EFFECTS OF ENVIRONMENTAL AND CLINICAL INTERFERENTS ON THE HOST CAPTURE EFFICIENCY OF IMMOBILIZED BACTERIOPHAGES

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ABSTRACT

The concept of using bacteriophages to control populations of pathogenic bacteria is gaining momentum, driven mainly by the growing global crisis over antibiotic resistance in both the natural environment and healthcare settings. Bacteriophages (phages) are natural predators of bacteria and are innocuous to humans, animals or plants. Functionalizing surfaces with phage offers the promise of designing devices that can actively capture and deactivate bacteria such as water filters, wound dressings or antibacterial coatings. Our laboratory has previously proven the feasibility of this idea in a clean water matrix, demonstrating that phage-functionalized surfaces are promising candidates for selective capture and inactivation of bacterial pathogens. However, the complex composition of many natural samples (e.g., surface waters, waste water, blood, etc.) can potentially interfere with the interaction of phage and its bacterial host, leading to a decline in the efficiency of the phage-functionalized surface. In this study, the bacterial capture efficiency of phage-functionalized surfaces was assessed in the presence of potential environmental and biomedical interferents. Two phage-bacteria systems were used in this study, namely PRD1 with Salmonella Typhimurium and T4 with Escherichia coli. The potential environmental interferents tested were humic and fulvic acids, colloidal latex microspheres (as a model for environmental colloids), extracellular polymeric substances (EPS), as well as a natural unfiltered groundwater. Albumin, fibrinogen, and blood serum were also tested as representative interferents of interest for biomedical applications such as wound dressings. The inactivation of the selected phages by the potential interferents was first evaluated for each phage suspended in aqueous media containing each interferent. Next, the bacterial (host) capture efficiency of a

phage-functionalized substrate was evaluated in the presence of each interferent. Interestingly, humic and fulvic acids reduced the capture efficiency of T4-functionalized surfaces by over 60%, even though they did not lead to inactivation of the suspended virions. Neither humics nor fulvics affected the capture efficiency of PRD1. EPS and human serum decreased the host capture efficiency for immobilized PRD1 and T4 by over 70%, and also impaired the infectivity of the non-immobilized (planktonic) phage, although to a much lower extent (less than 50%). The fundamental mechanisms leading to the observed decrease in performance of the phage-functionalized surfaces in the presence of selected interferents is discussed in detail in the thesis. These findings demonstrate the inadequacy of traditional phage selection methods (i.e., infectivity of suspended phage towards its host in clean buffer) for designing antimicrobial surfaces and further highlight the importance of taking into account the environmental conditions in which the immobilized phage is expected to function.

RÉSUMÉ

La résistance, de plus en plus avérée, aux antibiotiques a motivé l'intérêt d'utiliser des bactériophages pour le traitement, le contrôle et la détection de populations bactériennes pathogènes. Des bactériophages aux surfaces fonctionnalisées ont été mis au point pour une application à des surfaces antimicrobiennes, en tant que biocapteurs, dans des dispositifs médicaux permanents ou des pansements ainsi que dans des dispositifs pour la capture des bactéries. Dans cette étude, l'efficacité de capture des bactéries, de deux modèles de phage à surface fonctionnalisée, a été évaluée en présence de différents interférents potentiels environnementaux et biomédicaux. Les deux systèmes phages-bactéries utilisées dans cette étude sont le PRD1 de *Salmonella* Typhimurium et le T4 d'*Escherichia coli*. Les interférents potentiels testés comprenaient : les acides humiques et fulviques, les eaux souterraines naturelles, les microsphères de latex colloïdales, des substances polymères extracellulaires (SPE), l'albumine, le fibrinogène et le sérum. Tout d'abord, l'inactivation des phages sélectionnés par les interférents potentiels a été évaluée pour les phages en suspension dans des milieux aqueux. Ensuite, l'efficacité de capture des bactéries d'une surface de verre phage-fonctionnalisée a été évaluée en présence de différents interférents. Une SPE a été identifiée pour réduire l'efficacité de captage des PRD1 et des T4, et a également été trouvée pour réduire l'activité des PRD1 en suspension. Le sérum a diminué l'activité des deux phages en suspension, et a également abouti à une réduction de l'efficacité de capture bactérienne. L'addition d'acides humiques ou fulviques réduit l'efficacité de capture des surfaces T4-fonctionnalisées, mais n'a pas conduit à l'inactivation des virions en suspension. Ces résultats soulignent la

nécessité pour une surface potentielle de phage fonctionnalisée, d'être testée pour ses performances dans les conditions d'application finale.

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PREFACE AND CONTRIBUTION OF AUTHORS

This thesis was prepared in accordance with the McGill University thesis preparation guidelines in a manuscript-based format. Chapter 1 consists of a thesis introduction. Chapter 2 contains a manuscript published in *Langmuir* on the effects of environmental and clinical interferents on the host capture efficiency of immobilized bacteriophages. The authors are Daniel V. Dixon, Zeinab Hosseinidoust, and Nathalie Tufenkji.

The experimental work, data analysis, and manuscript drafting was performed by Daniel Dixon. Zeinab Hosseinidoust provided technical advice in the design of experiments and editing the manuscript. Nathalie Tufenkji provided guidance and supervision throughout the project and in manuscript editing.

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CHAPTER 1: INTRODUCTION

1.1 BACTERIAL PATHOGENS, A GLOBAL THREAT

Bacterial infections pose a worldwide threat to human health. The spread of pathogenic bacteria is facilitated by contamination of surfaces in healthcare and food industries, and through drinking water supplies, amongst others. Traditionally, bacterial infections have been treated with antibiotics, but with the rise of antibiotic resistant bacteria, the need for alternate treatments is rising. Novel methods for detection, control and treatment of pathogenic bacteria are desperately needed and have thus attracted high interest from researchers.

One proposed solution is the use of bacteriophages for bacterial population control and detection. Bacteriophages, viruses that infect bacteria, can be very specific to their target, even down to a specific subspecies, and are harmless to eukaryotic cells (such as human and animal cells).

1.2 BACTERIOPHAGES

Bacteriophage (or phage) were first suggested to exist in 1915 by British scientist Frederick Twort and also independently discovered in 1917 by French Canadian Felix d'Herelle.[1-2] There are currently thirteen recognized families of phage, and they exist in a variety of shapes (cubic, binary, helical, or pleomorphic), sizes (20 to 2000 nm), and with single or double stranded nucleic acids (ssDNA, dsDNA, ssRNA, or dsRNA).[3] Table 1.1 lists the current classifications of phage.

Table 1: Classification of Bacteriophages

Symmetry	Family	Nucleic acids	Structural Details
Binary (tailed)	*Myoviridae*	Linear dsDNA	Contractile tail
	Siphoviridae	Linear dsDNA	Long, noncontractile tail
	Podoviridae	Linear dsDNA	Short tail
Cubic	*Microviridae*	Circular ssDNA	Isometric
	Corticoviridae	Circular dsDNA	Complex capsid, lipids
	Tectiviridae	Linear dsDNA	Internal lipoprotein vesicle
	Leviviridae	Linear ssRNA	Isometric
	Cystoviridae	Linear dsRNA	Envelope, lipids
Helical	*Inoviridae*	Circular ssDNA	Filaments or rods
	Lipothrixviridae	Linear dsDNA	Envelope, lipids
	Rudiviridae	Linear dsDNA	Resembles Tobacco Mosaic Virus
Pleomorphic	*Plasmaviridae*	Circular dsDNA	Envelope, lipids, no capsid
	Fuselloviridae	Circular dsDNA	Spindle-shaped, no capsid

Bacteriophages, like other viruses, can only replicate by infecting host cells. A bacteriophage will adsorb to a host cell by attaching to specific receptors on the cell surface; it then transfers its genetic material into the bacterium. Lytic phage use the bacterium's own mechanisms for protein production to make new bacteriophages. The bacteria will be lysed after a number of new progeny phages are assembled, releasing them to infect other hosts. Lysogenic phage, will have their DNA lie dormant, integrated into the host DNA; lysis will only occur under special conditions or when external factors are met.[3] For use as an antimicrobial agent, lytic phages are preferred, as they will lyse the host without the need of an inducer.

Bacteriophages can be very selective toward a specific species or even strain of bacteria. They have evolved alongside their host bacteria over millions of years in a manner referred to by scientists as an "evolutionary arms-race"; when a bacterium develops resistance to a certain phage, phage will mutate to be able to infect the resistant bacterium.[4]

This selectivity can be advantageous or disadvantageous depending on the application. When considering phage as a treatment for bacterial infections, this selectivity would make preparation and selection of an appropriate phage more tedious, likely requiring a custom cocktail of different phages to ensure effective treatment or inactivation of pathogenic bacteria. On the other hand, with highly specific bacteriophages, a single bacterial strain may be targeted leaving other microbes unaffected. Highly specific phages can also be very advantageous in the food industry, where the taste, smell and texture of the food is dependent on certain bacteria. Broad spectrum biocides will destroy the pathogens and "good" bacteria, whereas when using phage, only selected pathogenic bacteria can be targeted. Furthermore, phages with a narrow host range can serve well in biosensor applications to detect a certain species or strain of bacteria.

1.3 Applications of Immobilized Phage

1.3.1 Biosensors

The development of biosensors for the rapid detection of pathogenic bacteria has been of interest in recent years. One major component of a biosensor is the recognition element which must interact specifically with the desired analyte. Antibodies are commonly used as recognition elements. Antibodies, also called immunoglobulins, are

large Y-shaped proteins produced by the immune system, which function to identify and help remove foreign antigens or targets such as viruses and bacteria in the body. This feature has been exploited by immobilization of antibodies onto various substrates to detect pathogens of interest.[5] A major disadvantage of antibodies is the cost associated with isolating and purifying them, only to be limited in the end to a narrow range of physical conditions where the antibody will remain effective.[6] This is where bacteriophages present a greater advantage. Phages can be very specific in targeting their host, but with the added benefit of potentially being much cheaper to manufacture and more stable under a wider range of conditions.[7] Like antibodies, phage can be immobilized on a sensor surface and be used as the detector for the bacterial pathogen of interest.

A number of biosensor platforms with phages as the recognition element have been developed. Some notable examples include the use of surface plasmon resonance (SPR) platforms for the detection of *E. coli* and Methicillin-resistant *Staphylococcus aureus* (MRSA) with chemically immobilized bacteriophages,[8-9] the use of a phage monolayer on a quartz crystal microbalance (QCM) for the detection of MRSA,[10] and the detection of *E. coli* with immobilized T4 using electrochemical impedance spectroscopy (EIS) with loop-mediated isothermal amplification (LAMP).[11] Phage can also be used for more than just the direct sensing element. Taking advantage of the ability to self propagate in the host, lytic phages have been used as an agent for signal amplification in sensors. For example, the use of phages immobilized on magnetic or cellulose beads to capture and detect bacteria has been demonstrated by performing a bioluminescent ATP assay after cell lysis is induced from the phage.[12]

1.3.2 ANTIMICROBIAL SURFACES

Bacteriophages can be immobilized on surfaces to capture and lyse unwanted bacteria. These functionalized surfaces can be used in indwelling medical devices (*e.g.,* catheters, stents, and implant), wound dressings, food packaging, and coatings on door handles amongst others. For many medical and food packaging applications, the specific nature of phage interactions can be very beneficial; bacteriophages or phage mixtures can be selected to target only the unwanted bacteria, leaving the harmless bacteria intact. Furthermore, the self-propagating nature of phage will allow for smaller initial doses, and more time between replacements, both factors that can drive down costs. Bacteriophage cocktails have been investigated for use as coating on catheters to control *Pseudomonas aeruginosa, Proteus mirabilis* and *Staphylococcus epidermidis* biofilm formation.[13-15] In the context of biocontrol on food, phage have been immobilized on a modified cellulose surface and used to control the growth of *Listeria monocytogenes* and *Escherichia coli* O157:H7.[16]

1.4 BACTERIOPHAGE IMMOBILIZATION

The basic premise of creating an antimicrobial surface or a sensor using bacteriophage is to immobilize the virions on the substrate of interest. Targeted bacteria are expected to be captured by the immobilized phage and subsequent lysis of the bacteria may occur. The immobilization of phage has been studied using a variety of techniques, each with its own advantages and disadvantages.

The simplest method to create a bacteriophage-functionalized surface is to attach phage to the substrate by simple adsorption. This method has been demonstrated by a number of

researchers to functionalize catheters in an effort to prevent biofilm formation.[13-15] While successful at reducing biofilm growth on catheters, this method required regular retreatment to remain effective.[13] This highlights some of the disadvantages of physical adsorption based approaches, the low interaction energy characteristic of physisorption means changes in chemical potential, shear stress, or ionic strength may result in phage detachment from the surface. Furthermore, physisorption of phage onto surfaces also results in a reduced surface density compared to selected chemisorption techniques.[17]

To further enhance adsorption in favour of bacteriophage immobilization on surfaces, some researchers have exploited the overall negative charge of phage virions by using cationic surfaces to take advantage of electrostatic attraction.[18] In one study, cellulose membranes were modified with polyvinylamine and treated with base to create a cationic surface; this surface was then exposed to a phage suspension to allow immobilization of phage.[16] Another advantage of using this method is that it is postulated that the bacteriophage will be more favourably oriented on the surface. Some phages have a charge difference between the head and the tail sections, where the head is more negative and the tail more positive, so by using a cationic surface, the head section would be attached to the surface while the tail would remain exposed to the environment where it is more accessible to interact with host bacteria.[16, 19]

Another common technique for phage immobilization is via covalent bonding of phage to the surface. A few researchers have attached phage to a glass surface by silanization of the surface with aminosilanes and then promoting a crosslinking reaction between the carboxyl groups on the phage with the amines on the surface. An aminosilane commonly used is (3-aminopropyl)triethoxysilane (APTES), this may react

with a carbodiimide, such as N-[3-(dimethylamino)propyl]-N'-ethylcarbodiimide hydrochloride (EDC) used in conjunction with an activating agent, such as N-hydroxysuccinimide (NHS), to activate the carboxylic acid on the phage.[20-21] Another method is to use a self-assembled layer of dithiobis(succinimidyl propionate) (DTSP) on gold, which can then directly bond with the amine groups on the bacteriophage.[9]

An alternative to chemical modification of the substrate would be chemical modification of the phage virion. Bacteriophages have a range of functional groups on their protein coat, making them amenable to chemical modification by various chemistries.[22-23] Phages have been biotinylated to take advantage of biotin-streptavidin interactions, creating a strong bond to the substrate.[24] Streptavidin is a protein that has an extremely high affinity for biotin and is commonly used for detection and purification of biomolecules.[25] The biotin may be chemically added to the phage with the streptavidin on the surface, but without a good way to control exactly where the biotin will bind to the phage there is a possibility of causing undesired phage orientation.[24] To improve on this method, favourable bacteriophage orientation has been promoted with the use of genetically modified bacteriophage that will express biotin acceptor-type proteins. These proteins can then be biotinylated through an enzyme-mediated reaction.[26] Using this approach, phage orientation was improved because the affinity tags for biotin were all expressed in the same location on the head of the phage, ensuring that when immobilized, the tails will point away from the streptavidin-coated surface.[26] The disadvantage of using genetically modified phage is that the process is labour intensive; this is especially problematic if tailored phage cocktails are needed, in which case a large number of phages must be genetically modified.

A summary of bacteriophage immobilization techniques is presented in Table 1.2.

Table 2: Summary and examples of selected bacteriophage immobilization techniques

Immobilization method	**Examples**
Physisorption	Adsorption of phage on neutral hydrogel coated catheter[15]
Electrostatic adsorption	Coating cellulose membrane polyvinylamine and absorption of phage onto the positive surface[16]
Covalent binding	EDC/NHS crosslinking of phage to aminosilanized glass[21] Phage binding to self-assembled monolayer on gold SPR chips[9] Biotinylated phage attached to streptavidin-coated beads[24, 26]
Genetic modification	T4 phage expressing biotin or cellulose binding modules on its capsid (head)[26]

1.5 Environmental Interferents

Immobilized bacteriophages are expected to remain infective in real life conditions, during use of the phage-functionalized surface. These conditions may be different from those in the environment that the phage was isolated from, and thus, various factors can potentially interfere with the functionality of the bacteriophage and, as a consequence, the efficiency of the bioactive substrate. The subject of interfering molecules/particles is of special interest when dealing with complex samples such as natural waters, wastewater, or blood. It is important to study how materials found in such complex environments can interfere with phage-host interactions. Very few studies have focused on determining the factors affecting efficiency of immobilized phage. Most of the research in this area is focused on the effect of environmental conditions (*e.g.,* pH, ionic strength, temperature, *etc.*) on the interaction of phage with the solid substrate on

which it is expected to adsorb [19, 27-28] rather than the interaction of immobilized phage with its host. In one of the very few instances in the open literature, bovine serum albumin (BSA) was found to inhibit non-specific binding of bacteria to the surface, while phage-functionalized surfaces remained uninhibited by BSA.[29]

Although reports in this area have been relatively scarce, valuable insight can be gained from the studies published on factors affecting efficiency of non-immobilized phage. Yates *et al.* used bacteriophage MS2 as a model for human viruses and studied a variety of factors affecting MS2 persistence in groundwater. These parameters included: temperature, pH, calcium concentration, magnesium concentration, ammonia concentration, nitrate levels, and total dissolved solids.[30] Of these parameters, temperature and calcium concentration were found to have a significant impact on MS2 infectivity.[30] Environmental factors affecting phage ability to inhibit bacterial growth and biofilm formation in *Pseudomonas aeruginosa* were also recently assessed by Knezevic *et al*.[31] The study tested a variety of carbohydrates, amino acids, as well as extracellular polymeric substances (EPS) and lipopolysaccharides (LPS) along with temperature, and pH. The results varied depending on the phage used, but each was shown to have some effect at concentrations of 500 mM for the carbohydrates and amino acids, and 200 μg/mL for the EPS and LPS[31]. A summary of these findings is presented in Table 1.3.

Table 3: Summary of inactivation parameters tested against selected phage

Parameter	Phage Tested	Results
Temperature[30]	MS2	Positive correlation with virus decay rate
Calcium concentration[30]	MS2	Positive correlation with virus decay rate
Contact Angle[28]	MS2	Inactivation occurs with increased contact angle
	ϕX174	No inactivation observed
pH[31]	δ, σ-1	>50% inactivation at pH 1-3
	J-1, 001A	>50% inactivation at pH 1-5
Glucose (0.5 M)[31]	δ, σ-1, J-1	Partial neutralization
Rhamnose (0.5 M)[31]	δ, σ-1	Partial neutralization
	J-1	No significant neutralization
Glucoseamine (0.5 M)[31]	δ, σ-1	Partial neutralization
	J-1	No significant neutralization
Mannose (0.5 M)[31]	δ, σ-1	Partial neutralization
	J-1	No significant neutralization
Alanine (0.5 M)[31]	δ, σ-1, J-1	Partial neutralization
Galactose (0.5 M)[31]	δ, σ-1	No significant neutralization
	J-1	Partial neutralization
Glutamine (0.5 M)[31]	δ	No significant neutralization
	σ-1, J-1	Partial neutralization
LPS (200 μg/mL)[31]	δ	No significant neutralization
	σ-1, J-1	Partial neutralization
EPS (200 μg/mL)[31]	δ, σ-1, J-1	Partial neutralization

The growing interest in the use of bacteriophage in the biotechnology industry and their ubiquity in the environment calls for in-depth research that can facilitate their large-scale production and use. Determining factors that can affect bacteriophage

infectivity and decay is critical in establishing the efficiency of any product based on immobilized phage and thus deserves attention from researchers.

Potential interfering molecules/cells/particles must be chosen based on the final application of the phage-functionalized substrate. For example, extracellular polymeric substances (EPS) are interferents of particular interest when designing a bioactive surface which must resist biofilm formation. EPS is a mixture of different biomolecules that is secreted by bacteria in a biofilm amongst other conditions. For a sensor designed to detect pathogens in groundwater, larger colloidal particles or natural organic humic and fulvic acids may associate with the nano-sized phage and thus interfere with their interaction with the host bacteria thereby decreasing the sensitivity of the sensor. If a bioactive surface is designed to be used as an indwelling medical device, the immobilized phage must be neutral to the potential effects of serum and blood clotting factors. An early study on the inactivation of non-immobilized T4 in animal serum has demonstrated that inactivation does indeed occur, with only partial reactivation.[32] Other studies with different phages have reported no deactivation in the presence of serum.[13, 15]

To summarize, there exists some information on the environmental factors affecting infection efficiency of free (suspended) phage, and there have been some studies on factors that influence the interactions of phage with surfaces, but no studies have yet reported on how immobilized bacteriophage perform in more complex real-life systems, where dissolved biomolecules and/or suspended particulate matter can interfere with the capture and inactivation of host bacteria. The effect of interferents on free and immobilized phage is expected to be different; free phage are able to diffuse in an aqueous medium and orient themselves in a manner to facilitate attachment to receptors

on the host bacteria and infection of the host. In contrast, both diffusion and orientation are inhibited for immobilized phage and it is the bacteria that must approach the phage to be captured.

1.6 Thesis Objectives

The purpose of this study was to determine the bacterial capture efficiency of phages when immobilized on a surface in the presence of selected environmental interfering agents. To attain this objective, a number of select interferents of relevance to environmental or clinical applications were chosen. The effect of the chosen interferents was studied on non-immobilized (suspended) phage (as indicated by loss of phage infectivity) and on covalently immobilized phage (as indicated by loss of host capture efficiency). The details of the experimental methods and the obtained results are presented in Chapter 2.

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CHAPTER 2: EFFECTS OF ENVIRONMENTAL AND CLINICAL INTERFERENTS ON THE HOST CAPTURE EFFICIENCY OF IMMOBILIZED BACTERIOPHAGES

2.1 INTRODUCTION

Bacteriophages, viruses that infect bacteria, have attracted considerable attention in the past years as an alternative solution for the control of bacterial populations. This interest has been further fueled by the global crisis over antibiotic resistance. Bacteriophages (phage) offer many advantages over antibiotics and many other common biocides; namely, they can be very specific to their target host, even to the level of a specific subspecies, and are harmless to eukaryotic cells (such as human cells).

Bacteriophages can be immobilized on various substrates for the design of bioactive surfaces. These surfaces have many potential applications such as indwelling medical devices,[1-3] food packaging,[4] and water filtration systems.[5] Furthermore, phage-functionalized surfaces can be used in biosensor designs using platforms such as surface plasmon resonance,[6-7] quartz crystal microbalance,[8] or dip-stick assays.[9]

In an earlier study, we demonstrated the efficacy of a phage-functionalized surface for the capture and deactivation of bacteria.[10] The findings emphasized how the orientation of the immobilized phage, specifically the position of its host binding proteins on the surface, played a significant role in the capture of host bacteria.[10] To extend this work, the efficiency of phage-functionalized surfaces needs to be determined when applied outside of ideal laboratory conditions. Phage-functionalized substrates are

expected to remain functional (*i.e.,* able to capture and/or destroy host bacteria) when used in the presence of complex analytes such as those found in wastewater, groundwater or medical samples (*e.g.,* blood or urine). Regardless of the method of immobilization, or the substrate used, the environment that the immobilized phage encounters is expected to significantly affect the phage interaction with its host bacterium, consequently affecting the efficiency of the bioactive surface. The conditions encountered by a bioactive surface functionalized with phage may be different from the environment the phage was originally isolated from. Therefore, it is important to study how various biomolecules, particulate matter, natural colloids, or even cells found in such complex environments can affect the efficiency of phage functionalized surfaces.

Much of the research in this area has focused on the effect of environmental conditions on phage attachment to a substrate,[11-16] or on infectivity of non-immobilized (suspended) phage toward its host.[17-18] To our knowledge, there is no report on how environmental conditions may affect the interactions of surface-immobilized phage with its host. To address this gap in knowledge, a number of select interferents of relevance to environmental or clinical applications were chosen. The potential interfering molecules/particles were chosen based on potential applications for a phage-functionalized substrate. For example, extracellular polymeric substances (EPS), a mixture of biomolecules secreted by bacteria, are of interest when considering the development of a biofilm-resistant surface. Natural organic fulvic and humic acids are ubiquitous in environmental water samples and should be considered in biosensing or water treatment applications of phage-functionalized surfaces. Larger colloidal particles also present in aquatic environments or biomedical fluids (e.g., blood cells) may interfere

with the activity of nano-sized phage. If applications for indwelling medical devices are desired, the immobilized phage must be resilient to the potential effects of serum and blood clotting factors. Two phage/host systems were used herein; namely, PRD1 with *Salmonella* Typhimurium and T4 with *Escherichia coli*. The two chosen phages have very different shapes and modes of infection. T4 is an asymmetric tailed phage and is representative of >95% of known bacteriophages. PRD1 is an icosahedral phage and is completely symmetrical in terms of shape and location of its capture proteins. This study examines the effect of the chosen interferents on the infectivity of non-immobilized (suspended) phage and on the host capture efficiency of the same phages when covalently immobilized on a surface.

2.2 Materials and Methods

2.2.1 Reagents and Materials

The following chemicals were purchased and used without further purification: agar, ethanol, hydrochloric acid, magnesium sulfate heptahydrate, methanol, sodium hydroxide, sodium chloride, tris(hydroxymethyl)aminomethane, trypticase soy agar, and trypticase soy broth (TSB) (Fisher Scientific). (3-aminopropyl)triethoxysilane (APTES), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), formaldehyde, N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), human serum albumin (HSA), human sera, fibrinogen, poly(ethylene glycol), and sulphuric acid (Sigma-Aldrich). Suwannee River humic acid (SRHA) and Suwannee River fulvic acid (SRFA) were obtained from the International Humic Substances Society. Sulfate latex microspheres (1 μm) and SYTO 9 green nucleic acid stain were purchased from

Invitrogen. A sample of natural groundwater was collected and characterized as described by Petosa *et al*.[19]

2.2.2 Bacteria Culture and Bacteriophage Propagation

Salmonella enterica serotype Typhimurium LT2 (HER1023) and bacteriophages PRD1 (HER23) and T4 (HER27) were obtained from the Félix D'Hérelle Reference Centre for Bacterial Viruses (Université Laval, Québec, Canada). *Escherichia coli* BL21 (ATCC BAA-1025) was purchased from Cedarlane (Cedarlane Corporation, Burlington, Ontario, Canada).

To prepare the bacterial culture, an inoculum from a frozen glycerol stock (-80˚C) was streaked on a trypticase soy agar (TSA) plate and incubated overnight at 37˚C. A single colony from the plate was used to inoculate 10 mL of TSB which was incubated overnight (37˚C, 150 rpm). A 75 µL aliquot was taken from the overnight culture and diluted 100 fold into fresh TSB and incubated until a bacterial concentration of 10^9 CFU/mL was reached.

Bacteriophage propagation was performed using the soft-agar overlay technique.[20] Bacteriophages were purified by PEG precipitation[21] followed by centrifuge filtration (Millipore 100 kDa centrifugal filter unit). Final phage concentrations were adjusted to 10^{11} plaque forming units (pfu)/mL in saline-magnesium buffer (SM buffer: 50 mM Tris-HCl, 100 mM NaCl, 8.1 mM $MgSO_4$, pH 7.5).

2.2.3 EPS Extraction

Extracellular polymeric substances (EPS) of host bacterium were extracted by the formaldehyde-NaOH method.[22] Formaldehyde was added to 10 mL of bacterial culture to

a final concentration of 0.22% (v/v). The suspension was then incubated at 4˚C for 1 h followed by the addition of 4 mL of 1 M NaOH. The suspension was incubated at 4˚C for 3 h and then centrifuged at 20000*g*. After centrifugation, a 0.2 μm Millipore membrane filter was used to filter out larger particles and bacteria, and smaller molecules were separated by dialyzing against 1 L of DI water with a 3500 Da Slide-A-Lyzer dialysis membrane cassette (Thermo Scientific) at 4˚C for 24 h. The purified EPS was then freeze-dried for 24 h and the powder was used to prepare known concentrations of EPS solution.

2.2.4 Bacteriophage Inactivation Assay

Bacteriophage inactivation (for non-immobilized or suspended bacteriophage) in the presence of environmental molecules was assessed as described by Kropinski.[23] Serial dilutions of the potential interferents were made in SM buffer followed by the addition of a known concentration of phage. The phage-interferent suspension was then incubated at room temperature for 1 h. The suspensions were mixed with bacterial host and plated using the soft-agar overlay technique to determine the number of pfu formed. A control sample of phage mixed with only SM buffer without any potential interfering compounds was used to normalize the data.

2.2.5 Preparation of Phage-Functionalized Surfaces

Glass slides coated with aminosilane were prepared as a model substrate for bacteriophage functionalization as described by Hosseinidoust *et al.*[10] Briefly, glass discs (12 mm diameter) were sonicated consecutively in MeOH/HCl (1:1) and concentrated H_2SO_4. The glass discs were then washed with EtOH and dried under high purity N_2. The clean glass discs were subsequently dipped in a 10% (v/v) solution of APTES in EtOH

for 30 min, sonicated in EtOH for 30 min and dried for 1 h at 120˚C. Bacteriophages were cross-linked to the aminosilane surface using (EDC 5 mg/mL) and NHS (6 mg/mL) to couple carboxyl groups in the phage protein coat to amine groups on the disc surface. The phage-functionalized surface was then washed with SM buffer and blocked with 1 mg/mL BSA.

2.2.6 Immobilized Phage Capture Experiment

Bacterial suspensions were rinsed three times (3000*g*, 5 min), followed by resuspension in SM buffer containing the desired concentration of the potential interferent. The bacteriophage-functionalized surfaces were then immersed in the bacterial suspensions and left shaking at 150 rpm for 30 min after which the suspension was replaced with a buffer solution containing the green fluorescent nucleic acid stain SYTO 9 (excitation/emission 485 nm/498 nm) and left shaking at 150 rpm for 15 min, followed by a rinse with SM buffer. Bacteria attached to the surface were imaged using an inverted fluorescence microscope (Olympus IX71, Tokyo, Japan) with a filter set capable of illuminating SYTO9. Images were recorded with an Evolution VF cooled monochrome CCD camera (1392×1040 resolution with 4×4 binning) and analyzed using Image-Pro Plus, version 6.0. At least three discs were analyzed for each condition, and at least 30 images were recorded for each disc. Data for attached cells were analyzed by one-way analysis of variance followed by a posthoc Tukey multiple comparisons of means. P values < 0.05 were considered significant.

2.2.7 Electron Microscopy

Scanning electron microscopy (SEM) was used to image phage-coated surfaces with or without bacteria. Phage-functionalized surfaces were prepared as described for

immobilized phage capture experiments and selected discs were incubated with host bacteria to induce bacterial capture by immobilized phage. The slides were subsequently rinsed with SM buffer and fixed with 2.5% (v/v) glutaraldehyde. The slides were then dehydrated with a series of ethanol/water solutions (30% to 100% EtOH) followed by a series of amyl acetate/ethanol solutions (25% to 100% amyl acetate). The slides were air dried and coated with 50 Å of an Au-Pd coating (Hummer VI Au-Pd sputter coater). Samples were examined with a Hitachi SU-8000 field emission-STEM (FE-STEM).

2.3 Results and Discussion

2.3.1 Phage Activity In Suspension

Before assessing the bacteria capture efficiency of immobilized phage, experiments were carried out to gain a better understanding of whether the potential interfering agents would influence the infectivity of the phage while in suspension. The two phage-host systems used were PRD1 with *S.* Typhimurium and T4 phage with *E. coli*. Phage activity (infectivity) was evaluated using standard culture-based methods. The number of plaques formed after exposure to the interferents of interest was determined by plaque counts using the soft agar overlay method. Herein, humic acid, a major fraction of natural organic matter found in water consisting of high molecular weight polyaromatic compounds, fulvic acid, a more acidic, lower molecular weight compound similar to humic acid with higher oxygen content and more carboxyl groups, and EPS, naturally secreted biomolecules from bacteria, were selected as molecular interferents that may be present in natural water samples. Latex microspheres are often used as model colloids in environmental studies – negatively charged sulfate latex colloids were used here to

examine the effect of a 1 μm-sized non-biological colloid on phage infectivity. Finally, phage activity was also evaluated for phage suspended in a natural groundwater sample.

Figure 1 presents the phage infectivity data normalized as a percentage to the control group. The presence of fulvic acid, humic acid, groundwater, and latex particles (Figure 1a-d, respectively) did not result in any significant reduction in phage activity for either T4 or PRD1 over the concentration ranges tested. EPS was found to reduce the activity of PRD1 up to 45% at concentrations over 100 mg/L (Figure 1e). Various interferents of interest in a biomedical context were also selected to examine their effect on phage infectivity. Fibrinogen and albumin were chosen as these proteins are found in significant abundance in blood plasma. Phage activity was also evaluated for phage suspended in human serum as a medium relevant in applications such as wound dressings or indwelling medical devices. The presence of albumin and fibrinogen did not lead to a significant change in phage infectivity (Figure 1, panels g and h, respectively). For both PRD1 and T4 phage, a decline in phage infectivity was observed with increasing concentrations of serum (Figure 1f). In the presence of undiluted serum, the infectivity of PRD1 and T4 was reduced by 45% and 20%, respectively.

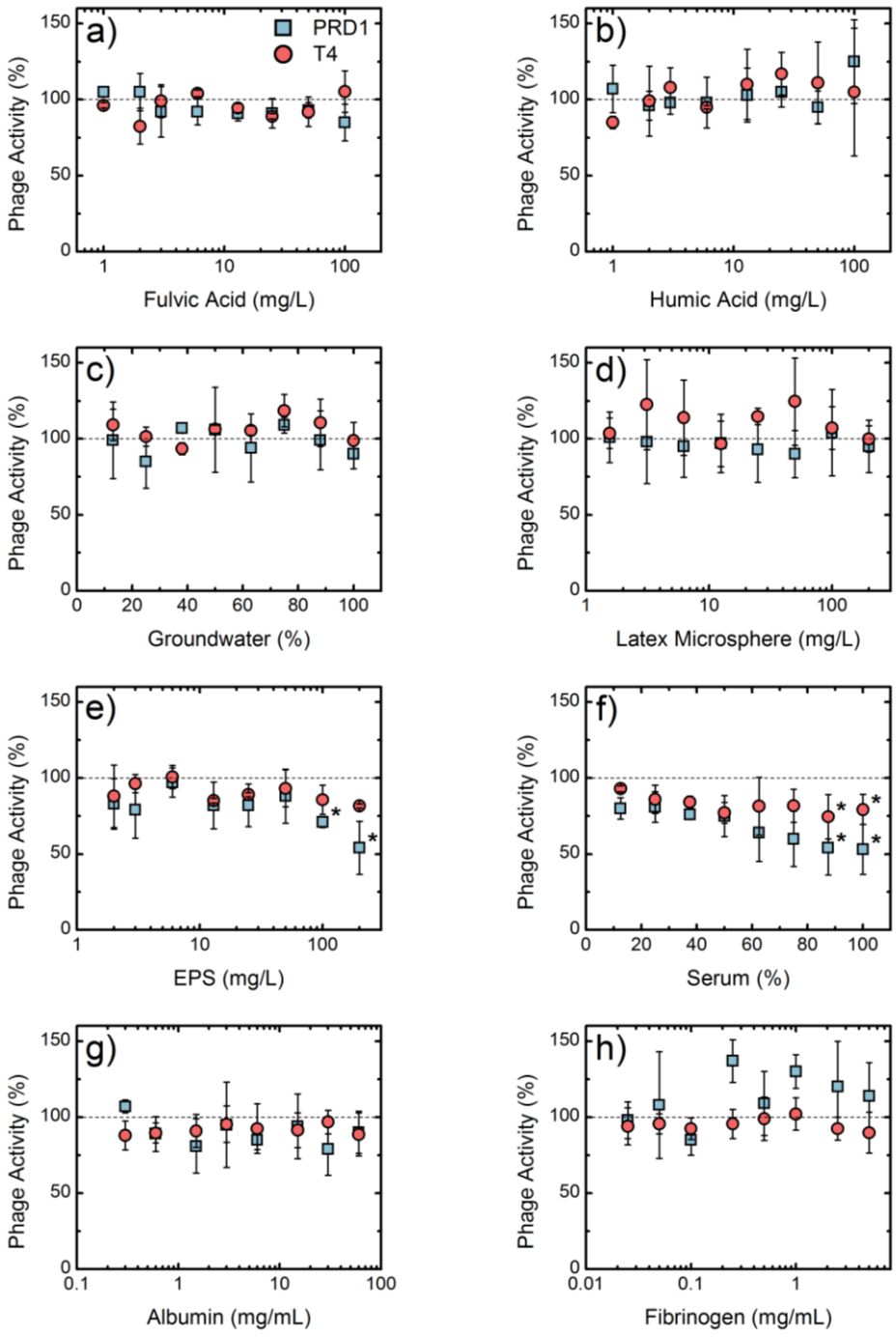

Figure 1. (a-h) Bacteriophage activity versus concentration of potential interfering substances. PRD1 (squares) and T4 (circles) activity was measured against hosts *S.* Typhimurium and *E. coli*, respectively. Data represent the mean ± standard deviation. Data significantly different from the control ($p < 0.05$) are indicated with *.

It is interesting to note that while albumin and fibrinogen, both major components of blood plasma, had no observed effect on the activity of either phage, serum did reduce phage infectivity. With albumin, the most abundant serum protein by mass, eliminated as the cause, naturally we can conclude there is another component in the serum that is causing the deactivation. The inactivation of phage by serum has been observed previously for two different T-even phages, where horse serum was believed to cause irreversible deactivation.[24]

The EPS extracted from each host bacterium had a different effect in each phage-host system. In the case of T4, there was a slight reduction in infectivity in the presence of EPS from *E. coli*, but the extent of inactivation was not significant when compared to the control. In contrast, significant inactivation of PRD1 was observed at high concentrations of EPS from *S*. Typhimurium. The different behaviors of the two phage in the presence of EPS can be explained by recalling the different structures, and mechanisms of binding, that are associated with each phage. The prepared EPS suspensions likely contain some lipopolysaccharides (LPS) and membrane proteins from the host bacteria,[25] which are good candidates for interacting with the phage in suspension. In the case of PRD1, the phage attaches to the host bacterium with its P2 protein via a receptor on the bacterial membrane.[26-27] This protein-receptor interaction has a high measured affinity with an irreversible binding process that triggers the injection of DNA into the host.[28] Thus, the presence of bacterial membrane proteins in the EPS preparations would then give a plausible explanation for the reduced titer of PRD1 in the presence of its host EPS: binding of proteins (present in the EPS mixture) to the PRD1 P2 protein may cause PRD1 to simply release its DNA in suspension instead of

infecting a host bacterium. In the case of T4 binding to *E. coli* B, the process is considered to occur in two steps with the first being the reversible binding of the long fibers to diglucosyl residues on the LPS in the bacterial cell wall.[29] Only after at least three long fibers have attached will the phage induce a conformational change and become irreversibly bound.[29] Therefore, due to the reversible nature of the first step in the T4 binding process, the inactivation of T4 by the LPS in the EPS mixture can conceivably be less pronounced than an irreversible binding process would be. The result is only the small reduction in activity that was observed.

Another likely explanation for the different effects of EPS on the infectivity of T4 versus PRD1 phage is the variable affinity of the different EPS molecules for the phages. Binding of different EPS components to the phage surfaces can lead to different electrostatic, electrosteric, and/or hydrophobic interactions between the phages and their respective host cells.

2.3.2 Bacteria Capture on Phage-Functionalized Surfaces

Surfaces functionalized with phages PRD1 and T4 were prepared as detailed in the Materials and Methods section. Interferents cannot be expected by default to affect free and immobilized phage similarly; free phages are able to diffuse through a liquid medium and orient themselves in a manner to facilitate attachment to receptors on the host bacteria and infect the host. In contrast, both diffusion and orientation are inhibited for immobilized phage and it is the bacteria that must approach the phage to be captured. Representative images of bacteria attached to the phage-coated surfaces are shown in Figure 2. A scanning electron micrograph of *Salmonella* attached to a PRD1-functionalized model substrate (glass disc) is depicted in Figure 2a. The electron

micrograph clearly indicates the presence of phage on the glass surface. The image is fairly representative of the whole sample and demonstrates high surface coverage with a uniform distribution over the entire surface. Figure 2b shows a fluorescence micrograph of *Salmonella* attached to the PRD1-functionalized surface, which is representative of fluorescence images obtained during the bacterial capture experiments. The presence of phage on the surface significantly enhanced the number of attached bacteria compared to a similar surface without any phage (Figure 2c). The bacteria on the surface were also found to be fairly uniformly distributed, likely an effect of the even distribution of the phage on the surface as well as the random nature of bacterial diffusion toward the phage-functionalized substrate.

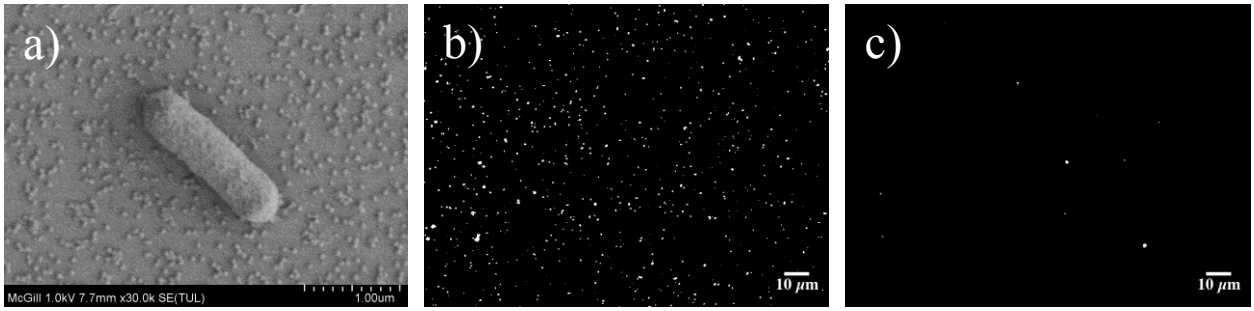

Figure 2. (a) Scanning electron micrograph and, (b) fluorescence micrograph of *S.* Typhimurium attachment to a PRD1-coated model substrate. (c) Fluorescence micrograph of *S.* Typhimurium attachment to a control surface without PRD1. The fluorescence images have been converted to black and white for clarity. The bacteria are shown in white over a black background.

Bacteria attachment to the phage-coated surfaces was quantified in the presence of a number of interferents of interest in environmental or biomedical applications. The results are summarized for two systems: PRD1 with *S.* Typhimurium (Figure 3a), and T4

with *E. coli* (Figure 3b). Both systems were evaluated in the presence of EPS from the respective bacterial host, humic acid, fulvic acid, a 1 μm model colloidal polystyrene latex particle, and natural groundwater. The systems were also evaluated against simple and complex biological interferents, namely human serum, albumin, and fibrinogen. As a control treatment, bacteria attachment to a phage-functionalized surface was assessed in clean buffer free of any potential interferents. Each condition was also tested with a blank; namely, bacteria attachment was assessed on a surface without any bacteriophage.

It is interesting to note that while humic and fulvic acids did not cause phage inactivation (Figure 1a, b), they did reduce the capture efficiency of immobilized T4 (but not PRD1) by over 60% in each case (Figure 3). This suggests that humic and fulvic acids may not be damaging phage T4, but rather preventing bacterial attachment to the immobilized phage. While no published study has reported on the interactions of T4 with humic or fulvic acids, there have been reports on other viruses. Bacteriophage MS2 has been observed to form reversible complexes with fulvic acid,[11] and poliovirus has been found to interact with humic and fulvic acids.[30] Thus, it is likely that these organic acids can interact with T4 in a manner that would interfere with the ability of the T4 receptors to attach to their host bacteria. The reversibility of this interaction would also explain why no effect was seen when determining suspended phage inactivation when the phage and bacteria were able to diffuse more freely to a favorable binding position. Humic acid and fulvic acid, both complex natural organic acids and well known chelating agents have some key differences. Fulvic acid, relatively smaller in molecular weight, is an organic compound with higher oxygen content mostly manifesting itself in the form of carboxyl groups thereby making fulvic acid more acidic.[31] Despite these differences, both fulvic

acid and humic acid were found to behave similarly in this study with regards to their effects on capture efficiency. These differences would mostly manifest themselves as differences in electrostatic attraction, steric hindrance, and hydrophobicity. From this study, we can conclude that either the differences were too subtle to detect, or they do not play a large role in the interaction between the phage, bacteria, and humic and fulvic acids. Additional studies would be required to further explore this phenomenon.

For both PRD1 and T4, the presence of the host EPS in suspension during bacterial capture on the surface resulted in fewer bacteria attached when compared to the control. Also, a higher concentration of EPS resulted in greater interference in bacterial binding. For PRD1-functionalized surfaces, this corresponded to a 20% reduction in bacterial capture at 20 mg/L and a 70% reduction at 100 mg/L of EPS. For T4-functionalized surfaces, the reduction was 50% and 80% compared to the control for the respective EPS concentrations at 20 mg/L and 100 mg/L. The effect observed with PRD1 may be explained by the irreversible attachment of EPS proteins to the phage as described in the evaluation of phage inactivation (Figure 1e). The occupied receptors on PRD1 would prevent bacterial attachment. When considering the T4-functionalized surface, a similar mechanism is likely taking place; the LPS in the EPS mixture is likely binding to the phage fibers, thereby blocking the attachment to bacteria. There was a greater effect of EPS on immobilized T4 bacteria capture efficiency (Figure 3b) than there was on T4 inactivation (Figure 1e). This difference may occur because, when suspended, T4 is free to diffuse and favorably interact with its host cell, but when immobilized, the T4 is unable to reorient itself, thereby preventing bacterial attachment.

The presence of a model environmental colloid (latex microsphere) did not interfere with the bacterial capture efficiency of either phage (Figure 3). Likewise, exposure to a natural unfiltered groundwater did not lead to a measurable change in bacterial capture. It has been hypothesized that some tailed phages may be polarized in charge, with the tail fibers exhibiting positive charge and the phage head exhibiting negative charge.[32] If we accept this notion of polarity, it is expected that the negatively charged latex particles attach to and block the capture proteins on the positively charged tail fibers of T4, affecting its capture efficiency. The lack of such an effect could be interpreted as the absence of this polarity for T4 or the relative weakness of the positive charge on the tail fibers, such that the electrostatic attraction is overcome by the shear induced by rinsing the phage-functionalized surface.

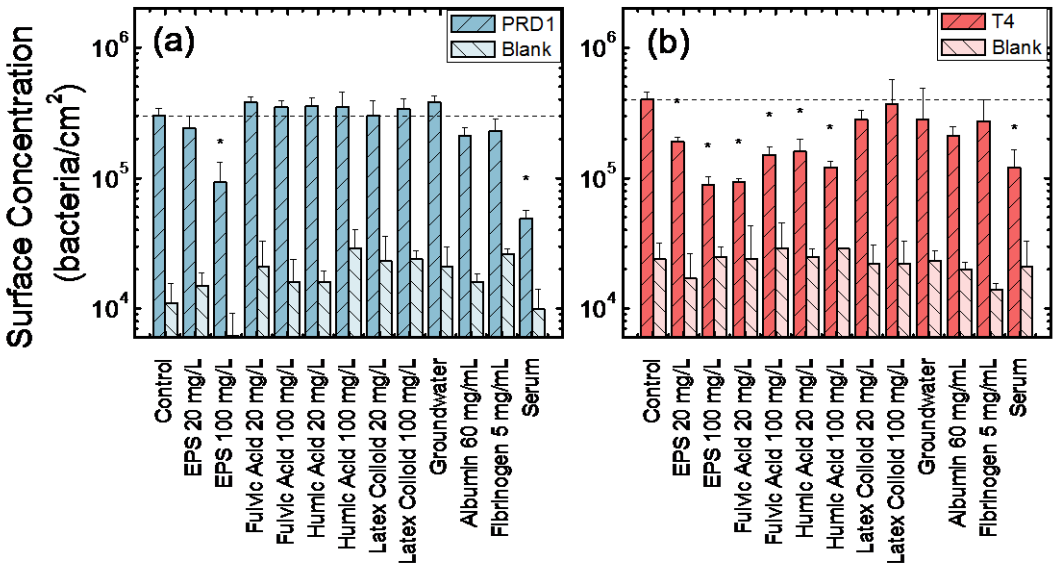

Figure 3. Number of bacteria attached to the covalently immobilized bacteriophage for (a) PRD1 and (b) T4, in the presence or absence of potential interferents of interest. Blanks are the same treatment in the absence of bacteriophage. Data significantly

different from the control ($p < 0.05$) are indicated with *. Data represent the mean ± standard deviation.

For both the PRD1/*S.* Typhimurium and T4/*E. coli* systems, the presence of serum led to an 85% and a 70% reduction in the amount of bacterial attachment to the phage-functionalized surface, respectively. This result is likely related to the direct inactivation of the phage, as observed in the phage inactivation assay (Figure 1f). Indeed, a greater extent of bacterial capture interference is noted for PRD1 (Figure 3a), which correspondingly experienced greater inactivation in the presence of serum (Figure 1f): a 45% reduction in PRD1 activity is observed versus a 20% reduction for T4. The presence of fibrinogen or albumin did not lead to a significant reduction in the bacteria capture efficiency of either phage (Figure 3).

In examining the results presented in Figure 3, it appears that the presence of interferents affects the capture efficiency of T4 phage more than PRD1. This could be explained by the difference in shape, mode of infection and molecular composition of the phage capsid for the two phages. T4 and PRD1 have different capture proteins with affinities for different molecules on the bacterial host cell surface. Therefore, certain interferents may bind to one and not the other. Furthermore, PRD1 contains lipids in its protein coat and is thus expected to be relatively hydrophobic whereas T4 is a hydrophilic phage.[33] The relative hydrophobicity of PRD1 could decrease its interaction with the interferents in the medium[34], hence explaining the lower effect of interferents on the efficiency of immobilized PRD1. Moreover, T4 is an asymmetric phage that must be immobilized on the surface with its tail fibers oriented away from the surface to capture bacteria. The orientation of the T4 population immobilized on the surface cannot be

controlled with conventional methods and hence is a mixture of three possible orientations (tail facing away from the surface, tail attached to the surface and phage lying on the side).[10] Therefore, a portion of the T4 population is completely inactive (tail attached to the surface) and another portion has a completely favorable orientation (tail pointing away from the surface). A third subpopulation of the immobilized T4 phage is lying sideways on the surface and can still function in capturing bacteria (although with a lower efficiency). The presence of macromolecular interferents in the medium can readily interfere with the already weak capture ability of the latter subpopulation of T4 phage by introducing steric hindrance and electrostatic repulsion.

2.4 CONCLUSIONS

The efficiency of phage-functionalized surfaces was assessed when subjected to biomolecules/particulate matter found in environmental or biomedical samples for two model bacteriophages, PRD1 and T4. The aim of this study was to determine whether potential applications involving phage-functionalized surfaces would face challenges when used under more complex environmental or biomedical conditions. Overall, PRD1 and T4 performed well, with an exception being in the presence of serum. Serum was found to reduce the activity of both phages and resulted in fewer bacteria attaching to their respective phage-functionalized surfaces. EPS from the host bacterium also diminished the ability for the immobilized phages to capture their hosts. The natural organic materials, humic and fulvic acids, both reduced the capture efficiency of T4 when immobilized, but did not cause any irreversible inactivation. These results demonstrate that environmental and clinical interferents can affect the efficiency of phage-functionalized bioactive surfaces even if phage infectivity is not affected. Furthermore,

this research highlights the need for these bioactive substrates to be tested under conditions relevant to the final application.

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CHAPTER 3: CONCLUSIONS AND SUGGESTED FUTURE WORK

This thesis determined the bacteriophage host capture efficiency of two model phage-functionalized surfaces, PRD1 with *Salmonella* Typhimurium and T4 with *Escherichia coli*, in the presence of potential environmental and biomedical interferents. Serum was found to reduce the efficiency of both immobilized phage systems, and directly inactivate these phages in suspension. Extracellular polymeric substances (EPS) from the host bacteria were also found to decrease bacterial attachment to both phage-functionalized surfaces. Humic and fulvic acids did not cause phage inactivation, but were found to reduce the bacteria capture efficiency of the T4 coated substrate. This emphasizes the importance of testing phage-functionalized surfaces and not just phage in suspension because there is more to the phage-host interaction on surfaces than the measured inactivation kinetics.

While model surfaces are excellent proofs-of-concept, the development of a potentially applicable bioactive surface would allow for the collection of even more relevant data and move the creation of bioactive bacteriophage products closer to reality. This study has helped foster an appreciation for the complexity involved when designing phage-coated surfaces, and all the factors that need to be considered. From phage immobilization and orientation, phage-host interactions, physical and chemical properties of phage, potential interfering agents, and the environmental and ecological impact of the rise of phage products there is a great deal of room for fundamental and applied studies in almost every aspect needed for the design of bioactive surfaces and biosensors.