireference electrode used was calibrated with the ferrocene/ferrocenium redox couple using $E^0_{\text{Fc}^+/\text{Fc}} = + 0.64 \text{ V}$ vs. NHE. All potentials reported are referenced to NHE.

Laser excitation was provided by a supercontinuum laser (WhiteLase SC-400-4, Fianium, Beverly, MA). A center excitation wavelength of 498.2 nm was spectrally separated from the broadband emission by a computer-controlled acousto-optical tunable filter (AOTF, Fianium). A Z488rdc dichroic mirror (Chroma, Rockingham, VT) projected the pulsed laser excitation (20 MHz repetition rate, 24 μW out of the objective) towards the water-immersion objective (UPLSAPO 60XW, N.A. = 1.20, Olympus). The confocal observation volume was focussed between 20 and 80 μm above the electrode surface, as controlled by a piezoelectric objective scanner (PIFOC P-721 controlled by an E710, Physik Instrumente (PI), Auburn, MA). The emission in a 500–580 nm spectral window (ET540/80 bandpass filter, Chroma) was collected by the same objective, passed through a 100 μm diameter pinhole positioned at the image plane, and split onto two avalanche photodiode detectors (APDs; SPCM-AQR-14, Perkin Elmer Optoelectronics, Vaudreuil, QC) with a 50:50 non-polarizing beamsplitter in pseudo-cross-correlation format.³⁶ This configuration was used to observe microsecond dynamics that are obscured by detector afterpulsing in a single-detector setup.

6.7. Supporting Information

The Supporting Information contains the fluorescence enhancement of chemically oxidized H₂B-PMHC, electrochemical characterization of H₂B-PMHC and the effect of O₂ under electroreductive conditions.

6.7.1. Fluorescence Enhancement of Chemically Oxidized H_2B -PMHC

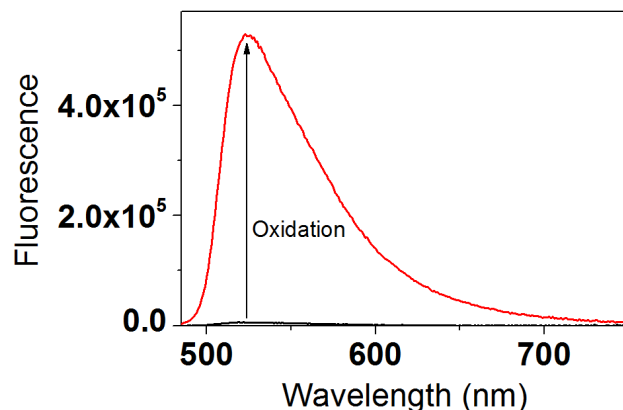


Figure 6.5. Fluorescence enhancement observed in acetonitrile solutions upon chemical oxidation of H_2B -PMHC (1 μ M) by 2 eq. of cerium(IV) ammonium nitrate. An 83-fold fluorescence enhancement is calculated by integrating the intensities between 485 – 675 nm following emission correction for background. $\lambda_{ex} = 480$ nm.

6.7.2. Electrochemical Characterization of H_2B -PMHC

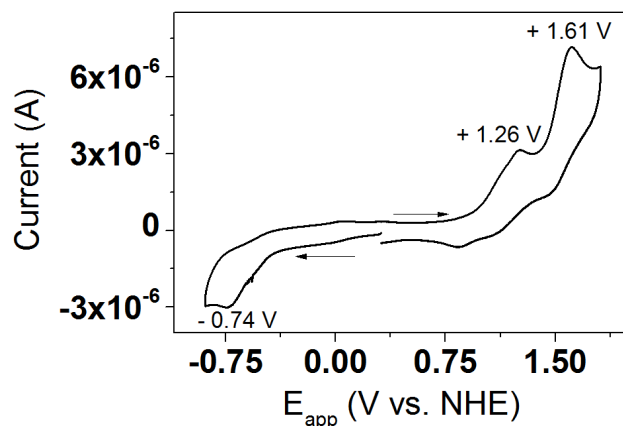


Figure 6.6. Cyclic voltammogram of H_2B -PMHC (54 μ M) in argon-purged 0.1 M Bu_4NClO_4 acetonitrile solution. The experiment was run in the SMS-EC cell: Pt/ITO served as the working electrode, AgCl-coated Ag wire as the quasireference electrode and a Pt wire as the counter electrode. The anodic peaks at 1.26 V and 1.61 V are assigned to the oxidation of the chromanol and BODIPY core, respectively. The cathodic peak at -0.74 V is assigned to the reduction of the BODIPY core, in accordance with reduction potentials values for similar BODIPY dyes (~ -0.8 V).²⁷ The potential sweep rate was 0.1 V/s.

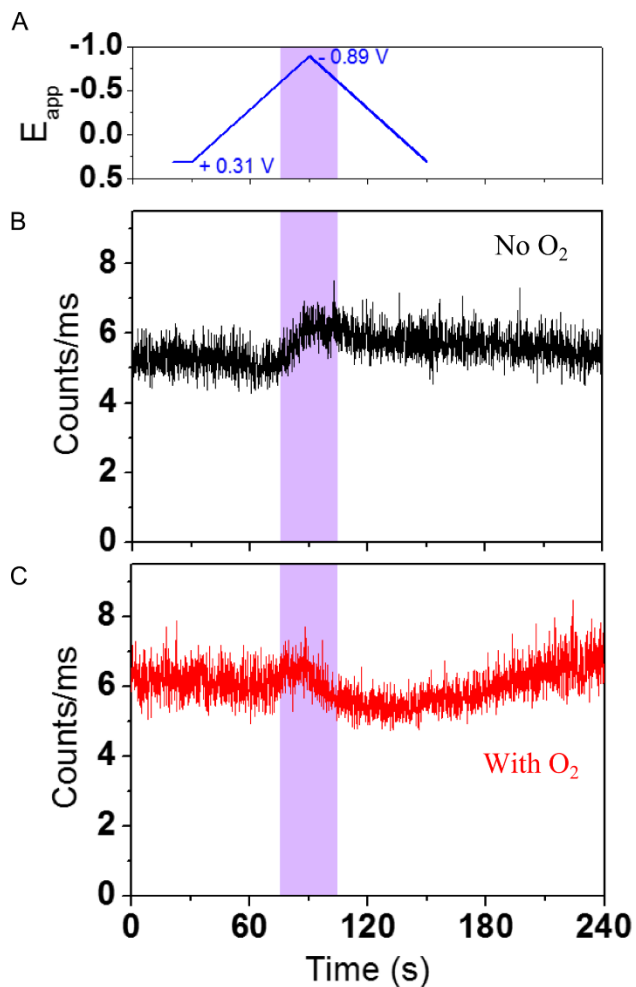
6.7.3. Effect of O_2 under Electroreductive Conditions

Figure 6.7. Effect of the presence of oxygen under reductive conditions. A) Applied electrochemical potential. The potential scan rate was 20 mV/s. B and C) Fluorescence trajectories of H_2B -PMHC under influence of the bias shown in A). B) Argon-purged conditions. C) Air-equilibrated conditions. Times where $E_{app} \leq -0.6$ V are highlighted in purple.

6.8. References

- (1) Yuan, Z.-Y.; Su, B.-L., Insights into Hierarchically Meso-Macroporous Structured Materials. *J. Mater. Chem.* **2006**, *16*, 663-677.
- (2) Nelson, J. J.; Amick, T. J.; Elliott, C. M., Mass Transport of Polypyridyl Cobalt Complexes in Dye-Sensitized Solar Cells with Mesoporous TiO_2 Photoanodes. *J. Phys. Chem. C* **2008**, *112*, 18255-18263.
- (3) Campbell, F.; Compton, R., The Use of Nanoparticles in Electroanalysis: An Updated Review. *Anal. Bioanal. Chem.* **2010**, *396*, 241-259.
- (4) Inzelt, G., Kinetics of Electrochemical Reactions. In *Electroanalytical Methods*, Scholz, F.; Bond, A. M.; Compton, R. G.; Fiedler, D. A.; Inzelt, G.; Kahlert, H.; Komorsky-Lovrić, Š.;

Lohse, H.; Lovrić, M.; Marken, F.; Neudeck, A.; Retter, U.; Scholz, F.; Stojek, Z., Eds. Springer Berlin Heidelberg: 2010; pp 33-53.

(5) Ciszowska, M.; Stojek, Z., Voltammetry in Solutions of Low Ionic Strength. Electrochemical and Analytical Aspects. *J. Electroanal. Chem.* **1999**, *466*, 129-143.

(6) Amatore, C.; Pebay, C.; Sella, C.; Thouin, L., Mass Transport at Microband Electrodes: Transient, Quasi-Steady-State, and Convective Regimes. *ChemPhysChem* **2012**, *13*, 1562-1568.

(7) Amatore, C.; Pebay, C.; Thouin, L.; Wang, A.; Warkocz, J. S., Difference between Ultramicroelectrodes and Microelectrodes: Influence of Natural Convection. *Anal. Chem.* **2010**, *82*, 6933-6939.

(8) Zoski, C. G.; Wijesinghe, M., Electrochemistry at Ultramicroelectrode Arrays and Nanoelectrode Ensembles of Macro- and Ultramicroelectrode Dimensions. *Isr. J. Chem.* **2010**, *50*, 347-359.

(9) Zoski, C. G.; Simjee, N.; Guenat, O.; Koudelka-Hep, M., Addressable Microelectrode Arrays: Characterization by Imaging with Scanning Electrochemical Microscopy. *Anal. Chem.* **2004**, *76*, 62-72.

(10) Dungchai, W.; Chailapakul, O.; Henry, C. S., Electrochemical Detection for Paper-Based Microfluidics. *Anal. Chem.* **2009**, *81*, 5821-5826.

(11) Palacios, R. E.; Fan, F.-R. F.; Bard, A. J.; Barbara, P. F., Single-Molecule Spectroelectrochemistry (SMS-EC). *J. Am. Chem. Soc.* **2006**, *128*, 9028-9029.

(12) Palacios, R. E.; Chang, W.-S.; Grey, J. K.; Chang, Y.-L.; Miller, W. L.; Lu, C.-Y.; Henkelman, G.; Zepeda, D.; Ferraris, J.; Barbara, P. F., Detailed Single-Molecule Spectroelectrochemical Studies of the Oxidation of Conjugated Polymers. *J. Phys. Chem. B* **2009**, *113*, 14619-14628.

(13) Palacios, R. E.; Fan, F.-R. F.; Grey, J. K.; Suk, J.; Bard, A. J.; Barbara, P. F., Charging and Discharging of Single Conjugated-Polymer Nanoparticles. *Nat. Mater.* **2007**, *6*, 680-685.

(14) Lei, C.; Hu, D.; Ackerman, E. J., Single-Molecule Fluorescence Spectroelectrochemistry of Cresyl Violet. *Chem. Commun.* **2008**, 5490-5492.

(15) Lei, C.; Hu, D.; Ackerman, E., Clay Nanoparticle-Supported Single-Molecule Fluorescence Spectroelectrochemistry. *Nano Lett.* **2009**, *9*, 655-658.

(16) Xu, W.; Shen, H.; Kim, Y. J.; Zhou, X.; Liu, G.; Park, J.; Chen, P., Single-Molecule Electrocatalysis by Single-Walled Carbon Nanotubes. *Nano Lett.* **2009**, *9*, 3968-3973.

(17) Shen, H.; Xu, W.; Chen, P., Single-Molecule Nanoscale Electrocatalysis. *PCCP* **2010**, *12*, 6555-6563.

(18) Zhang, G.; Xiao, L.; Chen, R.; Gao, Y.; Wang, X.; Jia, S., Single-Molecule Interfacial Electron Transfer Dynamics Manipulated by an External Electric Current. *PCCP* **2011**, *13*, 13815-13820.

(19) Zhao, J.; Zaino Iii, L. P.; Bohn, P. W., Potential-Dependent Single Molecule Blinking Dynamics for Flavin Adenine Dinucleotide Covalently Immobilized in Zero-Mode Waveguide Array of Working Electrodes. *Faraday Discuss.* **2013**, *164*, 57-69.

(20) Oleynik, P.; Ishihara, Y.; Cosa, G., Design and Synthesis of a Bodipy- α -Tocopherol Adduct for Use as an Off/on Fluorescent Antioxidant Indicator. *J. Am. Chem. Soc.* **2007**, *129*, 1842-1843.

(21) Krumova, K.; Friedland, S.; Cosa, G., How Lipid Unsaturation, Peroxyl Radical Partitioning, and Chromanol Lipophilic Tail Affect the Antioxidant Activity of α -Tocopherol: Direct Visualization Via High-Throughput Fluorescence Studies Conducted with Fluorogenic α -Tocopherol Analogues. *J. Am. Chem. Soc.* **2012**, *134*, 10102-10113.

- (22) Godin, R.; Liu, H.-W.; Cosa, G., Ambient Condition Oxidation in Individual Liposomes Observed at the Single Molecule Level. *Chem. Sci.* **2014**, *5*, 2525-2529.
- (23) Lee, S. B.; Lin, C. Y.; Gill, P. M. W.; Webster, R. D., Transformation of α -Tocopherol (Vitamin E) and Related Chromanol Model Compounds into Their Phenoxonium Ions by Chemical Oxidation with the Nitrosonium Cation. *J. Org. Chem.* **2005**, *70*, 10466-10473.
- (24) Parkhurst, R. M.; Skinner, W. A., Chromanols and Tocopherols. In *Chemistry of Heterocyclic Compounds*, John Wiley & Sons, Inc.: 2008; pp 59-137.
- (25) Wei Yao, W.; Mei Peng, H.; Webster, R. D., Electrochemistry of α -Tocopherol (Vitamin E) and α -Tocopherol Quinone Films Deposited on Electrode Surfaces in the Presence and Absence of Lipid Multilayers. *J. Phys. Chem. C* **2009**, *113*, 21805-21814.
- (26) Nair, V.; Deepthi, A., Cerium(IV) Ammonium Nitrate A Versatile Single-Electron Oxidant. *Chem. Rev.* **2007**, *107*, 1862-1891.
- (27) Krumova, K.; Cosa, G., Bodipy Dyes with Tunable Redox Potentials and Functional Groups for Further Tethering: Preparation, Electrochemical, and Spectroscopic Characterization. *J. Am. Chem. Soc.* **2010**, *132*, 17560-17569.
- (28) Molina, A.; Serna, C.; Martínez-Ortiz, F.; Laborda, E., Potentiostatic Voltammetry at Spherical Electrodes and Microelectrodes in the Presence of Product. *J. Electroanal. Chem.* **2008**, *617*, 14-26.
- (29) Oja, S. M.; Guerrette, J. P.; David, M. R.; Zhang, B., Fluorescence-Enabled Electrochemical Microscopy with Dihydroresorufin as a Fluorogenic Indicator. *Anal. Chem.* **2014**, *86*, 6040-6048.
- (30) D. Webster, R.; M. Bond, A., Different Mechanisms for the Reaction of Disubstituted Aromatic Esters and Thioic S-Esters with Electrochemically Generated Superoxide. *J. Chem. Soc. Perkin. T. 2* **1997**, 1075-1080.
- (31) Matsuo, M.; Matsumoto, S.; Iitaka, Y., Oxygenations of Vitamin E (α -Tocopherol) and Its Model Compound 2,2,5,7,8-Pentamethylchroman-6-ol in the Presence of the Superoxide Radical Solubilized in Aprotic Solvents: Unique Epoxidations and Recyclizations. *J. Org. Chem.* **1987**, *52*, 3514-3520.
- (32) Nanni, E. J.; Stallings, M. D.; Sawyer, D. T., Does Superoxide Ion Oxidize Catechol, α -Tocopherol, and Ascorbic Acid by Direct Electron Transfer? *J. Am. Chem. Soc.* **1980**, *102*, 4481-4485.
- (33) Sawyer, D., The Redox Thermodynamics for Dioxygen Species (O_2 , $O_2^{\cdot-}$, $HOO^{\cdot}$, $HOOH$, and HOO^-) and Monooxygen Species (O , $O^{\cdot-}$, $\cdot OH$, and ^-OH) in Water and Aprotic Solvents. In *Oxygen Radicals in Biology and Medicine*, Simic, M.; Taylor, K.; Ward, J.; von Sonntag, C., Eds. Springer US: 1988; Vol. 49, pp 11-20.
- (34) Nishikimi, M.; Machlin, L. J., Oxidation of α -Tocopherol Model Compound by Superoxide Anion. *Arch. Biochem. Biophys.* **1975**, *170*, 684-689.
- (35) Cordes, T.; Blum, S. A., Opportunities and Challenges in Single-Molecule and Single-Particle Fluorescence Microscopy for Mechanistic Studies of Chemical Reactions. *Nat. Chem.* **2013**, *5*, 993-999.
- (36) Yu, J.; Lammi, R.; Gesquiere, A. J.; Barbara, P. F., Singlet-Triplet and Triplet-Triplet Interactions in Conjugated Polymer Single Molecules. *J. Phys. Chem. B* **2005**, *109*, 10025-10034.

7. Concluding Remarks and Outlook

7.1. Concluding Remarks

Single molecule spectroscopy (SMS) is a very versatile tool that has tremendous potential for advancing the fields of biology, chemistry and physics. Specifically for chemistry, we believe that we have only seen a fraction of the advancements possible through SMS studies. The body of work presented in this thesis highlights our progress in monitoring redox processes in heterogeneous systems at the single molecule (SM) level. We focused on two different themes: the biological system of lipid peroxidation and the material-oriented application of state-of-the-art single molecule spectroelectrochemistry (SMS-EC). Our studies, centered in these two themes, demonstrate that changes in redox states, either chemically induced by reactions or controlled by an electrochemical potential, may be monitored at the SM level. This opens the opportunity to study a diverse range of heterogeneous systems and enables the evaluation of the distribution and changes in key parameters such as the turnover frequency of a catalyst, the electrochemical potential of a redox process, and electron transfer rates. Furthermore, utilizing SMS allows for significant advancements in knowledge that are not possible using traditional ensemble measurement techniques.

Lipid peroxidation is relatively-well understood from a chemical point of view. The steps involved in the autoxidation of lipids and their kinetics have been the subject of important research efforts over the past few decades. However, oxidation taking place in other heterogeneous and confined environments, such as low density lipoproteins (LDL), is poorly understood in comparison to lipid peroxidation. We sought to develop general SM methods to investigate oxidation processes that occur in heterogeneous media in order to better characterize, to name a few, oxidation that takes place under physiological conditions in LDL nanoparticles within

biological fluids¹ or in lipid particles (aerosols) in the atmosphere. To achieve this goal we monitored the fate of fluorogenic antioxidants, developed in our research group, under lipid peroxidation conditions within individual liposomes. This served as an excellent platform to demonstrate SM observation in the study of oxidation processes.

In Chapter 2, we describe the oxidation of the fluorogenic antioxidant H₂B-PMHC monitored at the SM level. This oxidation-sensitive probe is initially non-fluorescent in the reduced state, but emits bright fluorescence upon oxidation of its chromanol trap segment. Lipid peroxidation provided us with a heterogeneous oxidation system that is ideally positioned to demonstrate the advances made in detecting SM reactions. We visualized oxidation taking place in individual liposomes under oxidative conditions. The wide range of fluorescence enhancements observed reflects the heterogeneous nature of lipid peroxidation. We moreover showed that oxidation occurring during standard liposome preparation could be inhibited by the addition of antioxidants. A water-soluble antioxidant additive was overall more effective at preventing oxidation in comparison to a lipid-soluble antioxidant. We further discovered serendipitously the anomalous blinking behaviour of the probe H₂B-PMHC during oxidation.

Chapter 3 focuses on a rapid fluorescence correlation spectroscopy (FCS) method that we devised in order to determine the lipid partitioning preference of a dye or chemical probe under dilute conditions. Development of this method was prompted by preliminary observations that our activated (oxidized) fluorogenic probes were escaping the lipid environment of liposomes. Comparing ensemble methods (concentrated) and SM methods (highly dilute) revealed the influence of lipid concentration on the fraction of dye associated to the lipid membrane. Under dilute conditions, dyes have a greater probability of escaping from the lipid membrane and diffusing into the bulk aqueous phase, which risks complicating SM experiments. FCS and SMS

methods were also used to determine the influence of the oxidation reaction partner on the localization of the activated chemical probe. In our case, the preference for the lipid or aqueous phase of reactive oxygen species (ROS) was transferred to the adduct of the ROS and the probe.

We identified SMS-EC as an exciting state-of-the-art technique that warranted further development in our research group. The unique possibility of observing electrochemical processes one molecule at a time opens the door to exploring a plethora of intriguing research questions. As a way to demonstrate the breath of SMS-EC, we chose to study three distinct types of systems. SMS-EC provides a method to observe how the luminescence and charge transfer properties of conjugated polyelectrolytes (CPEs) are intertwined, knowledge that is crucial for their applications in optoelectronic devices. SMS-EC was also our method of choice to reveal how electronic density in a semiconductor influences interfacial charge transfer (ICT) in solar cells, a necessary step in the energetic conversion of sunlight. Finally, SMS-EC could determine the concentration profile of species that are electrochemically-generated on an electrode surface, confirming the validity of models developed to account for diffusion-limited mass transport.

As explained in Chapter 4, we developed a facile method to produce CPE nanoparticles (NPs). The optoelectronic properties of these nanoconfined heterogeneous systems were studied with SMS-EC to reveal the interplay between energy and charge transfer processes. We observed two distinct behaviours of the fluorescence when changes were applied to the electrochemical potential. We concluded that the two populations of NPs were characterized either by slow or rapid ion percolation, a result of more tightly or loosely packed chain conformations. Two-color imaging was also introduced to SMS-EC for the first time, which allowed us to relate the oxidation potential to polymer chain aggregation within individual NPs.

In Chapter 5, we describe how we applied SMS-EC to the study of ICT between dye sensitizers and semiconductors, an important process in the function of dye-sensitized solar cells (DSSCs). We modulated the rate of ICT by controlling the electron density in a thin film of SnO_2 semiconductor. Three important observations were made. Firstly, the photobleaching of a perylene dye reported on the rate of electron injection into SnO_2 . Secondly, the energetics of the sensitizers had little influence over the changes in ICT as the applied potential was changed; rather, the electronic occupancy of the semiconductor states was the dominant factor. Thirdly, we observed an active, non-productive ICT pathway resulting in the photoreduction of dye. Although SM observation has not yet been accomplished in this challenging system, our investigation further demonstrates the capabilities of fluorescence spectroscopy to report on ICT in solar cells.

Chapter 6 illustrates how SMS-EC was utilized to electrochemically oxidize a fluorogenic antioxidant to produce a fluorescent species and individually detect these diffusing fluorescent molecules. The experiments were performed in organic solvents, extending SMS-EC beyond the previously reported redox reactions of freely diffusing molecules in aqueous environment. We also demonstrated the use of SMS-EC to experimentally determine the concentration profile of electrochemically generated species emanating from a planar electrode. We found that our experimental data were well-described by previously developed models.

7.2. Outlook

The study of redox processes in heterogeneous systems is a challenging endeavour that involves combining the intricacies of monitoring redox states and the difficulties of determining chemical dynamics at interfaces. We anticipate that SMS will continue to enable significant advances in this field with further development of suitable probes and methodologies.

Highly sensitive fluorogenic antioxidants such as H₂B-PMHC are needed in order to elucidate oxidation pathways in heterogeneous media. We have shown that lipid peroxidation is a heterogeneous process by observing a wide range of fluorescence enhancements in individual liposomes. We also discovered the anomalous blinking behaviour of H₂B-PMHC under SM conditions. The conclusive observation of individual oxidation events, which would represent a significant advancement in our abilities to monitor oxidation processes, will require determination of the root cause of this blinking behaviour. This research is presently ongoing in our research group and the results will undoubtedly dictate future projects. Possible directions include developing methods to prevent the blinking behaviour (additives or improved probe design) or harnessing this blinking behaviour for the development of simple, super resolution imaging protocols.²

Continued development of SMS-EC techniques will enable in depth characterization of heterogeneous optoelectronic systems, allowing for optimization of devices. The different facets of SMS-EC allow for further detailed studies of the interplay between electronic and luminescence properties, and optical detection of controlled electrochemical transformations.

SMS-EC has been used to extract electrochemical parameters of individual conjugated polymers and evaluate their distribution. We have further shown that two-color SMS-EC can be used to link the electrochemical parameters not only with an individual NP, but also to observe the differences between deaggregated and aggregated polymer chains. The two-color SMS-EC approach would thus be of great use to evaluate procedures that aim to control the chain conformation of conjugated polymers.³ Two-channel SMS-EC could also easily be implemented to ratiometrically monitor redox-state switching that induces a change in emission spectra as opposed to an off/on fluorescence switching.

We also exemplified the opportunities for SMS-EC to further our understanding of ICT occurring in solar cells. The imaging of sensitized thin films of SnO_2 allowed us to visualize the changes in rates of ICT, and to determine the role of the semiconductor density of states (DOS) electronic occupancy. We envisage that true SMS-EC investigations of dye-sensitized interfaces, where individual dye sensitizers can be resolved, will lead to a wealth of information regarding the dynamics of ICT. Previous SMS studies of model sensitized semiconductor interfaces consisting of dyes sandwiched between the semiconductor and a protective polymer coating have already educated us on the distribution of energetic barriers for ICT and heterogeneous dynamics.⁴ Employing the SMS-EC platform we described, we may now build on these studies by moving to interfaces in direct contact with solution and controlling the Fermi level of the semiconductor. As a result, SMS-EC will allow detailed investigations of ICT at the SM level to elucidate the expected dynamic heterogeneity under an environment that closely mimics the working conditions of solar cells.

We advanced SMS-EC techniques by detecting individual electrochemically-generated fluorescent molecules for the first time in organic solvents. The study of chemical reactions at the SM level has been limited in part by difficulties associated with the use of organic solvents. We mitigated issues such as non-specific binding of fluorescent impurities by monitoring freely-diffusing reaction products in a confocal microscope instead of imaging an interface where impurities accumulate. Expanding the SM detection of electrochemical products to include organic solvents greatly multiplies the number of redox reactions and analytes that may be studied. We also demonstrated that SMS-EC accurately measures the concentration profile of electrochemically-generated species. While we limited ourselves to the simple geometry of a planar electrode, no limitations are in place regarding the geometry of the electrode that can be

studied by SMS-EC. Situations where mass transport is complicated by hampered diffusion or convection currents may now also be studied by SMS-EC to experimentally determine the flow of material during electrochemical experiments. Additionally, the optical detection of SMS-EC allows to probe the internal volume of porous electrocatalyst. Therefore, the catalytic activity of individual sites within materials such as electroactive metal-organic frameworks (MOF) could be specifically probed.

Tremendous opportunities are also available by combining SMS-EC with electrochemical imaging techniques such as scanning electrochemical microscopy (SECM).⁵ We envisage correlated imaging experiments where the femtoliter focal volume of an optical microscope traces the motion of the microelectrode, providing a direct visualization of the flow of chemicals to show their relationship with electronic currents. It may further enable dramatically improving the resolution of SECM by providing an optical window to the electric signal, and result in the precise study of light-initiated redox processes at the nanoscale. Ultimately, SM detection of redox processes taking place in heterogeneous systems holds remarkable potential to spur rapid development in a wide range of biological and material systems and will play a major role in contemporary chemistry going forward.

7.3. References

- (1) Parthasarathy, S.; Raghavamenon, A.; Garelnabi, M.; Santanam, N., Oxidized Low-Density Lipoprotein. In *Free Radicals and Antioxidant Protocols*, Uppu, R. M.; Murthy, S. N.; Pryor, W. A.; Parinandi, N. L., Eds. Humana Press: 2010; Vol. 610, pp 403-417.
- (2) Rust, M. J.; Bates, M.; Zhuang, X., Sub-Diffraction-Limit Imaging by Stochastic Optical Reconstruction Microscopy (STORM). *Nat. Methods* **2006**, *3*, 793-796.
- (3) Bolinger, J. C.; Traub, M. C.; Brazard, J.; Adachi, T.; Barbara, P. F.; Vanden Bout, D. A., Conformation and Energy Transfer in Single Conjugated Polymers. *Acc. Chem. Res.* **2012**, *45*, 1992-2001.
- (4) Lu, H. P., Single-Molecule Interfacial Electron-Transfer Dynamics. In *Handbook of Spectroscopy*, Wiley-VCH Verlag GmbH & Co. KGaA: 2014; pp 877-910.

- (5) Fan, F.-R. F.; Liu, B.; Mauzeroll, J., 12 - Scanning Electrochemical Microscopy. In *Handbook of Electrochemistry*, Zoski, C. G., Ed. Elsevier: Amsterdam, 2007; pp 471-XIII.