Use of Bioconjugation with Cytochrome P450 Enzymes

Julie Ducharme and Karine Auclair*
Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal, Quebec, Canada H3A 0B8;
*Corresponding author, karine.auclair@mcgill.ca

Abstract: Bioconjugation, defined as chemical modification of biomolecules, is widely employed in biological and biophysical studies. It can expand functional diversity and enable applications ranging from biocatalysis, biosensing and even therapy. This review summarizes how chemical modifications of cytochrome P450 enzymes (P450s or CYPs) have contributed to improving our understanding of these enzymes. Genetic modifications of P450s have also proven very useful but are not covered in this review. Bioconjugation has served to gain structural information and investigate the mechanism of P450s via photoaffinity labeling, mechanism-based inhibition (MBI) and fluorescence studies. P450 surface acetylation and protein cross-linking have contributed to the investigation of protein complexes formation involving P450 and its redox partner or other P450 enzymes. Finally, covalent immobilization on polymer surfaces or electrodes has benefited the areas of biocatalysis and biosensor design.

Introduction

Cytochrome P450 enzymes (P450s or CYPs) consist of a superfamily of heme proteins ubiquitous in all kingdoms of life. In humans, P450s are involved in a number of processes including steroid and lipid biosynthesis, as well as drug metabolism. [1,2] The heme prosthetic group harbored by P450s is central to enzyme activity. The typical reaction catalyzed by P450s follows the general stoichiometry:
$NADPH + H^+ + O_2 + R\text{-}H \rightarrow H_2O + ROH$,
where R-H is the substrate. However, P450 enzymes can perform a wide range of oxidation reactions including not only hydroxylation, but also heteroatom oxidation, epoxidation, rearrangement, Baeyer-Villiger reactions and more. [3,4]

In the process of oxidizing substrates, a molecule of oxygen needs to be cleaved, which requires two electrons to be transferred to the P450. The reducing equivalents are mostly provided by nicotinamide adenine dinucleotide (phosphate), i.e. NAD(P)H, but are typically transferred to the P450 enzyme by one or more redox partner proteins. P450s are separated in four general classes depending on the nature of their redox partner(s). Class I P450s include bacterial and mitochondrial enzymes, and require both an iron-sulfur protein (ferredoxin/adrenodoxin) and a flavin-containing reductase (ferredoxin/adrenodoxin reductase). Class II comprises microsomal P450s which get electrons from the flavoprotein cytochrome P450 reductase (CPR). Class III P450s are self-sufficient in that they also contain a reductase domain. Finally, Class IV P450s accept electrons directly from NADPH. [5,6]
The fields in which P450s are of interest are diverse and include agriculture,

bioremediation, bacteriology, drug development, biocatalysis and biotechnology. [7] Mammalian P450s have been studied extensively for their importance in drug metabolism, while microbial P450s have been mostly considered for their biocatalytic potential due to their higher activity, stability and solubility in aqueous environment. Together with their redox partner, microsomal P450s are membrane-bound proteins and tend to be much less stable than their prokaryotic counterparts. P450s have also attracted the interest of enzymologists for the ability of some to bind multiple substrates at once, leading to allosteric and/or cooperative behavior. [8]

The P450 field has progressed a lot with the recent improvements in techniques such as protein X-ray crystallography, mass spectrometry (MS), nuclear magnetic resonance (NMR), computational chemistry, stopped-flow and rapid mixing techniques, freeze quench experiments and Mössbauer spectroscopy to name some of the most notable ones. Another important method that has contributed significantly to the progress of the field is bioconjugation. The method, which consists of chemically modifying biomolecules, has proven to be an invaluable tool to study biological systems, and as a result, has become a burgeoning field. Rooted in this trend, the present review covers publications that report chemical modifications of P450s with the objective of highlighting how this approach can contribute to research and applications. P450 bioconjugates have been used to address numerous questions and the chemical strategies used are diverse and specific to the problem investigated. The material presented below is organized based on the overall goal of each study. The work covered in the first three sections is generally more fundamental in nature and aims at improving our understanding of P450 structure, dynamics and interactions with substrate(s) and redox partner(s). The last three sections of the review summarize how bioconjugation was used to enable various applications such as drug metabolism studies, biocatalysis and biosensing.

1) Protein structure and active site exploration

It is not uncommon for a single P450 enzyme to catalyze the formation of different regioisomeric products from the same substrate, suggesting that some substrates can have considerable freedom of motion within the active site. [9,10] In addition, some P450s accept several drastically different substrates, and even multiple substrates at once. This is especially common among drug metabolizing P450s. [11] Diverse techniques have been employed to characterize the active site of P450s and locate the substrate binding site(s). High resolution X-ray crystallography is one of the major tools used to this end however many of the substrate-promiscuous P450s are challenging to crystallize. [12] Moreover, protein crystallography provides static information and may only reveal a minor species instead of the major conformer. Other techniques such as spectroscopic analysis, [13] site-directed mutagenesis, [14–16] molecular modeling, [17] mechanism-based inactivation [18] and photoaffinity labeling [19] can be used as complementary methods to characterize enzyme-substrate complexes. Among these, photoaffinity labeling with substrate variants and mechanism-based inhibition involve chemical modifications, and have the advantage of directly providing information on the enzyme active site.

Covalent modification at the active site is often achieved via substrate analogues that become reactive upon enzyme transformation (mechanism-based inactivation or MBI, also referred to as suicide inhibition) [20] or exposure to light (photoaffinity labeling). In the context of P450s, MBI uses molecules designed to be intrinsically stable yet become reactive (typically electrophilic) upon oxidation by the enzyme. A nearby nucleophilic residue next reacts with the electrophile, leading to covalent attachment of the molecule in the enzyme active site. On the other hand, photoaffinity labeling uses a substrate analogue containing a photolabile group which becomes reactive when exposed to light. The reactive product next reacts with an adjacent amino acid residue. Interestingly, photoaffinity labels do not require a nearby nucleophile since photogenerated radicals or electron deficient species can rapidly react with usually unreactive aliphatic residues. Examples of common photolabile groups include azides, diazo compounds, enones and aryldiazonium salts. The status of both strategies as a tool to study the active site structure of P450s has been thoroughly reviewed in the early 2000's. [19,21] This section will therefore focus on more recent examples.

1.1) Photoaffinity labeling

Dus and coworkers reported the first photolabeling study of a P450 in 1979. They used a photolabile derivative of the $P450_{cam}$ inhibitor *N*-phenylimidazole, 1-(4-azidophenyl)imidazole (API, Figure 1a). [22] The method took advantage of the known coordination of imidazole moieties to the P450 heme iron (also called type II ligands). This ensured that the covalent modification would take place in the active site. In 2006, Trnka *et al*. reported the use of a similar strategy to label $P450_{cam}$ but with [^{3}H]4-benzoyl-*N*-[2-(imidazole-4-yl)ethyl]benzamide ([^{3}H]HBP, Figure 1b). [11] The fact that the imidazole anchor provides some information about the orientation of the ligand allowed them to draw conclusions about the structure of the substrate-binding pocket. The authors took advantage of the fact that $P450_{cam}$ is one of the best-characterized member of the P450 superfamily [23] and of the various $P450_{cam}$ ligand-bound crystal structures published. [24–26] Using [^{3}H]HBP, covalent adducts were identified at two residues, Met103 on the B'-C-loop (Figure 2) and at Met121 on the C-helix. The authors suggest that the adducts were formed from two specific orientations of HBP when ligated to the P450 heme, thus validating the specificity of their method. Of the 10 solvent accessible methionine residues, only Met103 and Met121 were photolabeled.

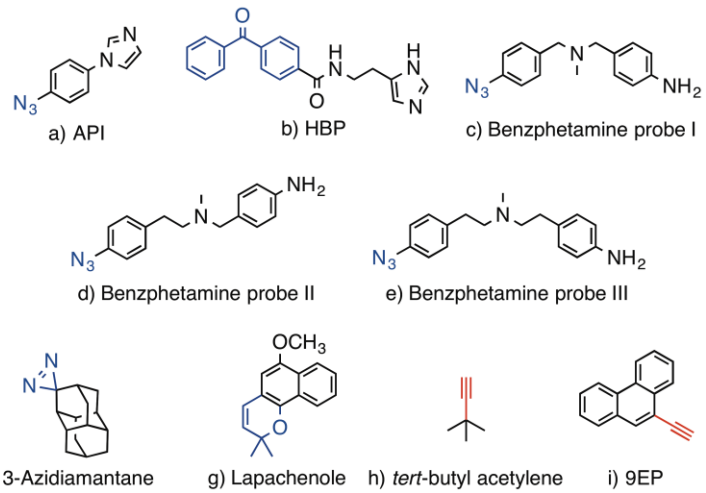

Figure 1. Photoaffinity labelling and MBI probes use with P450. The photoactive and mechanism-based reactive moieties of the molecules are respectively highlighted in blue and red

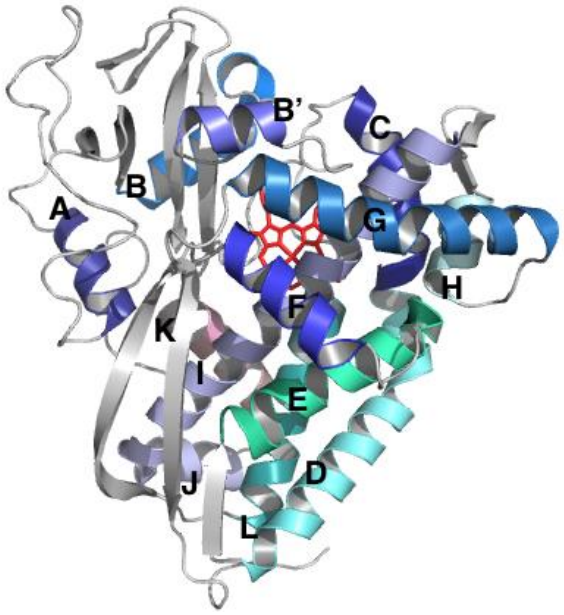

Figure 2. Crystal structure of $P450_{cam}$ with labeled helices (PDB: 2CPP)

Beside early studies on $P450_{cam}$, most photoaffinity labeling was performed on microsomal P450s. [27] Hodek *et al.* developed a homology model of rabbit P450 2B4 using the crystal structures of various soluble bacterial P450s and of a truncated mammalian P450 2C5/3 variant. [28] Despite the fact that a P450 2B4 crystal structure was available at the time, [29] the reliability of this structure was questionable for three reasons: 1) the enzyme *N*-terminus was truncated; 2) the crystal structure did not agree with previous results obtained from photoaffinity labeling; and 3) nor did it support results obtained with mutant enzymes in solution. [29,30] The homology model served as an additional source of information to help with the design of new photoaffinity probes. The authors used three benzphetamine-derived probes of different lengths containing a

photoactive azido group on one end and an amine (acting as a heme anchor) on the other (Figure 1c-e). Specific labeling was observed only for one of the probe and took place at Arg197, which is located ~16.5 Å away from the heme. The binding orientation of the probe matched exactly the prediction from the P450 2B4 homology model. This study also led to the identification of an enzyme substrate access channel.

A few years later, Hodek and coworkers investigated further this substrate access channel with photoaffinity labeling. [31] This time, 3-azidiamantane (Figure 1f) was employed as the probe to examine the substrate-binding site of rabbit microsomal P450 2B4. Following photolabeling, four labeled peptides were identified: Leu359-Lys373, Leu30-Arg48, Phe127-Arg140 and Arg434-Arg443. With this result in hand and the previously developed P450 2B4 homology model, [28] the group proposed a novel binding region outside the active site, with a possible substrate entrance point near Arg113.

While several photoaffinity labeling studies focused on the P450 1A and 2B families, [19] the first such study reported for P450 3A4 was only published in 2005. Nelson and coworkers successfully photolabeled P450 3A4 with the known substrate lapachenole to form a 1:1 adduct (Figure 1g). [12,32,33] Determination of the site of attachment was achieved by trypsin digestion and isolation of the fluorescently labeled peptide fragments before characterization by collision-induced dissociation (CID) mass spectrometry. Two modified residues were identified, Cys98 and Cys468. Using the P450 3A4 crystal structures available at the time [34,35] (none of which including an enzyme in complex with lapachenole) the authors were able to get a more complete view of the binding mode of lapachenole. Cys98 is located within the flexible B-B' loop, in close proximity to the proposed substrate recognition site 1 (SRS-1) of P450 3A4, while the Cys468 residue is located on the outer surface of the enzyme (Figure 3). By comparison with the crystal structure of P450 3A4 bound to progesterone, [35] lapachenole appeared to bind at a different site.

1.2) Mechanism-based inhibition (MBI)

Despite the fact that MBI is usually regarded as unusual, its occurrence arises with higher frequency with P450s than with other enzyme types. This is mainly attributed to the promiscuity (in terms of both substrates and products) of many P450s and to the oxygenated nature of the products. MBI is a valuable tool for mechanistic and structure-activity relationship studies. The use of MBI with P450s has recently been reviewed in detail, mostly from a pharmacology or medicinal chemistry perspective. [36–38] Additionally, more specialized reviews have also been published recently. For example Halpert and coworkers reviewed MBI as a tool for investigation of the active site of P450s of the 2B family, [39] while Liu *et al.* have focused on the known inhibitors, including mechanism-based inactivators, of P450 family 1 enzymes. [40] To our knowledge, the most recent review covering structural investigation via MBI was published in 2001 by Kent *et al.* [21] Since this, only a few new examples have been reported. They are described below.

In 2004, Blobaum *et al*. investigated the MBI of P450 2E1, wild type and T303A mutant,

by *tert*-butyl acetylene (Figure 1h). [41] Thr303 is believed to be involved in substrate orientation and in protonation of the distal oxygen atom of the ferric hydroperoxo intermediate. Inactivation of P450 2E1 by *tert*-butyl acetylene led to the generation of a novel reversible acetylene-iron complex (abs 485 nm).

Zhang *et al.* investigated the MBI of P450 2B4 by 9-ethynylphenanthrene (9EP) and determined the structure of the modified protein by X-ray crystallography (Figure 1i). [42] They found that 9EP was covalently attached to Thr302 via an ester bond. Interestingly, the rate of inactivation of P450 2B4 by 9EP exhibited sigmoidal kinetics. Moreover the fluorescence quenching upon 9EP binding of the unmodified vs. the 9EP-modified enzymes suggested two binding sites with different affinities: one in the active site, having the lower affinity, and one at the enzyme periphery. Using docking studies, the authors determined that the high affinity peripheral site was located at the entrance of the substrate access channel, and suggested its potential function as an allosteric site.

In conclusion, photoaffinity labeling and MBI have had an important impact in fine-tuning our understanding of P450 structures. Even with the increasing accessibility of more recent protein structure determination techniques such as NMR and cryo-EM, bioconjugation may remain a key tool for its complementarity to other methods.

2) Allostery, cooperativity and conformational transitions in P450s

The importance of protein dynamics in enzymatic function has become increasingly recognized. Native protein states can be described as a statistical ensemble of different conformers of similar free energies. [43,44] Our understanding of the P450 mechanism has shifted from a more static to a more dynamic model involving conformational rearrangements in part resulting from ligand binding. Moreover, it has been suggested that a few P450 enzymes such as P450 3A4 [45] may show a new type of cooperativity. Several models and hypotheses have been put forward to explain this phenomenon and one of the prevailing hypotheses is that the cooperativity observed with P450 3A4 may be related to its ability to bind multiple substrates simultaneously. Since P450 3A4 has a large and flexible binding pocket, the orientation of one ligand may be affected by the presence and nature of other ligands. [15,46–52] The crystal structures reported for the erythromycin-bound and ketoconazole-bound P450 3A4 demonstrate this unusual plasticity of the active site. [53] While homotropic cooperativity is observed for P450 3A4 with a great variety of substrates, [8,54–56] several examples of heterotropic cooperativity are also known for P450 3A4, of which a notable one is observed with α-naphtoflavone (ANF). [57–60] The possibility that the cooperative behavior is arising from an allosteric mechanism has also been studied and discussed thoroughly. [61–69] Observation by X-ray crystallography of a progesterone binding site located away from the heme, between the F-F' and G-G' loops [35,70,71] has led to the hypothesis that this site may play a role in P450 3A4 activation by steroids and other effectors. [72] Despite the large amount of data in support of P450 3A4 allosteric modulation, this topic remains controversial as interpretation of the evidences is complex, and further complicated by the prevalence of conformational heterogeneity in samples of drug metabolizing P450s. [58,62,73]

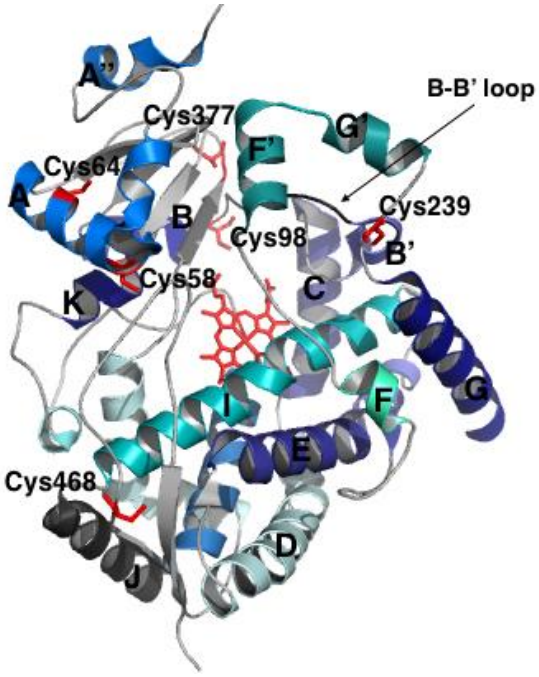

Figure 3. Crystal structure of P450 3A4 with labeled helices and all native cysteine residues except for the proximal iron ligand (PDB: 1W0F)

To distinguish between the different models of cooperative or allosteric binding, it is critical to be able to monitor enzyme-ligand interactions. This section combines some bioconjugation experiments designed for this purpose. Fluorescence resonance energy transfer (FRET), luminescence resonance energy transfer (LRET), and fluorescence-quenching have proven to be instrumental to this area. [74] Most studies focused either on locating the exact binding sites or on monitoring conformational transitions as a result of substrate and/or effector binding. Although some studies have looked at the cooperativity of P450eryF, P450 BM3 and P450 119, the majority of such studies have focused on P450 3A4.

2.1) Investigation of substrate binding sites in P450s other than 3A4

Davydov *et al.* established an approach to directly monitor enzyme-ligand interaction and resolve the first and second binding events of 1-pyrenebutanol to P450eryF, a soluble bacterial P450. [75] Cys154, the only accessible cysteine in this protein, was mutated to alanine or serine before introducing a new cysteine at Ser93 in the active site (B'-C loop). The S93C/C154A, S93C/C154S, and S93C/S154C mutants were characterized for their affinity toward 1-pyrenebutanol and for cooperative binding. Mutant S93C/C154S was selected as it retained most properties of the wild type enzyme. It was modified at the newly introduced Cys93 with various fluorescent probes including 7-ethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM), *N*-[2-(1-maleimidyl)-ethyl]-7-(diethylamino) coumarin-3-carboxamide (MDCC) and monobromo-bimane (mBBr) (Figure 4a-c). Mutant conjugation to either of CPM, MDCC or mBBr had no significant impact on the enzyme interaction with 1-pyrenebutanol and cooperativity. The authors monitored FRET between the substrate 1-pyrenebutanol and the fluorophores, which

allowed them to resolve the two binding events for 1-pyrenebutanol and the associated K_d values. They observed that the first binding event had no effect on the heme iron spin equilibrium but instead led to a decrease in amplitude and broadening of the Soret band of the low-spin state, which together suggest a conformational change. The authors interpreted their results as indicative of allostery, in which binding of an effector to P450eryF leads to a conformational rearrangement.

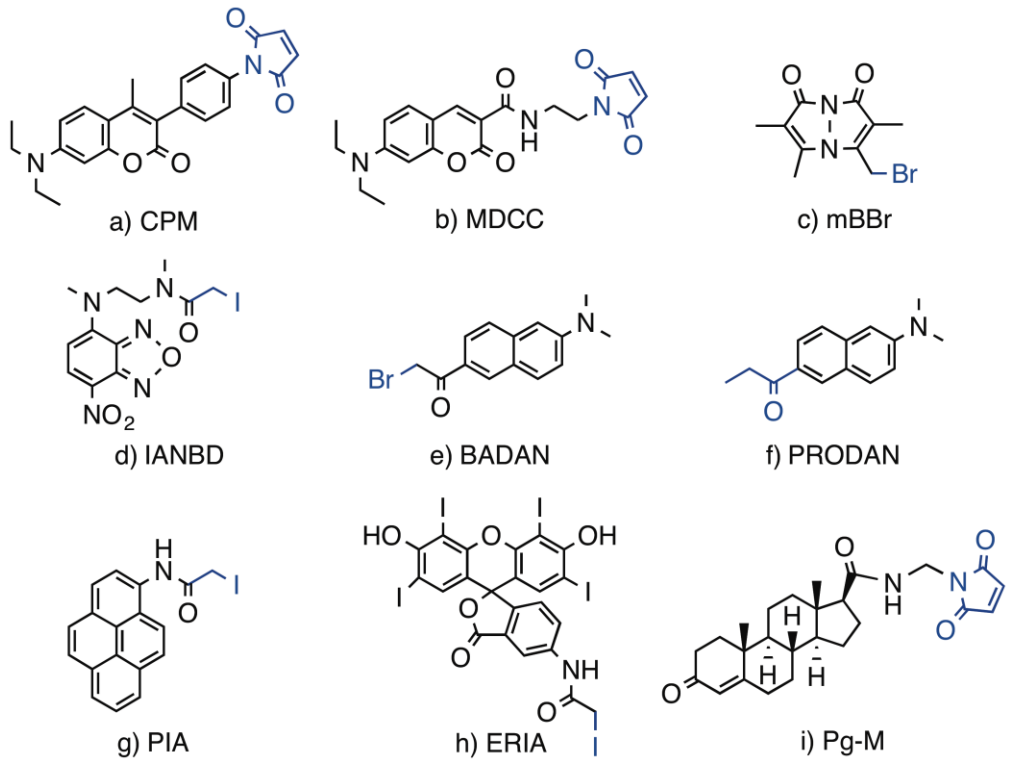

Figure 4. Molecular probes used with P450. The thiol-reactive moieties of the molecules are highlighted in blue

A few years later, Ferrero *et al.* reported a method to investigate the ability of P450 BM3 to bind multiple ligands at once. [76] It had previously been established that not all binding events show a detectable spin shift. [77] The authors used two known single cysteine mutants of P450 BM3 (C62S and C156S) [78] and labeled them with the fluorescent thiol reactive *N,N'*-dimethyl-*N*-(iodoacetyl)-*N*'-(7-nitrobenz-2-oxa-1,3-diazol-4-Yl)-ethylenediamine (IANBD) group (Figure 4d). The method was first validated using lauric and arachidonic acids because these substrates are known to induce a heme iron spin shift upon binding to the enzyme. IANBD fluorescence changes were next used to investigate potential spin-silent binding events that may occur with propranolol, chlorzoaxone or nifedipine. [79] Thus IANBD served as a fluorescent probe capable of sensing nearby protein conformational changes. Significant changes in fluorescence were obtained upon binding of these ligands (19 –130%), thus validating their approach. This method may find use to screen for new substrates, especially when ligand binding causes only small conformational changes.

Hof and coworkers [80] investigated the fluorescence quenching mechanism of the 2-(*N,N*-dimethylamino)-6-propionylnaphthalene dyes BADAN and PRODAN by a tryptophan residue of P450 BM3 and P450 119 (Figure 4e-f). BADAN was covalently attached to a cysteine residue introduced in the P450 active site. They found that in the absence of a tryptophan residue close to the dye, the fluorescence decayed under apparent triexponential kinetics with lifetimes of 100 ps, 700-800 ps and 3 ns. In contrast, when histidine residues close to BADAN were mutated to tryptophan, a shorter decay was observed, leading to the conclusion that fluorescence quenching of BADAN and PRODAN involves tryptophan oxidation by the excited dyes.

2.2) Investigation of cooperativity and allostery in P450 3A4

In 2006, Tsalkova *et al.* reported a study looking at the interaction of ANF with P450 3A4. [81] Wild type P450 3A4 contains seven cysteine residues, not all of which are accessible for chemical modification. The authors generated a partially cysteine-depleted mutant (C98S/C239S/C377S/C468A) to allow the site-directed incorporation of thiol reactive fluorescent probe at two sites (Cys58 and Cys64) on the A-helix. BADAN, CPM and mBBr were each separately linked to the P450 mutant and the change in their respective fluorescence was monitored in the presence of bromocriptine, 1-pyrenebutanol, testosterone or ANF. BADAN is known to be well suited for probing protein conformational changes as it exhibits an ample solvatochromic shift. [82,83] CPM and mBBr were also used to ensure that the florescence intensity decrease observed with BADAN, was not caused by change in FRET efficiency due to covalent binding to the enzyme. The substrates were selected based on their known behavior with P450 3A4: 1-PB and TST exhibit homotropic cooperativity, [15,84] BCT is known to exhibit no cooperativity, [85] and ANF is a heterotropic effector. While the addition of ANF resulted in a significant decrease in BADAN or CPM fluorescence, this was not the case for 1-pyrenebutanol, bromocriptine or testosterone, suggesting that ANF may have an additional binding site.

Previous reports had demonstrated P450 3A4 activation by glutathione (GSH) *in vitro*. [86–88] While looking at the activity of human liver and insect cell microsomes by monitoring the *O*-debenzylation of 7-benzyloxy-4-(trifluoromethyl) coumarin (BFC) or 7-benzyloxyquinoline (BQ), Halpert and coworkers observed that cooperativity was eliminated in the presence of 1-4 mM of GSH. [89] With that in mind, the authors next attached the fluorescent label BADAN at Cys64 of a cysteine-depleted mutant to further investigate the effect of GSH. Fluorescence change upon GSH addition suggested conformational changes in the A-helix region. The authors concluded that GSH might be involved in the allosteric regulation of P450 3A4 *in vivo*.

In a different study, the same group also looked at the mechanism of P450 3A4 ligand binding through FRET using the laser dye fluorol-7GA (F7GA) as a model substrate. [70] The FRET probe was a thiol-reactive pyrene iodoacetamide (PIA, Figure 4g), which allowed monitoring of fluorol-7GA binding. The authors showed that there were at least two binding sites for fluorol-7GA, with significantly different FRET efficiencies. To probe the location of these sites, FRET was next measured in a series of mutants bearing

the same pyrene group but at different positions in the binding pocket. The results suggested the presence of a high affinity-binding site on the enzyme periphery. With docking studies, they estimated its position to be in the vicinity of residues 217-220, which is in the same area as the peripheral progesterone binding site observed in the P450 3A4 crystal structure reported by Williams *et al.* [35] Interestingly, binding of F7GA to this peripheral site was reported to cause a heme spin shift, and this first binding event may be required before a second binding event can occur at the active site.

Additional results from Sineva *et al.* also support allosteric modulation of P450 3A4. [90] To study the effector-induced allosteric transitions of P450 3A4, the authors used LRET between probes covalently attached to various naturally occurring or introduced cysteine residues. A series of mutants, each containing only two reactive cysteines, were generated (C64/C468, C377/C468, and C64/C121). Each mutant was labeled with both erythrosine iodoacetamide (ERIA) as a phosphorescent probe (Figure 4h-i) and the near-infrared DY-731 maleimide fluorophore (DYM). These two probes were used as a donor/acceptor pair to sense conformational motions. Concurrent binding of the enzyme to both ANF and testosterone resulted in an increase (1.3 to 8.5 Å) in the distance between the probes depending on the effector and the point of attachment of the probes. The change observed for substrates that are not known to show cooperativity, like bromocriptine and 1-pyrenebutanol, was negligible. The spatial separation between the β- and α- domains observed upon binding of testosterone and ANF agrees with the suggested mechanism of allostery.

Polic *et al.* published this year a new approach to investigate P450 3A4 allostery. [91] Instead of using fluorescent or luminescent probes to look at conformational changes, they covalently attached a progesterone-maleimide (Pg-M, Figure 4j) regioselectively at the putative allosteric site, although the authors cannot eliminate the possibility that the progesterone group may reorient in the bioconjugate. Starting from a cysteine-depleted mutant, new cysteine residues were individually introduced in the allosteric pocket at positions Phe213, Phe215 and Asp217. Initial rates of 7-benzyloxy-4-(trifluoromethyl) coumarin and testosterone oxidation were monitored for the labeled mutants, revealing an increase when progesterone was attached at the allosteric site. The most pronounced effect was observed with the progesterone-labeled mutant F215C which showed an increase in k_{cat} of 30 fold with the substrate 7-benzyloxy-4-(trifluoromethyl) coumarin and four fold with testosterone. Results with these progesterone bioconjugates also suggest that binding at the allosteric site may have a positive effect on enzyme kinetic stability.

Overall bioconjugation has provided an alternative and elegant approach to investigate P450 ligand binding. These studies have often revealed phenomena not detected by other means.

3) Probing of P450 interactions with other P450s, CPR and membranes

Probing surface topology of proteins has proven its utility not only in investigating protein structure and function but also in understanding the nature of protein-protein

interactions. X-ray crystallography and multidimensional NMR are commonly used to investigate the fine 3D structure of proteins as they provide detailed information but are time consuming and not always successful with protein-protein complexes. Computational approaches offer a good alternative but rely on estimations. Chemical modification is a straightforward way of obtaining experimental data on the formation of complexes between P450s and other proteins or membranes. This approach is based on the assumption that the relative reactivity of amino acid residues on a protein reflects their surface accessibility and thus provides information about the nature of protein complexes.[92]

P450 bioconjugation has demonstrated great utility in mapping interactions between a P450 and its reductase, e.g. CPR in the case of Type II P450s. Electron transfer from the flavin-containing reductase involves protein recognition and formation of a binary complex with the P450. [93,94] The ratio of CPR to P450s in humans was estimated to be in the order of 1:5 respectively. [95] Since electron transfer requires formation of a 1:1 complex between P450 and CPR, how a single CPR can serve so many P450s remains bewildering. [96] The implication of charge-pairing interactions between P450 and CPR in protein complex formation is now widely accepted, [97][98] but still does not fully describe the interaction between CPR and P450s. [99] Chemical modification has also been applied more recently in probing P450-P450 interactions in the context of understanding oligomerization phenomenon. In 2011, Davydov *et al.* reviewed the different experimental methods reported in studies of P450 oligomerization. [100]

Overall, three main bioconjugation strategies have been used to study P450-CPR interactions: 1) altering the electrostatic charge of P450 through acetylation of amino residues; 2) introducing bulky groups, including fluorophores; and 3) cross-linking the two interacting proteins together.

3.1) P450 surface electrostatic charge modification

Before the widespread use of protein X-ray crystallography, acetylation of accessible amino groups was a common practice for topological probing of proteins because of its simplicity. Acetylation is not specific for lysine residues and can take place at other nucleophilic amino acid side chains including those of cysteine, tyrosine, serine, histidine and the *N*-terminus amine. However only amine functional groups form stable bonds upon acetylation [101] while acetylation of cysteine, tyrosine and histidine residues is reversible under aqueous conditions. [102]

Kunz *et al.* [103] looked at the surface charge of P450s using a method established almost 15 years before by H. Fraenkel-Conrat. [101] They used [^{3}H]acetic anhydride to radiolabel the ε-lysyl amino group of microsomal P450s (Figure 5a). The nature of the acetylated residues was confirmed through an established protein dansylation procedure. [104] The protein was stable to the acetylation conditions, but the modified enzyme was inactive towards the substrate ethoxycoumarin. The authors interpreted this result as suggesting the importance of polar residues for binding to CPR.

Strobel and coworkers further supported the predominant role of lysine in P450 2B1-CPR complex formation. [105] They found that acetylating lysine residues with [^{14}C]acetic anhydride inhibited the CPR-supported demethylation of benzphetamine but did not affect the enzyme activity when cumene hydrogen peroxide was utilized instead of CPR/NADPH. The lysine residues involved (positions 251, 384, 422, 433 and 473) were identified through trypsin digestion followed by HPLC separation and amino acid microsequencing. Interestingly, the authors also reported that chemical modification of arginine residues with phenylglyoxal and 2,3-butanedione did not affect the CHP-supported P450 demethylation of benzphetamine (Figure 5b-c). This result implied that arginine residues may not interact with CPR for this P450.

Technical developments and improvement in mass spectrometry (MS) have since facilitated studies of surface interactions. The most common methods used for this purpose have been reviewed. [106] In the P450 field, Nikfarjam *et al.* used MALDI-TOF MS to analyze acetylated P450 17α from guinea pig. [107] The acetylation was performed in the absence and in the presence of CPR. From tryptic fragments analysis, eight acetylated peptides were identified. It appeared that the presence of CPR prevented the acetylation of Lys326 and Lys327, suggesting their implication in this P450-CPR complex formation. Furthermore, the [^{3}H]-progesterone oxidation activity of the enzyme acetylated in the absence of CPR was decreased by 65% compared to the non-acetylated enzyme, while almost no inactivation was observed for the P450 17α acetylated in the presence of CPR.

Orientation of mammalian P450s relative to the membrane surface has been studied by different approaches including chemical modification and was reviewed in 1992. [108] Nowadays, other methods are preferred to study membrane topology but an example using bioconjugation was published not so long ago by Izumi *et al.* [109] They used protein surface acetylation to study membrane topology of P450 17α. The recombinant enzyme was first incorporated into liposomes. The remaining accessible lysine residues on the P450 surface were acetylated with acetic anhydride and the acidic residues were conjugated with glycinamide (Figure 5d) using the coupling agent 1-ethyl-3[3-dimethylaminopropyl]carboiimide hydrochloride (EDC, Figure 5g). The same chemical modifications were also performed on detergent solubilized P450 17α. The proteins were then digested and the peptides analysed with MALDI-TOF MS. By comparing the extent of modifications on both reconstituted proteins, the authors concluded that the F-G loop was near or in the membrane-binding domain of P450 17α.

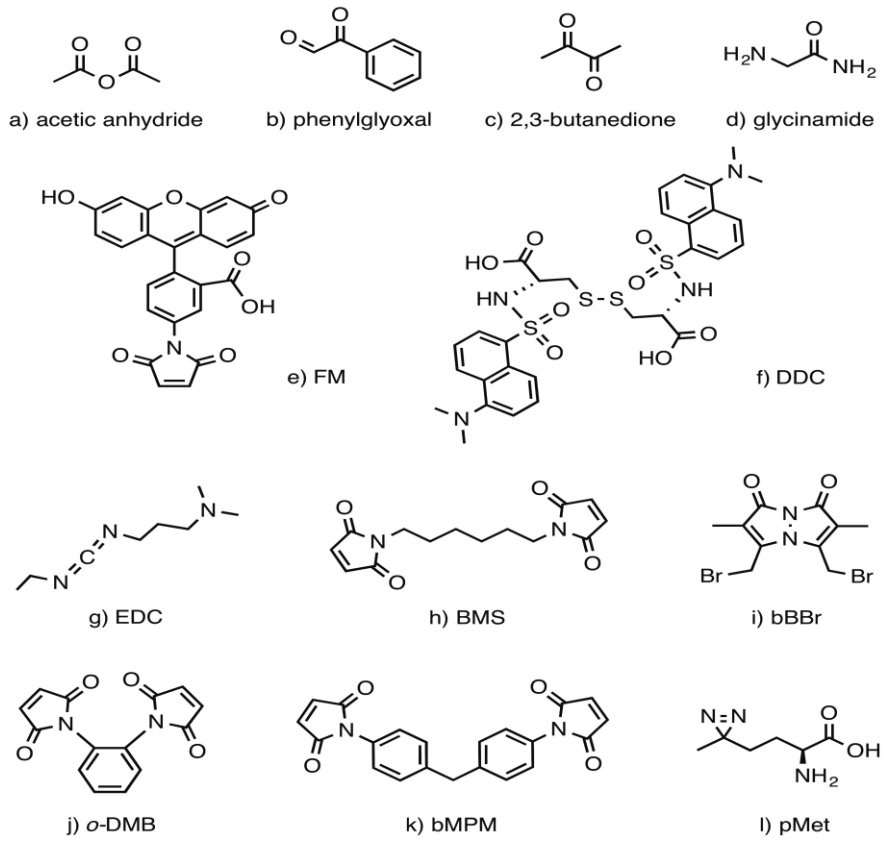

Figure 5. Probes and reagents used to study P450 interaction with P450, CPR and membrane.

3.2) P450 bioconjugation with bulky or fluorescent labels

In 1996, Davydov *et al.* reported a new method for monitoring the formation of P450-CPR complexes based on the expected fluorescence quenching of a fluorescent label on CPR by the heme prosthetic group of the P450. [110] In their study, the fluorescent probe CPM (Figure 4a) was covalently attached to CPR. Interaction of the modified CPR with P450 2B4 was next investigated before and after succinylation. The results suggested two distinct CPR contact regions on P450 2B4, only one of which is polar.

Kenaan *et al.* al utilized a similar strategy combined with stopped-flow analysis to study the role of hydrophobic residues in the P450 2B4-CPR interaction. [111] Single cysteine residues were genetically introduced at key positions on the surface of P450 2B4 (V267C, L270C, L420C, R133C, Y484C, H226C, and E60C) and each mutant was individually modified with fluorescein-5-maleimide (FM, Figure 5e). The K_d of the complex formation with CPR was calculated from fluorescence enhancement. The results showed that the modified sites on P450 2B4 exhibiting the greatest changes in fluorescence intensity upon CPR association are R133C, V267C, L270C and L420C.

In earlier work, Poulos and coworkers studied the interaction between the heme and FMN domains of P450 BM3. [112] From the crystal structure of P450 BM3, [113] they

identified positions 104 and 387 as being sites of close contact between the two domains. They introduced cysteine residues separately at each of these positions before reacting the cysteines with a bulky reagent, 1-dimethylamino-naphthalene-5-sulfonate-L-cystine (DDC, Figure 5f). This was expected to prevent interactions between the two domains. The rate of interdomain electron transfer was decreased by 10-20 fold with addition of the bulky group at Cys104, confirming that the area around residue 104 is critical for redox partner docking.

Davydov *et al.* were able to successfully generate a P450 BM3 variant bearing an aluminum-protoporphyrin IX (Al-BM3) group instead of a heme to study P450-substrate and P450-P450 interactions. This variant showed similar affinity for the substrates palmitic, arachidonic and cis-parinaric acids as the native enzyme. [114] The Al-BM3 enzyme fluoresces in the 550-650 nm region. The maximum intensity of this fluorescence was found to fluctuate upon substrate titration, suggesting the existence of a second binding site, which can't be detected by normal spin shift measurements of the native enzyme. Furthermore, after labeling of the flavoprotein domain of Al-BM3 with CPM, [110,115] the authors observed FRET, which suggests that the porphyrin domain of P450 BM3 may form a complex with its flavoprotein domain. The authors were able to derive a K_d of 5 μM at 0.06 M ionic strength.

One year later, Davydov *et al.* studied P450 3A4 oligomerization in the presence of ANF in Supersomes. Two cysteine depleted mutants (with only C58/C64 or C468 accessible) [116] were used to probe P450 oligomerization via LRET and the kinetic parameters were investigated by 7-benzyloxy-4-(trifluoromethyl) coumarin *O*-dealkylation. Mutant C56/C64 was labeled with the fluorescence donor ERIA (Figure 4i). The authors state that Cys58 has a poor accessibility for large probes which should favor site-specific labeling of ERIA at Cys64. Mutant C468 was labeled with the fluorescence acceptor DYM (Figure 4h). The two labeled mutants were incorporated into the Supersomes and oligomerization was monitored by fluorescence change in the presence and absence of ANF. According to their design, the formation of mixed oligomers of the labeled mutants would result in resonance energy transfer proportional to the degree of oligomerization. The amplitude of LRET measured in mixed oligomers exhibited a sigmoidal dependence on the surface density of the P450 3A4 in the Supersomes. Interestingly, the addition of ANF replaced this sigmoidal behavior for a linear one. These results suggest that modulation of P450 3A4 by ANF may be affected by P450-P450 interactions.

3.3) Cross-linking of P450s- to their redox partner(s)

Cross-linking of a P450 to its redox partner has also been used to study the interaction between the two enzymes. Typical cross-linking agents favor the formation of amide bonds between complementary charged residues. This strategy was already an important method in the 70's. For example, a method reported by Sheehan *et al.*, [117] which uses EDC (Figure 5g), has proven useful in the cross-linking of P450scc (P450 11A1) to its reductase. [118]

Müller *et al.* wanted to discriminate between different electron transfer models involved

during steroid hydroxylation in the adrenal cortex. This system generally consists of a membrane-bound adrenodoxin reductase (AR), a soluble electron transport protein, adrenodoxin, and a membrane-bound P450 (e.g. P450scc). [119] The group reported the zero-length cross-linking of bovine adrenodoxin and AR, as well as adrenodoxin and P450scc, using EDC as the coupling agent. [120] Edman protein degradation, mass spectrometry and X-ray crystallography were used to identify the residues involved in the cross-linking. In the adrenodoxin-AR complex, cross-linking occurred between Asp39 and Lys27 respectively, while adrenodoxin Asp79 was linked to Lys40 in the adrenodoxin-P450scc complex. The adrenodoxin-P450scc bioconjugate was inactive in steroidogenic hydroxylase activity, revealing that the cross-linking is not favorable for activity.

In 2006, Gao *et al.* reported cross-linking P450 2E1 to cytochrome b_5 also using EDC. Tandem mass spectrometry coupled to ^{18}O-labeling was employed for detection of the cross-linked peptides after protein digestion. It was determined that Lys428 and Lys434 of P450 2E1 were covalently linked to Asp53 and Glu56 of cytochrome b_5 respectively. These findings provided the first experimental information about the orientation of the complex between a microsomal P450 and its redox partner. A few years later, the same methodology was used to investigate the interaction between P450 2B6 and CPR. The residues involved in protein-protein interaction were located in the C-helix of P450 2B4, and in the connecting domain between the FAD and FMN domains of CPR. [121]

In an effort to improve electron transfer to $P450_{cam}$, Nagamune and coworkers have combined genetic fusion and bioconjugation to regioselectively link $P450_{cam}$ to both putidaredoxin (Pdx) and Pdx reductase (PdxR). [122] First, $P450_{cam}$ was genetically fused to PdxR with a linker containing a trans-glutaminase-reactive glutamine residue (Qlinker). A CKtag, which is recognized by the trans-glutaminase and reacted at its lysine residue, was genetically engineered at one end of Pdx. Finally trans-glutaminase was used to catalyze the bioconjugation between the two constructs. The resulting branched structure showed higher catalytic activity and coupling efficiency than the equimolar reconstitution system. The authors propose that bioconjugation may increase local concentration of proteins in a way that facilitates electron transfer.

On the other hand, Poulos and coworkers used cross-linking to investigate the preferred $P450_{cam}$ conformer involved in its interaction with Pdx. [123] From a $P450_{cam}$-Pdx molecular model, the authors identified residues that might be involved in the complex formation of the two proteins. [124] Lys344 in $P450_{cam}$ was suspected of interacting with Cys73 in Pdx and thus was mutated to another cysteine in order to use the homobifunctional maleimide reagent 1,6-bismaleimidohexane (BMS, Figure 5h). A 1:1 covalent complex was successfully generated using this reagent and the bioconjugate was crystallized (2.15 Å) in the presence of excess camphor substrate. The 3D structure demonstrated that Cys73 of Pdx was covalently attached to His355 (and not the newly introduced C344) of $P450_{cam}$. The crystal structure also revealed that $P450_{cam}$ adopted an open, substrate-free conformation.

Protein cross-linking was also used to study the functional consequences of

oligomerization of P450 3A4. [125] Davydov *et al.* generated three P450 3A4 cysteine-depleted mutants with accessible cysteines at positions C58/C64, C468 or C166. Following incorporation into microsomes, the mutants were cross-linked together using the thiol-reactive bifunctional reagents dibromobimane (bBBr), 1,2-dimaleimidobenzene (*o*-DMB) and bis(4-maleimidophenyl) methane (bMPM, Figure 5i-k). The authors observed a clear effect of protein oligomerization on catalysis and ligand binding.

Recently, Ječmen *et al.* published an article reporting the use of photo-initiated crosslinking to investigate the interaction between P450 2B4 and cytochrome b_5. [126] Unlike cross-linking with EDC, photo-initiated cross-linking does not require small molecule reagents which may perturb protein-protein complex formation. The authors first genetically engineered a cytochrome b_5 mutant to harbor a diazirine-containing methionine analog (pMet, Figure 5l). After UV irradiation, the activated pMet reacted with nearby residues of P450 2B4. Mass spectrometric analysis of the bioconjugates revealed the existence of at least two P450-cytrochrome b_5 complexes with different orientations.

In summary, bioconjugation is a versatile and rapid tool to map interaction surfaces.

4) P450 biocatalysis

Enzymes are increasingly used in the chemical industry. [127] P450s are among nature's most versatile biocatalysts. They are well known for their ability to address a major challenge in organic synthesis, the regio- and stereoselective hydroxylation of unactivated C-H bonds, which makes them appealing for various applications. [128–131] The range of chemical transformations catalyzed by P450s is substantial and has even been extended further through enzyme engineering. [132] In this respect, several reviews cover the recent progress made in P450 engineering, whether it is to increase substrate scope, modify cofactor preference, or increase enzyme stability. [133–135] Perspectives on synthetic applications of P450s and more general reviews have also been published. [129,136,137] Despite their potential, there are a number of recurring challenges limiting large-scale implementation of P450 processes. These include the dependence on both a cofactor and one or more redox partner(s) for most P450s, their low stability, and poor activity towards industrially relevant molecules. [138] Martinez *et al.* have reviewed P450 bioreactor design and challenges associated to large scale use of P450s. [139] Industrial applications of P450s have been for the most part restricted to whole-cell systems which can solve the cofactor regeneration problem. [138] In a recent review, Lundemor *et al.* have summarized whole-cell industrial P450-utilizing processes, [140] while Munro and Girvan have reviewed the applications of microbial P450s in biotechnology and synthetic biology. [132]

Bioconjugation has contributed to this field mainly as a tool to anchor enzymes on solid supports. Immobilization of enzymes has proven to be useful in biotransformations as it can facilitate product separation and improve operational and storage stability of enzymes. [141–145] Immobilization can be covalent or non-covalent. Examples of the former with P450s are few; they are summarized below.

4.1) Non-selective immobilization

The first covalent immobilization of a P450 was reported in 1975 and served to study drug metabolism. [146] The method was originally developed by Wilchek [147] and consisted of covalently attaching the enzymes contained in a microsomal extract (i.e. P450 and CPR) to a cyanogen-bromide-activated Sepharose 4B resin. Later on, Eremin *et al.* [148] reported the immobilization of a purified P450 from rabbit liver onto albumin-modified Sepharose and Biogel P-300 and noted a substantial increase in stability.

Axarli *et al.* engineered a series of P450 BM3 mutants with altered substrate specificity and reported efficient immobilization of their best P450 BM3 variant on epoxy-activated sepharose CL6B. [141] To this end, sepharose CL6B was activated with bis-oxirane 1,4-butanediol diglycidyl ether (Figure 6a). The amino groups on the surface of the enzyme reacted covalently with the epoxide functional group on the resin. The authors tested the enzyme activity with the substrate sodium dodecyl sulfate (SDS) and reported that the immobilized P450 BM3 exhibited 81% of the activity observed for the enzyme in solution. Stability of the immobilized BM3 variant was investigated over a period of 30 days. The rate of inactivation for the immobilized and the soluble enzyme were 2.2×10^{-3} and 12.2×10^{-3} respectively, showing that immobilization significantly stabilizes this P450 BM3 variant.

Focusing on bioreactor design, Wollenberg *et al.* developed a method for the immobilization of P450 2C9 on poly(methyl methacrylate) (PMMA) in plug flow bioreactors. The goal was to produce sufficient amounts of drug metabolites for toxicity assays. [149] PMMA chips and bioreactors surface were activated with EDC and *N*-hydroxysulfosuccinimide (S-NHS) (Figure 6b). P450 2C9 was attached to the surface through reaction at the *N*-terminus of the enzyme. The coupling reaction was performed in the presence of a substrate in order to preserve enzyme activity. [150] These bioreactor channels were tested for metabolite production by running a solution of the substrate diclofenac, CPR, and NADPH through them. It appeared that the production of 4'-hydroxydiclofenac was larger for the *N*-terminus-modified enzyme than for the free enzyme, showing that immobilization enhanced P450 activity. The authors also showed that immobilization improved enzyme stability.

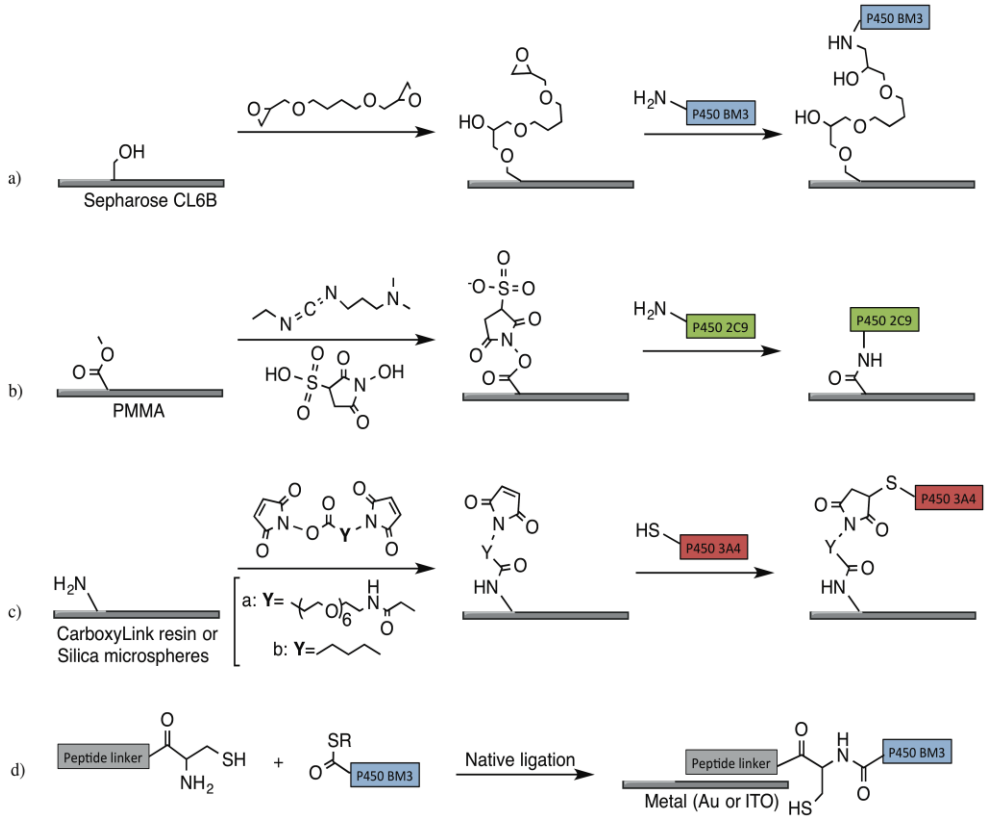

Figure 6. P450 immobilization strategies for biocatalysis purposes

4.2) Regioselective immobilization

In all of the above cases, the P450 was immobilized in a non-specific manner. In 2012, Auclair and coworkers reported the oriented covalent immobilization of P450 3A4 through cysteine/maleimide chemistry. [151] The method was also shown to be effective to regioselectively introduce fluorophores. The authors generated a series of cysteine-depleted mutants (C98S, C98S/C377S/C468G, C98S/C239S/C468G and C64S/C98S), each with a few different cysteine residues remaining. Mutant C98S/C239S/C468G was found to react solely at C64. This mutant was successfully immobilized onto a maleimide-functionalized CarboxyLink resin and onto silica microspheres (Figure 6c, Y = linker a). The activity of the CarboxyLink-immobilized enzyme towards the substrate 7-benzyloxy-4-(trifluoromethyl) coumarin was retained upon immobilization when compared to that of the free enzyme. Furthermore, enzyme thermodynamic stability was also retained over a 16 h period. As for the microspheres, they were first aminosilanized and treated with a cross-linker (Figure 6c, Y = linker b). The result was a PEG-maleimide surface analogous to those used to immobilize biomolecules on glass slides. [152] P450 3A4 was reacted with the modified microspheres using the same conditions as for immobilization onto the CarboxyLink resin. The activity of P450 3A4 on microspheres was assayed with two substrates, testosterone and 7-benzyloxy-4-(trifluoromethyl) coumarin, and the immobilized P450 3A4 retained 60% and 40% of its activity respectively compared to the unmodified mutant. Immobilization on silica microspheres

can find use in enzyme microarray production for example.

Zernia *et al.* have recently developed an immobilization method for linking a P450 BM3 variant to a metal surface. [153] Their approach consists of using a peptide linker between the enzyme and the metallic support, thus providing sufficient distance between the two and avoiding denaturation by contact with the metal (Figure 6d). Two different peptides were designed to contain a C-terminal group with affinity either for gold (Au-binding peptide) or indium tin oxide surfaces (InSn-binding peptide) and a cysteine at the *N*-terminus to enable native chemical ligation. P450 BM3 was expressed with a chitin-binding tag (IMPACT system) allowing one-step purification and resulting in a thioester-functionalized protein. Native chemical ligation was used to introduce P450 BM3 at the *N*-terminus of each peptide. The ligation efficiencies were 40% and 70% for the Au-binding and the InSn-binding peptides respectively. The peptide-modified P450 BM3 bioconjugates were next immobilized onto gold and indium tin oxide surfaces. Using the BROD enzyme activity assay, the authors found that the k_{cat} of the enzyme increased after immobilization for both metal surfaces.

In summary, while covalent immobilization can help stabilize P450 enzymes, it can also be detrimental. No specific strategy is guaranteed to work; various solid supports, sites of attachment, linkers and chemical reactions must be tested.

5) P450 electron transfer

As mentioned above, P450s require a source of electrons to function catalytically, and the electrons are often transferred from NAD(P)H to the P450 enzyme via a reductase. The electroactive component of P450s is an iron-protoporphyrin IX, which harbors iron(III) in the resting state. Transfer of the first electron yields a heme iron(II) at the active site which can bind a molecule of oxygen. Following a second electron transfer and oxygen protonation, the O-O bond is cleaved and the resulting ferryl species (compound I), a strong oxidant, is generated in the active site as demonstrated by the seminal work of Green and coworkers. [154–156]Despite the large functional and structural diversity of P450s, studies with different P450s suggest a similar catalytic cycle. Numerous methods have been used to study electron transfers to P450s although only studies that use bioconjugation are summarized here.

In 2005, Bistolas *et al.* published a review summarizing the P450s and electrodes used in biosensors, with an emphasis on the theoretical background. [157] More recently, Schneider reviewed the same topic but focused on different immobilization strategies and covered in detail the published examples of P450 immobilization onto gold electrodes. [158] Two general strategies have been used to achieve electron transfer to the heme redox center of P450s from alternative electron sources: 1) the use of a mediator to shuttle electrons from an electrode, or from light, to the protein, and 2) direct covalent attachment of the protein to the electrode with or without a self-assembled monolayer in between. [159,160]

5.1) Use of mediators for electron transfer to P450s

The use of mediators is interesting since it can affect the redox properties of the enzyme. The Hill group had developed a method to chemically modify several proteins and peptides (e.g. β-lactamase I and glutathione) with *N*-(2-ferrocene-ethyl)maleimide (Fc-Mi, Figure 7a). [161][162] Using this method, $P450_{cam}$ was modified by covalent attachment of Fc-Mi to various cysteine residues on its surface. [163] The authors started from a $P450_{cam}$ mutant (C334A) bearing one less surface-exposed cysteine. After bioconjugation with Fc-Mi, two ferrocene moieties were found covalently linked to the enzyme, one at Cys85, which is in the camphor-binding site, and the other at Cys136, located on the protein surface. The crystal structure of the modified enzyme was determined at 2.2 Å resolution and was compared to the wild-type enzyme. [26,164–167] Significant conformational differences were observed for the labeled versus unlabeled $P450_{cam}$. Direct-current cyclic voltammetry experiments were carried out on the bioconjugate and showed a clear signal, originating both from the heme and the bound ferrocene molecules. The authors measured a shift in the redox potential of the heme going from -380 mV for the camphor-bound unmodified $P450_{cam}$ to -280 mV for the Fc-Mi labeled protein.

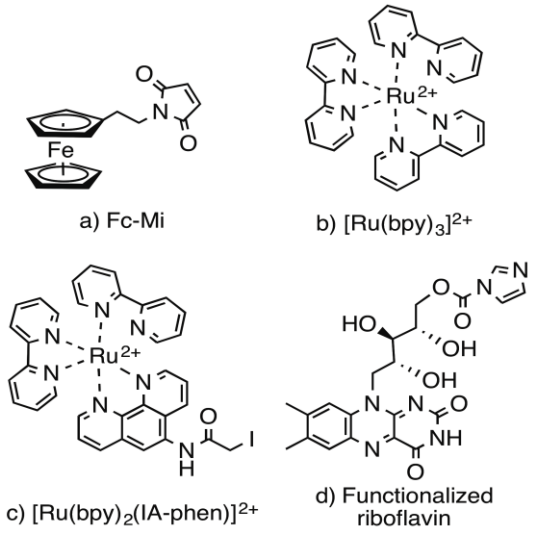

Figure 7. Electron transfer mediators bioconjugated to P450

The Hill group later reported a similar experiment but with improved control over regioselectivity. [168] This was achieved starting from a $P450_{cam}$ mutant with all five surface cysteines mutated to alanine, and with a new cysteine residue introduced separately at different positions (R72C, R112C, K344C, or R364C). The same redox-active Fc-Mi was covalently attached to the best-expressing mutant, K344C. The conjugated $P450_{cam}$ showed comparable electrode-mediated camphor hydroxylation activity (in the absence of Pdx/PdxR/NADH) as the wild type enzyme in the presence of Pdx/PdxR/NADH, suggesting that the ferrocene moiety was not detrimental to enzyme activity. Upon conjugation, the redox potentials of the heme and of the ferrocene were increased by 33 mV and 27 mV respectively. The authors did not investigate the reason for these changes of potential and cannot rule out the presence of an interaction between the two iron centers.

The group of Cheruzel recently reviewed and highlighted the key achievements in light-driven biocatalysis of Ru(II)-diimine-functionalized metalloproteins. [169] This work took root in the theoretical framework for electron transfer between distant redox centers initially reported by Marcus in 1986. [170] The unique photochemical properties of $Ru(bpy)_3^{2+}$ (where bpy is 2,2'-bipyridine) analogs are well established [171] and make these complexes of particular interest for tracking electron transfers (Figure 7b). [172] Building on the theory elaborated by Marcus, the Gray group pioneered the use of ruthenium complexes in the investigation of electron transfer with modified proteins. [173–175]

Inspired by the work of Sevrioukova [176] and Katz [177], Gray and coworkers reported a method to anchor P450 BM3 on basal plane graphite (BPG) electrode. [178] Sevrioukova *et al.* reported that position 387 (mutated to N387C) of the enzyme was an ideal site for the covalent attachment of a ruthenium diimine moiety for mimicking the interaction of the reductase. On the other hand, Katz *et al.* reported the successful oriented immobilization of *N*-1-(pyrene)iodoacetamide (Py) on a carbon electrode also for the purpose of protein (non-P450) immobilization. Finally, combining all this information, Gray and coworkers designed a P450 BM3 mutant containing a single accessible cysteine at position 387, onto which the Py moiety was attached before electronically connecting this bioconjugate to the BPG electrode (Figure 8). Rapid electron transfer between the electrode and the P450 BM3 heme domain was successfully achieved and the protein integrity was confirmed by spectrophotometric measurements.

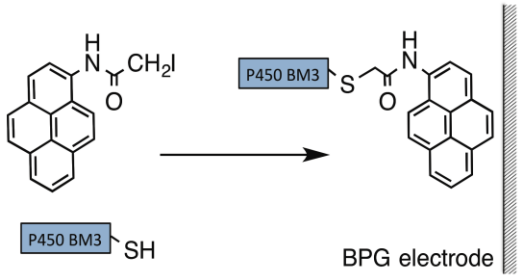

Figure 8. P450 BM3 immobilization on BPG electrode strategy by Gray and coworkers [178]

Efforts have also been focused on finding alternative routes to the P450 compound I and II reaction intermediates. The Cheruzel group employed free $[Ru(bpy)_3]^{2+}$ to generate compound I and II in a bimolecular flash-quench photochemical oxidation of microperoxidase-8 and horseradish peroxidase. [179,180] The same method applied to P450 was however unsuccessful, which was attributed to its deeply buried heme. To overcome this problem, Ener *et al.* covalently attached a ruthenium-diimine photosensitizer to a non-native cysteine residue introduced on the surface of the P450 BM3 heme domain. [181] The authors had to first remove two native surface cysteines (C62A, C156S) and add a cysteine at position Lys97. The triple mutant was attached to the complex $[Ru(bpy)_2(IA\text{-}phen)]^{2+}$ where IA-phen is 5-iodoacetamido-1,10-phenanthroline (Figure 7c), and characterized by mass spectrometry, UV and luminescence spectroscopies, and X-ray diffraction. The authors reported that the bioconjugate undergoes rapid photooxidation of the resting ferric heme state to

compound II in flash-quench experiments without the need for reactive oxygen species (O_2 or H_2O_2) or NADPH/CPR.

The same group subsequently tested different positions for the attachment of the ruthenium photosensitizer. Thus a series of bioconjugated mutants were generated (photosensitizer-label at K97C, Q397C, Q109C and L407C) and tested for the hydroxylation of lauric acid. [182] When labeled with $[Ru(bpy)_2(IA\text{-}phen)]^2$, mutant L407C showed the most interesting behavior. This P450 BM3 heme domain mutant had a turnover number of 140 after 150 min of reaction with lauric acid, in the presence of light and absence of cofactor. They next reported an optimized bioconjugate with the new ruthenium ligand, 4,4'-dimethoxy-2,2'-bipyridine ($(OMe)_2$bpy), which showed not only improved stability but also a larger total turnover numbers of 935. [183]

The photochemical reduction of flavins by simple electron donors such as amines (e.g. EDTA) has been described previously [184] and can be used to design photoreceptors or mediators of light-induced electron transfer. [185] The group of Shumyantseva exploited this reaction in several P450 studies. [186–189] They demonstrated that free flavin in solution can be reduced photochemically and next used to transfer electrons to a modified P450 2B4 bearing a covalently attached riboflavin group. The riboflavin moiety was introduced using an analog functionalized with carbonyldiimidazole (Figure 7d) which reacted with the P450 ε-amino groups. They investigated this P450 2B4 conjugate with free electron mediators such as FMN, FAD and riboflavin. With the use of NADPH, these semisynthetic flavocytochromes were shown to possess both reductase and oxygenase activities. The authors next attempted to immobilize riboflavin-modified P450s (P450 102, P450 2B4, and P450scc) onto rhodium-graphite screen-printed thick film electrodes functionalized with glutaraldehyde in the design of small biosensors. They reported one enzyme-electrode system that was able to catalyze *N*-demethylation of aminopyrine, *p*-hydroxylation of aniline, *O*-dealkylation of 7-alkoxyresorufin and side chain cleavage of cholesterol. They also showed that the reaction rates for the electrode-immobilized P450s were similar to those obtained for the free enzymes in the presence of the natural redox partner and NAD(P)H. In 2005 Shumyantseva *et al.* published a comprehensive review on the electrochemical reduction of P450s, including their own work on riboflavin as electron mediator and the bioconjugation methods. [190]

5.2) P450 immobilization on electrode

In attempts to obviate the need for redox partner(s) and NAD(P)H, P450s have been directly immobilized onto electrodes. [191–194] This has proven to be an important strategy to develop self-sufficient P450 platforms. Over the years, much attention has been dedicated to P450 bioelectrode development for different purposes such as analytical, [195] bioremediation [196] and production of important drugs and metabolites. [197,198] The use of P450 immobilization, specifically in the design of biosensors, is reviewed in section 6 below.

Proteins can be attached to electrodes through electrostatic, hydrophobic or covalent interactions depending on the surface attributes, but only the last one is discussed here.

[199] Enzyme stability and selectivity are strongly affected by the immobilization strategy. [200] Direct enzyme attachment on the electrode can sometimes prohibit substrate binding or product release due to steric constraints. [201,202] Moreover, enzyme concentration and orientation on the electrode surface, [203,204] choice of electrode material, presence of anchoring spacers and the distance between the redox center and the electrode surface, all contribute significantly to the enzyme activity. [170,203,205]

The covalent attachment of P450s to gold surfaces has the advantage of leading to stable and well-oriented immobilized proteins. Thiol groups have a high affinity for gold and one strategy involves the formation of a covalent bond between cysteine thiol groups of the protein surface and gold atoms. [206,207] This has been applied directly to wild type $P450_{cam}$. [208] However, the direct linkage of proteins to a gold electrode often results in protein denaturation. [209] Flexible spacer molecules, which self-assemble into monolayers on gold surfaces, have proven to be quite useful in solving protein stability issues. [210–213]

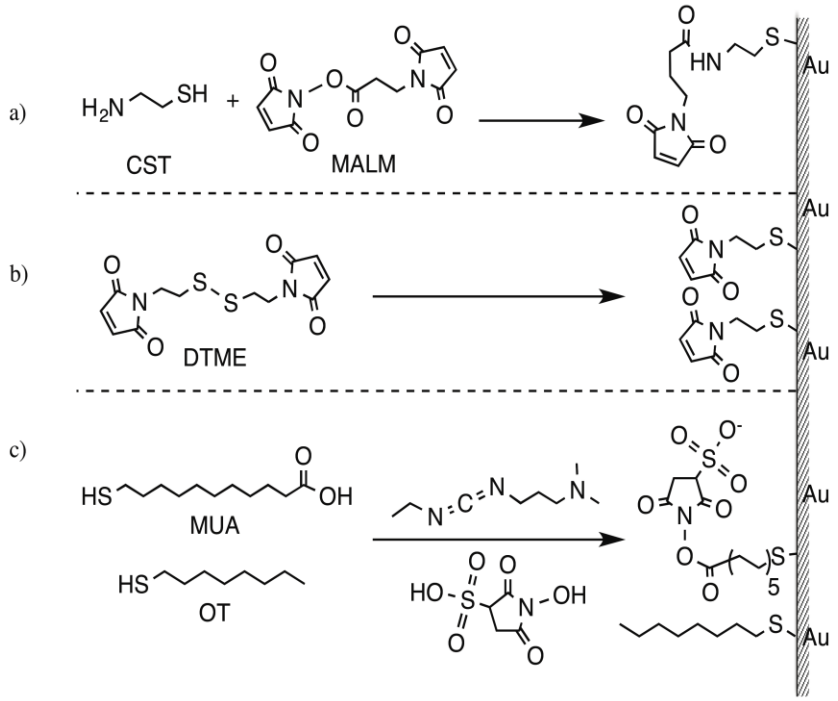

Figure 9. Gold electrode surface activation strategies previous to P450 immobilization

Ferrero *et al.* reported a method for immobilizing P450 BM3 on a gold electrode functionalized with one of two different spacers: cystamine-*N*-succinimidyl 3-maleimidopropionate (CST-MALM) or dithio-bismaleimidoethane (DTME), resulting in the formation of maleimide-terminated monolayers (Figure 9a-b). [78] The exposed maleimide groups displayed on the electrode served to anchor P450 BM3 via a naturally occurring surface cysteine, Cys62 or Cys156. For regioselective control two mutants were generated (C62S and C156S) before covalent attachment to the functionalized gold electrode. A measurable current was only obtained for the immobilized C156S mutant,

suggesting that Cys62 is a privileged point of attachment for efficient electron transfer. With tapping mode atomic force microscopy, the authors also noticed that use of the CST-MALM spacer resulted in a more homogenous enzyme monolayer on the electrode as opposed to the DTME spacer.

Mak *et al.* demonstrated that direct immobilization of human P450 2E1 on gold via surface cysteines led to enzyme inactivation but was successful when using a cystamine-maleimide spacer. [210] In 2010 the same group reported an improved oriented immobilization of P450 2E1 on gold electrode. [214] Following depletion of all accessible cysteine residues but one (either Cys261 or Cys268), the enzyme was covalently attached to a gold electrode modified with DTME. The activity of the immobilized enzyme was determined using *p*-nitrophenol as a substrate with the electrode serving as an electron source. It was found that the immobilized P450 2E1 formed 2 to 3-fold more product than the free mutant enzyme in solution in the presence of CPR and NADPH.

Gannett *et al.* have also linked the *N*-terminus of P450 2C9 to a gold surface. To ensure proper spacing of the enzyme molecules, the gold surface was first treated with a 1:3 mixture of octanethiol (OT) and 11-mercaptoundecanoic acid (MUA) (Figure 9c). The carboxylic acid groups were then activated with EDC/NHS before reaction with P450 2C9. [150] The system was catalytically active in the presence of NADPH and CPR. A few years later the authors reported that this P450 2C9-electrode conjugate could catalyze the transformation of warfarin to 7-hydroxywarfarin in the absence of NADPH or CPR. [215] Other studies with electroactive immobilized P450 have been reported but with much lower activity compared to the free enzyme in solution. [201,216–220]

Structural changes induced by immobilization remain mostly unknown. Using surface enhanced resonance Raman spectroscopy (SERR), Todorovic *et al.* looked at the effect of immobilization on the structure of $P450_{cam}$. In particular, they looked at the effect of immobilization on the conformational transitions and redox potential shift. [221] The enzyme was immobilized on SAM-coated silver electrodes prepared from mercaptoacetic acid of different chain lengths (C_2-C_{16}). [222] EDC was used for covalent cross-linking between the P450 surface lysine residues and the carboxylate groups displayed by the SAM on the electrode. The electroactivity of the immobilized enzyme was confirmed by an increase in SERR of the heme marker bands, which underwent frequency upshifts upon electrode potential increase. The authors were able to track the heme oxidation state and the ligation pattern, with and without the substrate camphor, and found that all the immobilization conditions tested led to the formation of some denatured enzymes. They also pointed out that immobilization affected the level of hydration of the enzyme as well as the strength of the coordinative bond to the axial ligand. Both the tightly bound water molecules and the ligand field are known to affect the enzyme redox potential. Moreover, the electric field from the electrode was suggested to potentially induce destabilization of the heme ferrous state. This study also nicely highlighted the potential of SERR to probe the integrity of the P450 active site.

Electrochemistry is arguably the area to which P450 bioconjugation has contributed the most. This is further elaborated on in the section below.

6) P450 Biosensors

Human P450s play a central role in Phase I xenobiotic metabolism. Five P450s (1A2, 2C9, 2C19, 2D6 and 3A4) are responsible for the metabolism of ~95% of all drugs in the clinic. [223] Metabolic stability of a drug is one of the major factors that determine its concentration in the systemic circulation. Drug candidates must be tested both for their transformation by human P450s and for their ability to inhibit these enzymes, not only to estimate blood concentration but also to predict potential harmful drug interactions. [158] Serious drug-drug, drug-food and drug-natural health product interactions can lead to hospitalization and even death. [224,225] There is a need for screening methods to rapidly evaluate P450 drug metabolism and P450 inhibition.

6.1) P450 immobilization in biosensor and biodevice design

Tanvir *et al.* reported a methodology for the covalent co-immobilization of P450 2E1 and glucose-6-phosphate dehydrogenase (G6PD) on porous alumina membranes containing microchannels. [226] P450 2E1 is known to metabolize pharmaceuticals and xenobiotics such as chlorzoxazone, tamoxifen, paracetamol and ethanol, and to activate many toxic substances. [227,228] In this context, G6PD was used to regenerate NAD(P)H. [229] Aluminium oxide membranes are relatively inert towards biological molecules but can be derivatized to make them more reactive. [230,231] In this application, the aluminum oxide surface was reacted with (3-aminopropyl)triethoxysilane (APTES) to display amino groups that were modified both with glutaraldehyde for the immobilization of G6PD, and with *N*-succinimidyl-3-maleimidoproprionate for the immobilization of P450 2E1 (Figure 10a). The surface accessible amino groups of G6PD and the cysteine residues of P450 2E1 were covalently reacted with the aldehyde and maleimide groups respectively. The authors showed that the immobilized P450 fully retained its activity towards the substrate 3-cyano-7-ethoxycoumarin. Moreover, the system showed reusability and improved stability (3-4 freeze-thaw cycles).

Giovannozzi *et al*. generated a P450 BM3-based biosensing platform for arachidonic acid detection, a known biomarker involved in pain and inflammation processes. [232,233] The authors used porous silicon films that were activated with (3-aminopropyl)-trimethoxysilane (APTMS) followed by immersion in a glutaraldehyde solution and then reacted with a P450 BM3 bioconjugate bearing an IANBD fluorescent probe at Cys62. When reacting with glutaraldehyde, the amino groups of APTMS formed aldehydes which reacted with lysine residues on the P450 BM3 surface to form imines. [234] The fluorophore label served to achieve a more specific response of the enzyme-substrate interaction, as inspired by the work of Ferrero *et al*. [76] Binding of arachidonic acid led to a change in the fluorophore environment resulting in a fluorescence enhancement proportional to the concentration of the bound ligand. The system acted like an arachidonate biosensor and was successfully used to test several blood plasma samples. A linear response was observed in the micromolar range of concentrations, even in the

presence of various additives. The extracellular concentration of arachidonic acid has been shown to be in the order of nanomolar to micromolar, [235,236] and hence further improvement in the sensitivity is warranted.

Fantuzzi *et al.* reported the first integration of a P450-electrode into a microfilter plate format for rapid determination of affinity parameters of known drugs. [237] For this purpose, some of the most relevant drug-metabolizing P450s, 3A4 2D6 and 2C9, [238] were each covalently attached to a gold electrode via a self-assembled monolayer (SAM) of 1:1 10-carboxydecanethiol and 8-hydroxyoctanethiol respectively, at the bottom of each well of the plate. The SAM carboxylic acid groups were activated with EDC and *N*-S-NHS. A protein solution containing either P450 3A4, 2D6 or 2C9 was next added to the well plate to fully cover the gold working electrode and form amide bonds between the protein lysine residues and the S-NHS-ester of the SAM-coated electrode. The authors extracted K_m values from the current generated as the enzyme used electrons from the electrode for catalysis. The observed current increased as a function of substrate concentration with a typical hyperbolic, Michaelis-Menten behavior. [239,240] The biosensor was validated by screening a total of 30 known drugs with K_m values ranging from ~1 μM to a little over 100 μM.

Asturias-Arribas *et al.* developed a P450 2D6-based electrochemical biosensor for the detection of codeine. [241] Using lysine-EDC/NHS chemistry as previously described, [242] the enzyme was covalently bound (no linker) to the carbon surface of a working electrode. Chronoamperometric measurements of electrode electron transfer to the P450 were collected against a Ag/AgCl reference electrode giving rise to a signal that was proportional to the codeine concentration in solution. The authors successfully applied their biosensor to determine the concentration of codeine in urine and pharmaceutical samples.

Construction of multi-enzyme complexes to mimic natural metabolism pathways *in vitro* has attracted significant interest. Using an electrochemical approach, Lu *et al.* recently reported the successful design of a system with two different P450s immobilized on Au nanoparticle/chitosan/reduced graphene oxide nanocomposite sheets (Au/CS/RGO). [243] To mimic the complementary action of P450 1A2 and P450 3A4 on clopidogrel, both enzymes were immobilized on a Au/CS/RGO nanosheet-modified glass carbon electrode. P450 1A2 was first introduced covalently at the primary amine site of chitosan via cross-linking with glutaraldehyde, while P450 3A4 was attached at the hydroxyl sites of chitosan through a carbamate bond formed with the help of carbonyldiimidazole (CDI, Figure 10b). With this system the authors studied the electron transfer between each enzyme and the electrode, the enzymatic activity towards clopidogrel and the synergistic behavior of the two-enzyme modified electrode. They determined the apparent K_m and k_{cat} in the cascade metabolism of clopidogrel. When used *in situ*, the sensitivity of the two-enzymes system was defined as 143.4 $\mu A m M^{-1}$ and the detection limit for clopidogrel concentration was 0.63 μM.

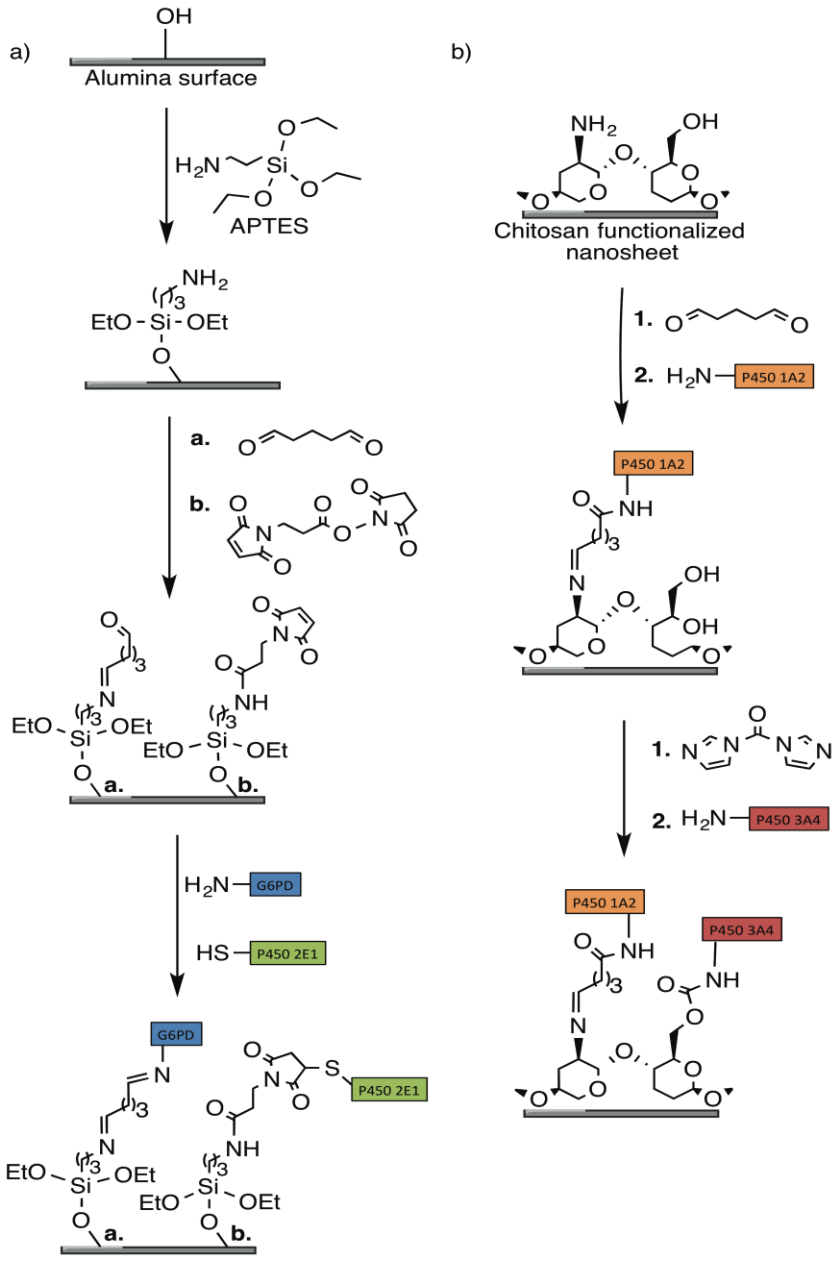

Figure 10. P450 immobilization in biosensor design

In contrast, P450s can sometimes be necessary for prodrug activation. This motivated Quester *et al.* to design a P450 bioconjugate as a means to help deliver the enzyme to breast cancer tumor tissues and thus improve local drug activation. [244] This biodevice concept was demonstrated with tamoxifen. [245] Activation of tamoxifen by P450 3A4 in non-targeted tissues results in undesired healthy tissue damage. The authors used the F87A mutant of P450 BM3 as a model for P450 3A4. [35,246] This mutation is known to increase the enzyme efficiency for tamoxifen bioactivation. [247] Since PEG bioconjugation of proteins is increasingly used as a strategy to improve therapeutic protein delivery, [248] the mutant P450 BM3 enzyme was functionalized by reaction of

its surface lysine groups with folic acid polyethylene glycol$_{5000}$-*N*-hydroxysuccinimide (NHS-PEG-FA). The folic acid groups serve to target the modified P450 to MCF-7 breast cancer cells (known to overexpress folic acid receptors on their surface). Upon binding to the cancer cells via the folic acid receptors, the PEG functionalized P450 molecules are expected to enter the cells via the formation of vesicles. Once inside the cells, the PEG groups are hydrolyzed off, rendering the enzyme active for tamoxifen activation to 4-hydroxytamoxifen. Interestingly, the P450 bioconjugate significantly reduced the dose of tamoxifen needed to kill MCF-7 cancer cells and did not show immunogenic activity.

6.2) P450 profiling using activity probes

Activity-based protein profiling (ABPP) has emerged as a key technique in functional proteomics. ABPP relies on the development of active site-directed covalent probes to provide the basis for quantitative analysis of the functional state of individual enzymes in complex proteomes. The probes are usually designed based on the scaffold of known substrates, mechanisms-based inhibitors and electrophiles. Evans and coworkers have recently reviewed mechanism-based enzyme profiling [249] and Cravatt *et al.* reviewed the state of the field and described the most recent advances. [250] This review will only cover publications that were not covered in the earlier reviews.

In the context of pharmaceutical development, drug metabolism is typically evaluated *in vitro*. To facilitate detection of P450 activities in more complex biological systems, the group of Cravatt developed a series of new P450-directed ABPP probes. One of the probes is based on the scaffold of the broad spectrum mechanism-based inhibitor 2-ethynylnaphthalene, but bearing an extra alkyne moiety. Following enzymatic transformation of one of the probe's alkyne group to a ketene, a nucleophilic residue of the enzyme attacks the ketene to form a covalent bond. Detection is achieved by addition of an azide-containing reporter group which reacts with the remaining alkyne on the probe (Figure 11a-b, probe **1**). [251,252] This probe was used to profile P450 activities in native liver proteomes Although the designed probe was successful at labeling several mouse liver P450s, it is unlikely that it could universally mark all mammalian P450s. To expand the coverage of P450s, Cravatt and coworkers designed eight more probes (Figure 11b, probes **2-9**) based on the structure of other well-known P450 mechanism-based inhibitors. [21,253,254] These ABBP probes were screened against 14 human P450s (1A1, 1A2, 1B1, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 2J2, 3A4, 4A11, 4F2 and 19A1) co-expressed with CPR in insect cells. While some probes were more promiscuous and targeted multiple P450s (probe **1–3** and **5**), probe **6**, **7** and **8** showed selectivity for 2D6, 1B1/2J2 and 1A2 respectively. P450 2C9, 2D6 and 3A4 generally exhibited a broad reactivity with the ABPP probes reflecting their flexible active site structures.

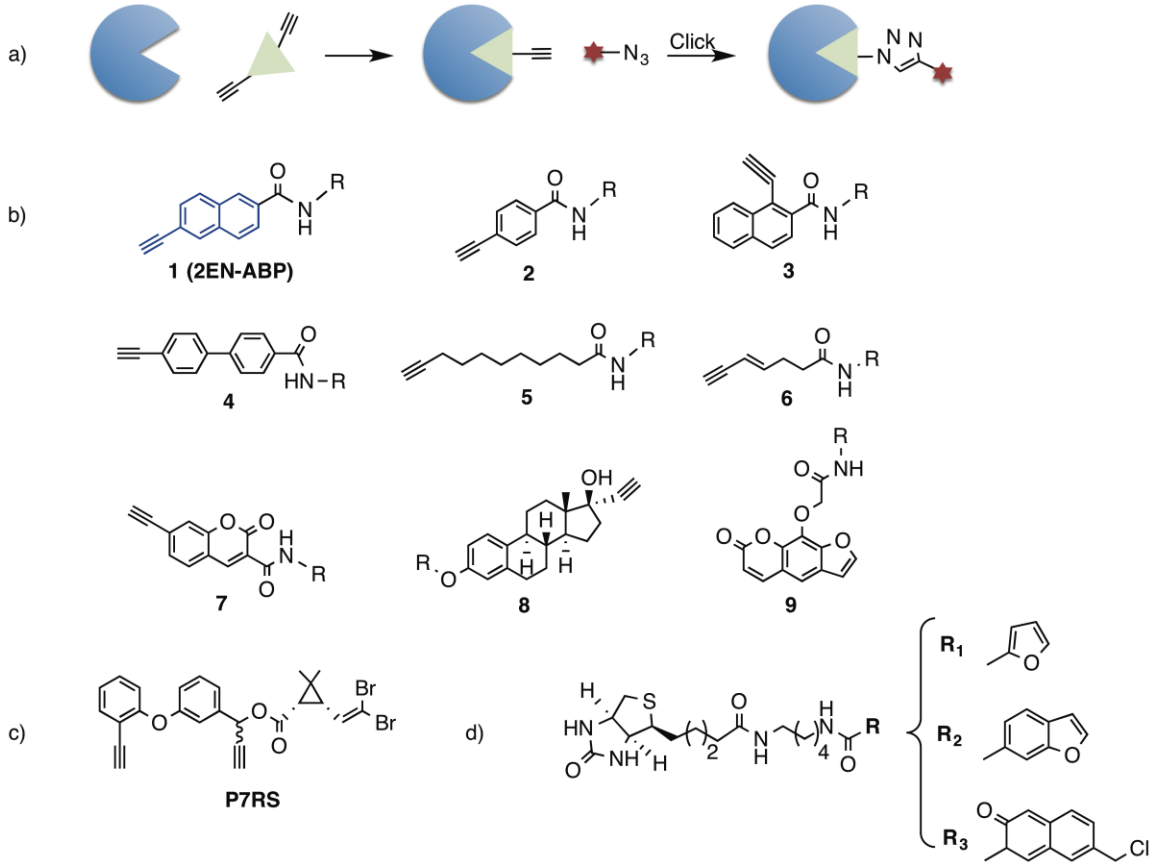

Figure 11. Activity-based probes for P450 profiling. The blue part on probe 1 is the 2-ethynylnaphthalene moiety.

In 2013, Ismail *et al.* developed pyrethroid-based probes for profiling P450 enzymes involved in insecticide metabolism. [255] Seven pyrethroid-mimicking probes were designed based on the deltamethrin scaffold. The authors tested the probes on mosquito P450 6M2 and P450 6Z2, which are strongly associated with pyrethroid resistance in the malaria-transmitting species *Anopheles gambiae*. [256,257] The authors also used the probes to profile mouse and rat liver microsomal P450s. Probe P7RS (Figure 11c) produced the strongest fluorescent signal in all experiments.

In an attempt to design probes that may be specific to one P450 enzyme, Sellars *et al.* [258] recently reported a new group of activity-based probes, with some inspired from furanocoumarins which are irreversible inhibitors of P450s. [253,259–262] Three probes were synthesized and their potential to inactivate P450 3A4 and/or P450 1A2 was evaluated with the Vivid assay. [263] The authors report that two of them (Figure 11d, R_1 and R_2) were selective for P450 3A4.

P450s are versatile catalysts with a great, yet hardly explored potential for use in biosensors. Advances in bioconjugation methodologies will help future sensor design.

Conclusion and future directions

Photoaffinity labeling and mechanism-based inhibitors have demonstrated utility to probe the P450 active site. Covalently-attached fluorescent probes have been used to investigate conformational motions, understand cooperativity and allostery, and further understand ligand binding to P450s. Together, the chemical modification of P450 surfaces and protein-protein cross-linking have provided a mapping of P450 interactions with other proteins and membranes. The covalent immobilization of P450 on electrode has found use in electron transfer studies as well as biosensor design. Finally, the interest in using P450s in biocatalysis continues to grow yet their implementation is challenged by a number of issues, some of which have been solved by P450 immobilization.

Across the studies summarized in this review, the maleimide-thiol chemistry has played a central role, especially when regioselectivity was desired. Amide bond formation has at times afforded a regioselective product *(N*-terminus), but has most often served as a non-selective yet efficient method. It is noteworthy that incorporation of non-canonical amino acids into P450s for the purpose of bioconjugation has not been reported, except in one instance [91] where the copper-assisted azide-alkyne cycloaddition was shown to be detrimental to the activity of a propargyl-tyrosine-containing P450 3A4 variant. In summary, bioconjugation has played an important role in our understanding and applications of P450s and we believe that the development of new biorthogonal reactions can fuel further breakthrough in this field.

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