Controlled Fabrication of Bacteriophage Based Interfaces: Understanding and Applications

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Bacteriophages have emerged as a tool for functional material development, such as bacterial sensors, capture systems and antimicrobial surfaces. The fabrication and application of bacteriophage based interfaces however still remain a challenge due to the lack of understanding in the mechanism of virus-surface interactions and the contribution of such interactions to the end-point applications. This thesis reports the use of controlled surface modification for the adsorption of bacteriophage with the ultimate aim of being able to better understand the mechanism and critical factors involved in virus-surface interactions, as well as the influence of such interactions to the end-point applications.

The work starts with the control surface modification of planar indium tin oxide (ITO) as a model substrate for T4 bacteriophage adsorption to establish the mechanism and surface physico-chemical properties involved in the virus-surface interactions. The T4 bacteriophage adsorption to planar and particulate ITO suggest the significance of substrate physical configuration to the adsorption of bacteriophage. The importance of surface chemical properties is also reflected on the adsorption of T4 onto bare, $-NH_2$ and -COOH functionalised planar ITO.

The second part of the thesis focuses on the use of bacteriophage conjugated magnetic particles (Fe_3O_4) for the rapid capturing and isolation of *Escherichia coli*. It was revealed for the first time that a successful bacteria capturing requires not only an optimum tailoring of the particle's surface physicochemical properties for high bacteriophage loading, but also an in-depth understanding of how external factors, such as temperature and solution chemistry influence the subsequent bacteriophage-bacteria interactions.

The last part of the thesis explores the antimicrobial activity of bare and functionalised ITO conjugated with T4 bacteriophage to *E. coli*. The variation in bacteriophage loading appears to have minimum influence to the antimicrobial activity, which reveals the paramount importance of understanding the mechanism of action of the end-point applications as it provides an insight into factors that count to such activity. The presence of food components, such as casein and starch did not interfere with the antimicrobial activity of ITO/T4, meanwhile the variations in solution pH, specifically at pH 5 was found to significantly impede the antimicrobial activity of ITO/T4.

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Abstract

Bacteriophages are viruses that have emerged as a tool for functional material development, such as bacterial sensors, capture systems and antimicrobial surfaces. The fabrication and application of bacteriophage based interfaces however still remains a challenge due to the lack of understanding of the mechanism of virus-surface interactions and the contribution of such interactions to the end-point applications. This thesis reports the use of controlled surface modification for the adsorption of bacteriophage with the ultimate aim of being able to better understand the mechanism and critical factors involved in virus-surface interactions, as well as the influence of such interactions to the end-point applications.

The work starts with the control surface modification with amine ($-NH_2$), carboxylic (-COOH) and methyl ($-CH_3$) surface functional groups of planar indium tin oxide (ITO) as a model substrate for T4 bacteriophage adsorption to establish an understanding on the mechanism and surface physico-chemical properties involved in the virus-surface interactions. A comparative T4 bacteriophage adsorption study between functionalised planar and particulate ITO suggests the significance of substrate physical configuration to the adsorption of bacteriophage. The importance of surface chemical properties is also reflected on the adsorption of T4 onto bare, $-NH_2$ and -COOH functionalised planar ITO, which suggests the presence of a unique binding behaviour involving specific functional groups on planar ITO surfaces.

The second part of the thesis focuses on the use of bacteriophage conjugated magnetic particles (Fe_3O_4) for the rapid capturing and isolation of *Escherichia coli* (*E. coli*). The investigation of T4 bacteriophage adsorption to silane functionalised Fe_3O_4 with amine, carboxylic and methyl surface functional groups reveals the domination of non-specific net electrostatic and hydrophobic interactions in governing bacteriophage adsorption to particle. It was revealed for the first time that successful bacteria capturing requires not only an optimum tailoring of the particle's surface physicochemical properties for high bacteriophage loading, but also an in-depth understanding of how external factors, such as temperature and solution chemistry influence the subsequent bacteriophage-bacteria interactions.

The last part of the thesis explores the antimicrobial activity of bare and functionalised ITO conjugated with T4 bacteriophage to *E. coli*. The mechanism of their antimicrobial activity was found to be due to the release of T4 bacteriophage from ITO/T4. The variation in bacteriophage loading appears to have minimum influence to the antimicrobial activity, unlike the above findings. This reveals that paramount importance of understanding the mechanism of action of the end-point applications as it provides an insight into factors that count to such activity. The presence of food components, such as casein and starch did not interfere with the antimicrobial activity of ITO/T4, meanwhile the variations in solution pH, specifically at pH 5 was found to significantly impede the antimicrobial activity of ITO/T4.

This thesis shows that fundamental understanding of virus-surface interactions is paramount in the fabrication and application of bacteriophage based interfaces. It also reveals that factors influencing the efficacy of bacteriophage based interfaces varied depending of the mechanism of action of the end-point applications. These knowledge should provide a scaffold for better fabrication of bacteriophage based interfaces for a variety of applications.

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List of Publications

Journal Publications:

1. Liana AE; Chia EW; Marquis CP; Gunawan C; Gooding JJ; Amal R. Adsorption of T4 Bacteriophages on Planar Indium Tin Oxide Surface via Controlled Surface Tailoring. Journal of Colloid and Interfaces Science. 468: 192-9. (DOI: 10.1016/j.jcis.2016.01.052)
2. Liana AE; Marquis CP; Gunawan C; Gooding JJ; Amal R. T4 Bacteriophage Conjugated Magnetic Particles for *E. coli* Capturing: Influence of Bacteriophage Loading, Temperature and Tryptone. Colloids and Surfaces B: Biointerfaces. (Accepted with minor revision)
3. Liana AE; Marquis CP; Gunawan C; Gooding JJ; Amal R. Antimicrobial Activity of T4 Bacteriophage Conjugated Indium Tin Oxide (ITO). ACS Applied Materials and Interfaces. (In Preparation)

Conference Proceedings:

1. Liana AE; Lim M; Marquis C; Gooding JJ; Amal R, 2014, 'Understanding the role of Surface Functionalisation of Magnetic Iron Oxide on T4 Bacteriophage Attachment', in ICONN 2014 - Proceedings of the 2014 International Conference on Nanoscience and Nanotechnology, Australian Nanotechnology Network, Adelaide SA Australia, 2nd – 6th February 2014
2. Liana AE; Lim M; Marquis C; Gooding JJ; Amal R, 2012, 'Immobilization of T4 bacteriophages on modified magnetic nanoparticles surface', in ICONN 2012 - Proceedings of the 2012 International Conference on Nanoscience and Nanotechnology, Australian Nanotechnology Network, Perth WA Australia, 5th – 9th February 2012

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Chapter 1 Introduction

Bacteriophages are viruses that infect bacteria and use their host bacterium as a factory for their own replication.[1] They were first discovered independently between 1915 to 1917 by Frederick Twort and Felix d'Herelle.[2-3] Their ability to infect bacteria immediately attracted the attention of many researchers who were looking for effective cures for infectious diseases. It was reported that the use of bacteriophage in the water supply to control cholera outbreaks in India from 1933 to 1935 resulted in a significant reduction in the outbreak length to 48 h from the average length of 26 days.[3] Nonetheless, the bacteriophage research was abandoned during 1940s largely due to the discovery of penicillin as an antimicrobial agent and the lack of sufficient knowledge on the mechanism behind bacteriophage-mediated bacteria killing.[3]

The rise of multi-drug resistant bacteria has rekindled interest in bacteriophage research, with bacteriophage now being exploited beyond their potential therapeutic benefits. Many studies have placed more emphasis on the development of bacteriophage based interfaces, such as bacteriophage based biosensors,[4-5] phage-based biosorbents for bacteria concentration[6-8] and bacteriophage based antimicrobial surfaces[3,9] that exploited their high degree of specificity,[10] and their ability to infect[9-10] and identify viable from non-viable bacteria.[11-12]

The fabrication of bacteriophage based interfaces, such as biosensors, antimicrobial surfaces and phage-based biosorbents for bacterial concentration necessitates a fundamental understanding of virus-surface interactions; in particular, those that govern the adsorption of virus onto surfaces in a controlled manner. However, many studies have focused solely on the end results and put less emphasis on understanding the mechanisms and factors that influence their performance. As a result, there is abundant literature on the applications of bacteriophage based interfaces, however, commonly the reported results are contradictory. For instance, higher wild-type T4 bacteriophage loading has been reported to increase the bacteria capture density.[13] Interestingly, when similar surface functionalisation was employed to improve the loading of methicillin-resistant *Staphylococcus aureus* bacteriophage, there was no increase in the bacteria capture density as reported by Tawil and co-workers.[14] Furthermore, Tolba and colleagues reported a comparable high loading of genetically engineered T4 bacteriophage to magnetic beads as that of the cellulose beads.[6] However, bacteriophage conjugated cellulose beads did not show any bacteria capturing ability, unlike bacteriophage conjugated magnetic beads. It remains unclear as to whether the inconsistency observed arises from the different applications or the fabrication method of bacteriophage based interfaces itself or possibly both.

Similarly, many reports that focus on the fabrication of bacteriophage interfaces and their interactions are often unexpected. For instance, the high surface hydrophobicity and isoelectric point (IEP) were reported to be responsible for the adsorption of M13 filamentous bacteriophage onto a planar carbon grid.[15] On the contrary, high surface

hydrophobicity had limited influence on the adsorption of similar bacteriophage to a hydrophobic gold coated surface.[16] High adsorption of dengue virus to low IEP SiO_2 wafer was reported, [17] meanwhile the low IEP of SiO_2 surface was found to induce poor M13 filamentous bacteriophage adsorption.[15]

With the lack of comprehensive understanding of virus-surface interactions and their influence on end-point applications, it is therefore challenging to improve the performance of bacteriophage based interfaces. The work undertaken in this project hence revolves around addressing the central issues to better understand the mechanisms and critical factors involved in virus-surface interactions and the influence of such interactions to the end-point applications. The aims and objectives are fully enunciated below.

1. To devise a model surface using indium tin oxide (ITO) with organosilane grafting for controlled surface tailoring of its physico-chemical properties, which include ITO's physical configuration, surface roughness, different chemical moieties, surface charge and surface hydrophobicity and investigate the mechanism and the pre-dominant ITO surface physico-chemical properties that factor into the adsorption of T4 bacteriophage to bare and functionalised ITO surface. Herein, ITO is chosen due to its stability under physiological conditions and tuneable physico-chemical properties via surface modifications.[18-20]

2. To study the applicability of controlled surface functionalisation of iron oxide magnetic particles (Fe_3O_4) conjugated with T4 bacteriophage as a magnetic separation system that is culture independent towards bacteria capturing. Iron oxide magnetic particles serve as a scaffold for tailored surface modifications. The influence of virus-surface interactions and other external factors, such as temperature and solution chemistry to bacteria capturing performance will also be determined.

3. To determine the antimicrobial activity of bare and functionalised ITO conjugated with T4 bacteriophage and study their mechanism of antimicrobial activity and critical parameters, such as pH and the presence of food components (i.e. casein and starch) that govern their activity.

A review of the current literature focusing on structure and applications of bacteriophage, current state of the art of virus-surface interactions and factors influencing their interactions is presented in Chapter 2 to establish the current state of understanding. The controlled functionalisation and conjugation of ITO with T4 bacteriophage to study the influence of surface physico-chemical properties to T4 bacteriophage adsorption and their mechanism of interactions is discussed in Chapter 3. Chapter 4 describes the applicability of controlled functionalisation of Fe_3O_4 conjugated with T4 bacteriophage in capturing bacteria and the factors involved to achieve successful bacteria capturing was addressed. In Chapter 5, the antimicrobial activity of ITO conjugated T4 bacteriophage developed in Chapter 3 was investigated. The mechanism of antimicrobial

activity was assessed and the variations in factors governing the antimicrobial activity of ITO/T4 were compared to the findings in Chapter 4. Chapter 6 concludes the work with a discussion on major findings and the contribution of this thesis, followed by the scope for future exploration on the fabrications and applications of bacteriophage based interfaces.

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Chapter 2 Literature Review

2.1 Introduction

Bacteriophages (phages) are bacterial viruses which can have a broad host range or infect specific species or even strains of bacteria.[1] They are parasites that lack their own metabolism and are ubiquitous in nature.[2-3] Recent years have witnessed the increasing use of bacteriophages for various applications, namely biosensors,[4-7] phage based biosorbents,[8-9] antibacterial surfaces[10-11] and membrane filtration.[12] These bacteriophage based interfaces have exploited the inherent merits of bacteriophage, such as their specificity, ability to infect bacteria and their physicochemical properties, such as surface charge and surface hydrophobicity.[10,13] Such applications require the fabrication of materials with controlled surface properties that promote or inhibit bacteriophage adsorption.

This chapter will first review the interesting structures and compositions of bacteriophages, followed by relevant applications of bacteriophage based interfaces. An extensive discussion on the fundamentals of virus-surface interactions, such as electrostatic interactions, van der Waals and hydrophobic interactions is introduced. Particular emphasis is also placed on the current understanding of factors that elicit

different behaviour of virus adsorption to surfaces. Surface modification techniques are also presented as they are an essential tool to understand virus-surface interactions.

2.2 Structure and Compositions of Virus/Bacteriophages

A bacteriophage consists of a protein envelope known as a capsid, where the bacteriophage's genome is packaged, and receptor-binding proteins.[14-17] In some bacteriophages, however, the protein capsid is additionally enclosed by an envelope consisting of both glycol- and lipoproteins.[15-16] Bacteriophages may also have different shapes and sizes, which include tailed, filamentous and icosahedral (Figure 2-1).[1]

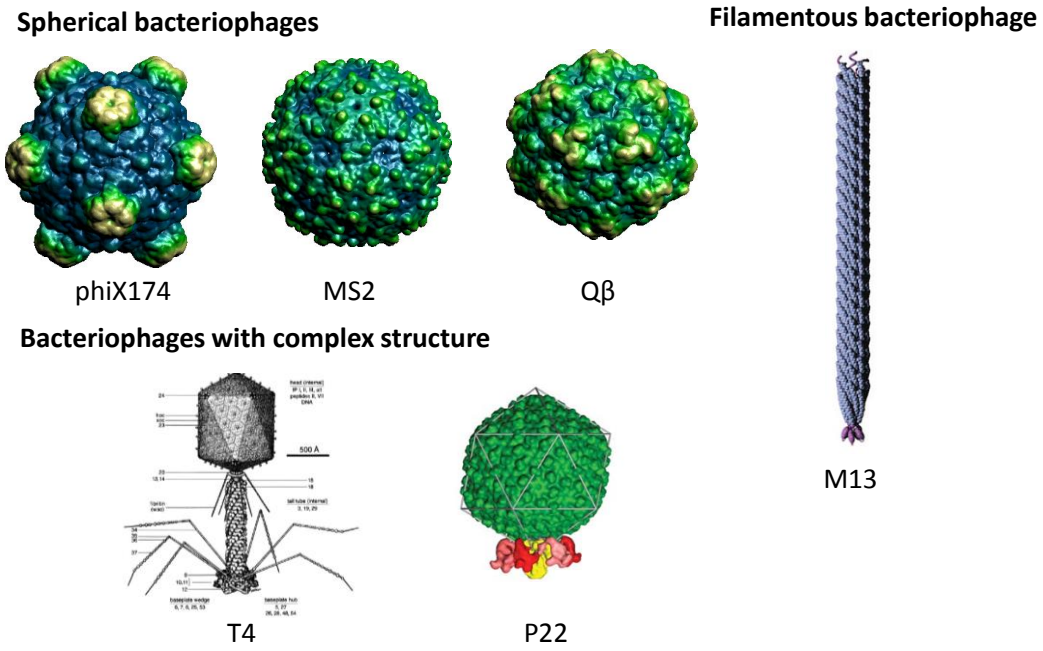

Figure 2-1 Different types and morphology of bacteriophages. Images of the Qβ, MS2 and phiX174 were reproduced from the VIPER database.[18] The structure of T4 bacteriophage, M13 and P22 were reprinted from Ref. 19,20 and 21, respectively.

The specificity of phages is mediated by the receptor-binding proteins, which recognise a defined surface molecule on susceptible cell target. The location of the receptor-binding proteins varies; it could be located on a pole for filamentous phages, all around the capsid, located asymmetrically on one vertex or at the tip of the tails.[1] Tails are protein structures that attach to the capsid and assist in the binding to the bacteria cell (Figure 2-2). A majority of the bacteriophage are tailed, with a variety of configurations; for instance long or short, rigid or flexible, contractile and non-contractile.[16]

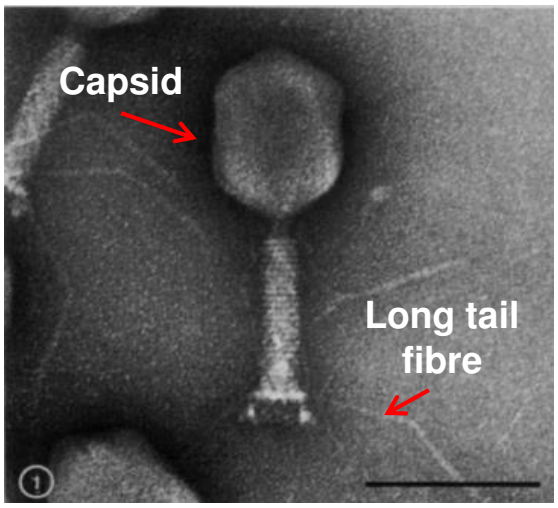

Figure 2-2 Morphology of tailed T2 bacteriophage.[22]

Bacteriophage infects its host bacterium by two different pathways, lytic or lysogenic cycle, which are largely dependent on both bacteriophage and host bacterium properties and conditions.[3,23] A lytic cycle involves the bacteriophages puncturing the bacterium cell wall, releasing its DNA or RNA, rapidly multiplying within the host bacteria cell and eventually releasing bacteriophage progeny at the expense of host bacteria death and lysis. The general lytic cycle for tailed phages is shown in Figure 2-3. The bacteriophage encounters the bacterial host during random movement and infection is initiated when

specialised adsorption structures, such as tail fibers or spikes, which contain the receptor binding domain, bind to the specific surface molecules or capsules on the target bacteria, namely oligosaccharides, teichoic acid, peptidoglycan, protein and lipopolysaccharide.[3,24] The attachment of some phages often involves a two-step process and two receptors. For instance, T4 phage has to bind with at least three out of six of its tail fibers to the primary receptor, in order to trigger the base plate rearrangement, which unravels the short tail fiber. This then leads to irreversible binding to the second receptors.[24] After irreversible binding, the bacteriophage genome penetrates into the cell and transcribed by host cell RNA polymerase, producing 'early' mRNA, that leads to the arrest of host cell transcription and re-directing it to produce new bacteriophage components.[3,25] These components are assembled into new bacteriophages. Following the complete assembly, the new bacteriophages are released from the host cell through host cell lysis that requires two enzymes, lysin and holing. Lysin is an enzyme that is capable of cleaving one of the main bonds in the peptidoglycan matrix, whereas holing is a protein that disrupts the peptidoglycan matrix allowing lysin to degrade the peptidoglycan and eventually cause the release of the bacteriophages.[3,24]

Unlike lytic cycle, lysogenic cycle does not bring about rapid lysis (Figure 2-3), instead the bacteriophage DNA is integrated into the host cell DNA. This DNA will be inherited by the daughter cell upon several rounds of host cell replication until certain external factors, such as exposure to ultraviolet light or presence of antibiotics induce the lytic cycle.[3,23,26]

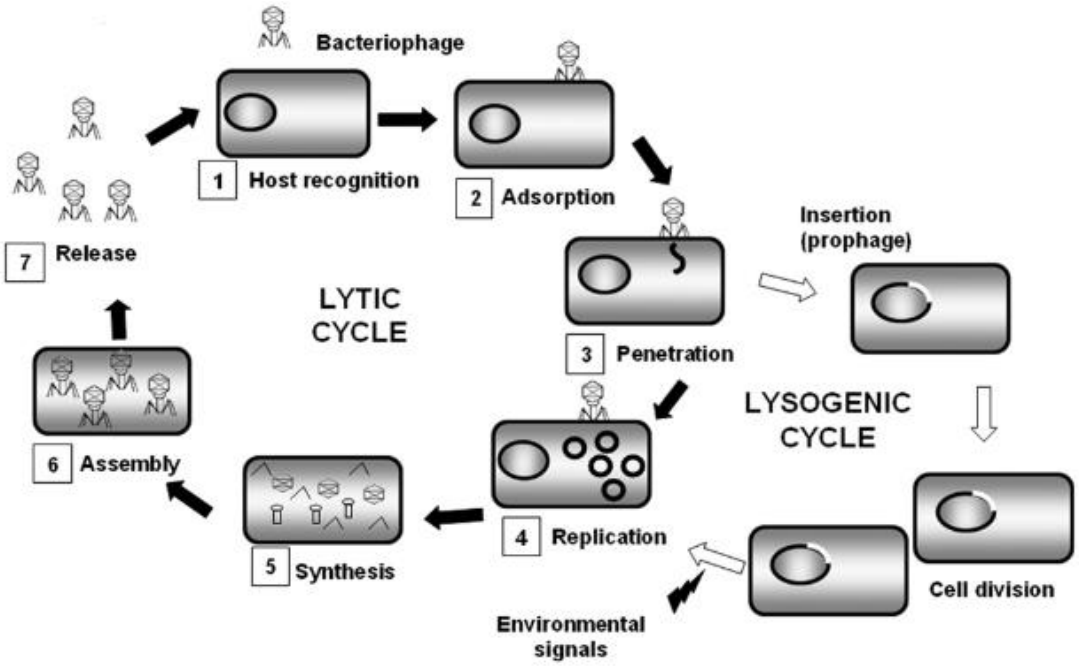

Figure 2-3 An overview of tailed bacteriophages lytic life cycle.[26]

2.3 The Advances in Bacteriophage Applications

2.3.1 Bacteriophages as an Antimicrobial Surface

With regard to the rising issue of surface contamination in healthcare environment,[10,27] microbial invasion towards open wound tissues[28] and indwelling medical devices infections,[29] there has been a surge in the development of antimicrobial surfaces. However, with the emerging crises of antimicrobial coating induced toxicity[10,27] and the presence of multi-drug resistant bacteria,[30] bacteriophage modified surfaces have emerged as a promising alternative due to bacteriophage self-reproducing ability that allows their multiplication as long as the host bacteria are still present, as well as their low toxicity towards eukaryotic cells and tissues.[29,31] The bacteriophages described herein are immobilised onto a surface to facilitate their controlled release over a long period of time.

The successful use of bacteriophage based antimicrobial surfaces was demonstrated by Curtin and Donlan on a neutrally charged hydrogel coated catheters.[32] The adsorption phage 456, coagulase negative staphylococcus phage to the catheters reduced the growth of *Staphylococcus epidermidis* by a 4.47-log reduction in the presence of divalent cations, as also evident by the scanning electron microscopy (SEM) images, which showed a decrease in the number of *S. epidermidis* in the biofilm (Figure 2-4B). A similar success was also reported by Kaur and associates[29] and Cooper and associates.[30]

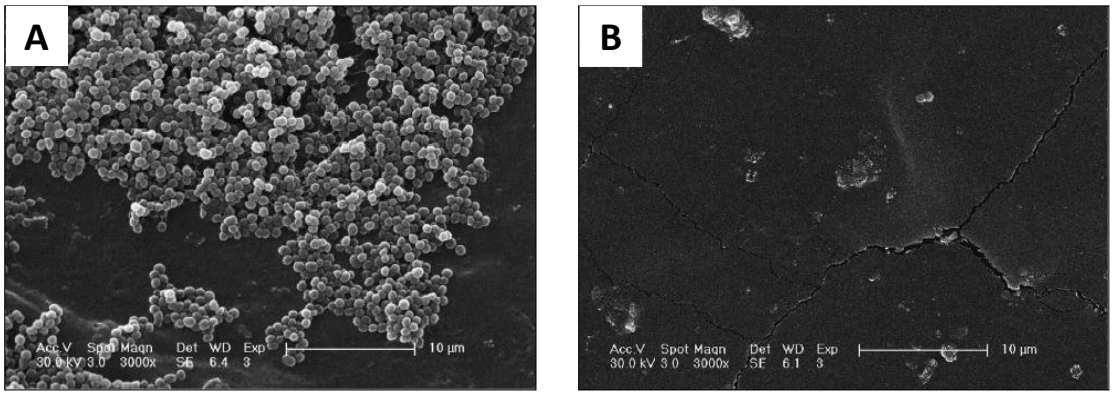

Figure 2-4 Scanning electron microscopy of a surface section of (A) catheter and (B) phage 456 coated catheter in the presence of divalent cations after *S. epidermidis* biofilm formation.[32]

Despite the success of bacteriophage as an antimicrobial agent, there is still a lack of fundamental understanding of their performance. The issues often center on the orientation of the receptor-binding protein on bacteriophage and bacteriophage loading upon immobilisation to surfaces.

Hosseinidoust and associates investigated the antimicrobial activity of different structure of bacteriophages, which were covalently bound to amine functionalised glass coverslips.[10] It was found that the position of receptor-binding protein on bacteriophage is critical in maximising the contact between bacteriophage and its host. For instance, high capturing of *Salmonella typhimurium* was achieved due to the availability of PRD1 and PR772 receptors on all vertices (Figure 2-5B). Interestingly, MS2 bacteriophage exhibited higher *Escherichia coli* capture than T4 bacteriophages, although both

bacteriophages have only one attachment site. The authors argued that MS2 has better accessibility to *E. coli* receptors than T4.

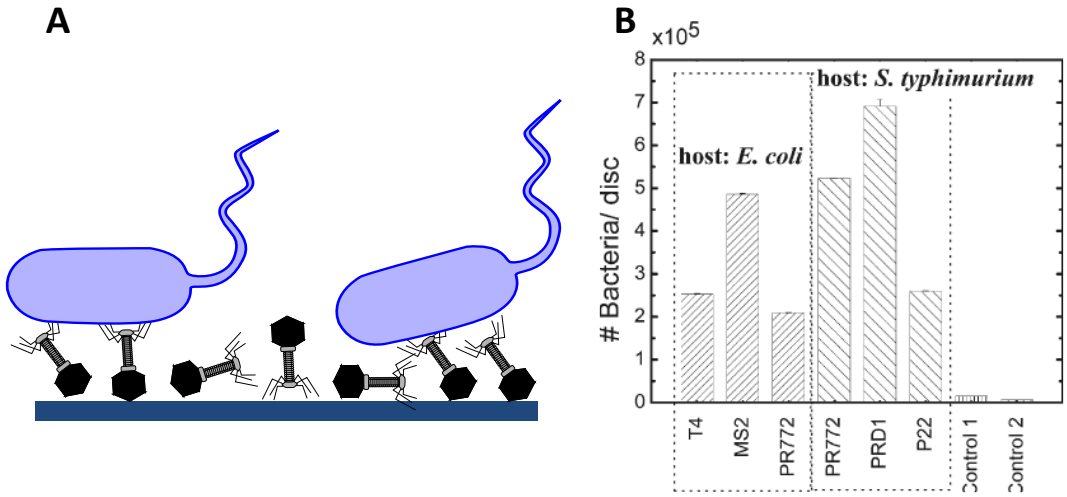

Figure 2-5 (A) Graphric representation of bacteriophage based antimicrobial surface and (B) the concentration of host bacteria captured by bacteriophage based disk.[10]

Meanwhile, the influence of bacteriophage loading on bacteriophage antimicrobial activity remains unclear. Anany and co-worker reported two different immobilisation strategies to promote the adsorption of bacteriophage by employing neutral and positively charged membrane.[33] At least a 6-fold increase in phage adsorption to the positively charge membrane compared to the neutrally charged membrane was observed. The increase in bacteriophage density on positively charged membrane however did not show any improvement in the antimicrobial activity, with both types of membrane showed similar activity.

2.3.2 Bacteriophages as a Surrogate Entity for Evaluating Virus Removal and Inactivation

Apart from their antimicrobial benefits, bacteriophage is often used to model human enteric viruses. With increasing incidence of enteric virus associated diseases, such as gastroenteritis, meningitis, hepatitis and heart anomalies that are commonly transmitted through surface water,[34] membrane processes are of increasing interest for the removal of waterborne viruses. However, testing the membrane performance in virus removal using the native viruses is far from practical. This is largely due to the difficulties in producing these viruses *in vitro*, hazards in handling pathogenic viruses and difficulty in analysing these viruses.[12] Therefore, many viral inactivation and removal studies prefer to employ "model viruses" which mimic the properties of potentially pathogenic viruses.

Bacteriophages have been used as a surrogate due to simple large scale production and well-established characterisation techniques. MS2 and Qβ (Figure 2-1) are F-specific single stranded RNA bacteriophages that have been extensively employed as a surrogate virus by the virtue of their similar morphological and structural resemblance to the human enteric viruses.[12,35] Other notable features include their inability to multiply in water environments and resistance to disinfection processes, such as sunlight,[36] chlorination,[37] chemicals and heat treatments.[35] MS2, in particular, makes an ideal worst-case strain for membrane performance studies, due to its small size, being highly negatively charged and hydrophobicity.[12,38]

The selection of membrane pore size is considered to be a critical aspect of virus removal using membrane filtration, since it is believed that the removal mechanism is predominantly due to size exclusion. There are several membranes with different pore size and molecular weight cut-off that are suitable for virus removal – virus sizes range between 0.01 - 0.1μm.[12,34,39] Microfiltration (MF) membranes have a pore size of ≥ 0.1 μm, whilst ultrafiltration (UF) membranes have a lower pore size of 0.001 - 0.1 μm. Nanofiltration (NF) membranes and reverse osmosis have a nominal pore size of 1 - 10 nm and ≤ 0.1 μm, respectively.[12] Although UF, NF and RO membranes showed good virus removal, complete removal of virus remains difficult.[12] For instance, ElHadidy and colleagues reported the better removal of MS2 bacteriophage compared to phiX174 despite the smaller size of the former, is postulated to result from electrostatic repulsion between MS2 and the membrane surface.[39] Other studies reported bacteriophage removal with MF membranes despite their large pore size. Van Voorthuizen and co-workers reported higher removal of MS2 achieved by hydrophobic MF membrane in comparison to the hydrophilic membrane at pH 3.9 and 7 with similar 0.22 μm nominal pore size.[38]

Anthony and colleagues reported that the intricate mechanism of virus removal is influenced by the interaction between the virus and membrane surface as well as the operating conditions, such as pH, solution environment and membrane charge.[12] A positively charged membrane will promote electrostatic attraction with net-negatively charged virus, meanwhile removal of virus by net-negatively charged membrane will require the presence of cations to form a salt bridging with the virus.[40] With varying

operating conditions being used, deriving a comprehensive mechanism of interactions between virus and surfaces remains a challenge.

2.3.3 Bacteriophages as a Recognition Agent

- **Biosensor Applications**

Due to their inherent specificity to certain strains of bacteria, bacteriophage have emerged as suitable recognition agents in applications such as biosensors and phage based biosorbents for bacterial isolation. A biosensor is an integrated analytical device that converts a biological response into an electrical signal. It potentially offers an alternative over the traditional pathogen detection methods, which may include high sensitivity, tuneable specificity, minimal sample preparation, cost effectiveness and a reduction in analysis time.[41-42] In general, a biosensor consists of a bio-recognition element, transducer and amplifier (Figure 2-6A). The specificity of the biosensor is dependent upon the type of bio-recognition agents used (Figure 2-6B). Recent advances in biosensors have utilised bacteriophages as a bio-recognition agent due to their high specificity even towards different strains of bacteria,[2,43] stability over a broad temperature and pH range,[2] resistance to enzyme degradation[43] and their ability to differentiate viable from non-viable pathogens.[8,13] The latter is especially crucial in order to avoid false positive (suggesting the presence of bacteria in the sample when it was not) or false negative (failing to detect the presence of pathogenic bacteria in the sample) detection. Bacteriophage derived biosensors have been used for detection of pathogens in food, including milk,[44-45] ground beef[45] and fresh tomato[46] with success.

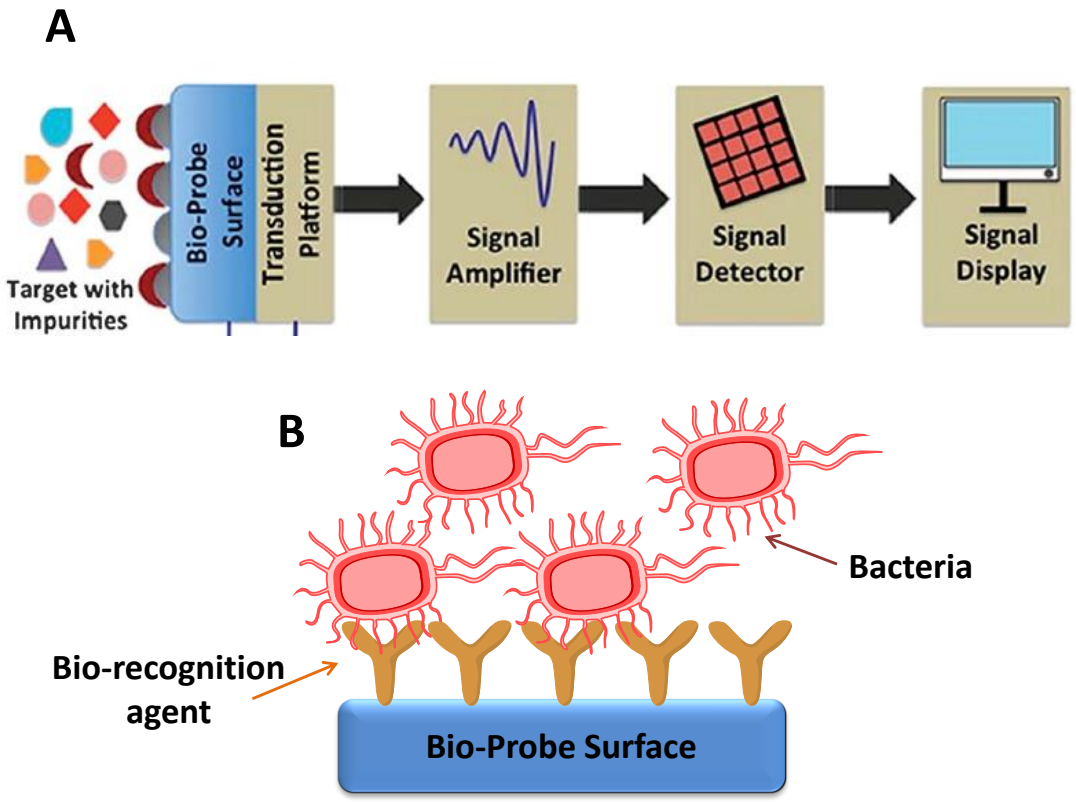

Figure 2-6 Schematic illustration of (A) biosensors[43] and (B) bacteria detection by bio-recognition agent on bio-probe surface.

Similar to bacteriophage based antimicrobial surfaces, effective immobilisation of bacteriophage to the transducer platform is a critical design element. Immobilisation techniques should promote bacteriophage loading and correct bacteriophage orientation, whereby its receptor-binding proteins remain exposed to recognise the target bacteria. Many studies have explored different methods of bacteriophage immmobilisations, from direct adsorption to bare surfaces[44,47] to chemical functionalisation of surfaces[4,48] to genetic engineering of bacteriophage surface,[49] however the results are often quite unexpected.

Balasubramanian and colleagues reported the detection of *S. aureus* with a detection limit of 10^4 colony forming unit (cfu)/mL using surface plasmon resonance, whereby lytic bacteriophages were adsorbed onto a bare gold surface of surface plasmon resonance sensors.[47] Nanduri and co-workers reported a comparable to a superior performance of adsorbed β-galactosidase specific bacteriophage based ELISA in comparison to the antibody based ELISA in detecting β-galactosidase from *Escherichia coli*.[6] Other studies have also demonstrated the success of adsorbed bacteriophage for detection of bacteria in fat free milk,[44] in *Bacillus anthracis* suspension[50] and on the surface of fresh tomatoes.[46]

Another strategy to promote the adsorption of bacteriophages to biosensor interfaces is via chemical functionalisation of the substrate. Arya and co-workers reported the adsorption of T4 bacteriophage to dithiobis(succinimidyl propionate) functionalised surface. This method of surface functionalisation promoted the bacteriophage loading via covalent bonding of T4 bacteriophages onto a gold surface.[5] Upon integration with surface plasmon resonance, this method achieved a specific detection of *E. coli* as low as 7×10^2 cfu/mL.

Further, Singh and colleagues revealed that chemical functionalisation of gold substrate with cysteine to provide amine functional groups on the surface led to a 7-fold improvement in bacteriophage attachment compared to direct adsorption to bare gold.[4] Further activation of the cysteine-gold surface with glutaraldehyde resulted in a dramatic 37-fold improvement in bacteriophage attachment compared to adsorption to bare gold

surface. The enhanced bacteriophage attachment has led to a 9-fold improvement in the bacteria capture density compared to bacteriophage conjugated bare gold surface. In contrast, Tawil and co-workers did not observe significant improvement in methicillin-resistant *Staphyloccocus aureus* bacteriophage loading and the respective bacteria capture efficiency on similarly cysteine-glutaraldehyde functionalised surface compared to its bare gold surface.[51]

- **Bacteria Isolation**

The use of bacteriophage as a recognition agent also extended to phage-based biosorbent for bacteria capture and isolation. This capture system is developed to aid the separation and concentration of target bacteria prior to bacteria detection step. This is especially useful in food applications, whereby the complexity of the food matrix, the presence of low level target bacteria and other microflora in food and water have placed a significant challenge for many rapid detection techniques, such as ELISA, polymerase chain reaction (PCR) and biosensors, which often rely on the availability of well-cleaned and concentrated samples.[52-56] Meanwhile, the conventional sample preparation technique, which involves sample enrichment steps are very lengthy and laborious.[52-53]

Magnetic particles have been used to magnetically control the separation and concentration of low level target chemical or biological materials with success.[57-61] Immunomagnetic separation technique exploited the use of bio-functionalised magnetic particles to selectively capture target bacteria through immunological reactions between the target bacteria and the bio-recognition agents.[62-63] The integration of bacteriophage as

a recognition agent and magnetic particles to isolate and concentrate target bacteria has been previously reported with a poor bacteria recovery (~20%).[9,64] Chen and co-workers recently reported 60 - 70% recovery of *E. coli* K12 using T7 bacteriophage conjugated with a mixture of iron and cobalt oxide nanoparticles (FeCo).[65]

Similar to bacteriophage based antimicrobial surfaces and biosensors, the immobilisation of bacteriophage to the particle surface is a critical pre-requisite. Several studies have exploited the bacteriophage immobilisation through genetic engineering of bacteriophage and surface chemical functionalisation. Although a massive improvement in bacteriophage loading has been observed, it did not always translate to better bacteria capture efficiency.

Edgar and co-workers demonstrated the immobilisation of phage using biotin tagged phage on quantum dot (QD) nanocomplexes for detection of *E. coli* (Figure 2-7).[66] The QDs were labelled using streptavidin and biotin interaction and attachment with bacteria was analysed under fluorescence microscopy. QD was able to detect as few as 10 bacterial cells per millilitre and a 100-fold improvement was observed after 1 h.

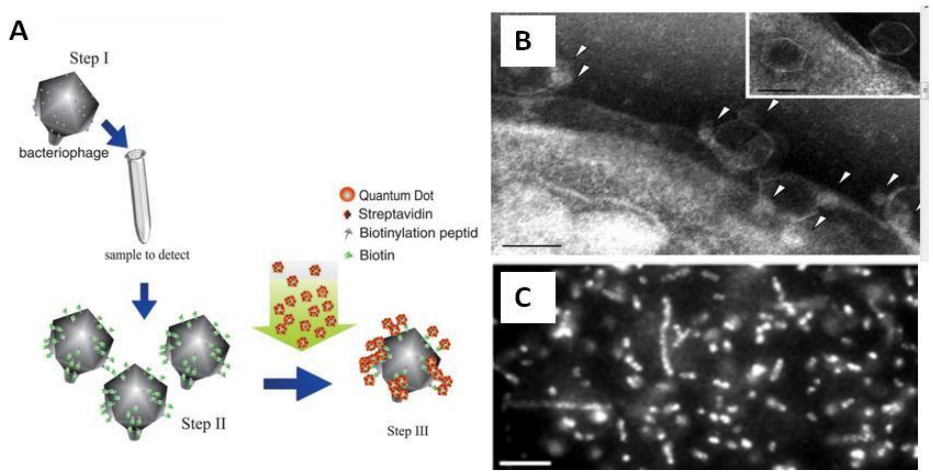

Figure 2-7 (A) Schematic attachment of quantum dots to biotin tagged bacteriophages, (B) Transmission Electron Microscopy (TEM) image of bacteriophage bound to streptavidin quantum dots, (C) fluorescent micrograph of cells with a 100-fold excess of bacteriophage on quantum dot nanocomplexes.[66]

Tolba and co-workers modified the soc capsid proteins of T4 bacteriophage with biotin carboxyl carrier protein (BCCP) and cellulose binding modules (CBM) to display a strong affinity peptide that bound to streptavidin coated magnetic beads and cellulose beads, respectively.[8] Both genetically engineered T4 bacteriophages resulted in at least 60% improvement in binding to their respective particles compared to the wild-type T4 bacteriophage. Interestingly, high bacteriophage loading did not translate to better capturing as evident by the undetectable level of bacteria captured by CBM-T4 on cellulose beads. Meanwhile, there is at least a 2-fold (~70%) improvement in bacteria captured by BCCP-T4 on magnetic beads at *E. coli* concentration of 10 to 10^5 cfu/mL compared to the wild-type T4 on magnetic beads. It is postulated that the poor orientation of immobilised T4 bacteriophage resulted in low capturing efficiency of CBM-T4.

Several reports on the use of bacteriophage as recognition agent for bacteria capture have utilised covalent linkage between bacteriophage and particle surface. For instance, Shahbani and co-workers reported the immobilisation of T4 bacteriophage to carboxylic functionalised magnetic beads via carbodiimide activation (EDC).[67] The resulted T4 bacteriophage conjugated magnetic beads had a binding efficiency between 70 - 80% when exposed to 10^4 - 10^8 cfu/mL *E. coli* concentration. The use of carboxylic acid functionalised magnetic beads to covalently bind T7 bacteriophage have also been reported by Chen and co-workers (Figure 2-8), with the calculated number of T7 phage per particle to be 1,871 plaque forming unit (pfu).[68] It has also been reported that an excessive 40-fold increase in bacteriophage loading per particle resulted in only one-fold increase in bacteria caputring.[63]

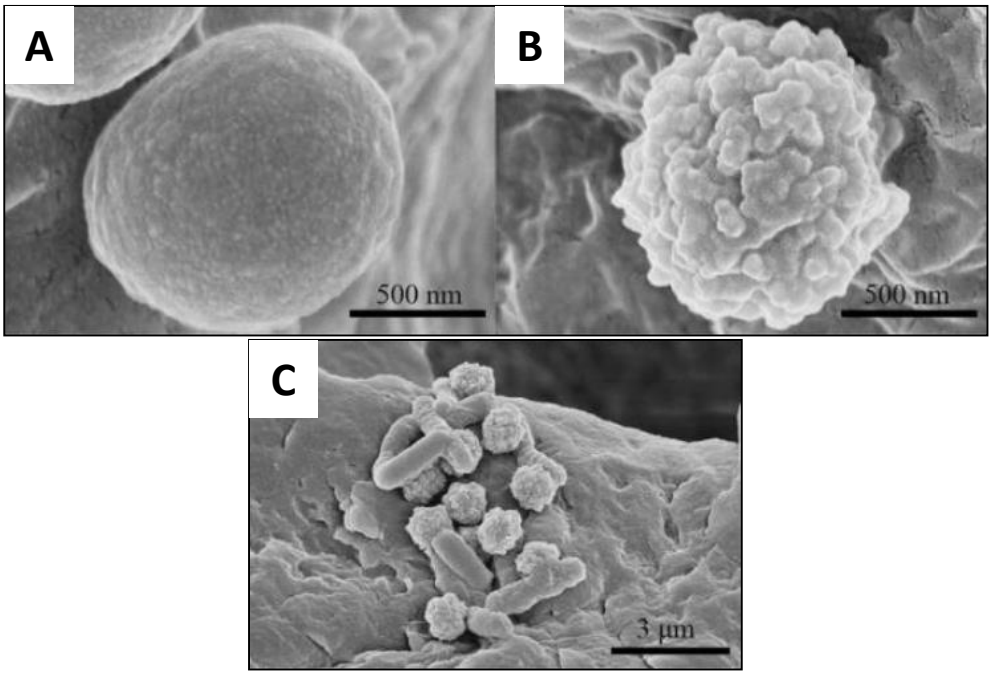

Figure 2-8 SEM images of (A) carboxylic acid functionalised magnetic bead (B) T7 bacteriophage conjugated magnetic bead and (C) *E. coli* attached to T7 bacteriophage conjugated magnetic beads.[68]

2.4 Challenges in Bacteriophage Based Interfaces

The above studies highlight a good success of various applications of bacteriophage based interfaces. However, it is also clear that there are some unexpected findings that are shared across varying applications. The variations in immobilisation techniques employed to improve bacteriophage loading and orientation did not always translate to better performance of bacteriophage based interfaces. For instance, the similar immobilisation technique employed by Tawil and co-workers[69] did not yield the same improvement in bacteriophage loading and bacteria capture density as mentioned by Singh and co-workers.[4] Such variability may originate from the lack of comprehensive understanding of bacteriophage-surface interactions and their influence to the end-point applications. The general understanding of virus-surface interactions are discussed below.

2.5 Virus and Surface Interactions: Current State of the Art

2.5.1 General Mechanisms of Virus Adsorption onto Surfaces

Many studies have previously attempted to investigate the mechanism behind virus-surface adsorption phenomena. It is believed that electrostatic interaction is the primary mechanism that drives the interactions of viruses with material surfaces. However, in quite a number of cases, the adsorptive behaviour cannot be solely explained by electrostatic interaction. Hydrophobic interaction has also been shown to play a key role in virus-surface interactions. Herein, the contribution of electrostatic interaction based on Derjanguin-Landau-Verwey-Overbeek (DLVO) theory and hydrophobic interaction will

be outlined. These should provide a conceptual framework to understand virus or bacteriophage adsorption mechanism.

2.5.1.1 Derjanguin-Landau-Verwey-Overbeek (DLVO) Theory

Bacteriophages or virus interactions, in general, have often been modelled in the same way as colloidal particle interactions with surface in a medium.[14,70-71] The attachment of colloid particles to surfaces is dependent upon the hydrodynamic transport of colloid to the surface and the surface chemical interactions between the two bodies.[70] The attractive and repulsive forces that exist between bacteriophages and surfaces can be described in the profiles of inter-surface potential energy by Derjanguin-Landau-Verwey-Overbeek (DLVO) theory. This profile accounts for the potential energies of double layer repulsion or attraction (electrostatic force, G_{EL}) and van der Waals attraction (G_{VdW}).[14,72]

$$G_{TOT}(d) = G_{VdW}(d) + G_{EL}(d) \qquad \textbf{2-1}$$

Charged surface or particles in water will be neutralised by the accumulation of a diffused counter charge near the surface that is known as a diffuse double layer or stern layer.[14,73] The double layer repulsion or attraction arises from the overlap of the diffuse double layer of two bodies. If both surfaces carry the same charge, the electrostatic interaction will be repulsive, whereas electrostatic attraction will occur for oppositely charged surfaces. The electrostatic force between an infinite plate and a sphere and sphere-sphere geometry can be calculated by equations 2-2 and 2-3 , respectively:[72]

$$G_{EL} = \frac{128\pi r_1 n_\infty kT}{\kappa^2}\gamma_1\gamma_2\exp(-\kappa h) \quad \textbf{2-2}$$

$$G_{EL} = \frac{128\pi r_1 r_2 n_\infty kT}{(r_1 + r_2)\kappa^2}\gamma_1\gamma_2\exp(-\kappa h) \quad \textbf{2-3}$$

$$\gamma = \tanh\frac{ze\varphi}{4kT} \quad \textbf{2-4}$$

where, r_1 and r_2 are the radius of virus particle and particle, respectively (m), n_∞ is bulk number of ions (ions m^{-3}), k is the Boltzmann constant (1.38 x 10^{-23} J/K), T is temperature (K), κ is Debye-Huckle reciprocal length (m^{-1}), γ is reduced surface potential, z is valence of symmetrical electrolyte, φ is electrical surface potential that is commonly approximated by zeta potential (V), e is electron charge (-1.602 x 10^{-19} C), h is separation between surfaces (m) and C_i is ion concentration (mol/dm^3)

The van der Waals attraction is an electrical force between instantaneous dipole moments within the different molecules.[71] This energy at a given distance is expressed by the Hamaker constant. The Hamaker constant between organic colloids is weak, due to similar Hamaker constant to water. The inorganic matters however tend to have larger Hamaker constants, hence having a stronger attraction for virus. The van der Waals attraction for sphere-plate and sphere-sphere geometry can be calculated by the equation 2-5 and 2-6, respectively:[72]

$$G_{VdW} = -\frac{A_{132}}{6}\left(\frac{r_1}{h} + \frac{r_1}{h+2r_1} + \ln\frac{r_1}{h+2r_1}\right) \quad \textbf{2-5}$$

$$G_{VdW} = -\frac{A_{132}}{6}\left(\frac{2r_1r_2}{h^2+2r_1h+2r_2h} + \frac{2r_1r_2}{h^2+2r_1h+2r_2h+4r_1r_2} + \ln\frac{h^2+2r_1h+2r_2h}{h^2+2r_1h+2r_2h+4r_1r_2}\right) \quad \textbf{2-6}$$

where, A_{132} is the combined Hamaker constant for the sphere (1) and plate (2) in a medium (3).

DLVO theory provides a conceptual framework to understand interactions of bacteriophage to solid surfaces. Loveland and colleagues have reported an agreement between experimental results of PRD1 bacteriophage attachment to quartz and ferric oxyhydroxide coated quartz surfaces with DLVO theory.[70] Similarly, Chattopadhyay and Puls also reported that the attachment of T2, MS2 and phiX174 to clay sorbents appeared to agree well with DLVO theory.[74] In contrast, Attinti and co-workers reported that there were some discrepancies between DLVO theory and the experimental results for the attachment of phiX174, MS2 and Aichi virus to oxide-removed, goethite-coated and aluminium oxide coated sand.[75] They found that despite higher energy barrier of phiX174 than MS2 and Aichi virus (both less than 1% adsorption), a 10% adsorption of phiX174 to oxide sands was observed.

DLVO theory is formulated for a geometrically smooth, well-defined and chemically homogeneous surface.[14,76-77] In practice, however, all surfaces are likely to have heterogeneous chemical compositions and a rough surface with irregular shape. Thus, discrepancies between theoretical predictions and experimental data are somewhat expected. Huang and colleagues explained in details that surface roughness might cause a dramatic reduction in the magnitude and range of interactions between two bodies.[77] For instance, electrostatic interactions for a rough surface could be weakened by a constant factor in comparison to a smooth surface. Suresh and Walz reported the attenuation of van der Waals attraction caused by a decrease in the repulsive energy barrier that subsequently led to larger adsorption rates than that predicted by DLVO theory.[76] Song and colleagues revealed that the interaction between colloidal particles with heterogeneous charged surfaces varies dramatically to the interaction with homogeneous charged surfaces.[78] By using patchwise and random distribution models, they showed that minor amounts of charge heterogeneity on otherwise an unfavourable surface had caused orders of magnitude higher colloidal particles deposition than similar surface with no charge heterogeneity.

2.5.1.2 Hydrophobic Interactions

Due to the discrepancy between DLVO theory and experimental data, Van Oss developed an extension of the theory by including the hydrophobic/hydrophilic interactions.[79] According to Stillinger, the hydrophobic interactions are seen as a result of an entropy-driven net attraction between two non-polar components in water.[80] Solubilisation of a non-polar component in water is accompanied by an entropy decrease,

which is attributed due to re-ordering of hydrogen bond network around the non-polar domain of the component. When two non-polar components are close together, their joint hydrogen bond network involves less entropy decrease than when each of these components solubilised in water. Hence, it is thermodynamically more favourable for two non-polar particles to interact with each other than with water molecule.

As reviewed by Gerba, hydrophobic interaction might play a significant role in the adsorption of viruses due to their hydrophobic domains.[71] Farrah and co-workers demonstrated that hydrophobic interactions are the primary forces involved during attachment of poliovirus 1 to membrane filters at high pH, at which this observation could not be explained by simple net electrostatic interactions.[81] A similar finding was also reported by Shields and Farrah.[17] The presence of antichaotropic salts, such as magnesium sulphate and metal chelators, such as citrate ions, which promote hydrophobic interactions was found to promote the adsorption of MS2 phage to membrane filters via strengthening the hydrophobic interactions.[82] Bales and co-workers revealed that hydrophobic interaction played a major role in the attachment of MS2 to silica particles coated with C_{18}-trichlorosilane, which showed a 40-fold improvement than bare silica particles at any pH.[83] However, it was argued that such improvement arose due to a decrease in double layer repulsion, rather than hydrophobic interactions.[70]

2.5.2 Factors Influencing Virus Adsorption to Surfaces

Many studies highlighted in section 2.5.1 have been performed using a variety of viruses, experimental conditions and surfaces, which may explain the variation observed in subsequent studies. The factors influencing virus adsorption to surfaces are summarised in the section below.

2.5.2.1 Type of Viruses

The variations in viruses' adsorption behaviour to surfaces are believed to result from the differences in the viruses charge and hydrophobicity. Most viruses are encapsulated with polypeptide coats that contain acidic, basic, neutral, hydrophilic and hydrophobic amino acids, such as glutamic acid, aspartic acid, histidine and tyrosine.[15] Depending on the pH of the environment, some amino acids are ionisable, which will give the virus a net electrical charge. At a certain pH, known as the isoelectric point (IEP), virus will carry a zero net charge.[15,71] The virus will carry a net positively charge below the pH of IEP and a net negatively charge above the IEP. The IEP data however cannot yield data concerning the localised pockets of positive and negative charges that may exist across the virus surface.[71,84] The IEP of virus has been known to vary not only by the type, but also by strain as highlighted in Table 2-1. Further, the polypeptide coat protein might fold in such a way to allow hydrophilic residues of the amino acids to associate with surrounding water molecules, whilst the hydrophobic residues are likely to be buried within the core protein.[71] Since complete protein folding to conceal the hydrophobic residues is difficult, some hydrophobic residues will still be exposed to the surrounding solution.

Table 2-1 Isoelectric point (IEP) of different species of bacteriophage.[15,85]

Bacteriophage species	Isoelectric point (IEP)
MS2	**3.1 - 4.0**
PRD1	**3.8 - 4.2**
Qβ	**5.3**
phiX174	**6 - 7.4**
T4	**4 - 5**

Gerba and co-workers did an extensive study to correlate virus adsorption behavior to various soils with their corresponding isoelectric point (Table 2-2).[86] Viruses with lower IEP (group 2) appeared to strongly adsorbed onto the soils, whereas the less adsorbing viruses (group 1) were viruses with higher IEP. The study also observed different adsorption behaviour were exhibited by different strains of viruses. This work shows that under defined conditions, IEP may be useful to predict the virus adsorption. A similar study was conducted by Shields and Farrah on two defined surfaces of ion exchange gel, which were designed to promote electrostatic interactions (DEAE-sepharose) and hydrophobic interactions (octyl-sepharose).[87-88] MS2 phage was found to exhibit the weakest electrostatic interactions and the strongest hydrophobic interactions, whereas phiX174 was the least hydrophobic of the viruses tested. These findings signify the importance of virus's IEP and hydrophobic properties in predicting virus adsorption to surfaces.

Table 2-2 Virus adsorption to soils.[86]

Group 1 (weakly adsorbed)	Group 2 (strongly adsorbed)	Group 3 (very weakly adsorbed)
phiX174	T4	f2
MS2	T2	
Coxsackie B4 (V216)	Coxsackie B3 (Nancy)	
Coxsackie B4 (V240)	Echo 7 (Wallace)	
Echo 1 (Farouk)	Poliovirus 1 (LSc)	
Echo 1 (V212)		
Echo 1 (V239)		
Echo 1 (V248)		

Several studies also show the importance of IEP in predicting the adsorption behaviour of viruses to charged surfaces.[89-91] Chen and co-workers reported high MS2 and PRD1 bacteriophage adsorption (> 95%) to amine functionalised silica particles.[90] This is largely due to both bacteriophages carried a net negatively charged since their IEP (pH 3.8 - 4.2) were below the solution pH of 7.3, whereas amine functionalised silica particles (IEP of pH 7) carried a net positively charged surface at the same solution pH, which induced an electrostatic attraction of two liked-charges. Meanwhile, the adsorption of both bacteriophages were significantly reduced (< 13%) to a net negatively bare silica (IEP of pH 3), which resulted from electrostatic repulsions of two like-charges. A similar observation is reported by Meder and colleagues, whereby high adsorption of low IEP

MS2 bacteriophage (pH 3.9) to a high IEP amine functionalised alumina particles (pH 11) was due to electrostatic interactions of two liked-charges.[89] Interestingly, the MS2 adsorption to carboxylic functionalised particles with the same IEP (pH 3.2) at a solution pH of 7.3 was not completely excluded. It was postulated that non-complete exclusion may result from different protonation state of the carboxylic functional groups.

2.5.2.2 Solution Chemistry

The adsorption of virus to surfaces is also greatly influenced by the solution chemistry, namely the pH of the solution, ionic strength and presence of cations in the solution. As earlier described in section 2.5.2.1, both viruses and surfaces will carry different net surface charge at varying pH values depending on their IEP, which can promote or inhibit virus adsorption to surfaces.

The distance at which the electrostatic double layers of two bodies begin to overlap is dependent on the ionic strength of the solution.[92] At high ionic strength, the shielding of the surface charges occurs, which subsequently reduces the surface potential and the thickness of the double layer. This allows the virus to come to a closer proximity to the surface, which allows the short range attractive forces to overcome the electrostatic barrier.[88,92] Penrod and co-workers reported the increase in ionic strength from 10 to 100 mM NaCl resulted in higher adsorption of MS2 and λ bacteriophages to quartz at pH 5.[93] Furthermore, Harvey and Ryan also reported that an increase in ionic strength at solution pH above the IEP of PRD1 resulted in higher adsorption to negatively charged geological media despite the electrostatic repulsion between like charges.[92] A similar finding was

also reported by Bales and co-workers, whereby the changed in eluent concentration from 0.5 to 0.005 M resulted in more MS2 and poliovirus being released from silica columns.[94]

The influence of cations in the background solution to virus adsorption has been studied on membrane filters. There are several proposed mechanisms to describe the enhanced virus adsorption to these filters. It is postulated that these cations serve as a salt bridge between virus and the filter. Wallis and co-workers observed that the addition of cations was paramount in the adsorption of viruses to cellulose nitrate membrane.[95] It was found that trivalent cations (Al^{3+}) are more effective than divalent cations (Mg^{2+}) with only 1% the divalent cations concentration required for the trivalent cations. The influence of salt bridging is also reported by Stagg.[96] It is reported that the MS2 phage adsorption onto nitrocellulose membrane at pH 7 in the presence of 0.05 M $MgCl_2$ was greatly dependent on the presence of Mg^{2+} cations. Elution of MS2 phage could not be achieved by simply introducing high pH eluent that are often used to promote charge reversal between phage and surface. A chelating agent (EDTA) was needed to bind the Mg^{2+} cations and elute the adsorbed MS2.[96]

Besides acting as a salt bridge between virus to filters, cations could also be adsorbed onto filter surface and cause charge reversal.[88] It was reported that the adsorption at neutral pH was still observed between negatively charged virus and filter surfaces. It is believed to be due to the adsorption of multivalent cations that reduced the repulsion between similar charges.[97]

Farrah and co-workers reported the significance of salts in influencing hydrophobic interactions between virus and surfaces.[81-82] Chaotropic salts in solution are used to disrupt hydrophobic interaction. It works by disordering the water molecules, which in turn reduced the thermodynamic barriers of non-polar groups in polar environment, hence enhanced the ability of the solutions to accommodate non-polar groups.[71] They are often large and singly charged ions, for instance iodide, thiocyanate and trichloroacetate.[81-82] On the contrary, antichaotropic salts, such as fluoride, citrate, calcium and magnesium, have been found to promote hydrophobic interactions. This is due to its ability to increase water structure and therefore reduce the ability of the polar solution to accommodate non-polar groups.[71,88] Farah and co-workers demonstrated that antichaotropic salts, such as NaF and Na_2HPO_4 appeared to promote the adsorption of poliovirus 1 to Zeta plus C-30 membrane at high pH of 9.5 even though both surfaces were negatively charged. However, in the presence of chaotropic salts or buffer alone, the adsorption of poliovirus was retarded at similar pH. The authors suggested that the virus adsorption to this filter was dominated by hydrophobic interaction at pH 9.5.[81] A similar finding is also observed on MS2 phage adsorption to a cellulose acetate and cellulose nitrate mix membrane in the presence of a antichaotropic salt, $MgSO_4$.[82] Meanwhile, the addition of urea, a chaotropic agent, reduced the adsorption of MS2 phage.

2.5.2.3 Nature of the Surface

Another pivotal aspect that also influences virus adsorption are the properties of surface used. The knowledge of such will aid in predicting and controlling virus adsorption. The role of surface's charge has been widely documented in the adsorption of virus to filter and soils[71] and briefly highlighted in section 2.5.2.1. The development of membrane

filtration system for virus concentration has provided an insight to the knowledge of virus-surface interactions. Previously, electronegative membranes, made of cellulose nitrate, fiberglass and fiberglass-epoxy were used to remove virus. However, due to electrostatic repulsion between net negatively charged viruses and surfaces, positively charged cations were added to the medium to promote salt bridging or charge neutralisation. For instance, 50% poliovirus 1 removal was achieved using 0.45 μm HA Millipore filter in the presence of Ca^{2+} ions.[98] A similar virus removal strategy was also reported elsewhere.[99-100] These studies have led to the development of an electropositive membrane that does not require pre-conditioning of the water used. Sobsey and Jones observed the virtue of electropositive charged filters in concentrating poliovirus from water than negatively charged filters.[101] These studies have indirectly highlighted the great importance of the substrate's surface charge in virus-surface interactions.

Apart from the surface macroscopic characterisations mentioned above, such as surface charge, many studies conducted on geological medium, such as soil, sediment and clay minerals revealed the significance of detailed microscopic information towards the overall surface properties. The heterogeneous composition of geological media, which often composed of different mixtures of oxides, carbonates and organic matters, give rise to the different region of the same material that are favourable for different type of viruses. For instance, the adsorption of neutral or slightly negatively charged poliovirus 1 to negatively charged montmorillonite clay was found to be preferential to the positive regions along the edge of the clay, which was rich in aluminium ions, instead of to the dominant negatively charged silica regions.[102] Moreover, the adsorption of mixed

population of reovirus 3 and T1 bacteriophage to clay materials was also found to be adsorption site specific, whereby the adsorption of reovirus occurred primarily to the negatively charged regions and T1 bacteriophage adsorbed to positively charged patches of clay.[103-105]

The importance of the microscopic surface parameters is also demonstrated in the interaction between bacteriophage and its host, which are often employed to provide a better model to investigate virus-surface interactions. As both bacteriophage and its host cell carry a net negative charge, a macroscopic surface characterisation, such as IEP often fails to provide a theoretical prediction on their attachment.[84] It was reported that the adsorption of bacteriophage to the host cell involves a specific electrostatic interactions of certain chemical groups.[106-108] Positively charged amino groups on T2 bacteriophage were found to interact with negatively charged carboxylic groups on *E. coli* surface.[107-108] However, such interaction did not occur in reverse between carboxylic groups on T2 with amino groups on *E. coli* surface. Puck and co-workers reported that the same specific chemical grouping that promote bacteriophage-host interactions on their surface is required for bacteriophage-inorganic surface interactions.[106]

2.6 Advancing the Understanding and Fabrication of Bacteriophage Based Interfaces

An approach to decipher the complex bacteriophage-surface interactions is through the modification of surfaces that will allow control tailoring of the surface physico-chemical properties, which may induce a different response of virus adsorption. Hence, the factors influencing the interactions and its mechanism can be elucidated. Two approaches to surface modifications, namely plasma polymerisation and self-assembled monolayer will be discussed.

2.6.1 Plasma Polymerisation

Plasma polymerisation is well-established technique whereby the substrate is irradiated together with the organic monomer, which are activated to form radicals by electric glow discharge generated plasma.[109-110] Hence, it is often known as glow discharge plasma polymerisation. Unlike the common polymerisation process, plasma polymers do not contain repeating monomer units, instead they comprise of heavily cross-linked, fragmented and re-arranged units of monomers.[111]

The advantages of plasma polymerisation manifest from its time efficient process that eliminates the several stages of conventional polymer coating process (i.e synthesis of polymer, preparation of coating solution, substrate cleaning, coating process, drying and curing) and ultra-thin film deposition.[112-113] Plasma polymerisation is also able to produce an active surface with amine, carboxyl, hydroxyl and aldehyde functional groups that are important moieties in interfacial immobilisation of biomolecules or bioagents (i.e cells

and proteins).[109] For instance, amine-based plasma polymerisation can be achieved by the application of these monomers, such as allylamine, diaminocyclohexane, 1,3-diaminopropane, heptylamine, propylamine and butylamine. In addition, a carboxylated polymer surface can be produced by utilising acrylic acid and propionic acid.[109] Those advantages have led to diverse application of coatings prepared by this method, for instance as a protective coatings,[114-116] sensors,[117-118] and biomaterial coatings.[111,115-116,119]

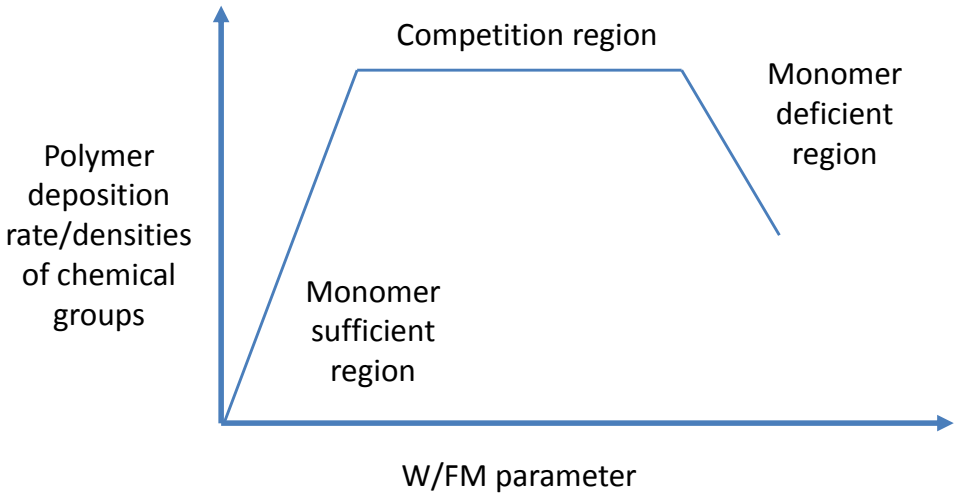

Figure 2-9 Different regions of plasma polymer deposition.

Despite the merit of plasma polymerisation, the quality of the polymer layer depends heavily on the experimental parameters. The influence of those parameters can be correlated in terms of W/FM ratio, with W corresponds to the discharge power, F refers to the flow rate of monomer and M attributes to the molecular weight of the monomer.[109,120] In some other studies, volume of the plasma reactor and pressure are often included. The different value of W/FM may fall into three regions, namely

monomer sufficient/discharge power deficient, competition region and discharge monomer deficient/discharge power sufficient (Figure 2-9). Most studies employed parameters to achieve the monomer sufficient/discharge power deficient region, whereby excess monomers are present and the less power translates to less fragmentation of monomers that subsequently led to higher density of chemical groups.[109] As illustrated in Figure 2-9, there is no increase in deposition rates with increasing discharge power or decreasing flow rate in the competition region. In the monomer deficient region, there is sufficient discharge power to fragment the monomers; hence a further increase in the discharge power will not lead to higher deposition rate.[109]

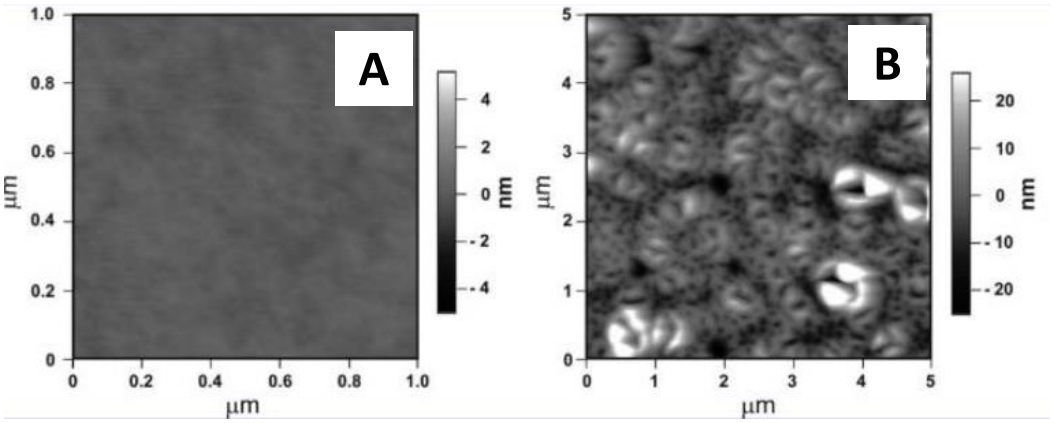

Figure 2-10 AFM images of *n*-heptylamine plasma polymer (A) as deposited and (B) after immersion in water for 24 h after deposition.[121]

Furthermore, the complex compositions of the plasma and different possible reaction pathways often lead to the formation of undesirable functionalities on the surface.[122] For instance, *n*-heptylamine was used to introduce amine surface functional groups, however a wider variety of nitrogen-containing functional groups (i.e -NH) would be obtained.[110,122] Furthermore, the plasma polymer has been reported to undergo swelling when exposed to solution, which results in partial delamination of the film.[110] In some scenarios, the swelling may cause significant reduction in thin film density and the increase in porosity.[121] The immersion of *n*-heptylamine plasma polymer in water resulted in the formation of tubular channels that resemble a honeycomb pattern (Figure 2-10B) in comparison to freshly deposited film (Figure 2-10A).[121] Vasilev and co-workers postulated that the limited water uptake by the film due to its slight hydrophobic nature, which ultimately induced shrinkage of the film. Such shrinkage might be relieved by microstructural alteration in the form of void formation as seen in Figure 2-10B. In contrast, Martin and colleagues did not observe swelling on *n*-heptylamine layer on mica after immersion in aqueous media for 4 h.[123] Despite the above challenges in instability of the polymer layer and tedious optimisation of the plasma parameters, plasma polymerisation remains of interest as one of surface coating methods.

2.6.2 Self-Assambled Monolayers (SAMs)

Self-assembled monolayers (SAMs) comprise of molecules with a head and terminal group, with the head group attaches to the surface due to its high affinity to the surface and leaving the tail group protruding to the solution, which then defines the chemical functionality of the surface.[110,124] The attractiveness of SAM lies on the availability of different tail groups for easy alteration of the surface chemistry (Table 2-3), it can produce well-defined packing and density of the specific molecules and it is able to incorporate more than one type of molecule.

Table 2-3 Surface Properties of Different Tail Groups of SAMs. [110]

Functionality	Properties	
	Net Charge	**Wettability**
OH	Neutral	Hydrophilic
NH_2	Positive	Hydrophilic
COOH	Negative	Hydrophilic
CH_3	Neutral	Hydrophobic
CF_3	Neutral	Hydrophobic

One of the most popular SAMs is organothiol (R-SH) that could spontaneously adsorb onto gold, silver, platinum and copper. Sulphur containing compounds, such as alkanethiols, dialkyl disulphides and dialkyl sulphides adsorbed to the metal via rapid chemisorption. An oxidative addition of S-H (2-7) or S-S (2-8) bond to, for instance a gold surface followed the reaction pathway below.[125]

$$R\text{-}S\text{-}H + Au_n^0 \rightarrow R\text{-}S\text{-}Au^+ \cdot Au_n^0 + \tfrac{1}{2} H_2 \qquad \textbf{2-7}$$

$$R\text{-}S\text{-}S\text{-}R + Au_n^0 \rightarrow 2\ R\text{-}S\text{-}Au^+ \cdot Au_n^0 + H_2 \qquad \textbf{2-8}$$

Kinetic studies on the organosulphur SAM formation reveal two distinct adsorption kinetics; the initial phase of assembly is achieved within a few minutes and it will reach a thickness of 80 - 90% of its maximum.[126] The final phase involves slow re-organisation of the molecule due to intermolecular Coulomb and van der Waals interactions between the molecules-surfaces and molecules-molecules to produce an extended, all *trans* close packed conformation.[110,126-127] The degree of order, packing density and surface coverage decreases as the chain length of alkanethiols decreases.

The simplicity and reliability of the preparation technique, as well as the flexibility in providing a wide variety of surface functional groups and even chain length are the success factors of organosulphur based SAM, nevertheless SAM layers often face with stability issues.[124,126] Although alkanethiols are stable at temperature below 100 °C, the formed gold-thiolate bond is prone to oxidation to either sulphinates ($-SO_2$) or

sulphonates ($-SO_3$). The rate of photo-oxidation has been reported to vary with length of molecule, with shorter chain SAMs oxidise at a much faster rate than longer chain SAMs.[124] The stability of issue can be address via employing organosulphur with two thiols.

Organosilane based SAM is also another commonly exploited self-assembly system, which are obtained by reacting organosilane with a hydroxylated surface, such as glass, poly(vinyl alcohol), aluminium, zinc oxide, indium tin oxide and iron oxide.[124] The general reactions of the SAM formation are illustrated in Figure 2-11. The organosilane molecules, typically alkoxysilanes undergo hydrolysis to produce a reactive intermediate, which then condense with another intermediate to form a polymer matrix that is linked together by -Si-O-Si- bonds.[128] The silane network interacts with the surface hydroxyl (-OH) groups on the surface and the condensation reactions occur on the surface to covalently anchor the silane network to the surface via heat or vacuum applications to remove water.[128]

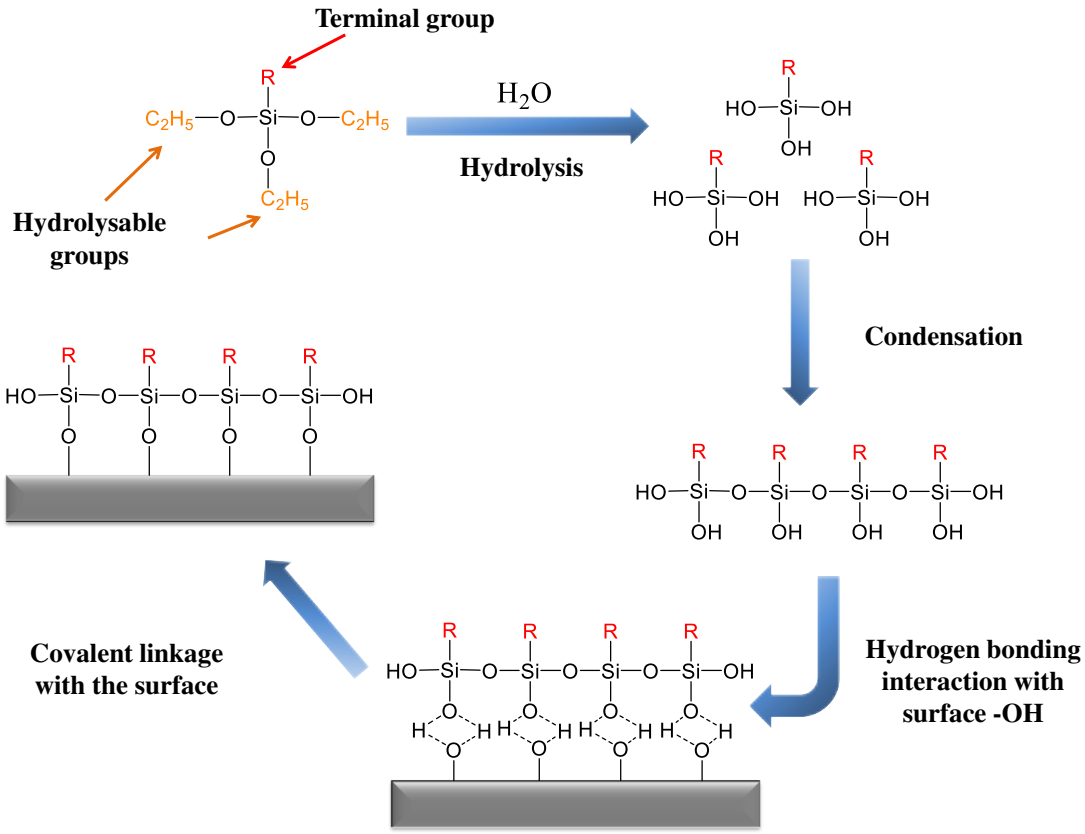

Figure 2-11 Organosilane based SAM formation on hydroxylated surface.

The quality of the organosilane layer is dependent on various experimental parameters. For instance, a multilayer is resulted from organosilane grafted in a manner illustrated above (Figure 2-11).[128] This is due to alkoxysilanes, such as ethoxysilane is allowed to firstly hydrolyse in the presence of water and condense to form a siloxane polymer network prior to binding with the substrate, which induces the formation of multilayer network. Multilayer formation may be avoided by the use of methoxysilanes and chlorosilanes that are reactive even without prior hydrolysis with water. This thus allows each of the organosilane to couple directly with the hydroxyl groups on the substrate. Besides the influence of water content, the resulting layer of organosilane SAM is also affected by the concentration of silane, temperature and incubation time. Howarter and

Youngblood reported the deposition of 3-aminopropyltriethoxysilane (APTES) on a silicon wafer surface at varying APTES concentration, incubation time and temperature (Figure 2-12).[129] It was found that an increase in APTES concentration and incubation temperature resulted in smoother layer as measure by Atomic Force Microscopy (AFM). A dramatic increase in the thickness was observed with increasing incubation time (1 nm to ~20 nm).

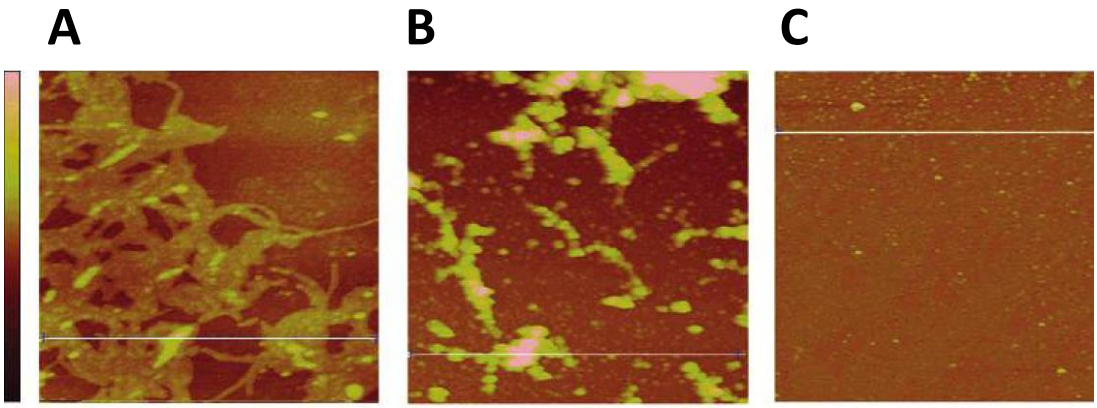

Figure 2-12 Atomic Force Microscopy (AFM) images of 3-aminopropyltriethoxysilane layer deposited on silicon wafers. (A) Multilayer-island formation deposited at 1% - 1 h - 75 °C, (B) rough film deposited at 10% - 24 h - 75 °C and (C) smooth layer deposited at 33% - 24 h - 75 °C. Height scale of 10 nm.[129]

The grafting of the organosilanes, such as APTES has been reported to be affected by pH of the solution as it will influence the orientation of the organosilane on the surface. The adsorption of APTES molecule to an oxidised iron surface at pH 8 was reported through the amino groups of APTES, instead of the silanol groups.[130] This is evident from the emergence of protonated amine peak ($-NH_3^+$) that was attributed from the intermolecular hydrogen bonding between amino groups and surface hydroxyl groups.[131] The adsorption of amino groups to the surface can be identified through angle resolved

X-ray Photoelectron Spectroscopy (XPS) or K-edge Near Edge X-ray Absorption Fine Structure (NEXAFS). Angle resolved XPS will provide atomic concentrations data at varying sampling depth through varying the take-off angle, which is defined as the angle between the sample surface and the directions of photoelectron emission. This infers that the orientation of APTES molecule can be deduced by analysing the change in atomic concentration of nitrogen and the corresponding nitrogen species at the bulk or near the surface. For instance, the adsorption of APTES onto a polished silicon surface at pH 10.4 showed an increase in NH_3^+ composition near the surface (43%) of the sample than on the bulk sample (28%) as shown in Figure 2-13B. This suggests that considerable number of amino groups interacting with the surface silanol groups.

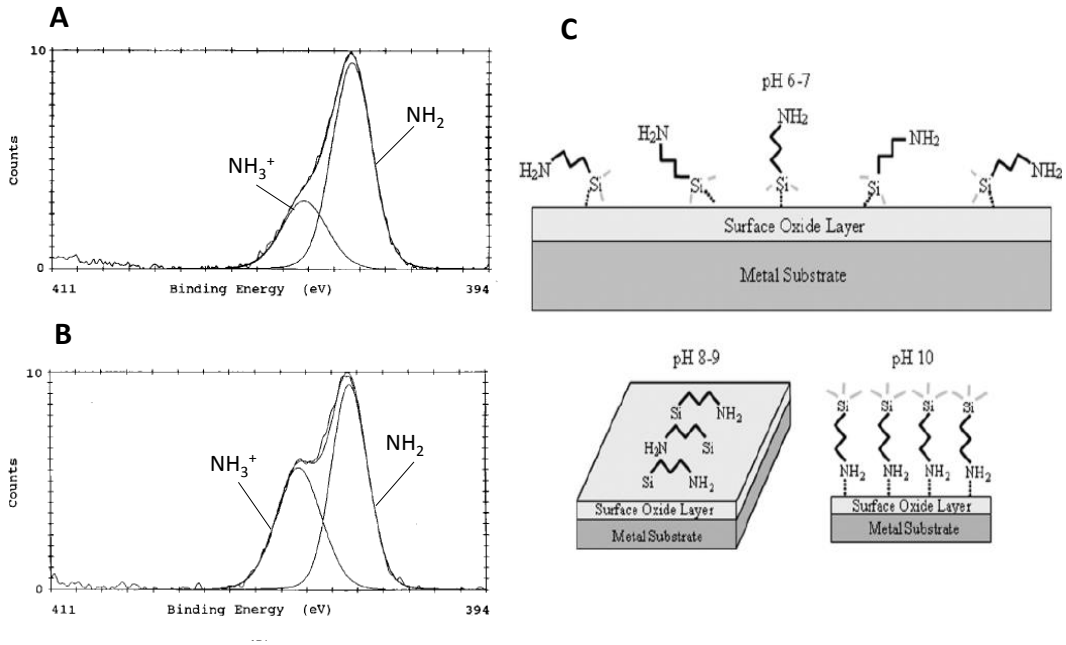

Figure 2-13 N1s narrow spectra of polished silicon coated with APTES at pH 10.4 at (A) the bulk of the sample and (B) near the surface.[131] (C) Schematic illustration of APTES orientation on zinc oxide surface at varying pH of adsorption.[132]

Further, Thomsen and co-workers employed K-edge NEXAFS, which is a well-established technique to determine the orientation of molecules at the surface to characterise the orientation of APTES on zinc oxide at different solution pH (Figure 2-13C).[132] The zinc oxide surface with an IEP of pH 8 - 9 will carry a net positive surface charge at pH 6 - 7, meanwhile silanol (pKa of 3 - 4) and amino groups (pKa of 8 - 11) on APTES will possess a net negative and net positive surface charge, respectively. The above properties will promote an orientation with silanol groups at the interface as depicted in Figure 2-13C. The zinc oxide surface will harbor a neutral surface charge at the solution pH 8 - 9, which is close to the IEP of zinc oxide. Thus, there is no preferential orientation expected.[132] Meanwhile, the anchoring of APTES molecule through amine

moieties is expected when the pH of the solution is higher than IEP of the substrate, whereby the surface will carry a net negative surface charge that attracts positively charged amine moieties. The knowledge on the variation of organosilane orientation over pH should be cautiously considered during the surface functionalisation as the moieties that protrude to the solution will define the properties of the surface.

2.7 Summary

An overview of the development in bacteriophage based interfaces and the challenges encountered across different applications was presented herein. This reveals the significance to establish a comprehensive understanding on the mechanisms and factors influencing bacteriophage adsorption to surfaces and their contributions to the end-point applications. Many studies performed on virus-surface interactions have used a variety of surfaces, viruses and adsorption conditions, which results in varying adsorption results with the subsequent studies. Hence, a better approach needs to be firstly establish in order to elucidate virus-surface interactions. This is the challenge this thesis will attempt to address via designing surfaces with control over their physio-chemical properties that will allow an in-depth investigation on factors influencing the virus adsorption to the surface and the mechanisms behind it. This study is also directed to explore the influence of such interactions towards different end-point applications.

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Chapter 3 Adsorption of T4 Bacteriophages on Planar Indium Tin Oxide Surface via Controlled Surface Tailoring

3.1 Introduction

A useful strategy to better understand the influence of physicochemical properties of adsorbing surfaces towards virus adsorption is through a comparative study that employs different surfaces with defined physicochemical properties. Previous studies have turned to modifying the surface chemistry of particles. It is found that virus-particle interaction is largely influenced by net electrostatic interactions between two oppositely charged components.[1-4] However, with the expansion of bacteriophage applications beyond the use of particulate based substrates, such as biosensors and antibacterial surfaces, an understanding of the extent to which substrate physical configuration contributes to the virus-surface interactions is yet to be established.

Indium tin oxide (ITO) coated glass is a thin film conductive substrate. It has been widely used due to its desirable properties, such as its stability under physiological conditions, transparency and conductive properties.[5-6] ITO surface can be easily modified to provide different surface functional groups or alter its physico-chemical properties, hence it has been used as a working biosensor electrode,[5,7] adhesion platform for biomolecules,[5] solar cells[8] and model substrate to investigate biological processes.[8]

T4 bacteriophage is a non-enveloped virus with linear-double stranded DNA tailed virus that infects *Escherichia coli* (*E. coli*) that has been widely used as a benchmark for viral research due to its virulent nature, rapid growth and its well-understood properties.[9-10] It has a complex structure that consist of a 120 nm long and 85 nm wide capsid, 100 nm contractile tail and six long tail fibers.[10-11] T4 bacteriophage has an isoelectric point (IEP) of pH 2 - 4[12] and T4 bacteriophage is reported to have a good hydrophobic binding potential as T2, however it is less than other commonly used bacteriophage, such as MS2.[13]

This work aims to provide a comparative study that investigates the significance of surface physico-chemical properties via controlled surface modification of a planar indium tin oxide surface as model substrate, on the adsorption behaviour of T4 bacteriophages. The study employed surface functionalisation via organosilane grafting which allows proper presentation of different chemical moieties, namely amine ($-NH_2$), carboxyl (-COOH) and methyl ($-CH_3$) groups, as well as the variations in their surface charge and hydrophobicity.

3.2 Materials and Methods

3.2.1 Reagents and Materials

Planar indium tin oxide (ITO) coated cover slips (18 x 18 mm, 15 - 30 Ω) were obtained from SPI Supplies. 3-aminopropyltriethoxysilane (APTES), octadecyltrimethoxysilane (ODTMS), bovine serum albumin (BSA), ITO nanopowder (referred as particulate ITO) and sodium citrate tribasic dihydrate were supplied by Sigma-Aldrich. Analytical grade methanol, ethanol, glacial acetic acid, *N,N*-dimethylformamide (DMF), sodium chloride, sodium hydroxide pellets, dichloromethane, Tris-HCl, glycerol and D-glucose were purchased from Ajax Chemicals. Phosphate buffered saline tablets (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4 and 0.2 g/L KH_2PO_4, pH 7.2-7.4, Ionic strength 298 mmol/L), beef extract, tryptone, yeast extract and agar bacteriological were obtained from Oxoid. Bacteriophage T4 (ATCC 11303-B4) and its host *E. coli* ATCC 11303 were procured from the ATCC.

Luria Bertani (LB) broth was prepared by dissolving 10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride in Milli-Q water. Tryptone agar was made by adding 10 g/L tryptone, 8 g/L sodium chloride, 2 g/L sodium citrate tribasic dihydrate, 3 g/L D-glucose and 10 g/L agar bacteriological in Milli-Q water. Media were sterilised by autoclaving at 121 °C for 20 min.

3.2.2 Preparation and Modification of Planar and Particulate ITO

All ITO substrates were cleaned by immersing the ITO substrates in dichloromethane, followed by methanol for 10 min each and in 0.5 M K_2CO_3 in Milli-Q water/MeOH (1:3, v/v) for 30 min under constant sonication. The ITO substrates were rinsed thoroughly with a copious amount of Milli-Q water and dried at 110 °C.

The amine terminated ITO was prepared by immersing the cleaned planar ITO surface or 0.3 g cleaned particulate ITO into a solution containing 5 mL anhydrous methanol, 0.25 mL water, 0.5 mL glacial acetic acid and 30 mL glycerol. The solution mixture was sonicated using an ultrasonic probe (Misonix) for 1 min in a three-necked round bottom flask. A mixture of 0.5 mL APTES in 5 mL of anhydrous methanol was titrated into the flask and the solution was heated to 120 ^{0}C for 15 h under a N_2 environment. After modification, the surface was subjected to repeated washing in Milli-Q water followed by ethanol to remove any weakly bound molecules and dried under vacuum. The bare ITO was prepared by following the protocol above and replacing the 0.5 mL APTES with 0.5 mL anhydrous methanol. The bare ITO was used as a control sample.

To introduce the carboxylic functional group, amine terminated planar ITO surface or 0.08 g of particulate ITO-NH_2 was added to a succinic anhydride (10%) in anhydrous DMF. The mixture was stirred for 3 h under a N_2 environment. The substrate was washed repeatedly in DMF followed by Milli-Q water and dried under vacuum.

ITO-CH_3 was prepared by immersing pre-cleaned planar ITO surface or 0.08 g of cleaned particulate ITO in 0.5% (v/v) n-butylamine in anhydrous methanol. 5% (v/v) ODTMS was added slowly into the solution. The sample was sonicated for 60 min at 20% amplitude. It was then left to incubate for 30 mins, before being washed three times in methanol and acetone. The sample was dried overnight under vacuum.

3.2.3 T4 Bacteriophage Adsorption

All immobilisations were performed in 10 mM phosphate buffer that had been adjusted to pH 6. The T4 bacteriophage titre was adjusted to a final concentration of 10^{10} pfu (plaque forming unit)/mL by dilution with 10 mM phosphate buffer pH 6 and the same concentration was used for all the phage immobilisation studies. The modified substrate was incubated in 0.5 mL of T4 bacteriophage solution for 16 h at 30 ^{0}C in a shaker incubator (Incu). The substrate was thoroughly washed three times in 50 mM Tris-HCl (pH 8), followed by three times washing in 0.1% BSA in 10 mM phosphate buffer pH 6 and rinsed four times in 10 mM phosphate buffer pH 6. The washing supernatants were collected and the phages were enumerated via a double agar overlay plaque assay.[14] Bacteriophage in 10 mM phosphate buffer pH 6 was used as a positive control.

The density of phages on the substrates was calculated by the following equation 3-1:

$$C = \frac{\left[\frac{C_i - \sum_0^{10} C_w}{C_i}\right]}{S.A\ of\ ITO} \qquad \textbf{3-1}$$

where C is the concentration of T4 phage on the substrate (pfu/cm^2), C_i and C_w are the initial bacteriophage concentration and bacteriophage concentration in washing supernatant collected, respectively. *S.A (cm^2)* is the surface area of planar or particulate ITO, which is 0.1 cm^2 and 2 cm^2, respectively.

3.2.4 Surface Characterisations

Contact Angle Goniometry. The static contact angle (CA) of water was measured using a Rame-Hart 100-00 goniometer, using a drop of Milli-Q water (3.5 μL). All samples were prepared in triplicate with at least four separate spots being measured for each sample.

X-ray Photoelectron Spectroscopy (XPS). XPS measurements of bare and differently modified ITO surfaces were acquired using an ESCALAB 220iXL spectrometer with a monochromatic Al Kα source (1486.6 eV). The spectra were accumulated at a take-off angle of 90^0 at a pressure of less than 10^{-8} mbar with pass energy of 20 eV. All spectra were referenced to the C1s signal (285.0 eV). Spectra were analysed using Avantage 4.88 software. The elemental analysis was also performed at different take-off angles, which was set to 30^0 (near to surface) and 90^0 (bulk). The take-off angle is defined as the angle between the surface and the directions of the photoelectron emission.

Zeta Potential Analysis. The zeta potential of particulate ITO was measured using phase analysis light scattering (Malvern Zetasizer Nano ZS) at varying pH in 10 mM NaCl. The pH was adjusted using 0.1 M HCl and 0.1 M KOH.

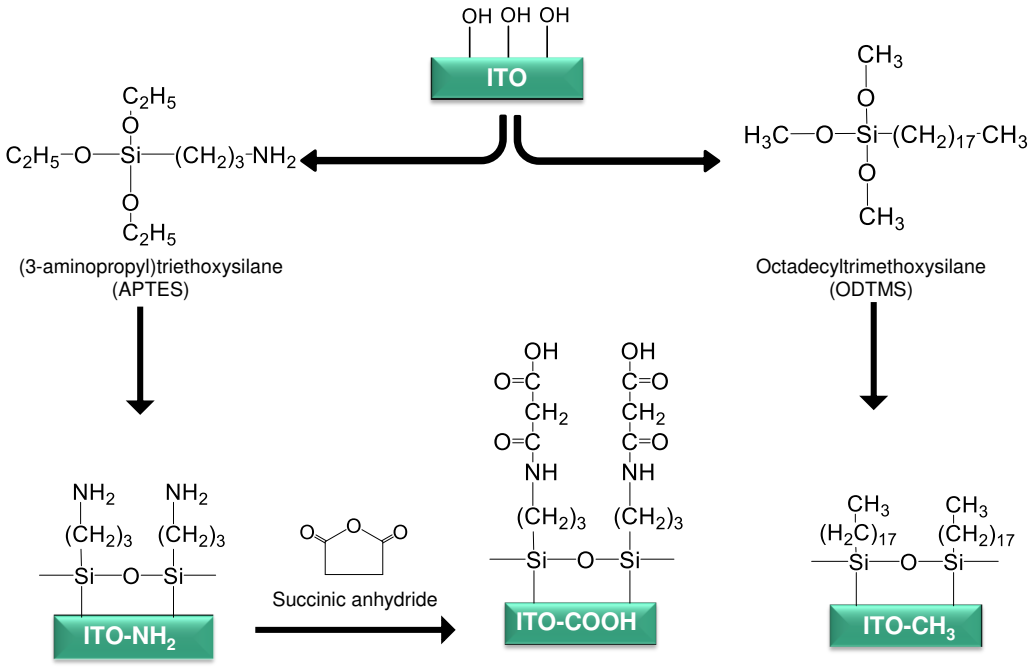
Scanning Electron Microscopy (SEM). The binding of T4 bacteriophage to planar ITO surfaces was characterised by SEM. The substrates were fixed with 2% glutaraldehyde in buffer for 30 min. The samples were washed in glutaraldehyde-free buffer twice for 5 min each. The specimens were dehydrated in a series of ethanol solutions (50%, 60%, 70%, 80%, 90% and 4 x 100%) for 15 min each and dried via critical point drying (Leica CPD030). The dried specimens were then mounted on aluminium support stubs using conductive carbon paint and coated with a 10 nm layer of platinum (Edwards Auto 306 Magnetron Sputter coater). The SEM imaging was done using a JEOL 7500FESEM operated at 5 kV at a working distance of 8 mm and using a spot size setting of 7.

Atomic Force Microscopy (AFM). The 3D surface topography and roughness were determined by Bruker Dimension ICON SPM using contact mode. The height and phase images (1 μm x 1 μm) were collected simultaneously and analysed using NanoScope Analysis software. All the roughness values refer to root-mean-square (rms) roughness.

3.3 Results and Discussions

3.3.1 Surface Functionalisation of Planar and Particulate ITO

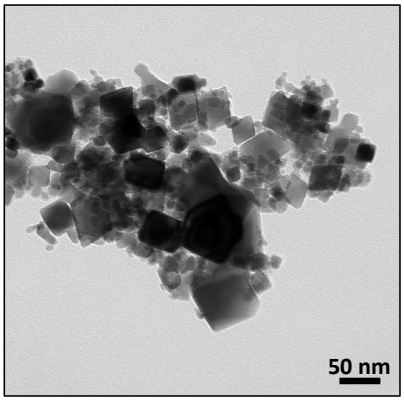
In the first step into the study of T4 adsorption behaviour onto planar surfaces, the planar ITO surfaces were functionalised with several functional groups, each designed to create unique surface characteristics. Apart from the obvious variation in the chemical species, the surface functionalisation of $-NH_2$, -COOH and $-CH_3$ groups on ITO (via organosilane grafting, see Scheme 3-1) also provided variations in the surface charge and surface hydrophobicity, which had been reported previously to influence virus adsorption.[1-2] Similar surface functionalisations were also done on particulate (as opposed to planar) ITO to provide an insight on the influence of physical properties of the substrates, as well as its chemical properties towards T4 bacteriophage adsorption.



Scheme 3-1 Schematic of surface functionalisation of ITO with amine (-NH_2), carboxylic (-COOH) and methyl (-CH_3).

Bare particulate ITO has a wide size distribution ranging from 8 nm to 60 nm (Figure 3-1A). Similar organosilane grafting as planar ITO was conducted on particulate ITO (see section 3.2.2). The surface functionalisation with -NH_2, -COOH and -CH_3 resulted in a similar hydrodynamic diameter of 0.9 to 1.6 µm as bare particulate ITO when suspended in 10 mM phosphate buffer pH 6 (Figure 3-1B). The aggregation of the particulate ITO is likely due to the compression of double layer in high ionic strength solution, which resulted in a reduction in inter-particle electrostatic repulsion.[15]

A



B

ITO particles	Hydrodynamic diameter (μm)
Bare ITO	1.5 ± 0.1
ITO-NH_2	1.4 ± 0.1
ITO-COOH	1.0 ± 0.1
ITO-CH_3	1.5 ± 0.1

Figure 3-1 Properties of particulate ITO. (A) HRTEM images of bare particulate ITO at 97000x magnification and (B) hydrodynamic diameter of bare and functionalised particulate ITO in 10 mM phosphate buffer pH 6.

The planar and particulate ITO surfaces were successfully functionalised as characterised by the XPS narrow spectra in Figure 3-2 and Figure 3-3, respectively. The (3-aminopropyl)triethoxysilane (APTES)-modified planar and particulate ITO surface were indicated by the emergence of a Si-O peak at 100.6 - 101.9 eV, which was attributed to the silica backbone of APTES.[16-18] The successful APTES grafting on planar ITO was also evident by the presence of amino groups in the form of free amine (NH_2) and protonated amine (NH_3^+) at 399.5 eV and 401.6 eV, respectively.[16-18] The emergence of NH_3^+ was found to be due to the intermolecular hydrogen bonding or coordination between amino groups with the surface hydroxyl groups.[19-20] Similarly, the emergence of a peak in N1s spectra was also observed on APTES modified particulate ITO, which corresponds to NH_2 at 399.27 eV (Figure 3-3). Note that an increase in carbon to indium ratio from 0.25 to 0.4 (Table 3-1) following APTES modification on planar was also

observed, which might be attributed from the addition of propyl group of APTES. To further investigate the nature of APTES grafting, an angle resolved XPS analysis was carried out for planar bare ITO and ITO-NH_2 (Table 3-2). The XPS analyses at lower take-off angle revealed increasing Si-O to indium and NH_2 to indium ratios closer to the surface of ITO-NH_2, which suggest a multilayer silane formation that resulted in some NH_2 buried closer to the surface.[19,21]

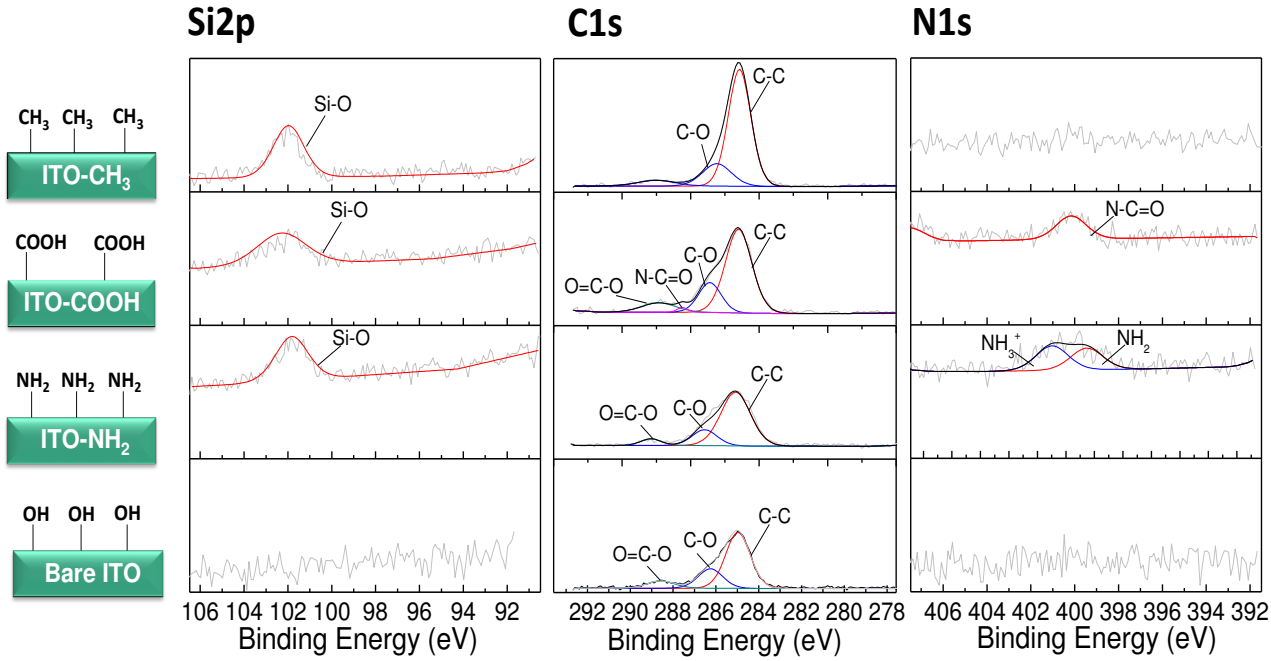


Figure 3-2 XPS narrow spectra of bare (-OH), amine (-NH_2) functionalised, carboxylic (-COOH) functionalised and methyl (-CH_3) functionalised planar ITO.

The subsequent conversion of amine on ITO-NH_2 to carboxylic groups by succinic anhydride (as described in Scheme 1) resulted in the formation of a peptide bond as demonstrated by the pronounced peak shift to 400.03 eV of N1s spectra, which corresponds to N-C=O (Figure 3-2).[20,22] An appreciable increase in the O-C=O peak was also observed in C1s spectra of particulate ITO, which suggest the successful conversion of amine to carboxylic group on particulate ITO (Figure 3-3). An additional peak is also observed in C1s spectra of carboxylic functionalised planar ITO, which is attributed to N-C=O (Figure 3-2).[23] The amide bond formation is in agreement with an increase in the relative carbon to indium intensity from 0.4 to 0.83 when compared to planar ITO-NH_2 as shown in Table 3-1.

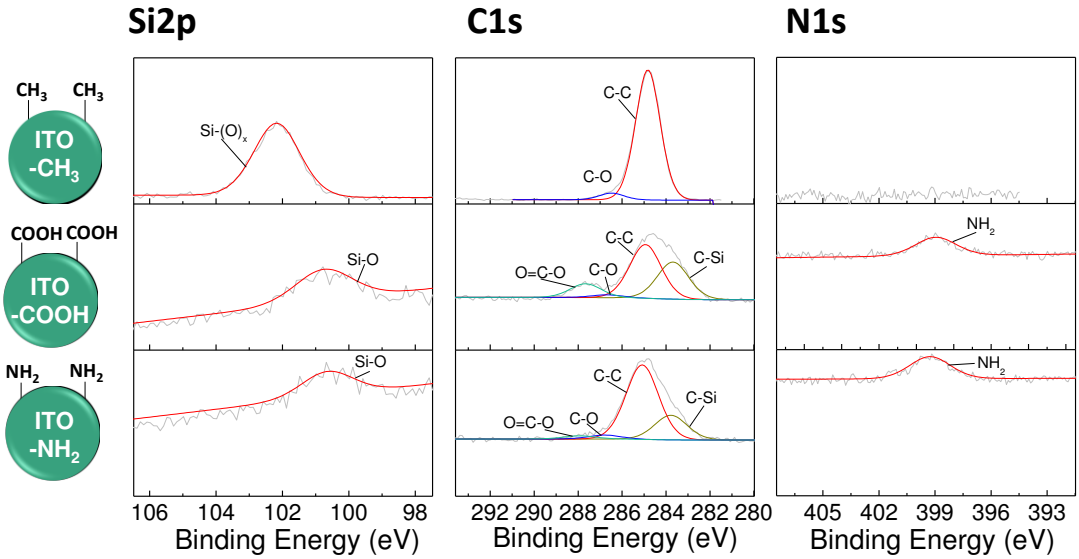


Figure 3-3 XPS narrow spectra of amine (-NH_2) functionalised, carboxylic (-COOH) functionalised and methyl (-CH_3) functionalised particulate ITO.

Evidence for the surface modification of ITO with octadecyltrimethoxysilane (ODTMS) comes from the pronounced Si-O peak at 101.9 eV and a substantial increase in C-C peak at 285 eV as evident in Si2p and C1s spectra, respectively in Figure 3-2 and Figure 3-3. Further, the dramatic increase in the relative carbon to indium intensity (Table 3-1) was also observed for methyl functionalised planar ITO which is mainly contributed from C-C peak (from 0.17 to 0.8) from the long alkyl chain in ODTMS.

Table 3-1 Elemental ratio of carbon species, nitrogen and silicon over indium of different functionalised planar ITO.

	C-C/In	C-O/In	N-C=O/In	O-C=O/In	N/In	Si/In
Bare ITO	**0.17**	**0.06**	**N/A**	**0.02**	**N/A**	**N/A**
ITO-NH_2	**0.33**	**0.04**	**N/A**	**0.03**	**0.02**	**0.06**
ITO-COOH	**0.59**	**0.14**	**0.02**	**0.08**	**0.01**	**0.08**
ITO-CH_3	**0.80**	**0.19**	**N/A**	**0.07**	**N/A**	**0.1**

The influence of the surface functionalisation of ITO surfaces to their physical and chemical properties is reflected in their surface hydrophobicity as characterised by surface water contact angle. It was found that after functionalisation planar ITO-NH_2 and ITO-COOH remained hydrophilic in nature, similar as planar bare ITO (Figure 3-4B). The contact angle obtained for planar ITO-NH_2 is concordance with other amino-silanised surfaces.[24-25] However, it is lower than data reported by Aissaoui and co-

workers (~70°).[26] The variation in contact angle is postulated to be due to the fraction of hydrophobic alkyl chain exposed to the surface that will inevitably increase the contact angle.[27] The acylation of planar ITO-NH_2 to form ITO-COOH resulted in a slight decrease in water contact angle to 33 ± 1.8°, which is comparable to other carboxylic terminated surfaces.[24-25] The functionalisation of planar ITO with ODTMS appears to increase the water contact angle to 97 ± 0.9°,[16,28-29] which infer that planar ITO-CH_3 has the least affinity to water in comparison to other functionalised surface.

Table 3-2 Elemental ratio of nitrogen and silicon over indium at different take off angle for planar bare and amine functionalised ITO. Take-off angle is defined as the angle between the surface and the directions of the photoelectron emission.

	Bare ITO		ITO-NH_2	
	90^0 (Bulk)	**30^0 (Closer to surface)**	**90^0 (Bulk)**	**30^0 (Closer to surface)**
N/In	**-**	**-**	**0.9%**	**2.2%**
NH_2/In	**-**	**-**	**0.6%**	**1.8%**
NH_3^+/In	**-**	**-**	**0.3%**	**0.4%**
Si-O/In	**0%**	**0%**	**1.7%**	**5.5%**

The silane surface modification has brought about changes in the net surface charge, which is demonstrated by the isoelectric point of bare and functionalised particulate ITO. As seen in Figure 3-4A, the introduction of the high pKa (9.8) amino groups caused the particulate ITO-NH_2 to be more positively charged than bare ITO, as indicated by the positive shift in isoelectric point (IEP) from pH 7.5 to 8.3.[30] In contrast, the subsequent conversion of amino groups to carboxylic groups (Scheme 3-1) lead to an opposite shift in IEP from pH 8.3 to 6.8, which leads to more negatively charged particles than particulate ITO-NH_2 and bare ITO. The shift in IEP to lower pH after amine functional groups conversion to carboxylic groups was also observed on iron oxide particles.[17] Due to the low polarity of particulate ITO-CH_3, it could not be protonated or de-protonated upon pH adjustment from acidic to basic pH range.

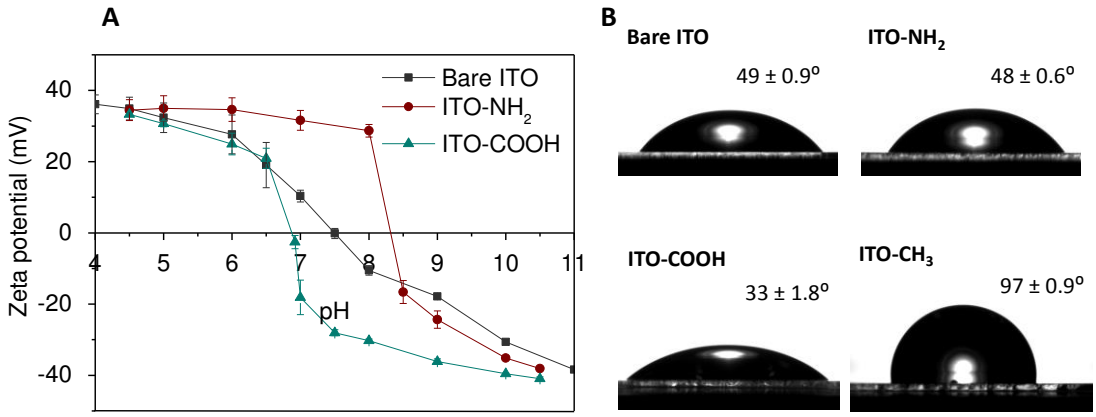


Figure 3-4 (A) Zeta potential profile of bare and functionalised particulate ITO in 10 mM NaCl. (B) Water contact angle of bare and functionalised planar ITO.

The surface topography of the bare and functionalised planar ITO was characterised under AFM. Contact mode images were acquired following each of the steps of surface modifications. Figure 3-5 presents the 3D images of the surfaces of bare and differently functionalised planar ITO. The 3D image of planar bare ITO reveals a grainy structure of the ITO surface with a measured root-mean-square (rms) of 1.8 nm. This agrees well with rms of 1.3 - 1.9 nm reported for clean planar ITO by Chockalingam and co-workers[31] and Choi and colleagues.[32] The APTES modification of bare planar ITO resulted in a relatively smooth layer with no island formation and an rms of 1.8 nm. This value however contrasts the relatively higher rms of APTES deposited silicon wafer (larger than 2 nm) reported elsewhere.[33-34] The high rms was due to prolonged APTES exposure, which signifies the formation of island from APTES agglomeration. The 3D topography of planar ITO-NH_2 observed in this study is in fact similar to the topography of well-rinsed APTES layer on glass substrate reported by Metwalli and co-workers.[35] The post functionalisation of planar ITO-NH_2 with succinic anhydride to provide carboxylic end surface functional groups resulted in a slight decrease in the surface roughness from 1.8 to 1.1 nm (Figure 3-5). This infers that the surface remained smooth after post functionalisation. A similar finding is also reported by Lee and co-workers.[24]

A significant increase of a measured rms from 1.8 to 3.9 nm was observed for non-ionised methyl functionalised planar ITO. This may be ascribed to the longer silane linker of ODTMS. Note that ODTMS molecule is six times longer than APTES molecule. The 2-fold increase in rms was also observed on silane grafted glass substrate, which molecule is three times longer than APTES.[35]

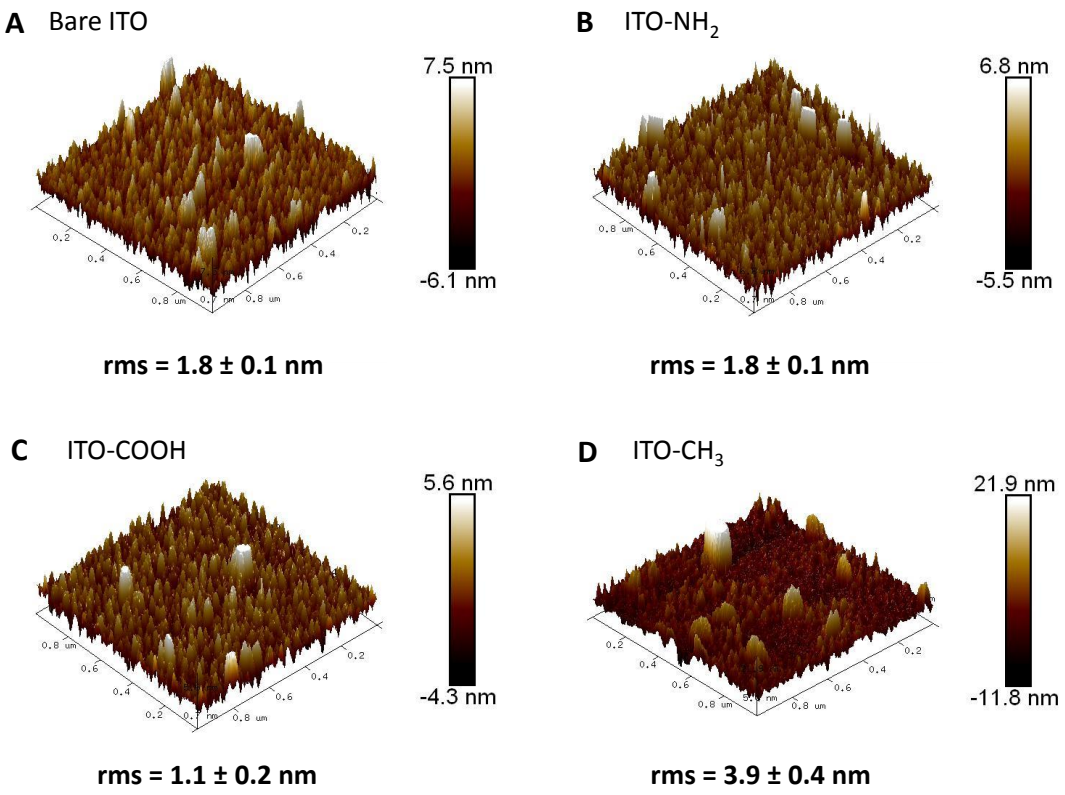


Figure 3-5 AFM 3D images of surface topography of bar and functionalised planar ITO. All data acquired with a scan size of 1 µm x 1 µm. The root-mean-square roughness is denoted by rms.

3.3.2 Adsorption of T4 Bacteriophages on Planar and Particulate ITO

The T4 adsorption behaviour towards the differently functionalised planar ITO surfaces was investigated by exposing the functionalised surfaces to T4 bacteriophage (10^{10} pfu/mL) for 16 h at 30 ^{0}C under constant mixing. Note that 10^{10} pfu was chosen based on theoretical calculations to achieve a complete T4 bacteriophage monolayer. Post-incubation, unbound and loosely-bound phages were removed by repeated washing of the surfaces with 50 mM Tris-HCl pH 8, followed by washing with 0.1% BSA in 10 mM phosphate buffer pH 6 to inhibit any non-specific binding on the planar ITO surface.

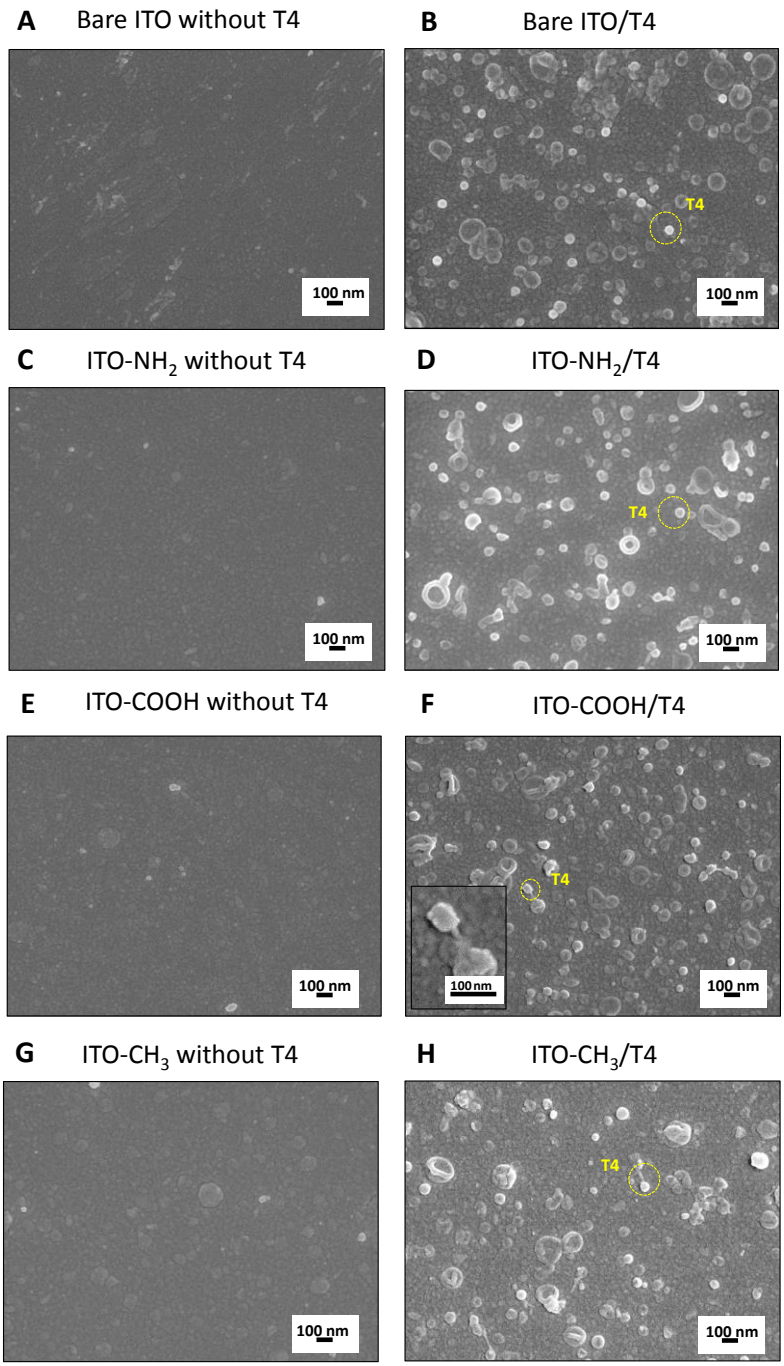


Figure 3-6 SEM images of planar (A, B) bare ITO, (C, D) ITO-NH_2, (E, F) ITO-COOH and (G, H) ITO-CH_3 without and with the presence of T4 bacteriophages, respectively. Yellow dotted circles denote T4 bacteriophage.

Figure 3-6 shows the SEM images of bare and functionalised planar ITO with and without the presence of T4 bacteriophage. The bacteriophage distribution was found to be fairly uniform on bare and functionalised planar ITO. The activity of adsorbed T4 bacteriophage on planar ITO was determined by placing the planar ITO on an *E. coli* lawn. This method of analysis exploited the ability of active bacteriophage to infect bacteria and cause bacteria lysis. The same principle has been employed to enumerate bacteriophage (via double agar overlay assay).[14] The *E. coli* forms a lawn on tryptone agar and the presence of active T4 bacteriophage on the lawn will result in a transparent/localised clearing zone, due to the production of new bacteriophage and *E. coli* lysis (Figure 3-7B). Figure 3-7A presents the negative control, whereby 10 μL of 10 mM phosphate buffer pH 6 was dropped on the lawn (location was marked by the piercing on the lawn). Herein, no clearing zone was observed. In contrast, clearing zones could be clearly observed when 10 μL of 10^9 pfu T4 bacteriophage/mL was dispensed on the *E. coli* lawn (Figure 3-7B). This infers the presence of active T4 that infected the *E. coli* and caused lysis.

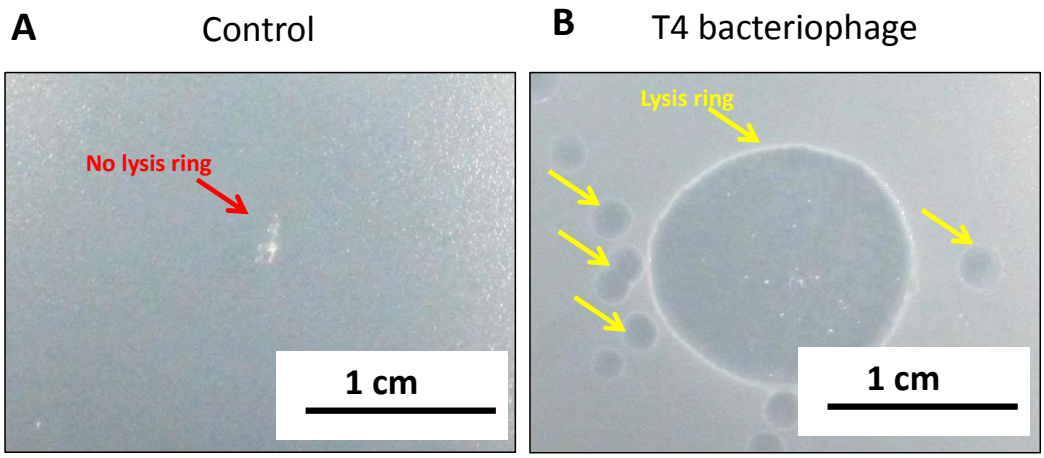


Figure 3-7 Activity test of (A) 10 µL of 10 mM phosphate buffer pH 6 and (B) 10 µL of 10^9 pfu T4 bacteriophage/mL on an *E. coli* lawn.

Figure 3-8 shows planar bare and functionalised ITO without and with the presence of T4 bacteriophage, respectively on the *E. coli* lawn. No clearing zone is observed on planar bare and functionalised ITO without T4 (Figure 3-8A, C, E and G). This implies that the variations in physico-chemical properties of bare and functionalised planar ITO did not bring about *E. coli* lysis. On the contrary, the presence of T4 bacteriophage on bare and functionalised planar ITO (as shown in SEM images in Figure 3-6A-H), shows the lysis ring formed around the ITO (Figure 3-8B, D, F and H), which suggests the adsorbed T4 bacteriophage is active.

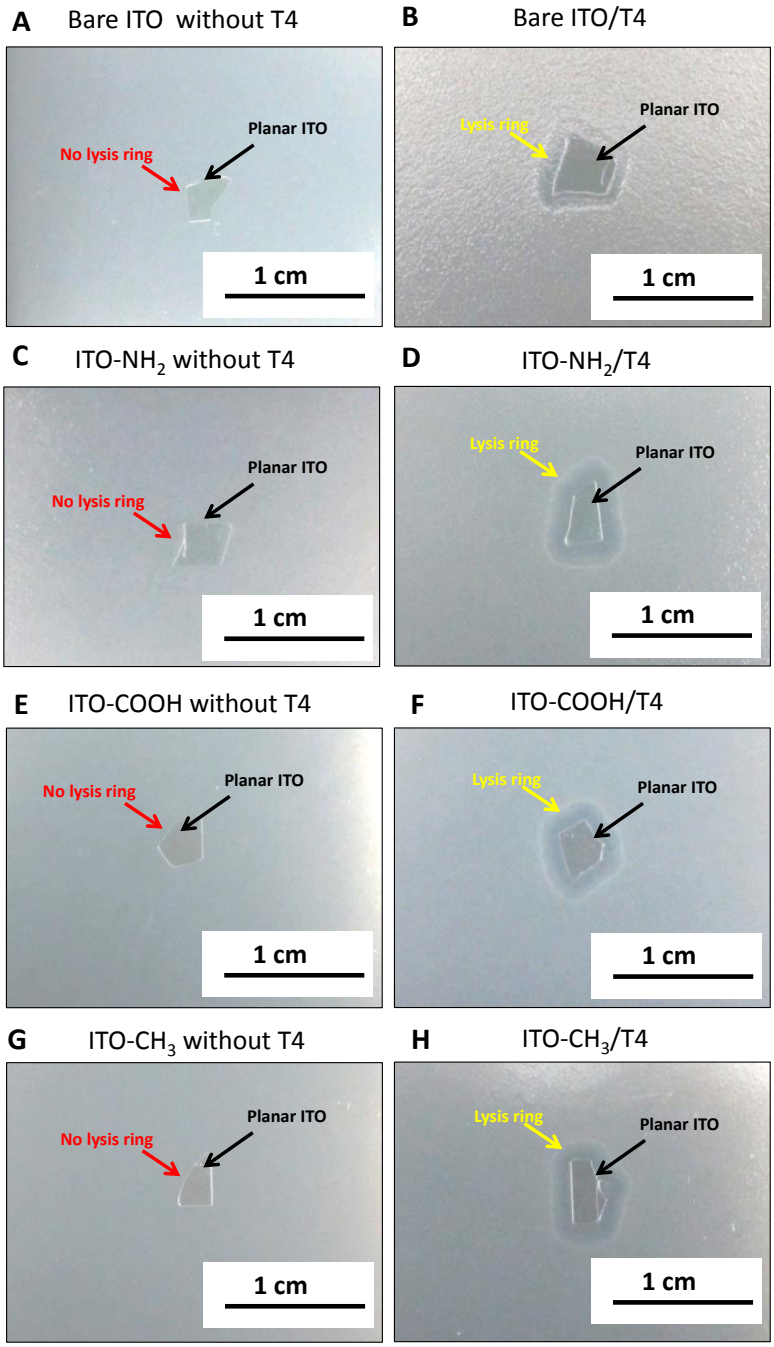


Figure 3-8 Activity test of (A, B) bare ITO, (C, D) amine, (E, F) carboxylic and (G, H) methyl functionalised ITO without and with the presence of T4 on an *E. coli* lawn. The presence of a lysis ring on B, D, E, F and H indicates the adsorbed T4 bacteriophage on planar ITO is infective towards *E. coli*.

The density of T4 bacteriophage adsorbed on planar ITO was enumerated via a double agar overlay plaque assay (see supporting information). Herein, distinct phage adsorption behaviour onto the differently-functionalised planar ITO surfaces (Figure 3-9) was observed. Unlike the adsorption behaviour typically observed on the particulate form of the substrate, the conversion of the hydroxyl group-rich planar bare ITO – to – ITO-NH_2 surfaces resulted in a substantial reduction in phage adsorption. The phage adsorption however, was increased following subsequent transformation of the planar ITO-NH_2 – to – ITO-COOH surface (Figure 3-9). These findings are in contrast to the well-documented virus adsorption behaviour on particulate substrates, whereby amine-functionalised particles are generally associated with a higher extent of virus adsorption in comparison to the carboxylic-functionalised particles.[2-4] Such a finding of higher phage adsorption on amine-functionalised particles was also corroborated in the current study with particulate ITO modified in an identical manner to the planar surfaces (inset in Figure 3-9). Interestingly, the extent of adsorption of T4 bacteriophage onto bare ITO and ITO-CH_3 did not vary between planar (Figure 3-9) and particular form of ITO (inset Figure 3-9). With comparable surface chemical compositions (Figure 3-2 and Figure 3-3) and phage exposure conditions between the two distinct forms of ITO, the variation in T4 adsorption behaviour is tentatively hypothesised to stem from different modes of phage mass transfer to the surface, which influences surface-phage contact frequency. The stationary nature of planar ITO surface induces lower collision frequency with T4 in comparison to the more mobile particulate ITO (hydrodynamic diameter = 1 - 1.5 μm, see Figure 3-1B). It has been reported that T4 bacteriophage requires the order of seconds to initiate binding with the host cell bacteria and the reduction in the contact time due to higher collision

frequency between T4 and its host cell has been shown to impede the T4 binding to its host.[36] Hence, the lower collision frequency exhibited between T4 and more stationary planar ITO might allow more contact time for T4 to initiate the necessary binding to the surface. In contrast, the higher collision frequency between T4 and mobile particulate ITO translates to less contact time available between T4. This difference in the extent of collision may therefore lead to different T4 adsorption behaviour. This study demonstrates the paramount influence of substrate physical configuration towards phage adsorption, which implies that the knowledge of virus-particles interactions is not directly transferrable to predict the virus adsorption behaviour to other form of substrate.

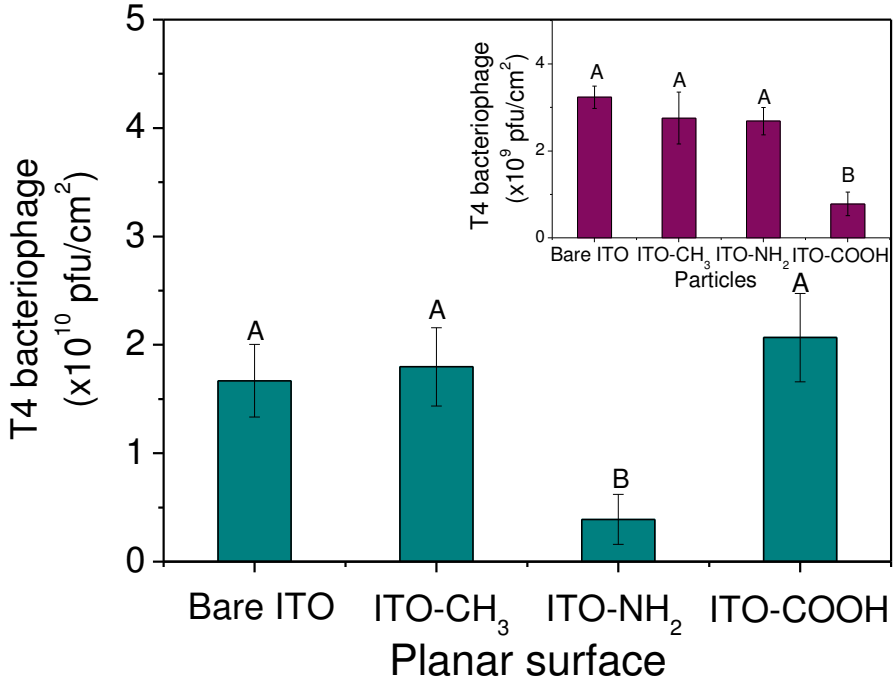


Figure 3-9 The calculated T4 bacteriophages concentration on bare and differently functionalised planar ITO based on double agar overlay plaque assay. The inset shows the calculated T4 bacteriophages on differently functionalised ITO particulate. Different letters indicate a significant difference (α = 0.05, by one-way ANOVA followed by Tukey test), from four independent replicates.

The functionalisation of planar bare ITO with -CH_3 groups with ODTMS resulted in a high degree of contact angle (~97°, Figure 3-4B) in comparison to bare and other functionalised planar ITO (33 - 49°, Figure 3-4B), which confirms the hydrophobicity of the surface. The modification also gave rise to the increase in surface roughness to almost double (root-mean-square roughness (rms) = 3.9 ± 0.1 nm, see Figure 3-5D) compared to bare planar ITO (rms = 1.8 ± 0.1 nm, see Figure 3-5A), which is attributed to the agglomeration of ODTMS molecule. Despite the significant changes in the surface

physical properties, planar ITO-CH_3 showed a similar high extent of T4 bacteriophage adsorption as compared to planar bare ITO (Figure 3-9). It appears that surface hydrophobicity and roughness influence the adsorption of T4 to planar ITO-CH_3. This finding is consistent with other study, which show high surface roughness and surface hydrophobicity promote the adsorption of fd phage to an amorphous carbon film.[37] Unlike planar ITO-CH_3, which possesses a high degree of contact angle (~97°, Figure 3-4B) and surface roughness (rms = 3.9 ± 0.1 nm, Figure 3-5D), bare ITO, ITO-NH_2 and ITO-COOH have similar contact angle (33° - 49°, Figure 3-4) and surface roughness (rms = 1.1 - 1.8 nm, Figure 3-5). Therefore, the surface hydrophobicity and surface roughness could not solely explain the variation in adsorption of T4 bacteriophage to planar bare ITO, ITO-NH_2 and ITO-COOH. This might suggest that there are other factors that influence the T4 adsorption behaviour on these three surfaces.

The phage-to-particle adsorption behaviour has been widely reported to result from electrostatic interactions between the phage and the substrate.[2-4] This is also corroborated by the adsorption of T4 phage to particulate ITO (inset in Figure 3-9). In this study, the introduction of the high pKa (9.8) amino groups caused the ITO-NH_2 particles to be more positively charged, as indicated by the IEP shift from pH 7.5 to 8.3 (Figure 3-4A) and in turn, rendering the particles highly favourable for adsorption of the net negatively charged T4 (IEP pH 2 - 4).[38-40] In contrast, the subsequent conversion of amino groups to carboxylic groups (Scheme 3-1) led to an opposite IEP shift from pH 8.3 to 6.8, which is in concordance with other reported literatures (pH 3.5 to 6.2).[17,41-44] The variations in reported IEP is largely due to the contribution of the native surface and the newly added

molecule.[41] The shift in IEP value leads to more negatively-charged particles that repulse the phage. The electrostatic interactions of the net oppositely charged components that dominate the bacteriophage-ITO particle interactions (inset Figure 3-9) however, appears to only have minimal effects on the phage adsorption behaviour to the corresponding ITO planar surfaces (Figure 3-9).

The modulation in T4 adsorption behaviour observed when transitioning from planar bare ITO – to – ITO-NH_2 – to – ITO-COOH surfaces suggests the prevalence of unique bacteriophage binding behaviour involving specific functional groups, beyond the non-specific electrostatic interactions. In contrast to that observed with ITO particles, a reduction in the adsorption of the net negatively charged T4 was observed when transitioning from planar bare ITO to the more positively-charged planar ITO-NH_2. Such interesting trend was also seen when transitioning from ITO-NH_2 to the more negatively-charged planar ITO-COOH, with a 5-fold increase in T4 adsorption (Figure 3-9). The unique phage adsorption behaviour on planar surfaces, in fact resemble those observed with *E. coli* host, which have been used as model systems to study bacteriophage-to-surface interactions.[45-46] The likely involvements of the surface amine and carboxylic functional groups, as also indicated in the current work, had been investigated by analysing the bacteriophage adsorption behaviour as a function of pH.[47] Note that the bacteriophage polypeptide is reported to comprise of carboxyl, amino or substituted amino, phenolic-hydroxyl and sulphydryl groups,[48] with similar functional groups also found on *E. coli* surface.[49-50] Optimum adsorption of T2 bacteriophage (structurally-alike to T4) was observed at pH 6.8 to 8.5 and dropped to essentially none at below pH 4 and

above pH 10.[47] The findings infer involvement of ionised surface functional groups – the carboxylic (-COO^-, pKa 3.1) and amine (-NH_3^+, pKa 8) moieties, and less likely, the phenolic-hydroxyl (-phenol-OH, pKa 10) or sulphydryl groups (-SH, pKa 8.5) at the optimum adsorption pH 6.8 to 8.5. Anderson reported similar pH dependency of the T4 bacteriophage adsorption to *E. coli* with an optimum adsorption seen at pH 6.8 to 9 and decreased outside the pH range.[51] The current work further extends the understanding of the involvement of the surface amine and carboxylic groups in the bacteriophage-surface interactions, as described herein.

A decrease in T4 adsorption following the blocking of hydroxyl groups on planar bare ITO (with APTES) to provide surface amine groups infers the reduction of favourable chemical binding site on planar bare ITO. This suggests the less significant role of $–NH_2$ compared to –OH groups in T4 adsorption. Such observation is in accordance with the minimal involvement of the *E. coli* host's surface amine groups in the adsorption of T2 to *E. coli*. T2 adsorption on *E. coli* was unaffected upon blocking of *E. coli*'s surface amine groups (with acetic anhydride) relative to the untreated cell.[47,52] The blocking of *E. coli*'s surface carboxylic groups on the contrary (with methanol and hydrochloric acid), resulted in significant reduction of T2 adsorption relative to the untreated cells.[47] The apparent role of the host's carboxylic functional groups is also indicated in the current work with a significant increase in the T4 adsorption when transitioning from planar ITO-NH_2 to ITO-COOH. Recalling the comparable macroscopic surface characteristics, such as surface roughness and hydrophobicity of planar bare ITO, ITO-NH_2 and ITO-COOH, it would be reasonable to deduce that the variations observed in bacteriophage adsorption

result from microscopic interactions involving surface functional groups of planar surface functional groups (potentially the -OH and -COOH). The findings denote the role of specific surface functional groups in the adsorption of bacteriophages on surfaces beyond the commonly accepted mode of net electrostatic interactions.

3.4 Summary

In this chapter, a comparative study to investigate the influence of surface physicochemical properties of ITO as model substrates for T4 bacteriophage adsorption is described. This study provides the first evidence of the role of the substrate's surface physical configuration (planar vs particulate) to bacteriophage adsorption behaviour, which implies that a phage-particle study can not necessarily be used as a model system to predict phage interactions to other form of surfaces. Importantly, the work presents evidence on the significance of planar surfaces' functional groups towards bacteriophage adsorption. Presence of specific functional groups, in this case the surface carboxylic and hydroxyl groups on planar ITO, was found to promote T4 bacteriophage adsorption. The presence of surface amine groups on planar ITO on the other hand, reduced the phage adsorption. This finding challenges the commonly accepted hypothesis whereby net electrostatic interactions dominate phage-surface binding.[2-3] These findings thus highlight the influence of chemical species beyond the widely postulated macroscopic surface parameters, such as net surface charge, surface roughness and surface hydrophobicity towards the adsorption of bacteriophage on planar surfaces.

Concerning the applications of bacteriophage-based interfaces, such as biosensors, antimicrobial surfaces and membrane filtration system, the findings presented herein will allow better design and fabrication of bacteriophage based interfaces via tailoring of the surface physico-chemical properties to achieve the intended applications.

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Chapter 4 T4 Bacteriophage Conjugated Magnetic Particles for *E. coli* Capturing: Influence of Bacteriophage Loading, Temperature and Tryptone

4.1 Introduction

The concept of concentration and isolation of target bacteria that is culture-independent has become increasingly popular,[1] as it allows isolation and concentration of target bacteria from other interferences and reduction of sample volume within a short period of time. The application of magnetic particles as a magnetically controllable material has revolutionised the isolation and separation of many chemical and biological materials especially when the concentration is low.[2-6] Immunomagnetic separation technique uses bio-functionalised magnetic particles to specifically capture target bacteria via

immunological reactions.[1,7] When introduced into food samples, the particles recognised and captured the target bacteria, separating them from the food debris through subsequent application of a magnetic field. The immunomagnetic separation may be introduced directly at enrichment step to reduce the time needed for enrichment.[8]

The specificity of the magnetic particles can be tuned by changing the recognition element on the particles. One potential example of the recognition element is bacteriophage. Bacteriophages are ubiquitous viruses with a broad or specific range of bacterial host.[9-11] The integration of bacteriophage and magnetic particles to isolate and capture target bacteria has not been well studied. Earlier studies have reported a very low bacteria recovery (less than 20%) of such system.[12-13] After almost two decades, a higher extent of bacteria recovery has only been reported by Chen and colleagues, with 60 - 70% recovery of *E. coli* K12 (10^5 cfu/mL) using T7 bacteriophage conjugated with mixed metal oxide nanoparticles containing 30% cobalt.[14] With limited studies available on the use of bacteriophage conjugated magnetic particles to capture and isolate target bacteria, the understanding of their performance still needs to be elucidated. Furthermore, the effectiveness of immobilised bacteriophage in infecting bacteria has been reported to reduce in the presence of environmental interferences, such as human albumin and extracellular polysaccharide.[15] Moreover, the presence of certain cations (Ca^{2+} and Mg^{2+}), solutions' pHs and temperatures have also been reported to significantly affect the interaction between bacteria and bacteriophage.[16-17] Yet, thus far there are no studies that investigate the influence of such variables to the performance of bacteriophage based

magnetic particles in capturing bacteria. The aim of this study is to begin to address that knowledge gap.

By exploiting the virus-surface interactions observed in Chapter 3, similar surface functionalisation strategy was applied on magnetic iron oxide nanoparticles (Fe_3O_4) for the adsorption of T4 bacteriophage. The influence of surface physico-chemical properties to T4 bacteriophage adsorption, as well as to the performance of the particles in capturing T4 bacteriophage's host bacteria, *Escherichia coli* (*E. coli*) were investigated. The critical importance of temperature and solution chemistry to the *E. coli* capturing performance by T4 conjugated Fe_3O_4 were studied.

4.2 Materials and Methods

4.2.1 Reagents and Materials

Iron (II,III) oxide nanopowder, 3-aminopropyltriethoxysilane (APTES), succinic anhydride, bovine serum albumin (BSA), octadecyltrimethoxysilane (ODTMS), phosphotungstic acid (PTA) and sodium citrate tribasic dihydrate were sourced from Sigma-Aldrich. Analytical grade methanol, ethanol, glacial acetic acid, sodium chloride, sodium hydroxide pellets, *N,N*-dimethylformamide (DMF), Tris-HCl, glycerol and D-glucose were purchased from Ajax Chemicals. Phosphate buffer saline tablets (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4 and 0.2 g/L KH_2PO_4, pH 7.2 - 7.4), tryptone, yeast extract and agar bacteriological were obtained from Oxoid. Bacteriophage T4 (ATCC 11303-B4) and its host *E. coli* (ATCC 11303) were supplied from the ATCC.

Luria Bertani broth was made by adding 10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride in Milli-Q water. Tryptone agar was prepared by dissolving 10 g/L tryptone, 8 g/L sodium chloride, 3 g/L D-glucose, 2 g/L sodium citrate tribasic dehydrate and 10 g/L agar bacteriological in Milli-Q water. All media were sterilised by autoclaving at 121 °C.

4.2.2 Surface Modification of Iron Oxide (Fe_3O_4) Nanoparticles

The amine functionalised Fe_3O_4 (Fe_3O_4-NH_2) was prepared by mixing 0.2 g of iron (II,III) oxide nanopowder with 5 mL anhydrous methanol, 0.25 mL water, 0.5 mL glacial acetic acid, and 30 mL glycerol. The solution was sonicated for 2 min at 20% amplitude (Misonix) and transferred into a three-necked round bottom flask. A mixture of 10% (v/v) of APTES in anhydrous methanol, and was slowly titrated and the solution was heated to 120 ˚C for 15 h under nitrogen. The particles were subjected to repeated washing after functionalisation in Milli-Q water and ethanol and dried under vacuum. The bare Fe_3O_4 was prepared as a control sample by following the same procedure with the absence of APTES.

The amine functionalised Fe_3O_4 was further derivatised to introduce carboxylic functional groups. An amount of 0.05 g of Fe_3O_4-NH_2 was added to 10% (w/v) of succinic anhydride in anhydrous DMF. The mixture was stirred for 3 h under nitrogen. The particles were washed in DMF, Milli-Q water and ethanol successively and dried under vacuum.

The methyl terminated Fe_3O_4 was obtained by dispersing 0.05 g bare Fe_3O_4 in 5% (v/v) n-butylamine in anhydrous methanol and 1 mL of ODTMS under sonication at an amplitude of 20% (Misonix) for 1 h. The suspension was left for 30 min and subjected to serial washing in methanol. The resulted particles were dried under vacuum overnight.

4.2.3 T4 Bacteriophage Propagation

The propagation of T4 bacteriophage was done by incubating 50 µL of 10^{10} plaque forming unit (pfu) per mL in 4 mL of fresh *E. coli* culture for 45 min at 37 °C. The mixture was then added to 200 mL log-phase *E. coli* culture and was incubated at 37 °C in a shaking incubator for 3 h. The solution was centrifuged twice at 5000 g for 20 min to pellet down the bacteria. The supernatant was then centrifuged twice at 25000 g for 2 h at 4 °C to pellet down the bacteriophages from the supernatant. The obtained pellet was re-suspended in 3 mL of 10 mM phosphate buffer pH 6 and stored at 4 °C until ready for use. Note that some bacterial debris may present in the stock solution, which might partially influence the bacteriophage density upon adsorption to surfaces. Size exclusion chromatography may be used to further purify bacteriophage solution.[18]

4.2.4 T4 Bacteriophage Adsorption

T4 bacteriophage was allowed to adsorb on to bare and differently functionalised Fe_3O_4 by incubating 0.15 mL T4 bacteriophage (~10^{10} pfu/mL) to 0.35 mL of the modified Fe_3O_4 (0.1 mg/mL) for 16 h at 30 °C in a shaker incubator (Incu). The particles were subjected to a series of washings in 50 mM Tris-HCl pH 8, followed by 0.1 % BSA in 10

mM phosphate buffer pH 6 and finally rinsed in 10 mM phosphate buffer pH 6 to remove unbound T4 bacteriophage and avoid any non-specific binding. The particles were suspended in 0.5 mL of 10 mM phosphate buffer pH 6. The collected washing supernatants were enumerated to determine the concentration of T4 bacteriophages remaining in the supernatant via a double agar overlay plaque assay.[18] A positive control of T4 bacteriophage in 10 mM phosphate buffer pH 6 was used.

The density of phages on the particles (C) was calculated using equation 4-1 by subtracting the control T4 bacteriophage (C_0) with the sum of T4 bacteriophages (C_w) remaining in washing supernatants. The calculated value was normalised with the corresponding surface area (SA, cm^2) of particles calculated from the hydrodynamic diameter obtained from dynamic light scattering measurement.

$$C = \frac{\left[\frac{C_0 - \sum_0^{10} C_w}{C_0}\right]}{SA\ of\ Fe_3O_4} \qquad \textbf{4-1}$$

4.2.5 T4 Bacteriophage Adsorption

T4 bacteriophage was allowed to adsorb on to bare and differently functionalised Fe_3O_4 by incubating 0.15 mL T4 bacteriophage (~10^{10} pfu/mL) to 0.35 mL of the modified Fe_3O_4 (0.1 mg/mL) for 16 h at 30 °C in a shaker incubator (Incu). The particles were subjected to a series of washings in 50 mM Tris-HCl pH 8, followed by 0.1 % BSA in 10 mM phosphate buffer pH 6 and finally rinsed in 10 mM phosphate buffer pH 6 to remove unbound T4 bacteriophage and avoid any non-specific binding. The particles were

suspended in 0.5 mL of 10 mM phosphate buffer pH 6. The collected washing supernatants were enumerated to determine the concentration of T4 bacteriophages remaining in the supernatant via a double agar overlay plaque assay.[19] A positive control of T4 bacteriophage in 10 mM phosphate buffer pH 6 was used.

The density of phages on the particles (C) was calculated using equation 4-1 by subtracting the control T4 bacteriophage (C_0) with the sum of T4 bacteriophages (C_w) remaining in washing supernatants. The calculated value was normalised with the corresponding surface area (SA, cm^2) of particles calculated from the hydrodynamic diameter obtained from dynamic light scattering measurement.

$$C = \frac{\left[\frac{C_0 - \sum_0^{10} C_w}{C_0}\right]}{SA\ of\ Fe_3O_4} \qquad \textbf{4-2}$$

4.2.6 Bacteria Capture Study

Fresh *E. coli* cultures were grown to a concentration of 1 x 10^9 colony forming unit (cfu)/mL. 0.2 mL of *E. coli* cultures were centrifuged and re-suspended in a desired media. The *E. coli* cultures were diluted in a desired media to a final concentration of 1 x 10^5 cfu/mL. The suspended solution of bare and differently functionalised Fe_3O_4/T4 was discarded and replaced by 0.5 mL of *E. coli* suspension (1 x 10^5 cfu/mL). The suspension was allowed to incubate for 10 minutes at either 4 °C or 37 °C under constant mixing. The particles were quickly separated using a magnet, which was then placed in an ice

bath to prolong the latent period.[16,20] The supernatants were enumerated using a plate counting assay to quantify the remaining *E. coli*.

4.2.7 Surface Characterisations

Dynamic Light Scattering. The hydrodynamic diameter of bare and functionalised Fe_3O_4 suspensions in 10 mM phosphate buffer pH 6 was determined using dynamic light scattering (Malvern Zetasizer Nano ZS). The samples were sonicated with an ultrasonic probe for 1 min at amplitude 20% (Misonix) prior to the measurement.

Zeta Potential. Phase analysis light scattering (Malvern Zetasizer Nano ZS) was used to determine the zeta potential of bare and functionalised Fe_3O_4 in 10 mM NaCl. The isoelectric point of bare and functionalised Fe_3O_4 was determined by measuring the zeta potential value at varying pH using 0.1 M HCl and 0.1 M KOH.

Transmission Electron Microscopy (TEM). The morphology of the bare and functionalised Fe_3O_4 was observed using TEM (FEI Tecnai G2) at an accelerating voltage of 200 kV. For all images, particles suspension was dropped onto a 200 mesh, carbon coated copper grid.

The biological sample for TEM was prepared by floating the carbon coated copper grid on a drop of sample solution for a few minutes. The excess fluid was drained from the grid by touching its edge with filter paper. The grid was stained with 2% PTA in 10 mM

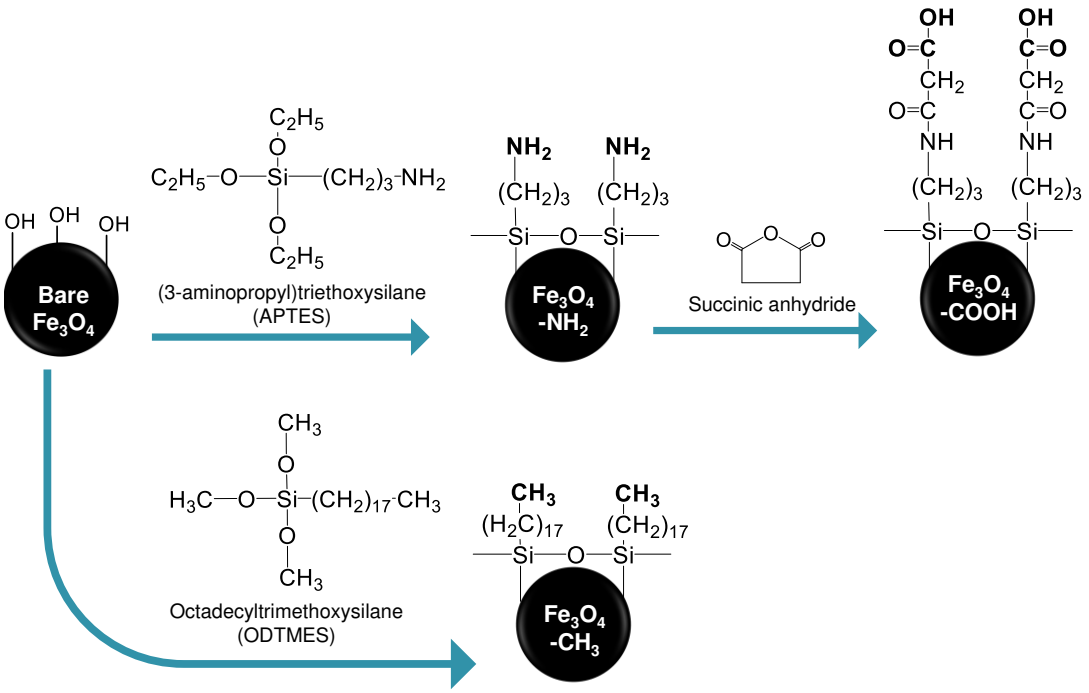
phosphate buffer saline. The grid was dried before examination under TEM (JEOL 2010) at an operating voltage of 200 kV.

X-ray Photoelectron Spectroscopy (XPS). Surface functionalisations were monitored using an ESCALAB220iXL spectrometer, operating with a monochromated Al-Kα radiation at 1486.6 eV and a source power of 120 W. The pass energy of 20 eV was used for narrow scans. All binding energies are referenced to the main carbon peak binding energy of 285.0 eV. Spectra were analysed using Avantage 4.88 software.

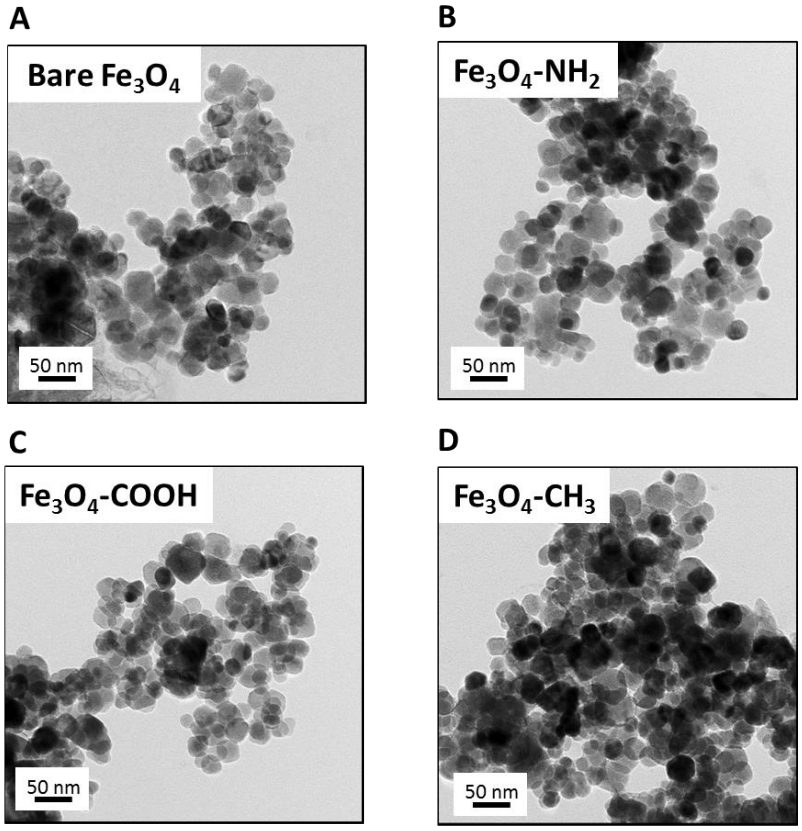
4.3 Results and Discussions

4.3.1 Controlled Surface Modification of Fe_3O_4 Nanoparticles

The surface modification of bare Fe_3O_4 by employing silane chemistry (Scheme 4-1) is to provide a scaffold to create tailored variations in the surface functional groups such as amine ($-NH_2$), carboxylic (-COOH) and methyl ($-CH_3$) groups, as well as variations in their surface charge. TEM images of bare Fe_3O_4 are shown in Figure 4-1A. The TEM images reveal that the iron oxide particles have a primary size particle of approximately 10 to 30 nm. There are no notable silane layers observed, nor morphological changes on the geometry of the particles following surface functionalisation (Figure 4-1B-D). Such scarcely visible silane layers on APTES and succinic anhydride modified iron oxide have also been reported by Li and co-workers.[21] Bare and functionalised Fe_3O_4 have similar hydrodynamic diameters (Figure 4-1E) when suspended in T4 bacteriophage suspending media (10 mM phosphate buffer pH 6). The immediate aggregation observed after suspension in high ionic strength solution is due to the compression of double layer, which subsequently reduces the electrostatic repulsion between particles.[22]



Scheme 4-1 Schematic of surface functionalisation of Fe_3O_4 with amine ($-NH_2$), carboxylic (-COOH) and methyl ($-CH_3$).



E

Samples	Hydrodynamic diameter (μm)
Bare Fe_3O_4	1.32 ± 0.05
Fe_3O_4-NH_2	1.42 ± 0.03
Fe_3O_4-COOH	1.28 ± 0.09
Fe_3O_4-CH_3	1.31 ± 0.08

Figure 4-1 (A-D) HRTEM images of bare, amine (-NH_2), carboxylic (-COOH), methyl (-CH_3) functionalised Fe_3O_4. (E) Hydrodynamic diameter of bare and functionalised Fe_3O_4 particles measured using dynamic light scattering. All samples were suspended in 10 mM phosphate buffer pH 6.

Figure 4-2 shows the XPS spectra of the bare and differently functionalised Fe_3O_4. For bare Fe_3O_4, the appearance of peak in Si2p narrow scan could be de-convoluted into two peaks centred at 101.2 eV and 99.3 eV. These peaks are attributed to Si-O peak and Fe3s, respectively.[23-24] The emergence of a tiny Si-O peak for bare Fe_3O_4 might be due to impurities in the carbon tape used to mount the sample for XPS analysis with similar observation also reported by Koh and co-workers.[23] The presence of impurities is also evident from the carbon contamination observed in the C1s narrow scan, which can be de-convoluted into fours peaks that are tentatively assigned to C-Si, C-C, C-O and O-C=O at 283.7 eV, 285.0 eV, 286.7 eV and 287.6 eV, respectively.[23,25] Amine functionalisation of Fe_3O_4 as shown in Scheme 4-1 resulted in the emergence of peaks in N1s narrow spectra (Figure 4-2), which can be de-convoluted into two peaks at 398.9 eV and 400 eV. These peaks are attributed to free amine (NH_2) and protonated amine (NH_3^+), respectively.[21,26-27] The emergence of NH_3^+ was attributed from the coordination or intermolecular hydrogen bonding between surface hydroxyl groups on particle surface with amino groups.[26] The adsorption of amino-silane molecules was also apparent by the increase in intensity of Si-O peak (101.0 eV), C-C (285.0 eV) and C-Si (283.7 eV) (Figure 4-2).

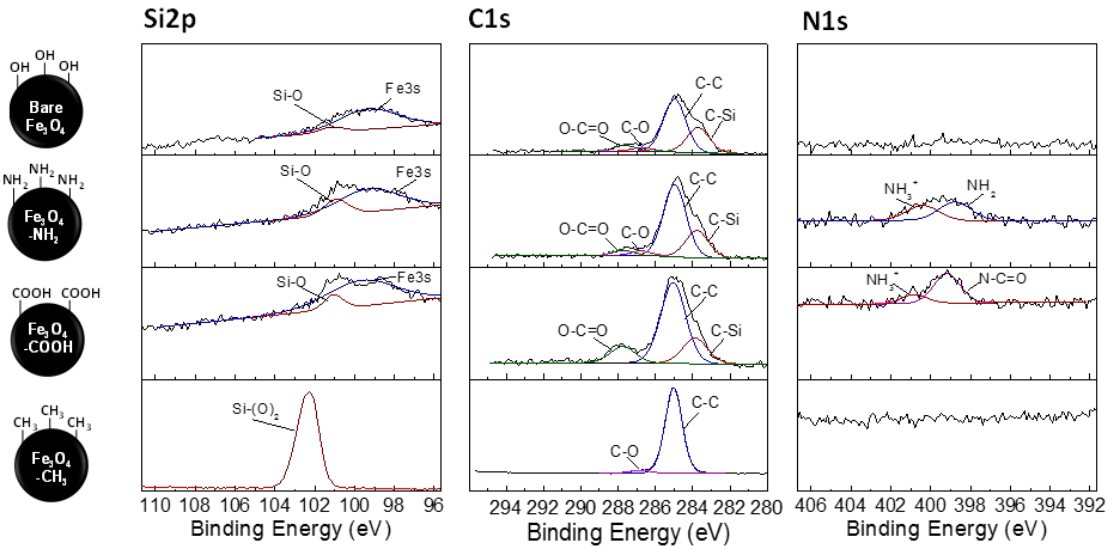


Figure 4-2 XPS narrow spectra of bare, amine (-NH_2), carboxylic (-COOH) and methyl (-CH_3) functionalised Fe_3O_4.

Carboxylic groups were introduced onto Fe_3O_4 via a nucleophilic reaction of -NH_2 with succinic anhydride that converts -NH_2 to N-C=O bond and yields carboxylic end functional groups (Scheme 4-1).[28-29] This conversion is evident from the disappearance of -NH_2 peak and the simultaneous appearance of an N-C=O peak at 399.2 eV (Figure 4-2).[29-30] The attenuation in O-C=O peak intensity in C1s narrow scan before and after reaction with succinic anhydride further confirming the successful conversion from Fe_3O_4-NH_2 to Fe_3O_4-COOH.

Silanisation using ODTMS grafting to obtain Fe_3O_4-CH_3 resulted in the dramatic attenuation in C-C peak (Figure 4-2).[31] A single peak is also observed at 102.3 eV in Si2p narrow scan, which is in agreement with the reported binding energy of 102.1 eV for a siloxane with different chemical state (Si-O_2).[32-33] The disappearance of Fe3s in Si2p

narrow scan as compared to other functionalised Fe_3O_4 might suggest that a thicker ODTMS layer formed on the Fe_3O_4 surface that exceeded the XPS sampling depth. A similar observation is also reported by Li and colleagues upon the silanisation using 3-mercaptopropyltrimethoxysilane (MPTMS) on Fe_2O_3.[21]

Apart from the different surface functional groups, the surface functionalisation also altered the surface charge of Fe_3O_4 particles. The presence of amine on Fe_3O_4 surface resulted in the positive shift in IEP (isoelectric point) from 6.8 to 7.8 (Figure 4-3), which is in agreement with other reported silanised particles.[21,34] This implies that Fe_3O_4-NH_2 particles exhibit a positive surface charge at pH less than 7.8. Following the conversion of amine moieties into carboxylic terminated groups, a left shift in IEP from 7.8 to 5.8 was observed (Figure 4-3).[21,35] The lower IEP is attributed to the presence of -COOH groups on the surface, which has a low pKa of 4.13.[24,36] These findings suggest that Fe_3O_4-NH_2 is more positively charged than bare Fe_3O_4 particles, whilst Fe_3O_4-COOH is more negatively charged than bare Fe_3O_4 particles. Fe_3O_4-CH_3 did not show any shift in IEP and the curve characteristic is also similar to bare Fe_3O_4. This is due to the neutral nature of methyl functional groups on the ODTMS that could not be protonated or de-protonated upon pH adjustment from acidic to basic pH. A similar observation was reported by Jesionowski and co-workers, whereby no shift in IEP was observed after silica particles was functionalised with methyl terminated octyltrimethoxysilane.[37]

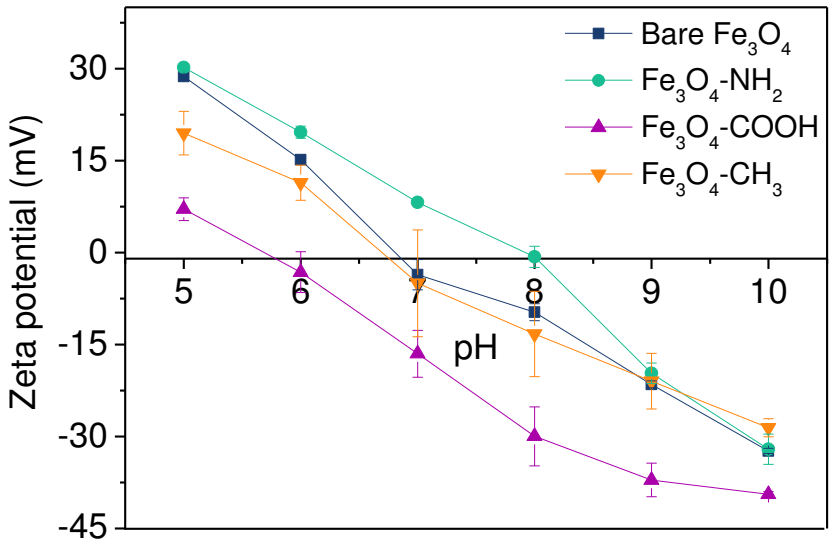


Figure 4-3 Zeta potential profile of bare, amine (-NH_2), carboxylic (-COOH) and methyl (-CH_3) functionalised Fe_3O_4 in 10 mM NaCl.

4.3.2 Effect of Surface Functionalisation on T4 Bacteriophage

T4 bacteriophage (Figure 4-4A) is a non-enveloped virus with protein polypeptide coat that comprises of various acidic, basic and hydrophobic amino acids.[38-39] The bacteriophage has an IEP between pH 2 - 4[40-41] and considered to be a hydrophobic virus.[42-43] The adsorptive behaviour of T4 bacteriophage to surfaces is determined by the interaction between virus protein coat and the surface physicochemical properties. Figure 4-4B shows the TEM image of bare Fe_3O_4 with T4 bacteriophage. The influence of the bare and differently functionalised Fe_3O_4 towards T4 bacteriophage adsorption is herein investigated.

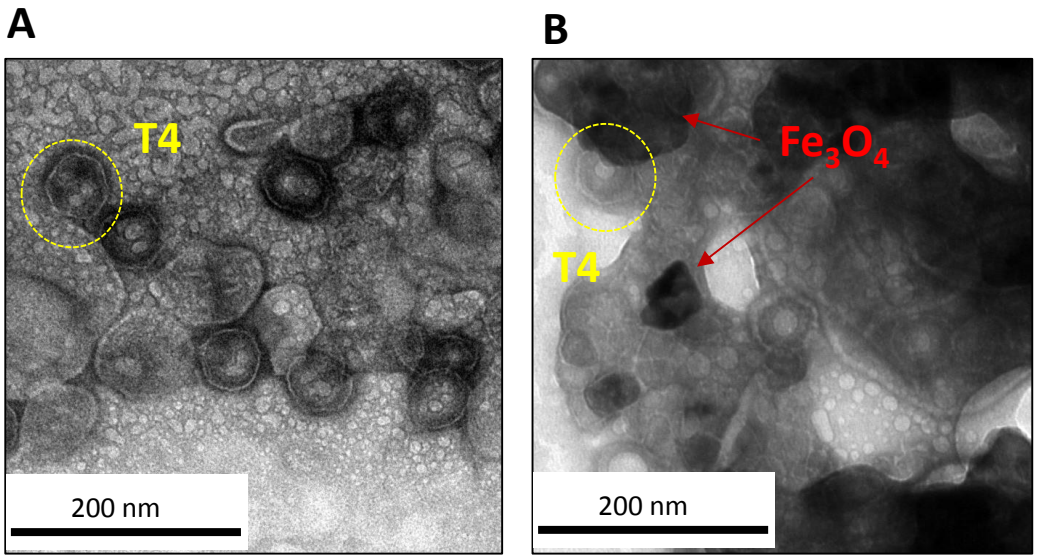


Figure 4-4 HRTEM images of (A) T4 bacteriophage and (B) T4 bacteriophage conjugated bare Fe_3O_4.

Figure 4-5 shows the T4 bacteriophage concentration adsorbed per surface area for the bare, amine, carboxylic and methyl functionalised Fe_3O_4 particles. The T4 bacteriophage

loading on bare Fe_3O_4, Fe_3O_4-NH_2 and Fe_3O_4-CH_3 was 3-fold higher than that on Fe_3O_4-COOH. As previously mentioned, bare Fe_3O_4 particles, which are rich in hydroxyl groups, have an IEP of 6.8, which suggests that they exhibited a net positive surface charge at the adsorption pH of 6. Meanwhile, T4 bacteriophage (IEP of pH 2 - 4) carried a net negative surface charge.[40] Hence, the high T4 adsorption on bare particles appears to result from an electrostatic attraction between the opposite net surface charge. This is concordance with the finding in Chapter 3, whereby electrostatic forces dominate the interactions of T4 bacteriophage with particulate indium tin oxide (IEP of pH 7.5).[44] Similarly, Meder and colleagues reported high MS2 phage (IEP of pH 4)[40] adsorption onto bare Al_2O_3 particles (IEP of pH 9.3) when incubated at pH 7.3.[45] The significance of the net surface charge of the particles, in dictating bacteriophage adsorption, explains the variation in its adsorption behaviour to other hydroxyl group rich particles. For instance, the poor adsorption of MS2 phage and PRD1 to SiO_2 particles is due to the low IEP of SiO_2 (IEP of ~pH 3), which repels the negatively charged MS2 phage and PRD1 phage at their immobilisation pH 7.2 - 7.4.[46] This suggests that amongst the macroscopic properties of the particle, the net surface charge predominantly dictates bacteriophage adsorption to the particles surface despite the similar presence of surface hydroxyl functional groups. Corroborating the role of electrostatic interaction, high T4 adsorption was also observed for amine functionalised Fe_3O_4 (Figure 4-5). The Fe_3O_4-NH_2 particles (IEP of pH 7.8, Figure 4-3) exhibit a net positive surface charge at the adsorption pH of 6, which promotes the adsorption of the net negatively charged T4 bacteriophage. High bacteriophage adsorption to amine functionalised particles has also been reported elsewhere.[45-47]

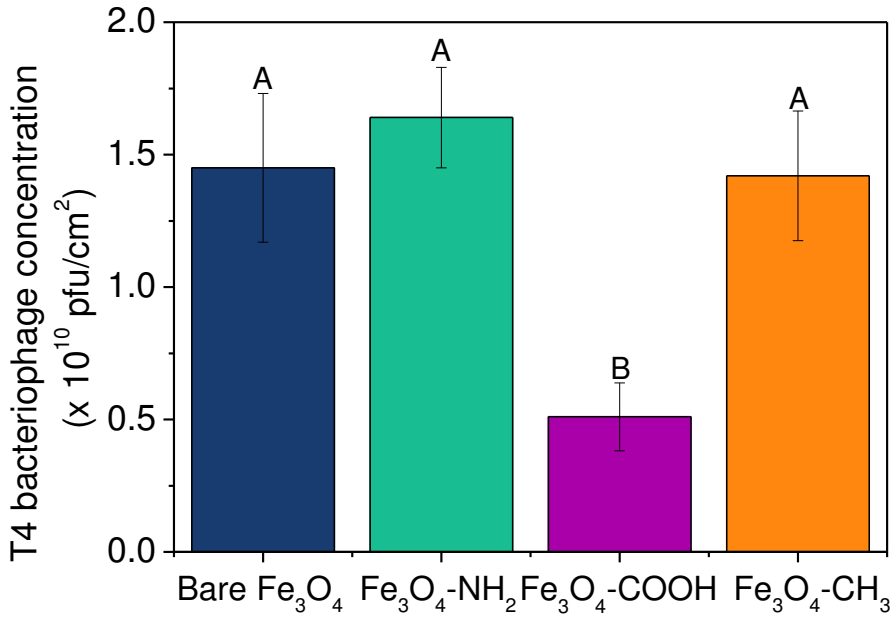


Figure 4-5 T4 bacteriophage concentration of bare and differently functionalised Fe_3O_4. Significant differences among different samples are indicated by different letters (α = 0.05, by one-way ANOVA followed by Tukey's test), from four independent replicates. 1 x 10^{10} pfu T4 bacteriophage /mL was incubated with 0.1 g Fe_3O_4/L.

The 3-fold reduction in T4 bacteriophage adsorption on Fe_3O_4-COOH compared to Fe_3O_4-NH_2 also appears to be due to an electrostatic repulsion between two like charges as evident by the negative shift in IEP from 7.8 to 5.8 for Fe_3O_4-COOH (Figure 4-3). This infers that both Fe_3O_4-COOH and T4 bacteriophage carry a net negative surface charge at pH 6. Such finding is also in agreement with studies conducted with other viruses, such as MS2, phiX174 and other phages to carboxylic modified particles.[45,47-48] Interestingly, the electrostatic repulsion between Fe_3O_4-COOH and T4 bacteriophage did not completely exclude T4 attachment. This observation is likely due to the complex

properties of T4 bacteriophage that allow a multitude of interactions to occur with hydrophilic positively charged, negatively charged and hydrophobic amino acid residues.[38-39] A similar observation was also reported by Meder and co-workers.[45]

Despite containing non-ionised groups, Fe_3O_4-CH_3 also exhibited high T4 bacteriophage adsorption (Figure 4-5). The addition of a large fraction of methyl groups by ODTMS grafting to surfaces has been found to increase the surface hydrophobicity,[31,44,49] which is likely to interact with the hydrophobic domains of the bacteriophage. The increase in surface hydrophobicity of silica particles following the grafting of octadecytricholorosilane has been reported to promote MS2 phage adsorption by 400-fold compared to the bare particles via hydrophobic interactions. Meder and colleagues also reported high MS2 phage adsorption to the more hydrophobic chloro-functionalised Al_2O_3 comparable to the bare Al_2O_3,[45] as also observed in the current work.

4.3.3 Bacteria Capture Study of Fe_3O_4/T4 Composites

In this study, the capabilities of bare and functionalised Fe_3O_4 particles with adsorbed T4 to capture *E. coli* were investigated. Fe_3O_4-CH_3 particles were not investigated due to the difficulty in dispersing the particles in *E. coli* suspending solution. Herein, the particles were incubated in various *E. coli*-containing solutions for 10 minutes at different temperatures under constant mixing. The particles were then separated from the *E. coli* suspension by applying a magnetic field. The *E. coli* supernatants were assayed using a plate counting assay to determine the *E. coli* capturing ability of Fe_3O_4/T4. Prolonged Fe_3O_4/T4-*E. coli* incubation is undesirable as *E. coli* lysis would commence at

approximately 30 minutes following T4 infection, as evident in Figure 4-6A, whereby a reduction in optical density at 600 nm (OD_{600}) was observed after 30 minutes.[50] It is also crucial to determine as to whether any potential traces of free T4 bacteriophage in the solution might cause a reduction in *E. coli* concentration that may lead to false positive data. To confirm this, ~10^7 pfu T4 bacteriophage/mL was incubated with ~10^7 cfu *E. coli*/mL for 10 minutes at 37 °C under constant mixing in Luria Bertani medium following similar experimental procedure as the *E. coli* capture experiments. We observed no reduction in *E. coli* concentration (Figure 4-6B), which implies that any changes in *E. coli* concentration exhibited by the Fe_3O_4/T4 systems are not due to possible traces of free T4 bacteriophage.

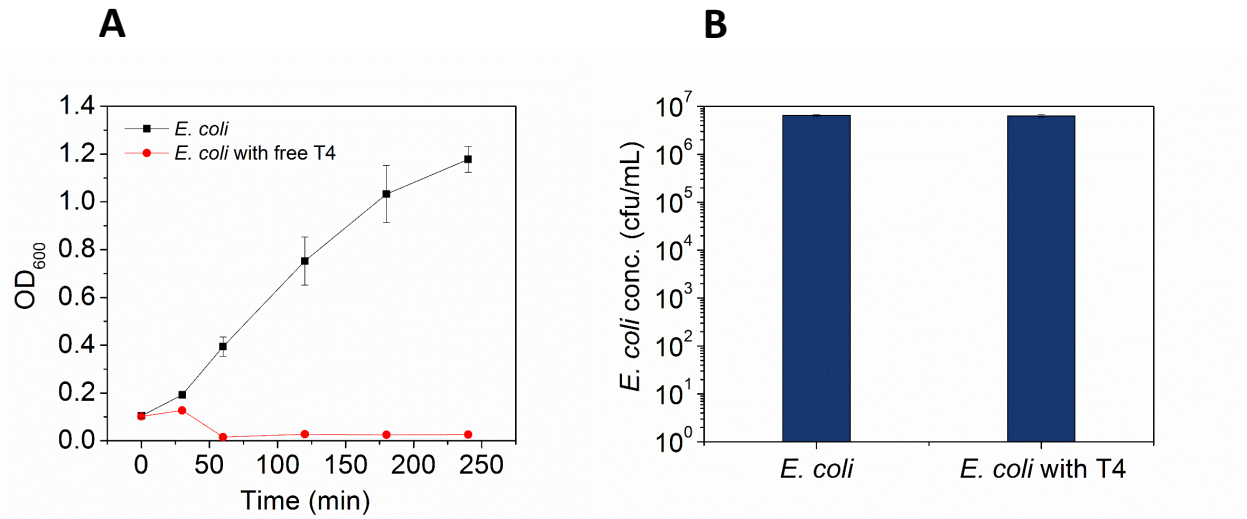


Figure 4-6 (A) Optical density measurement at 600 nm (OD_{600}) of *E. coli* with and without the presence of T4 bacteriophage. (B) Concentration of *E. coli* after incubation with and without T4 bacteriophage for 10 minutes, measured via plate counting assay. All experiments were conducted at 37 °C under constant mixing in Luria Bertani medium. ~10^7 pfu T4 bacteriophage/mL and ~10^7 cfu *E.coli*/mL were used. Data obtained from three independent replicates.

Figure 4-7A shows the percentage of *E. coli* captured by the T4 conjugated particle in Luria Bertani medium at 37 °C after 10 minutes incubation. It can be observed that ~30% capturing was achieved by bare and other functionalised Fe_3O_4 without T4, which suggests the contribution of non-specific binding. A ~3-fold increase in %*E. coli* captured for bare Fe_3O_4/T4 and Fe_3O_4-NH_2/T4 in comparison to their respective particle-only control suggests enhanced capturing capabilities in the presence of T4. This finding is in accordance with Chen and co-workers, who observed similar capturing efficiency for T7 conjugated bare mixed metal oxide (FeCo) nanoparticles.[14] In contrast, a low percentage of *E. coli* was captured by Fe_3O_4-COOH/T4 and no significant increase observed in

comparison to Fe_3O_4-COOH without T4. Herein, the low percentage of *E. coli* captured by Fe_3O_4-COOH/T4, which is 3-fold lower than bare Fe_3O_4/T4 and Fe_3O_4-NH_2/T4 appears to stem from the low T4 bacteriophage loading on Fe_3O_4-COOH. Figure 4-8 shows the relationship between T4 loading on the particles and the *E. coli* captured density. It can be observed that an increase in T4 bacteriophage density improve the *E. coli* capturing. This observation is in agreement with Naidoo and co-workers, who reported an increase in bacteria captured with higher phage loading on particles until it reaches the optimum point.[18]

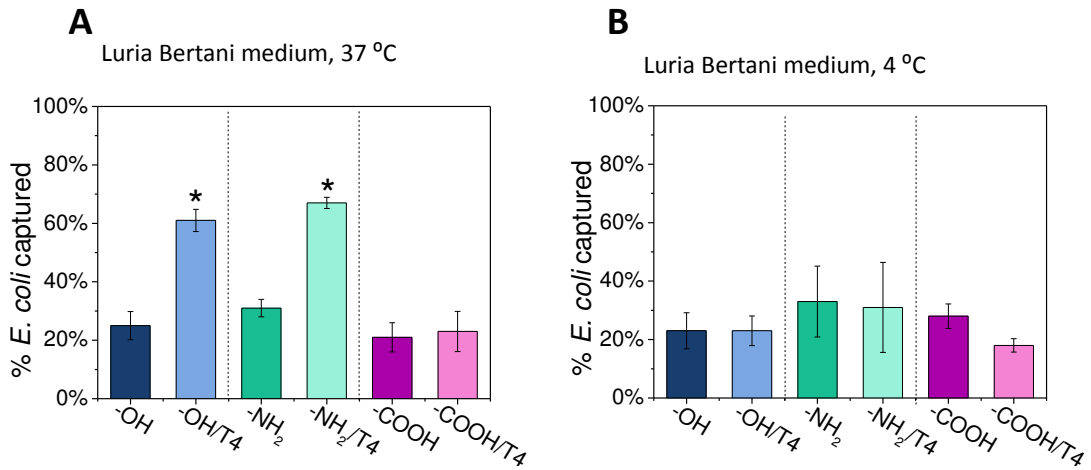


Figure 4-7 Percentage of *E. coli* captured by bare (-OH), amine (-NH_2) and carboxylic (-COOH) functionalised Fe_3O_4 particles with and without the presence of T4 in Luria Bertani at (A) 37 °C and (B) 4 °C. 10^5 cfu *E.coli*/mL were used. The * indicates a significant difference with the respective control sample (α=0.05, by two-way ANOVA followed by Holm-Sidak correction), from three independent replicates.

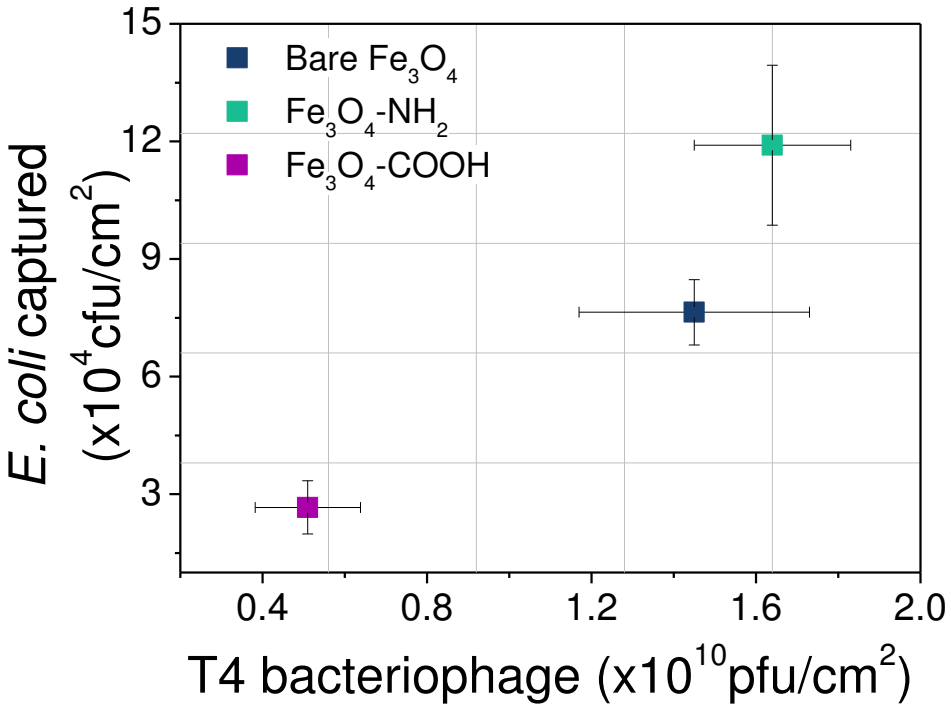


Figure 4-8 The relationship between *E. coli* captured density and the T4 bacteriophage loading on bare, amine (-NH_2) and carboxylic (-COOH) functionalised Fe_3O_4. 10^5 cfu *E. coli*/mL was used. Data collected from at least three independent replicates.

The effects of bacteriophage loading aside, it was found that the *E. coli* capturing capabilities of the T4 conjugated particles also varies with the incubation temperature. A lower incubation temperature of 4°C in Luria Bertani medium (Figure 4-7B) caused a reduction in *E. coli* capturing for both bare Fe_3O_4/T4 and Fe_3O_4-NH_2/T4, when compared to those at 37°C. Note that both Fe_3O_4/T4 and Fe_3O_4-NH_2/T4 exhibited comparable capturing percentages relative to their respective particle-only control at 4°C. This is most likely attributed to the retardation of irreversible T4 – *E. coli* binding at low temperature, as described herein. The binding of T4 to *E. coli* involves a two-step process that starts

with the reversible binding of the bacteriophage on *E. coli* surface via receptor identification, involving the distal end of T4 long tail fibres. This is followed by irreversible binding through T4 short tail fibres.[51] Unlike the reversible binding, the irreversible binding of bacteriophage to its host was found to be dependent on incubation temperature.[52] A clear example of this, the bound T1 bacteriophage was found to easily detach from *E. coli* surface at 3 °C due to dilution, which suggests the absence of irreversible binding at low temperature. A similar finding was also reported for T4 and *E. coli*.[53-54] Taken together, the findings suggest the need for T4 to establish irreversible binding with *E. coli* for capturing by bare Fe_3O_4/T4 and Fe_3O_4-NH_2/T4. In other words, the lowering of incubation temperature to 4 °C inhibits the necessary succession from reversible to irreversible binding of T4 to *E. coli*. This shows the importance of incubation temperature in dictating Fe_3O_4/T4 performance in capturing *E. coli*, even for particles with already high density of adsorbed bacteriophages.

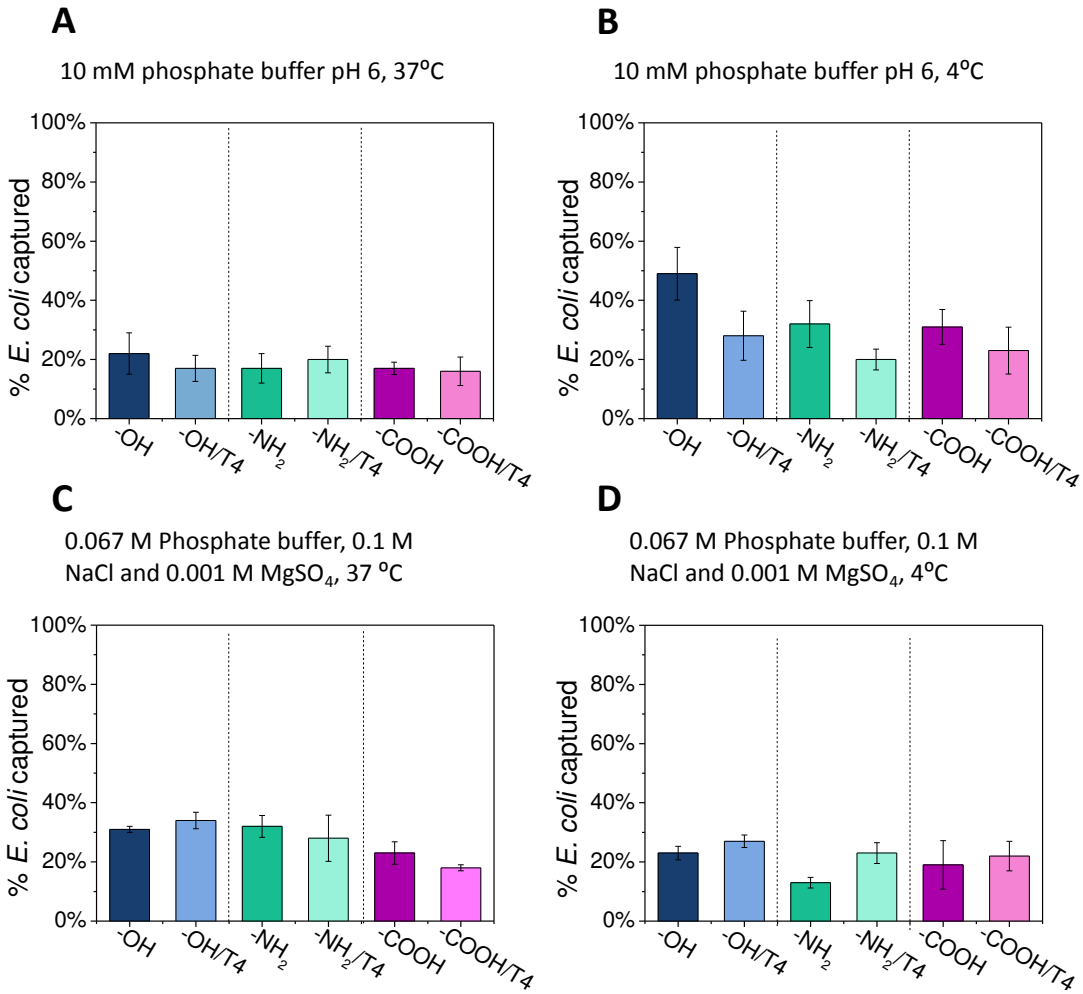


Figure 4-9 Percentage of *E. coli* captured by bare (-OH), amine (-NH_2) and carboxylic (-COOH) functionalised Fe_3O_4 particles with and without the presence of T4 in 10 mM phosphate buffer at (A) 37 °C and (B) 4 °C and in 0.067 M phosphate buffer, 0.1 M NaCl and 0.001 M $MgSO_4$ at (C) 37 °C (C) and (D) 4 °C. The statistical analysis revealed no significant differences compared to the respective control (α=0.05, by two-way ANOVA followed by Holm-Sidak correction), from three independent replicates.

As more efforts have been made to apply bacteriophage conjugated magnetic particles to capture and concentrate target bacteria from food samples, the efficacy of such application in different environments therefore need to be investigated. Previous studies have employed a variety of solution chemistry when investigating bacteriophage conjugated particles performance in capturing bacteria, such as phosphate buffer saline and Luria Bertani medium.[12,55-57] The influence of the solution chemistry on the capturing performance however, remains unclear.

Herein, the *E. coli* capturing performance was tested in 10 mM phosphate buffer pH 6. At the optimum temperature of 37 °C, poor *E. coli* capturing performance was observed for bare and other functionalised Fe_3O_4 with T4 in 10 mM phosphate buffer pH 6 (Figure 4-9A). Several bacteriophages have been known to require the presence of co-factors to establish interaction with their host bacteria or for multiplication inside their host bacteria. For instance, certain strains of T5 bacteriophage are dependent on the presence of Ca^{2+} to multiply,[58] whereas specific strains of T4 and T6 require presence of L-tryptophan to bind to its host bacteria.[58-59] Certain strains of T4 bacteriophage have been reported to require the presence of L-tryptophan to interact with its *E. coli* host; it is reported that six molecules of tryptophan are required to activate all the T4 tails one one phage for interaction.[60] Tryptone is one of the components in Luria Bertani medium and it is rich in tryptophan.[61] The absence of tryptone in 10 mM phosphate buffer might explain the reduction in *E. coli* capturing performance by bare Fe_3O_4/T4 and Fe_3O_4-NH_2/T4 when compared to those in Luria Bertani medium at 37°C.

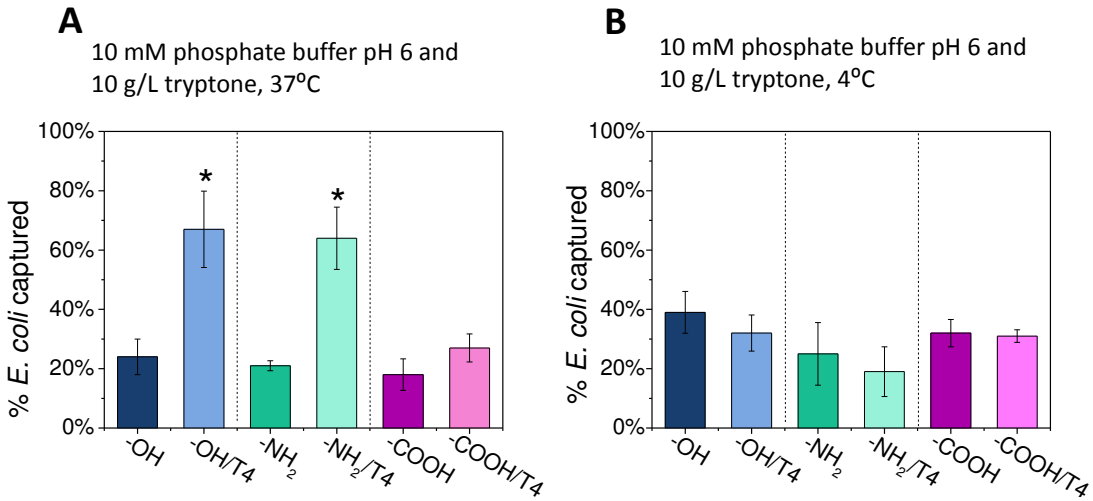


Figure 4-10 Percentage of *E. coli* captured by bare (-OH), amine (-NH_2) and carboxylic (-COOH) functionalised Fe_3O_4 particles with and without the presence of T4 in 10 mM phosphate buffer with 10 g/L tryptone at (A) 37 °C and (B) 4 °C. The * indicates a significant difference with the respective control sample (α=0.05, by two-way ANOVA followed by Holm-Sidak correction), from three independent replicates.

To validate the role of tryptone towards the *E. coli* capturing capabilities of the bare and functionalised Fe_3O_4/T4, 10 g tryptone/L was spiked into 10 mM phosphate buffer pH 6 system. Recalling the low *E. coli* capturing by bare Fe_3O_4/T4 and Fe_3O_4-NH_2/T4 (~20%) in the 10 mM phosphate buffer-only system at 37°C (Figure 4-10A), the addition of the tryptophan-rich tryptone significantly improved the bacterial capturing to ~70% (Figure 4-9A). In the absence of tryptophan, Kellenberg and colleagues observed the failure of T4 bacteriophage to bind onto *E. coli* due to retraction of T4 tail fibers in phosphate buffer at pH 7.[60] Harbouring the receptor binding domain on its tip,[62] the retraction of tail fibers will impede the interactions between T4 and *E. coli*.[63] This shows that the presence of tryptophan, (in this case supplied in tryptone), is vital in promoting

interactions between the bacteriophage and *E. coli* that ultimately leads to their irreversible binding at the optimum 37 °C for *E. coli* capturing.

The critical role of tryptone in promoting T4 – *E. coli* interaction is in line with further investigations of the effects of monovalent and divalent cations addition to *E. coli* capturing. The presence of monovalent (Na^+) and divalent (Mg^{2+}) cations has been widely reported to cause charge neutralisation and subsequently, promote bacteriophage adsorption to its host,[64] which results in *E. coli* capturing. Such addition of cations (0.1 M NaCl and 0.001 M $MgSO_4$) into the phosphate buffer system (0.067 M phosphate buffer pH 7) however, did not enhance the *E. coli* capturing of the bacteriophage conjugated particles relative to the control particles, even at 37 °C (Figure 4-9C). Unlike in the presence of tryptone, this finding infers that the *E. coli* capturing cannot be achieved through charge neutralisation via the addition of cations.

Interestingly, the presence of tryptone is benign to the T4 - *E. coli* interactions at low incubation temperature. The *E. coli* capturing capabilities of the bare and functionalised Fe_3O_4/T4 in Luria Bertani decreased at 4 °C (Figure 4-7B). The trend was also observed with the presence of tryptone in 10 mM phosphate buffer pH 6 at 4°C (Figure 4-9B). Apart from the already mentioned inability for T4 to establish irreversible binding with *E. coli* at low temperature, a couple of studies reported that adsorption of tryptophan to the T4 bacteriophage key binding site reached an optimum at 35 °C, with temperature above or below 35 °C was found to retard the adsorption of tryptophan that led to poor T4 adsorption on *E. coli*.[54,65] Therefore, the *E. coli* capturing by T4 bacteriophage

conjugated magnetic particles is unlikely at low temperatures even in the presence of tryptophan-rich tryptone. Similarly, poor *E. coli* capturing performance was exhibited by low T4 loading on Fe_3O_4-COOH (Figure 4-10A) even at the optimum conditions of 37 °C and the presence of tryptone in the media. Hence, it can be deduced that the presence of tryptone, the use of optimum incubation temperature (37 °C) or the high T4 loading alone is insufficient to promote *E. coli* capturing. These observations reveal a synergistic effect between temperature, the presence of tryptone and T4 loading in dictating the performance of bare and functionalised Fe_3O_4 in capturing *E. coli*.

4.4 Summary

In this study, the performance of T4 bacteriophage conjugated Fe_3O_4 in capturing and isolating *E. coli* was investigated. Herein, the variations in chemical surface properties introduced via organosilane grafting of the magnetic particles affected T4 bacteriophage loading onto the surface. In accordance with the previous finding in Chapter 3, high bacteriophage adsorption onto bare Fe_3O_4 and Fe_3O_4-NH_2 was found to result from the electrostatic interactions between the net positively charged particles with the net negatively charged T4. Meanwhile, electrostatic repulsion between the net negatively charged Fe_3O_4-COOH and T4 resulted in 3-fold lower bacteriophage adsorption. The adsorption of T4 onto non-ionised methyl functionalised Fe_3O_4 is attributed to hydrophobic interactions. The capturing performance is largely influenced by the bacteriophage loading, with a high percentage of *E. coli* capture (~70%) was achieved by

high T4 loading on bare Fe_3O_4 and Fe_3O_4-NH_2 after 10 minutes, while low T4 loading on Fe_3O_4-COOH resulted in poor *E. coli* capturing.

In addition to the bacteriophage loading, the important effects of incubation temperature and presence of tryptone on the *E. coli* capturing ability of the particles are also reported. Effective *E. coli* capturing requires an irreversible binding between T4 bacteriophage and *E. coli*, which occurs at 37°C in tryptone-containing media for the bare Fe_3O_4/T4 and Fe_3O_4-NH_2/T4. Poor *E. coli* capturing by both particles were interestingly observed at 37°C in the absence of tryptone and similarly, in the presence of tryptone at 4°C. The poor capturing is most likely caused by the inhibition of the irreversible binding at low temperature, with the absence of tryptone has been known to result in retraction of T4 bacteriophage tails where the receptor binding domain is located. The findings reveal that the presence of tryptone in the media or optimum incubation temperature alone is insufficient to promote bacteria capturing by particles, even with high bacteriophage loading. This study highlights that the success of bacteriophage conjugated particles in capturing bacteria is dependent on the surface physico-chemical properties to promote high bacteriophage loading and the contribution of external factors, such as temperature and tryptone to the subsequent bacteriophage-bacteria interactions.

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Chapter 5 Antimicrobial Activity of T4 Bacteriophage Conjugated Planar Indium Tin Oxide (ITO)

5.1 Introduction

Antimicrobial food packaging has recently gained more interest as a new form of food packaging that actively suppresses or inhibits the growth of pathogenic microorganisms, slows down food deterioration and increases food shelf life.[1-2] The traditional method that involves direct addition of antimicrobial agents to food via dipping and spraying has been well established due to its simplicity.[2] However, such practice was found to be less effective in suppressing the growth of microorganisms, due to their rapid diffusion, which dilutes the effective concentration of the antimicrobial agents and their possible denaturation upon contact with food components.[2-3] The incorporation of antimicrobial agents onto the packaging surface is expected to be more efficient as it allows a slow and

continuous release of antimicrobial agent to food, which in turn could prolong their shelf life.[2]

In an era where antibiotic resistant bacteria are on the rise,[4-5] bacteriophages possess an attractive alternative to the conventional antimicrobial agents, such as antibiotics. Bacteriophages are bacterial viruses which propagate by infecting their host bacteria and cause bacteria death by lysis to release new bacteriophages at the end of their infection cycle.[6] Owing to their ability to infect only a few strains of bacteria species or most rarely, to infect a closely related bacterial genus, bacteriophages offer minimal disruption to the other existing bacteria, unlike antibiotics which tend to infect a broader spectrum of microorganisms.[5] Their self-propagating ability allows multiplication of bacteriophage as long as the host bacteria is still present.[5,7] Bacteriophage also continuously adapt to bacterial mutation.[8] Some bacteriophages have been listed by US Food and Drug Administration (FDA) as safe to use to control *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* (*E. coli*) O157:H7 in ready to eat or raw meat.[9-10]

Comprehensive reviews on direct application of bacteriophage as an antimicrobial agent pre- or post-harvest have been extensively reported.[6,8] For instance, complete eradication of *Listeria monocytogenes* on artificially contaminated soft cheese by surface application of virulent phage P100 was reported.[11] The usefulness of A511 and P100 virulent phage for specific *Listeria monocytogenes* control in a range of foods (e.g. meat, seafood, dairy and plants) have also been reported.[12] However, incorporation of bacteriophage onto or within antimicrobial packaging is rarely reported. It has been

reported that the use of immobilised active phage against *Listeria monocytogenes* and *E. coli* on positively and neutrally charged cellulose membrane, with 24% more bacteriophage adsorbed onto positively charged membrane than to the neutral charged membrane.[13] However, both membranes exhibited similar 2-log units bacterial growth inhibition regardless to their loading. This finding is interesting as other bacteriophage based interfaces, such as bacteriophage based biosensor[14] and bacteriophage based conjugated magnetic particles for bacteria capture (Chapter 4) showed that higher bacteriophage loading is critical to achieve a good performance. Further, the performance of bacteriophage has been previously reported to be influenced by the presence of food components. The presence of fat globules had been reported to shield the bacteria surface and protect it from bacteriophages.[15] Dixon and colleagues reported a reduction in antimicrobial activity of immobilised PRD1 and T4 bacteriophage upon addition of environmental interferences, such as extracellular polymeric substances and human serum albumin.[16] There is however a limited research that focuses on understanding the mechanisms of antimicrobial activity exhibited by immobilised bacteriophage and factors contributing to their performance.

Thus, this work is aimed to investigate the antimicrobial activity and its mechanisms of bare (-OH), amine ($-NH_2$), carboxylic (-COOH) and methyl ($-CH_3$) functionalised indium tin oxide (ITO) surface conjugated with T4 bacteriophage that were presented in Chapter 3. ITO and T4 bacteriophage were used as a model substrate and bacteriophage. The influence of food components, modelled using casein and starch, as well as the variation

in solution pH towards bare and functionalised ITO/T4 antimicrobial activity were investigated.

5.2 Materials and Methods

5.2.1 Reagents and Materials

Bacteriophage T4 (ATCC 11303-B4) and its host *E. coli* (ATCC 11303) were procured from the ATCC. Bovine serum albumin (BSA), casein from bovine milk, starch from potato, phosphate buffer saline tablets (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4 and 0.2 g/L KH_2PO_4, pH 7.2 - 7.4) and sodium citrate tribasic dihydrate were sourced from Sigma-Aldrich. Sodium chloride, sodium hydroxide (NaOH) pellets, hydrochloric acid (HCl), Tris-HCl and D-glucose were purchased from Ajax Chemicals. Tryptone, yeast extract and agar bacteriological were obtained from Oxoid. SYBR® Gold was purchased from Life Technologies.

Luria Bertani medium pH 7 was made by adding 10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride in Milli-Q water. Luria Bertani medium pH 5 and 8 were prepared by adjusting the pH of Luria Bertani medium pH 7 with 10 M HCl and 10 M NaOH, respectively.

Tryptone agar was prepared by dissolving 10 g/L tryptone, 8 g/L sodium chloride, 3 g/L D-glucose, 2 g/L sodium citrate tribasic dehydrate and 10 g/L agar bacteriological in Milli-Q water. All media were sterilised by autoclaving at 121 °C.

30 mg starch/mL in Luria Bertani medium pH 7 was prepared by dissolving starch from potato in Luria Berani medium pH 7. The solution was heated slowly to completely dissolve the starch under constant mixing. The solution was let to cool to room temperature and used immediately.

Casein from bovine milk solution was prepared by dissolving 162 mg of casein in 5.4 mL of Luria Bertani medium pH 8. Due to the acidic nature of casein, the solution pH was frequently adjusted to pH 7 and it was also heated slightly in order to completely dissolve the casein.

5.2.2 Antimicrobial Activity of ITO/T4

The antimicrobial activity of ITO/T4 was assessed by preparing fresh *E. coli* culture with a concentration of 1 x 10^9 colony forming unit (cfu)/mL. The cultures were centrifuged and re-suspended in a desired media and further diluted to a final concentration of 1 x 10^5 cfu/mL. The suspended solution of bare, -NH_2, -COOH and -CH_3 functionalised ITO with T4 bacteriophage was discarded and 0.5 mL of *E. coli* suspension (1 x 10^5 cfu/mL) was added. The ITO/T4 and *E. coli* mixture was incubated at 37 °C under constant mixing (Incu). Samples were taken at different time interval to determine the concentration of the remaining *E. coli* and resulted T4 bacteriophage.

The above experiment was further extended to determine the ability of ITO/T4 to exhibit antimicrobial activity upon addition of another *E. coli* (1 x 10^5 cfu/mL) culture,

which is denoted as 2nd infection. The suspensions were sampled at different times to determine the concentration of *E. coli* and T4 bacteriophage.

All samples were enumerated to determine the concentration of *E. coli* and T4 bacteriophage via plate counting assay and double agar overlay plaque assay[17], respectively.

5.2.3 T4 Bacteriophage Leaching

The concentration of T4 bacteriophage being released from bare and functionalised ITO with T4 was determined by incubating bare and differently functionalised ITO/T4 in Luria Bertani medium pH 7 at 37 °C under constant mixing (Incu). Samples were taken at different time intervals and enumerated via double agar overlay plaque assay[17] to determine the concentration of T4 bacteriophage.

5.2.4 Stability of T4 Bacteriophage and *E. coli*

The stability of T4 bacteriophage in the presence of food components, namely casein and starch was determined by following the method by Kropinski.[18] Serial dilutions of food component were prepared in Luria Bertani medium pH 7 and a known concentration of bacteriophage was added to the solution. The phage suspensions were incubated at 37 °C for 2 h under constant mixing in a shaker incubator (Incu).

The T4 bacteriophage stability in Luria Bertani medium pH 5, 7 and 8 was determined by adding a known concentration of T4 bacteriophage to Luria medium adjusted to pH 5, 7 or 8. The phage suspensions were incubated at 37 °C in a shaker incubator (Incu) and the phage concentration was monitored overtime. A similar experiment was used to assess the stability and growth of *E. coli* in Luria Bertani medium of pH 5, 7 and 8.

All phage suspensions were enumerated via a double agar overlay plaque assay to determine the concentration of T4 bacteriophage after incubation.[17] *E.coli* concentrations were quantified using plate counting assay

5.2.5 Surface Characterisations

Fluorescence Imaging. Bare and differently functionalised ITO/T4 were imaged using Zeiss Axio Observer X.1 Spinning Disk and Total Internal Reflection Fluorescence (TIRF) Microscopy. The samples were incubated with 2.5 μL of SYBR gold per 1 mL of solution for 15 min prior to imaging.[19] The samples were imaged with an excitation and emission wavelength of 485 nm and 498 nm, respectively.

5.3 Results and Discussions

5.3.1 Antimicrobial Activity of ITO/T4

The surface functionalisation of ITO surface and the adsorption of T4 bacteriophage onto bare and functionalised ITO have been investigated in Chapter 3. To extend the knowledge of its efficacy as an antimicrobial surface, the antimicrobial activity of T4 conjugated ITO was investigated. Factors that were critical in dictating their performance were also determined.

The distribution of T4 bacteriophage on all surfaces (bare (-OH), $-NH_2$, -COOH and $-CH_3$ functionalised ITO) were characterised under fluorescence microscopy (Figure 5-1) and shown to be well distributed in all cases. The T4 conjugated ITO was immersed in an *E. coli* suspension in Luria Bertani medium at 37 °C and the concentration of viable *E. coli* density was monitored overtime. In Chapter 4, it was found that there is a synergistic influence between T4 bacteriophage loading on the surface, the presence of tryptone in the media and temperature in promoting T4 and *E. coli* interaction. Hence, Luria Bertani medium, which contains tryptone and a working temperature of 37 °C were used as a standard operating condition. A control experiment was prepared by incubating bare and functionalised ITO with the absence of T4 in an *E. coli* suspension.

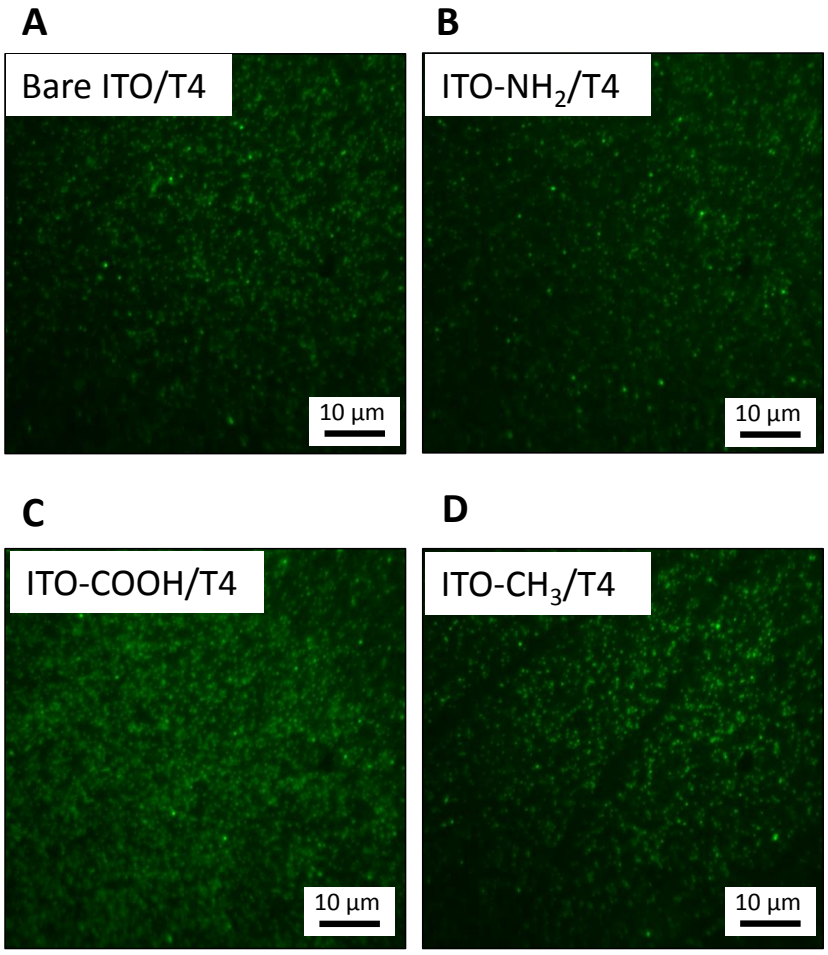


Figure 5-1 T4 bacteriophage on (A) bare, (B) -NH_2, (C) -COOH and (D) -CH_3 functionalised ITO. The green dots indicate T4 bacteriophage.

Figure 5-2A shows that the incubation of T4 conjugated bare, -NH_2, -COOH and -CH_3 functionalised ITO with *E. coli* resulted in more than a ~10^5-fold reduction in *E. coli* concentration after 2 h. Such reduction was not observed for bare and differently functionalised ITO in the absence of T4 bacteriophage, which signifies that the reduction in *E. coli* concentration is solely due to the presence of T4 bacteriophage, rather than the variations in bare and functionalised ITO's physicochemical properties. Interestingly, T4 conjugated bare, -NH_2, -COOH and -CH_3 functionalised ITO showed a comparable antimicrobial activity regardless the difference in their bacteriophage loading as previously described in Chapter 3. ITO-NH_2/T4 has a 3-fold less T4 loading in comparison to bare, -COOH and -CH_3 functionalised ITO surfaces. The independence of ITO/T4 antimicrobial activity to their bacteriophage loading suggest in significant role of bacteriophage loading in dictating their antimicrobial activity. This is in contrast to the previous finding reported in Chapter 4, whereby the performance of iron oxide (Fe_3O_4) conjugated with T4 bacteriophage to capture *E. coli* is highly dependent on the bacteriophage loading, with higher bacteriophage loading is required to achieve high *E. coli* capture.

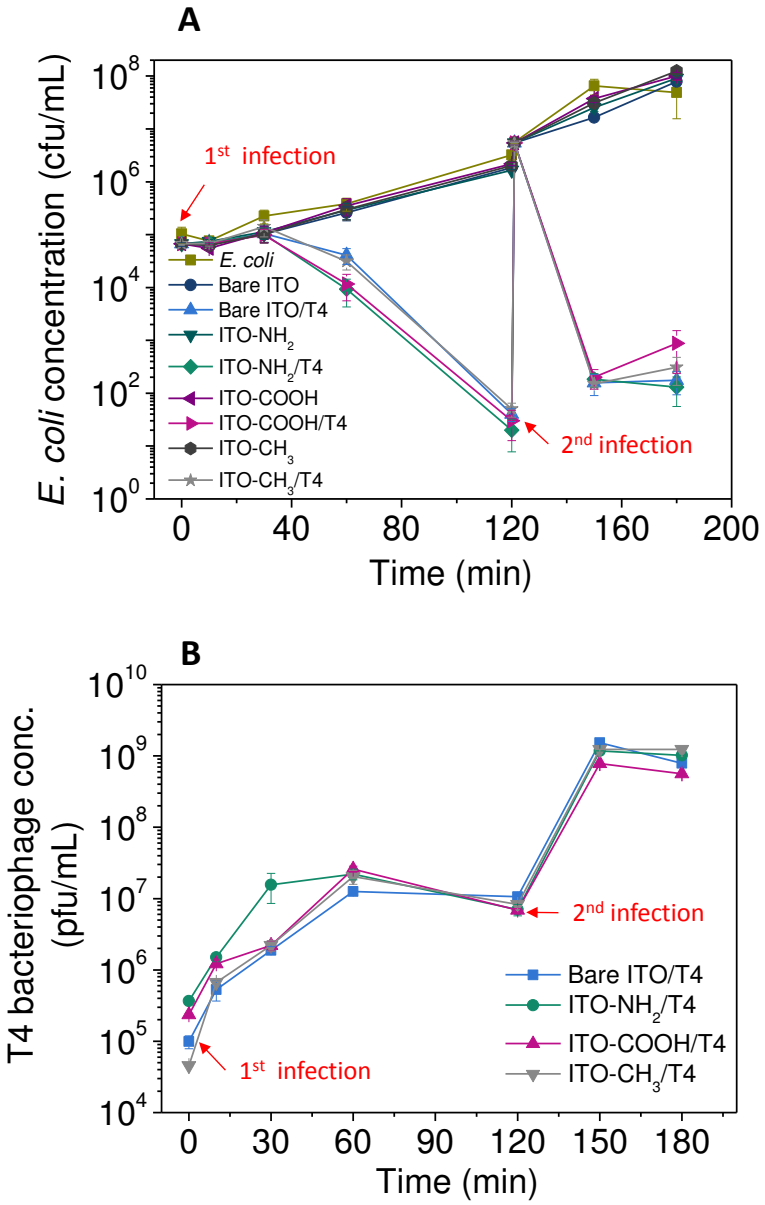


Figure 5-2 Antimicrobial activity of bare and functionalised ITO with and without the presence of T4 bacteriophages as monitored by (A) the *E. coli* concentration and (B) the resulted T4 bacteriophage concentration. 1st infection denotes the 1st addition of 1 x 10^5 cfu *E. coli*/mL and 2nd infection refers to the 2nd addition of 1 x 10^5 cfu *E. coli*/mL. Data obtained from three independent measurements.

To better understand factors, such as the influence of T4 bacteriophage loading, the mechanism of ITO/T4 antimicrobial activity needs to be firstly elucidated. Antimicrobial activity may be achieved in two ways, namely through the diffusion of *E. coli* to the bacteriophage on ITO/T4 surface or through the release and diffusion of free T4 bacteriophage from ITO/T4 surface to the surface of *E. coli*. This can be confirmed by investigating any potential release of T4 bacteriophage from ITO/T4 surface to the solution medium. Herein, it was found that there was an immediate release of less than 0.03% of adsorbed T4 bacteriophage (10^5 pfu/mL) upon incubation of the ITO/T4 in Luria Bertani medium (Figure 5-3). The release of T4 reached its maximum after 10 min and remained steady during the course of 3 h incubation. The release of T4 also appeared to be independent of their initial bacteriophage loading on ITO, whereby ITO-NH_2/T4 has a 3-fold less bacteriophage on its surface compared to other surfaces (Chapter 3).

The release T4 bacteriophage is therefore likely to be responsible for the ITO/T4 antimicrobial activity observed, since it allows the bacteriophage to diffuse to *E. coli* surface. The bacteriophage-bacteria interactions will then result in *E. coli* infection, immediate production of bacteriophage, followed by bacteriophage liberation at the expense of *E. coli* lysis or death. This cycle will continue until all *E. coli* have been infected (Scheme 5-1).

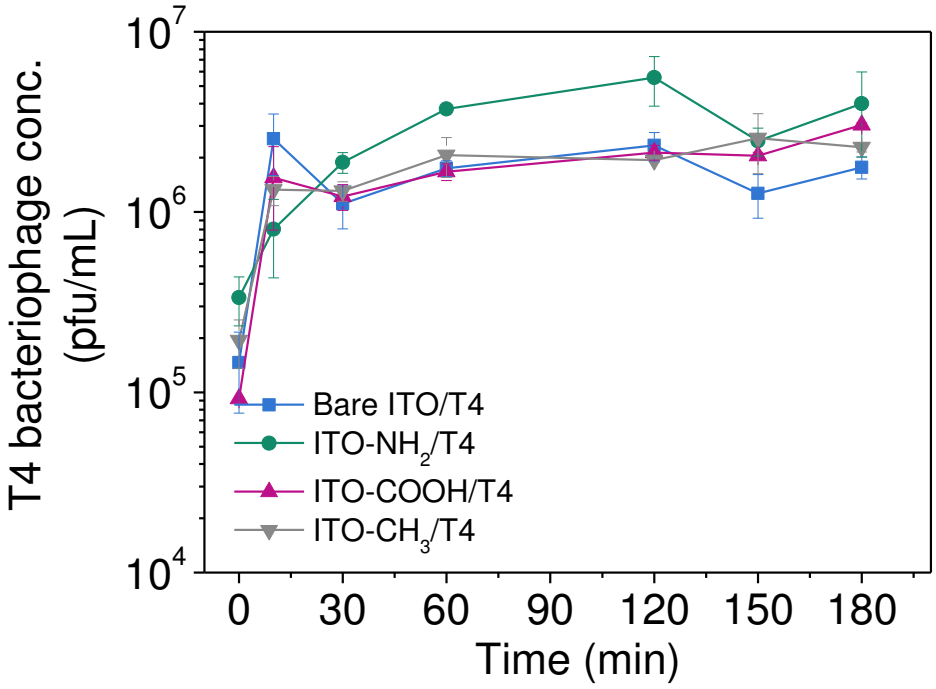
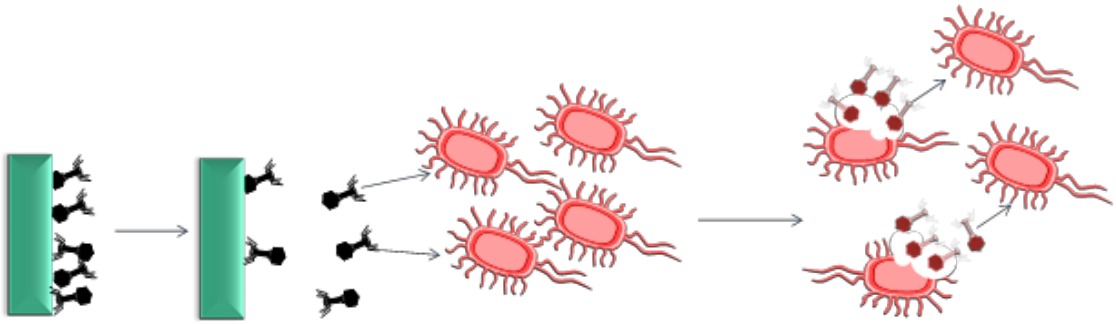


Figure 5-3 Concentration of released T4 bacteriophage from bare and functionalised ITO/T4 in Luria Bertani medium pH 7. Data obtained from three independent measurements.

Since the antimicrobial activity appears to be governed primarily by the release T4 bacteriophage, the similar concentration of T4 bacteriophage being released (Figure 5-3) might therefore explain the almost identical antimicrobial activity (Figure 5-2A) exhibited by bare, -NH_2, -COOH and -CH_3 functionalised ITO with T4. It is interesting to note that bacteriophage loading is less critical in dictating the activity of ITO/T4. Although this finding is in accordance with Anany and co-workers,[9] bacteriophage loading has been previously reported to play a significant role in the performance of other

bacteriophage based interfaces as reported in Chapter 4 and also by Naidoo and colleagues.[14] This hence shows that the performance of bacteriophage based interfaces is also dependent on the mechanism of the end-point applications. For instance, the above antimicrobial system relies on the concentration of T4 bacteriophage being released to the solution regardless of their loading. In contrast, in the application of bacteriophage conjugated magnetic particle system to capture bacteria (Chapter 4), the bacteria need to be in contact with the bacteriophage on the particle surface in order to be captured and isolated. Hence, the higher bacteriophage loading on the particle surface, the more bacteria are captured.



Scheme 5-1 Proposed mechanism of ITO/T4 antimicrobial activity.

One of the challenges in any antimicrobial system is the depletion of antimicrobial agent overtime, which might allow the re-growth of microorganism over time.[20] The depletion often results from the release of antimicrobial agent to the sample or the agent being utilised to inhibit the growth or eliminate the existing microorganisms.[20-21] To determine the ability of ITO/T4 to maintain its antimicrobial activity upon addition of more bacteria, which is denoted as 2nd infection cycle, a log-phase 10^5 cfu *E. coli*/mL was added to the ITO/T4 that had been previously incubated with *E. coli* for 120 min (Figure 5-2A). It can be seen that a similar ~10^5-fold reduction in *E. coli* concentration was observed after just 30 min incubation (t_{total} = 150 min) in Figure 5-2A. The much faster reduction in E. coli concentration was manifested from the amplification of T4 bacteriophage during the 1st infection which resulted in a 10-fold more T4 bacteriophage present in the suspension (Figure 5-2B). Thus, it allows more *E. coli* being infected at one given time. This finding infers that a single dose of T4 bacteriophage is sufficient to infect and cause bacteriophage amplification in the presence of their corresponding target bacterial host *E. coli*. The self-amplification ability of features a potential solution to the issues of antimicrobial agent depletion.[20]

5.3.2 Influence of Food Compositions and pH on ITO/T4 Antimicrobial Activity

The antimicrobial activity of ITO/T4 could be markedly affected by the different properties of food products, such as their compositions and pH. Dixon and co-workers reported the reduction in the antimicrobial activity of PRD1 and T4 bacteriophage in the presence of extracellular polymeric substances and human serum in water.[16] Similarly, O'Flaherty and colleagues reported the inhibition of bacteriophage K antimicrobial activity in raw milk.[15] It was found that the fat globules bound to bacteria surface and inhibited the interactions between bacteriophage and bacteria. Herein, casein, which is a major constituent of protein in milk (80%)[22] and starch were added to the Luria Bertani medium to act as model food substrates to investigate potential for interference.

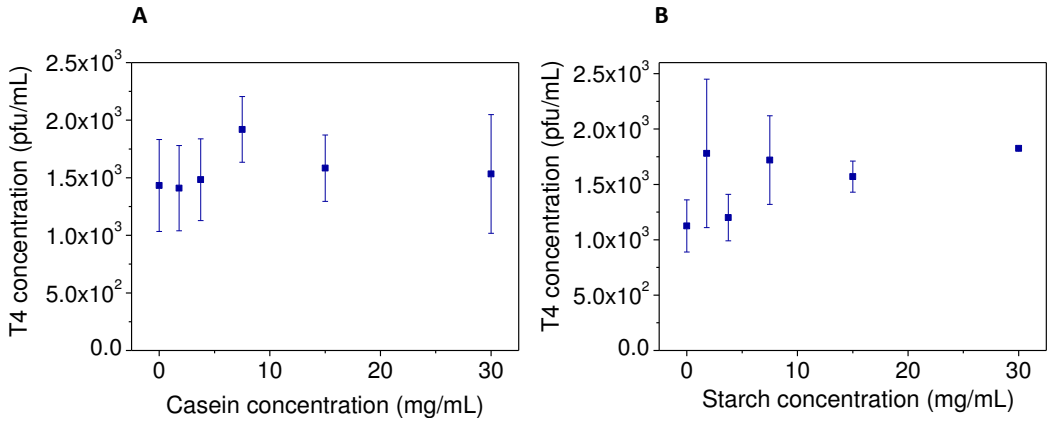


Figure 5-4 Stability of T4 bacteriophages incubation in (A) casein and (B) starch dissolved in Luria Bertani Medium pH 7. Data obtained from three independent measurements.

The stability of T4 bacteriophage in serially concentrations of casein and starch (0 to 30 mg/mL) at 37 °C was firstly determined. As shown in Figure 5-4A, T4 bacteriophage remained stable even at high concentration of added casein- note that 30 mg casein/mL is the highest concentration of casein in bovine milk.[23] Similarly, T4 bacteriophage also maintained its ability upon increasing concentration of starch (Figure 5-4B). The performance of ITO/T4 in 30 mg/mL of casein and starch were then investigated. It can be observed that bare and functionalised ITO/T4 maintained good antimicrobial activity in the presence of casein (Figure 5-5A) and starch (Figure 5-5B). These activities are similar to the observed antimicrobial activity of ITO/T4 in Luria Bertani medium only (Figure 5-6B). This finding demonstrates robust antimicrobial activity exhibited by ITO/T4 even in the presence of the model food components.

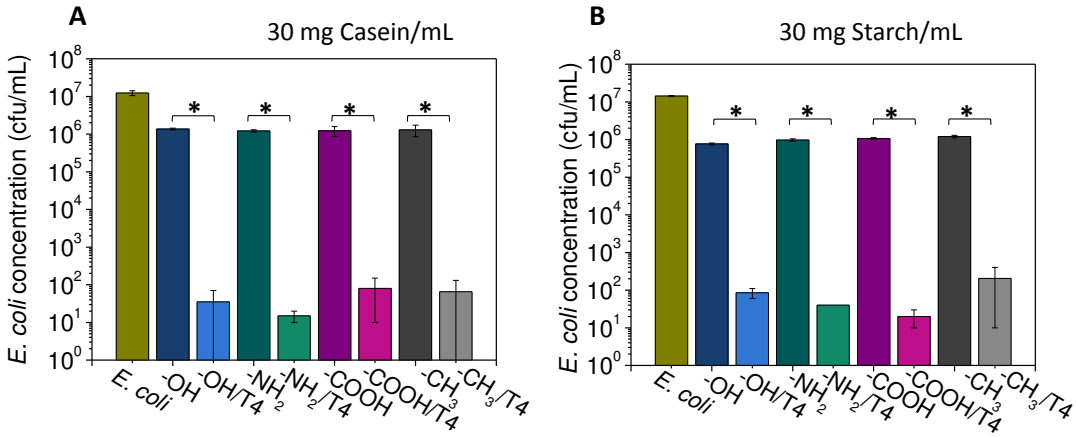


Figure 5-5 Antimicrobial activity of bare, amine (-NH_2), carboxylic (-COOH) and methyl (-CH_3) functionalised ITO with and without T4 bacteriophage in (A) 30 mg casein/mL and (B) 30 mg starch/mL in Luria Bertani medium pH 7 after 2 h incubation. The * indicates a significant difference with the respective control (α = 0.05, by two-way ANOVA followed by Holm-Sidak correction), from three independent replicates.

Apart from the presence of model food components, pH also plays a critical role as it often varies between one type of food to another. Many antimicrobial food agents change their activity with respect to pH.[20] Hence, ITO/T4 antimicrobial activity was investigated under different pH.

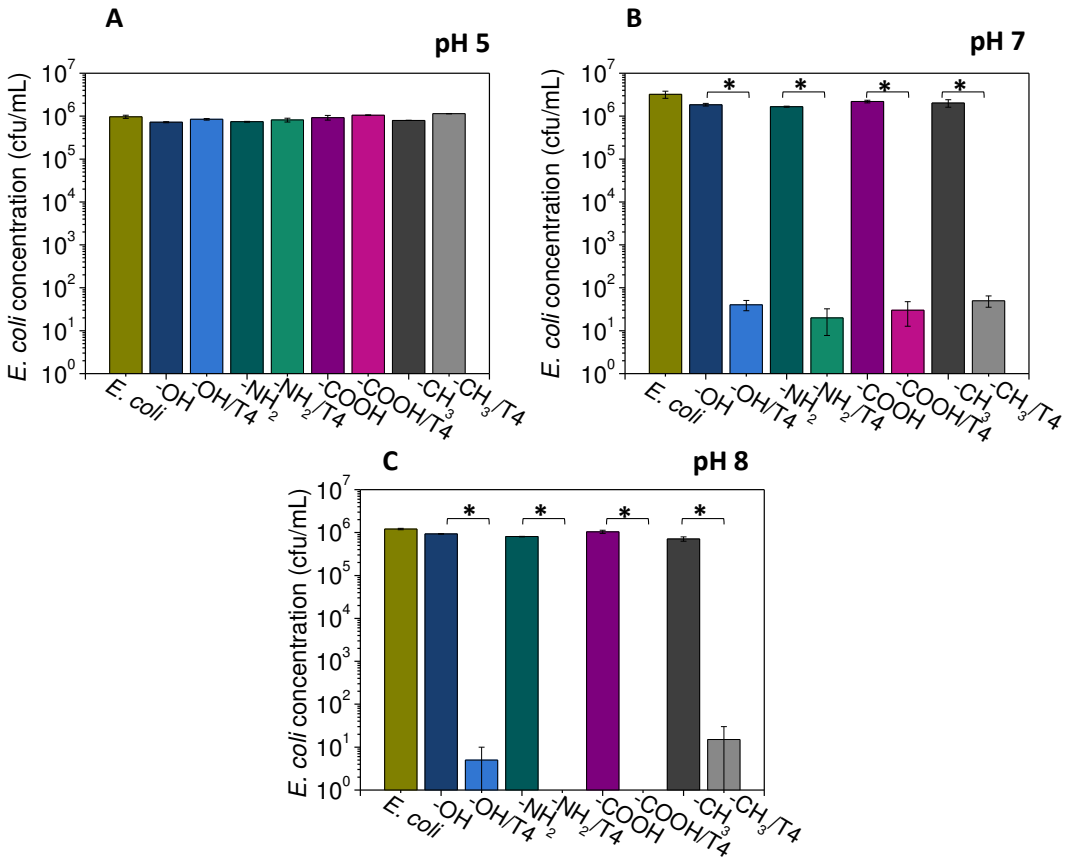


Figure 5-6 Antimicrobial activity of bare and functionalised ITO without and with T4 bacteriophage in Luria Bertani medium (A) pH 5, (B) pH 7 and (C) pH 8 after 2 h incubation. The * indicates a significant difference with the respective control (α = 0.05, by two-way ANOVA followed by Holm-Sidak correction), from three independent replicates.

The T4 bacteriophage conjugated bare and differently functionalised ITO were incubated with *E. coli* in Luria Bertani medium at pH 5, 7 and 8 for 2 h at 37 °C. Figure 5-6A shows that ITO/T4 exhibited poor antimicrobial activity at pH 5 after 2 h incubation. Meanwhile, a ~10^5-fold reduction in *E. coli* concentration was observed for ITO/T4 at pH 7 and 8 (Figure 5-6B and C, respectively). Jonczyk and colleagues reported that the stability of T4 bacteriophage reached its optimum at pH 6.0 to 7.5.[24] As observed in the current work (Figure 5-7A), however T4 bacteriophage remained stable during the course of 2 h incubation in the Luria Bertani medium at pH 5, 7 and 8. The undetectable antimicrobial activity of T4 conjugated bare and differently functionalised ITO at pH 5 may stem from the retardation in bacteriophage-*E. coli* adsorption and/or the slow intracellular development of new bacteriophage due to the slow growth of *E. coli*. Anderson reported a pH dependent T4-to-*E. coli* adsorption, with the optimum adsorption achieved at pH 6.8 to 9 and decreased outside the pH range.[25] Kellenberger and associates reported the retraction of T4 bacteriophage tail at pH 5.2, which resulted in less than 6% bacteriophage adsorption to bacteria.[26] The limited bacteriophage to bacteria adsorption might therefore explain the poor antimicrobial activity exhibited at pH 5. Meanwhile, the dramatic reduction in *E. coli* concentration as observed at pH 7 and 8 may be due to the optimum bacteriophage adsorption to bacteria.

Furthermore, as described earlier, the antimicrobial activity of ITO/T4 is dependent on the consecutive amplification of T4 bacteriophage that is greatly affected by the bacteria's physiological conditions. Faster bacteria growth rate has been known to result in faster bacteriophage adsorption to bacteria and higher production of bacterial ribosomes that

are vital in the synthesis and production of bacteriophage.[27-29] The above characteristics, in fact explain the dramatic reduction in *E. coli* concentration achieved at pH 7 and 8 (Figure 5-6B and C), whereby the *E. coli* growth rates for both pH values were much faster than at pH 5 (Figure 5-7B), thus resulted in faster bacteriophage production. Meanwhile, the *E. coli* growth profile at pH 5 was appreciable low (Figure 5-7B), which suggests the retardation in the synthesis and production of bacteriophage. This thus suggests that the antimicrobial activity of bare and functionalised ITO/T4 may vary depending on the solution pH.

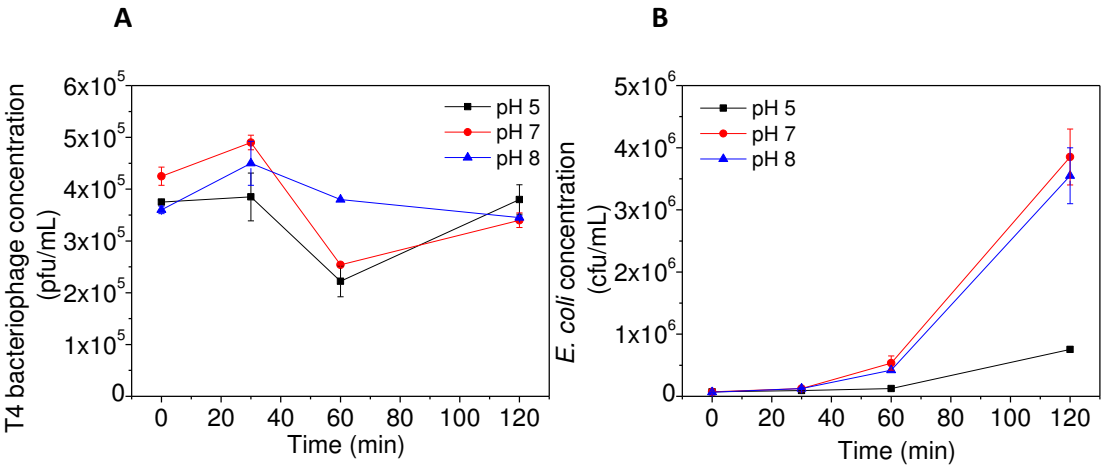


Figure 5-7 Concentration of (A) T4 bacteriophage and (B) *E. coli* overtime at different pH values in Luria Bertani media.

5.4 Summary

In this study, T4 bacteriophage conjugated bare, -NH_2, -COOH and -CH_3 functionalised ITO surfaces were investigated for their antimicrobial activity to *E. coli*. A ~10^5-fold reduction in *E. coli* concentration upon incubation with bare and functionalised ITO/T4 was observed following 2 h incubation. The antimicrobial activity was found to be due to the release of T4 bacteriophage from ITO/T4, which allowed T4 bacteriophage to diffuse to *E. coli* surface and subsequently cause bacterial infection. New bacteriophages were then released at the expense of *E. coli* lysis at the end of the infection cycle. Unlike the findings reported in Chapter 4 with T4 bacteriophage conjugated bare and functionalised Fe_3O_4 particles, the variations in bacteriophage loading on ITO surfaces only have minimum influence on the surface's antimicrobial activity. All bare and functionalised ITO surfaces exhibited comparable antimicrobial activity, regardless of their varying bacteriophage loading as previously observed in Chapter 3. This study highlights the close correlation between mode of antimicrobial activity to the factors influencing their performance.

The addition of a log-phase *E. coli* as a 2nd infection to the existing antimicrobial system of ITO/T4 revealed a similar yet much faster ~10^5-fold reduction in *E. coli* concentration in 30 min, which was due to the presence of a 10-fold more bacteriophage in the system. This self-amplification ability of bacteriophage in the presence of their corresponding host bacteria features a potential solution to the common issues of antimicrobial agent depletion.

Importantly, the antimicrobial activity of ITO/T4 remained unaffected in the presence of food components, such as casein and starch. The variation in solution pH however, was observed to significantly affect their antimicrobial activity with undetectable reduction in *E. coli* concentration observed at pH 5. Apart from the sub-optimal physiological state of the host *E. coli* at pH 5, the poor antimicrobial activity is also likely to result from the retardation in bacteriophage to *E. coli* adsorption at pH 5. The opposite was observed at pH 7 and 8, whereby the dramatic reduction in *E. coli* concentration may stem from the optimum bacteriophage adsorption to *E. coli* and/or the optimum *E. coli* growth rate compared to pH 5. The findings presented herein provide fundamental insight into the mechanisms and factors influencing the antimicrobial activity of bacteriophage conjugated surfaces that are important for better fabrication of bacteriophage based antimicrobial packaging and surfaces.

5.5 References

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Chapter 6 Conclusion and Recommendations

6.1 Conclusion

The overall aim of this dissertation was to establish a comprehensive understanding of the mechanisms and factors governing virus-surface interactions, as well as the influence of such interactions towards the performance of bacteriophage based interfaces. The study presented herein employed a controlled surface modification technique to carefully modulate different surface physico-chemical properties and the influence of resulted surface to bacteriophage interactions. This study reveals the significant importance of surface physico-chemical properties in governing virus-surface interactions. A comparative study between planar and particulate form of ITO with similar surface functionalisations and bacteriophage employed, showed a differing mode of bacteriophage adsorption to those surfaces. Bacteriophage-particle interactions appeared to be dominated by electrostatic interactions, whereas bacteriophage-planar surface interactions seemed to be influenced by the presence of specific surface functional groups. This signifies that a bacteriophage-particle study can not necessarily be used as a model system to predict bacteriophage interactions with other types of surfaces.

The presence of specific functional groups, in this case the surface carboxylic and hydroxyl groups on planar ITO, was found to promote T4 bacteriophage adsorption. The presence of surface amine groups on planar ITO on the other hand, reduced the bacteriophage adsorption. This highlights the critical influence of chemical species beyond the widely postulated macroscopic surface parameters, such as net surface charge, surface roughness and surface hydrophobicity towards the adsorption of bacteriophage on planar surfaces. These findings allow future research to better understand the parameters affecting virus adsorption to surfaces and ultimately improve the design and fabrication of bacteriophage based interfaces.

The knowledge of virus-surface interactions above was exploited to determine its influence on the efficacy of bacteriophage based interfaces for two different applications. Herein, T4 bacteriophage was conjugated with Fe_3O_4 magnetic particles by following similar surface functionalisation strategy as ITO. The performance of T4 bacteriophage conjugated Fe_3O_4 in capturing and isolating *E. coli*, as well as the external factors involved in the effective *E. coli* capturing were investigated. The variation in chemical surface properties was found to influence the bacteriophage loading on the particles. High bacteriophage adsorption onto bare Fe_3O_4 and Fe_3O_4-NH_2 was found to result from the electrostatic interactions between the net positively charged particles with the net negatively charged T4. In contrast, a 3-fold reduction in bacteriophage adsorption to the net negatively charged Fe_3O_4-COOH was due to electrostatic repulsion of like charges. The adsorption of T4 onto non-ionised methyl functionalised Fe_3O_4 is attributed to hydrophobic interactions. Variations in bacteriophage loading were found to influence

the *E. coli* capturing performance, whereby a high percentage of *E. coli* capture (~70%) was achieved using high T4 loading on bare Fe_3O_4 and Fe_3O_4-NH_2 after 10 min, while low T4 loading on Fe_3O_4-COOH resulted in poor *E. coli* capturing.

In addition to the bacteriophage loading, external factors, such as temperature and the presence of co-factors in the media, namely tryptone were found to be critical to achieve successful capturing of the target bacteria. The *E. coli* capturing requires an irreversible binding between T4 bacteriophage and *E. coli*, which occurs at 37 °C in tryptone-containing media for the bare Fe_3O_4/T4 and Fe_3O_4-NH_2/T4. Interestingly, poor capturing of *E. coli* by both particles were observed at 37 °C in the absence of tryptone and similarly, in the presence of tryptone at 4 °C. The poor capturing is most likely caused by the inhibition of the irreversible binding at low temperature and the absence of tryptone has also been known to result in retraction of T4 bacteriophage tails where the receptor binding domain is located. These findings show for the first time that the presence of tryptone in the media or optimum incubation temperature alone is insufficient to promote bacteria capturing, even with particles with high bacteriophage loading. This signifies that the efficacy of bacteriophage conjugated particles in capturing bacteria is not only dependent on the surface physico-chemical properties to promote high bacteriophage loading, but also requires the contribution of external factors, such as temperature and tryptone to facilitate the subsequent bacteriophage-bacteria interactions. These findings provide a scaffold for the application of bacteriophage conjugated magnetic particle for bacteria capturing in real samples.

In the second application, T4 bacteriophage conjugated bare and functionalised ITO prepared in Chapter 3 was investigated for their antimicrobial activity to *E. coli*. This study also explored as to whether varying bacteriophage based interface applications have different factors that influence their performance. Bare and functionalised ITO with T4 bacteriophage showed a ~10^5-fold reduction in *E. coli* concentration upon 2 h incubation. The antimicrobial activity exhibited by ITO/T4 was found to result from the release of T4 bacteriophage from ITO/T4, which allowed the bacteriophage to diffuse to *E. coli* surface and infect the bacteria. New bacteriophages were then released at the expense of *E. coli* lysis and death at the end of the infection cycle. The T4 conjugated bare and functionalised ITO surfaces exhibited comparable antimicrobial activity regardless of the variation in their bacteriophage loading, unlike the finding of T4 bacteriophage conjugated Fe_3O_4 particles. Such variations stem from the differing mechanism of action of the end-point applications, whereby the antimicrobial system herein relies on the concentration of released T4 bacteriophage to the solution regardless to their loading. Meanwhile, in the bacteria capturing system mentioned above, the bacteria need to be in contact with the bacteriophage on the Fe_3O_4 particle surface in order to be captured. Therefore, for the later system, the higher bacteriophage loading on the particle surface, the more bacteria are captured. The observations signify the importance in understanding the mode of action of the end-point applications, as it closely relates to the factors influencing their activity. This knowledge can assist in explaining the variations observed in factors influencing bacteriophage based interface's performance across different applications.

Further, the self-amplification ability of release T4 bacteriophage from ITO/T4 features a promising potential to the issues of antimicrobial agents depletion encountered in many applications. The addition of another dose of log-phase *E. coli* to the existing antimicrobial system of ITO/T4 revealed a similar and much faster ~10^5-fold reduction in *E. coli* concentration in 30 min, due to the presence of 10-fold more bacteriophage.

The ITO/T4 surfaces maintained their antimicrobial activity in the presence of model food components; namely casein from bovine milk and starch from potato in the media. However, variation in pH was observed to dramatically influence the antimicrobial activity with undetectable *E. coli* reduction observed at pH 5. The poor activity observed might manifest from the sub-optimal physiological state of the host *E. coli* and/or retardation in bacteriophage to *E. coli* adsorption at pH 5. The opposite was however observed at pH 7 and 8, whereby ITO/T4 exhibited a good ~10^5-fold reduction in *E. coli* concentration. Apart from the optimum bacteriophage adsorption to *E. coli* at pH 7 and 8, the marked reduction in *E. coli* concentration is likely to result from the optimum *E. coli* growth rate compared to pH 5. This work therefore provides an understanding of the mode of antimicrobial activity exhibited by bacteriophage based substrate and the influence of food properties (protein, starch and variations in pH) on the antimicrobial activity of bacteriophage based substrate.

6.2 Recommendations

The above studies show that the controlled surface tailoring to modulate different surface physico-chemical properties is a useful strategy in deciphering the mechanism and factors elicit virus-surface interactions. The implications of such factors on the efficacy of bacteriophage based interfaces were also found to be paramount. Understanding the mechanism of action of the end-point applications was proved to be important in establishing factors influencing the performance of bacteriophage based interfaces. The above knowledge will allow better design and fabrication of bacteriophage based interfaces for a wide range of applications. Recommendations for future research based on the findings in this thesis are presented below.

The significance of surface physico-chemical properties in determining the bacteriophage adsorption has been established in Chapter 3. However, the broad spectrum of other relevant target bacteria, such as *Salmonella*, *Campylobacter*, *Listeria*, *Bacillus* and *Staphylococcus* will require different bacteriophage to target those specific bacteria. For instance, P22 bacteriophage and Felix O1 are *Salmonella* specific bacteriophages, whereas P100 and A511 are *Listeria* specific bacteriophages.[1] As mention in Chapter 2, physico-chemical properties of bacteriophage vary not only by the type, but also by strains. Hence, to broaden the knowledge of bacteriophage-surface interactions and its implication to the end-point applications, further research employing relevant bacteriophages is a critical research interest.

Moreover, there have been few reports on the use of covalent binding to promote bacteriophage adsorption, however the superiority observed over their stability and high bacteriophage adsorption as compared to direct adsorption of bacteriophage to surfaces remains inconsistent throughout different studies.[2-4] A similar comparative study can be employed to establish a better understanding on the impact of covalent binding to the adsorption of bacteriophage and the efficacy of the resulted interface.

To further extend the understanding of virus-surface interactions, another parameter that needs to be studied is viral inactivation upon adsorption to surfaces and its implication to the end-point applications. Adsorption of certain viruses onto mineral surfaces and soils have been reported to induce bacteriophage inactivation.[5-6] The inactivation may perhaps explain some studies that observed high bacteriophage loading to two different surfaces, but did not result in a similar bacteriophage performance. The inactivation may be monitored by employing similar radio labelling technique used by Harvey and Ryan.[6] This study may provide an insight into other factors affecting the performance of bacteriophage based interfaces.

The successful application of bacteriophage conjugated Fe_3O_4 magnetic particles to capture bacteria in Chapter 4 has established three critical operating parameters that include bacteriophage loading, temperature and presence of tryptone, which will serve as a framework for the development of standard working procedure in pathogen separation. Nevertheless, extended research to optimise and deconvolute other influencing factors is recommended to warrant further investigation on the use of bacteriophage conjugated

Fe_3O_4 magnetic particles in real food samples. The sheer variety of food components might pose a significant challenge to the bacteria capturing ability in different applications. The different compositions of fat, particulate content and biochemical components in food,[7] require an extensive development work for each type of food. Hence, it is recommended to investigate the influence of food components to the bacteria capturing performance of bacteriophage conjugated Fe_3O_4 magnetic particles. A similar strategy may also be applied to medical applications. The rise in *E. coli* O157:H7 and other Verocytotoxin-producing *E. coli* incidence in human, which may cause adverse complications especially in children, requires a rapid bacteria sampling and detection from human stool sample in order to administer the appropriate treatments.[8] The use of bacteriophage conjugated Fe_3O_4 magnetic particles to aid bacteria sampling in medical applications however remains widely unexplored.

Further, the application of bacteriophage conjugated Fe_3O_4 magnetic particles can be extended to work in conjunction with current bacteria detection techniques. One of the critical challenges in the existing pathogen detection techniques is the inability to differentiate viable from non-viable bacteria. The use of bacteriophage conjugated Fe_3O_4 magnetic particles offers an added advantage as the bacteriophage allows differentiation of viable from non-viable bacteria, whereby the bacteriophage replication and the subsequent lysis only occur in the presence of viable bacteria.[9-10] Upon lysis, bacteria will release biochemical reagents, such as ATP and β-galactosidase,[11-13] which can be used as a target analyte for viable bacteria detection. The combination of these two technologies is therefore an attractive area of exploration.

The good antimicrobial activity exhibited by T4 conjugated bare and functionalised ITO in Chapter 5 provides a fundamental knowledge on the mechanism of the antimicrobial activity in a liquid sample. With less than 0.03% of T4 bacteriophage was released to the solution to exhibit their antimicrobial activity, it is therefore of great interest to explore the re-usability of such surface. An approach to determine substrate reusability is through the determination of substrate antimicrobial activity over several round of incubation in fresh bacteria suspension. Further, the concentration of release bacteriophage over several incubation cycles in fresh media should also be determined.

Aside from determining other food properties that may influence the antimicrobial activity of bacteriophage conjugated substrate, the utility of such interfaces in food applications must be extended beyond liquid samples. In solid food samples, the bacteria distribution is likely to lack of homogeneity[14] and the bacteriophage diffusion to target bacteria might also be limited. The increase in surface area of bacteriophage based substrate might offer a potential solution to this issue. Therefore, an investigation on the antimicrobial activity and its mechanism on solid sample needs to be further explored.

The success of T4 conjugated bare and functionalised ITO as an antimicrobial surface shows the potential of bacteriophage as an antimicrobial agent. However, the concern over the large spectrum of spoilage microorganisms,[15] such as yeast, mold and bacteria present in food samples require the use of a bacteriophage mixture or a possible coupling with other antimicrobial agents to deliver a synergistic antimicrobial effect. The effectiveness of bacteriophage coupling with a protein synthesis inhibitor in medical

application has been demonstrated by Kaur and colleagues.[16] Further work could be carried out by immobilising a mixture of bacteriophages or a combination of bacteriophage and other antimicrobial agents onto a substrate and investigate its antimicrobial activity for food applications.

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