Rational design of multifunctional antibacterial coatings

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MULTIFUNCTIONAL ANTIBACTERIAL
COATINGS**

HE TAO

**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

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Summary

The use of medical implants has significantly improved the survival and quality of lives of the world population and has been an indispensable procedure in health care industry nowadays. Biofilm formation, which is mainly composed of microorganisms within a protective extracellular matrix, is a major barrier in the long term utilization of the biomedical devices. Once biofilm is formed, it is extreme difficult to be removed due to its well-known drug resistance. Therefore, the best defensive strategy for combating medical device related infection is to prevent biofilm formation on the device surface. The anti-bacterial surface coating is one of the most effective ways for this purpose.

In this thesis, two novel kinds of antibacterial surface coating were developed. The first antibacterial coating is based on polyelectrolyte multilayer film (PMF) with the incorporation of silver nanoparticles, a widely used antimicrobial agent. The PMF has only one polyelectrolyte building block-polyethyleneimine (PEI) comparing with traditional two polyelectrolytes one, and silver (Ag) nanoparticles were incorporated into PEI multilayer via *in situ* reduction of Ag^+ . By varying PMF thickness and silver loading cycles, the mass of incorporated Ag nanoparticles can be controlled. It is shown that the PEI multilayer coating itself possesses considerable contact-killing properties against two types of bacteria. The loading of Ag nanoparticles not only enhanced the contact-killing effect, but also provided the coating with release-killing properties against bacterial.

The other type of antibacterial coating was equipped with mammalian cell adhesion ligand. The coating as mentioned above not only inhibited bacterial adhesion but also promoted host cell adhesion. This novel bio-selective antibacterial coating contains cell adhesion ligand on an anti-adhesive substrate. In order to choose the optimal ligand type, the biophysical responses of two types of bacteria including *Escherichia coli* (*E.coli*) and *Staphylococcus aureus* (*S. aureus*)) as well as mammalian cells (fibroblast) towards two classes of adhesive ligand, Arginine-Glycine-Aspartic acid (RGD) and collagen, on model device surfaces without anti-adhesive property are elucidated for the first time. The initial rate of deformation and adhesion energy result determined from confocal-reflectance interference contrast microscopy (C-RICM) shows that both collagen and RGD significantly enhanced fibroblast cell adhesion. By measuring of the detachment forces with atom force microscopy (AFM) and the bacterial density, it is found that RGD had no apparent effect on *E. coli* and *S. aureus* adhesion, while collagen increased the adhesion strength of *S. aureus*. The results herein strongly suggest that there is a highly intricate interplay between the type of adhesive ligand, bacteria strain, adhesion strength of bacteria and adhesion kinetics of mammalian cells which would be helpful for the design of advanced device surface.

Base on the studies of bacterial/mammalian cell adhesion strength on adhesive ligands bound on a model surface with anti-adhesive, novel surface coatings composed of RGD (10, 100, 1000 $\mu\text{g/ml}$) or collagen (1, 10, 100 $\mu\text{g/ml}$) coupled to anti-adhesive dextran sulfate (DS) and chitosan(CS) polyelectrolyte multilayer (PEM) were developed. Results show that collagen containing PEM surface promoted both fibroblast cell and *S.aureus* adhesion. What's more,

collagen promoted *S. aureus* adhesion at a low density no fibroblast cell can adhere. In contrast, RGD coupled DS/CS coating showed bio-selectivity. After RGD binding, DS/CS multilayers were adhesive to fibroblast cell but retained the anti-adhesive property against both *E.coli* and *S. aureus*. Therefore, the bio-selective surface, with RGD (1mg/ml) embedded in the DS/CS multilayers, are potential coating designed for medical devices. The use of such a multifunctional smart antibacterial coating may not only reduce the chance of biofilm related infection, but also induce host cell integration.

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Table of abbreviations

AgNO ₃	Silver Nitrate
AFM	Atomic Force Microscopy
APSi	3-Aminopropyl-Trimethoxysilane
ATCC	American Type Culture Collection
ATRP	Atom Transfer Radical Polymerization
CL	Collagen
CS	Chitosan
C-RICM	Confocal Reflectance Interference Contrast Microscopy
DLC	Diamond-like Carbon
DS	Dextran Sulfate
ECM	Extra Cellular Matrix
<i>E.coli</i>	<i>Escherichia coli</i>
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein Isothiocyanate
HA	Hyaluronan
HVVN	N-hexyl-N'-(4-vinylbenzyl)-4,4'-bipyridinium dinitrate
H ₂ O ₂	hydrogen peroxide
PBS	Phosphate Buffered Saline

PEI	Polyethylenimine
PEG	Poly(ethylene glycol)
PEO	Poly(ethylene oxide)
PI	Propidium Iodide
PMF	Polyelectrolyte Multilayer Films
PLL	Poly(L-lysine)(PLL)
PLL-g-PEG	Poly(L-lysine)-graft-poly(ethylene glycol)
PGA	Poly(L-glutamic acid) (PGA)
RGD	Arginine-Glycine-Aspartic acid
RMS	Root-Mean-Square Roughness (RMS)
<i>S.aureus</i>	<i>Staphylococcus aureus</i>
H ₂ SO ₄	Sulfuric acid
SEM	Scanning Electron Microscope
TiO ₂	Titanium oxide
TA	Terephthalaldehyde
Ti	Titanium
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
NHS	N-hydroxysulfosuccinimide sodium salt
NIH	National Institutes of Health
NO	Nitric oxide
NPVP	poly(4-vinyl-N-hexylpyridinium bromide)
NaBH ₄	Sodium borohydrate
XPS	X-ray Photoelectron Spectroscopy

Chapter 1 – Introduction

1.1 Background

The uses of implantable medical devices, such as prosthetic heart valves, cardiac pacemakers, orthopaedic joint replacement, dental implants, intravascular catheters and renal dialysis shunts, etc., have become an indispensable day-to-day practice of modern health care industry¹⁻⁹. However, the biggest problem in the application of medical devices is implant-associated infections¹⁻⁴. They are caused by bacterial adhesion onto the surface of implantable devices which may be followed by the formation of biofilms. According to the National Institutes of Health (NIH) in United States, biofilm formation is the major cause of over 80% of infections in human⁵.

Antibiotics have been most commonly used for combating device associated bacteria only at early stage of infection before a biofilm is developed. Once biofilms are formed, it is extremely difficult to remove it from the medical implant due to the well-know resistance to antibiotics⁶⁻¹². By far, there has been a lack of effective treatments against biofilms. The major solution to biofilm formation for most patients is to remove the infected medical implant, followed by a prolonged period of intensive antibiotics therapy. Although the removal of some implants is technically feasible, it makes the patients suffered from significant discomfort and creates a significant economic burden. It is estimated that the direct medical costs for treating device-associated infections exceed \$3 billion annually in U.S.A alone.

Therefore, prevention of biofilm formation on the medical device surface remains to be the best defensive strategy for combating medical device related infection. Thanks to following the recent advances in polymer sciences and surface chemistry, various classes of antimicrobial coatings have been developed. Surface functionalization is a relatively straight-forward and effective way to confer antibacterial property onto biomaterial surfaces with no changing on the bulk property properties. Another advantage is that it can prevent medical device-associated infections at implantation sites *in vivo* where antibiotics cannot easily reach easily.

1.2 Objective and Scope

The main research focus of this thesis is to develop antibacterial coating for rendering antibacterial property to model biomaterials. The techniques developed in this thesis have potential applications in several areas where bacterial contamination happens and infection controls are required, including but not limited to biomedical implants, water treatment, and food industry. The coating material is chosen related to the desired performance in the application. The coating materials selected in this project include synthetic polymer, inorganic materials and natural polysaccharide. Different types of surface functionalization were developed herein according to the material properties. The specific aims of this thesis were as follows:

- (1) To develop antibacterial coatings with both contact-killing and release-killing effect on bacteria.
- (2) To develop biophysical methods to quantify the interactions between bacteria/mammalian cell and surface, such as measurement of the adhesion

energy of bacterial/mammalian cell onto surface, and the detachment force of bacterial/mammalian cell from surface.

(3) To develop multifunctional coatings with bioselective ability that not only inhibits bacterial adhesion but also promotes mammalian cell adhesion. Such coating will be helpful in clinical applications due to the potential ability to integrate with host cells.

1.3 Outline

The thesis contains 6 chapters. In the first chapter, the background, scientific aim, and objectives of this PhD project are described. Chapter 2 contains the literature review which introduces the development of the research areas. In Chapter 3, silver nanoparticles incorporated polyethyleneimine (PEI) multilayers are developed. The process of building the multilayer and loading silver is studied. Various methods are used to investigate the anti-bacterial property of this polyelectrolyte multilayers (PEM) coating. In Chapter 4 and 5, another antibacterial coating with bio-selectivity inhibited bacterial adhesion but promoted host cell adhesion is developed. It is based on binding adhesive ligands (Arginine-Glycine-Aspartic acid (RGD) and collagen) on an anti-adhesive surface. In order to choose the optimal ligand type, in Chapter 4, the responses of bacteria and mammalian cells towards adhesive ligand on model surfaces without anti-adhesive property are elucidated by biophysical method, such as cell adhesion energy and bacterial detachment force. In Chapter 5, based on the results in Chapter 4, different densities of RGD and collagen are further coupled to anti-adhesive dextran sulfate (DS) /chitosan (CS) PEM surfaces. Cell and bacterial adhesion assays are used to check if the ligand bound PEM surface has

bio-selective property. In the last Chapter, conclusions are made and possible future work is discussed.

Chapter 2 – Literature review

2.1 Medical device-related infection

The use of indwelling or implantable medical devices, made from metals, polymers, ceramics in the forms of fibers or composites, etc^{1-9,13} has significantly improved the survival and quality of lives of numerous patients worldwide. Despite the increasing applications of medical implants and advances in biomaterials, medical implant-associated infections which happened during the usage of the biomedical devices remain to be serious issues in health care industry^{1-4, 14-17}. Almost all medical devices such as prosthetic heart valves, cardiac pacemakers, contact lens, orthopaedic joint replacement, stents, dental implants, intravascular catheters and renal dialysis shunts^{1-4, 18-21} can interact with bacteria.

The adhesion of bacteria onto the surface of implantable biomedical devices often leads to the formation of biofilms and triggers a cascade of pathophysiological responses of patients. The non-ideal responses as mentioned above ultimately lead to the devices failure⁶, and in some cases even causes the death of patients. Although strictly aseptic conditions are always maintained during the surgical implantation of medical device and systematic administration of antibiotics are implemented after surgical procedures, the incidence of device failure caused by bacteria remains to be significant^{7, 22-26}.

According to the National Institutes of Health in United States, biofilm formation accounts for over 80% of infections in human body, including

infections of oral soft tissues, teeth and dental implants, middle ear, airway/lung tissue, eye, urinary tract prostheses, peritoneal membrane and peritoneal dialysis catheters, indwelling catheters, pacemakers, prosthetic heart valves, etc. There are a lot of routes leading to the contaminations of medical device by infectious agents, such as airborne bacteria during surgical procedures, or from the contacts from clinical staff, or adhesion of micro-organisms to the newly inserted device through blood circulation.

Biofilm is extremely difficult to remove from medical implant once it is formed as they show strong antibiotic resistance⁶. Conventional antibiotics treatment are showing lower efficacy against implant associated infections because of above mentioned antibiotic resistance. Consequently, the only solution to biofilm formation for most patients is to remove the infected medical implant, followed by a prolonged period of intensive antibiotics therapy. Although the removal of some implants is technically feasible, it will make the patients suffered from significant discomfort, pain and depression and also economic burden to society. It is estimated that the direct medical costs for treating device-associated infections exceed \$3 billion annually in the U.S.²⁷.

The improvement in life span and medical technology will lead to increasing use of medical implants. As a result, biofilm-associated infections in medical implants will be increased at the same time. Despite considerable advances in our knowledge of biofilm, there have been a lack of innovative treatments against biofilms²⁸. Currently, the prevention of biofilm formation before the onset of infection remains to be the best defensive strategy for combating medical device related infection.

Until now, antibiotics have been most commonly used for killing device associated bacteria during the early stage of infection, before a biofilm is developed. The other problem is that long time use of antibiotics during surgery of device implantation may cause the development of antibiotics resistant microorganisms. A number of new strategies for inhibiting biofilm formation, such as device surface modification^{22, 29-32}, have been developed to combat biofilm associated infections. These alternative strategies may result in reducing the treatment cost and dosage of antibiotics and may prevent development of antibiotic-resistant microorganisms.

2.2 Drug Resistance of Biofilm

Biofilm is mainly composed of microorganisms embedded inside a protective extracellular matrix which contains polysaccharides, phospholipids, proteins, nucleic acids, and other polymeric substances hydrated up to 85% to 90%³³⁻³⁴. The development of a biofilm begins with the adsorption of proteins to form a conditioning film, followed by the bacterial adhesion and growth, then, extracellular polymeric substances are secreted by bacteria to enhance the anchorage of embedded microorganisms³⁵. Biofilm formation always triggers a significant resistance to antimicrobial agents, including antibiotics, biocides, and preservatives, compared with bacteria cultures in liquid suspension (planktonic)^{6, 36}.

Currently, there is no lack of understanding on the mechanisms behind the development of biofilm resistance to chemical reagents. Several theories³⁷ have been put forward to explain such broad range antimicrobial resistance, which

could be summarized as follows: (1) reduced infiltration or diffusion of antimicrobial agents into the biofilm; (2) decreased metabolisms of bacteria in the biofilm; (3) induced the expressions of resistance genes by the microorganisms inside the biofilm.

The exopolysaccharides matrix or glycocalyx of the biofilm has been known to delay and restrict the transport of antibiotics, such as high antimicrobial protein or lysozymes³⁸ and drugs like defensins, their analogues, and other antimicrobial peptides, through the entire regions of the biofilm³⁹. The negatively charged exopolysaccharides in the biofilm are extremely effective in reducing the diffusion rate of the positively charged aminoglycosides antibiotics³⁸. In addition to the retardation of antibiotics diffusion, there is also large amount of antibiotic-inactivating enzymes which accumulate inside the biofilm, which results in sharp concentration gradients of antimicrobial reagents inside the biofilm, and thus protects the bacteria in the interior of biofilm⁴⁰⁻⁴¹.

Antimicrobial agents, whose efficacy is dependent on DNA replications, kill fast growing bacteria more effectively than the slow growing ones. Moreover, the resistance towards antimicrobial agents decreases with the increase of bacteria growth rates^{6, 38}. Wentland et al. showed that the fast growing bacteria are mainly found on the surface of biofilm, while the slow growing ones predominantly located inside the biofilm⁴². The slow diffusion of nutrients and oxygen and accumulation of metabolic wastes make the bacteria grow slower. Based on the same reason, bacteria inside the biofilm favour anaerobic metabolism, which leads to further productions of acidic metabolites⁴³⁻⁴⁴. Another group has recently⁴⁵ claimed that the antimicrobial recalcitrance of biofilms is related to the presence of “persisters” in the cell population. Persisters

are not mutants, but phenotypic variants of the wild type. The metabolic activity of these persisters is very low and because of that, they are almost immune to antibiotics. Once the antibiotics level decrease, these “persisters” will be activated. They speculated that these persister cells contribute to drug resistance of biofilm, and drugs that are able to kill persisters should be effective in eradicating biofilms⁴⁵.

When bacteria start to form biofilms, some proteins, which are not found in free-floating bacteria, will be produced. The polysaccharide adhesin (PSA) controls the formation of intracellular contacts between adjacent bacteria which results in the formation of cell multilayer in biofilms⁴⁶. Rachid et al. showed that *ica* expression, which is a gene involved in synthesis of PSA, was increased and biofilm formation was accelerated when *S. epidermidis* was exposed to sub-inhibitory concentrations (1/70 to 1/2 of the Minimum Inhibitory concentration (MIC)) of tetracycline and quinopristin-dalfopristin (a semi-synthetic streptogramin commonly used as antibiotics)⁴⁷. Moreover, the contribution of efflux systems to the development of bacteria resistance against antimicrobial agents has been studied extensively in recent years^{38, 48-50}. The efflux pumps contribute a lot to quorum sensing and biofilm formation, so it is possible that antibiotic resistance of bacteria inside the biofilm may be induced by the efflux pump system which affects cell density, stress responses, and dormancy, rather than by direct drug effect.

2.3 Surface functionalizations for rendering surfaces antimicrobial

Once the biofilm forms on the surface of the medical device, due to the development of antibiotic resistance as discussed previously, it is not a simple task to eradicate it. Currently, the prevention of biofilm formation onto the medical device surface remains to be the best defensive strategy for combating medical device related infection.

Compared with other methods, surface functionalization is a relatively straight-forward strategy to confer antibacterial property onto biomaterial surfaces. Incorporation of antimicrobials in the bulk material that constitutes a device can be effective but it likely disrupts the bulk properties of the material. Various surface treatments are best alternative approaches because they are less expensive and maintain the bulk properties of the material. Moreover, surface functionalization can effectively render medical implant surface with antibacterial activities and at the same time, do not disrupt the bulk property properties of biomaterials. Another advantage is that it can prevent medical device-associated infections at implantation sites *in vivo* where antibiotics cannot reach.

Several surface functionalization methods have been used for rendering biomaterial surface anti-microbial: (a) grafting the surface with contact active polymer or antibacterial peptides, (b) coating the surface with microbe-repelling anti-adhesive polymer, (c) using composite coatings with controlled release biocides, (d) multifunctional coatings with two or three properties as mentioned above.

2.3.1 Contact active bacterial-killing coatings

Polycationic antimicrobial surfaces

The biocidal polymer containing alkyl quaternary amines⁵¹⁻⁵², such as alkylated poly(4-vinylpyridine)^{30, 53-54}, quarternized polyethylenimine⁵⁵⁻⁵⁶, quaternary derivatives of acrylic acid⁵⁷⁻⁵⁸ and viologen moieties⁵⁴ are the most commonly used antimicrobial agents for the surface modification of contact-active device^{55-63,30, 55, 64-69}. Most antimicrobial surfaces as mentioned above do not cause noticeable resistance to the representative bacterial strains including gram-positive *Staphylococcus aureus* (*S. aureus*) and gram-negative *Escherichia coli* (*E.coli*)⁶⁴.

Isquith et al. (1972) were the first group to report the antibacterial activity of alkyl quaternary amines coating on glass or cotton towards *S. faecalis* and *E. coli*⁷⁰. More recently, Flemming et al. (2000) has found that polyurethanes functionalized with methyl and ethyl quaternary amines has high antibacterial activity towards *S. aureus*⁷¹. Moreover, Gottenbos et al. (2002) has demonstrated the antibacterial activity of silicone rubber functionalized with alkyl quaternary amines⁵¹.

Klibanov's group has extensively studied these hydrophobic polycation as antibacterial coatings on different planar substrate such as glass, polypropylene, nylon, poly (ethylene terephthalate) (PET) and polyethylene of different density^{30, 55-68}. One of their recent work has shown that poly(vinyl-N-hexylpyridinium) derivative surfaces effectively killed both airborne and waterborne bacteria⁵⁹. Some other groups also showed that a surface concentration of 25nmol/cm² pyridinium groups on PET has high killing

efficiency against *E.coli*⁵⁴. At the same time, pyridinium-based polymers has low cytotoxicity⁵³.

Gelman and co-workers have recently synthesized a number of polystyrene-derived antibiotics that are cationic through N-quaternization and are not harmful to eukaryotic cell⁷². The result as mentioned above also suggested that reversible N-protonation leads to greater biocidal activity than irreversible N-quaternization.

Resell's group has recently applied atom transfer radical polymerization (ATRP) for grafting polymer chains of amino-2-(dimethylamino)ethyl methacrylate onto Whatman filter paper or glass slides⁵⁷. After quaternization with an alkyl halide, the surface show substantial antimicrobial activity towards either *E.coli* or *Bacillus subtilis*⁵⁷. The same group later discovered that antimicrobial activity is positively correlated with the charge density on the surface and the most biocidal surfaces had charge densities of higher than $1\text{-}5 \times 10^{15}$ accessible quaternary amine units/cm²⁵⁸.

Several reports have suggested that the antimicrobial activity of polycations is triggered by the disruption of bacteria membrane^{30, 55, 64-68}. Two mechanisms were proposed to explain the effect of polyquaternary amine in killing bacteria. One common hypothesis is that long cationic polymers acts like a needle in penetrating through the bacteria membrane^{30, 56, 64-65}. Alternatively, it was postulated that the disruption of bacteria membrane is induced by the displacement of cationic constituents in bacteria membrane with the highly charged surface of polycations⁷³. A recent study has shown that surface charge density of immobilized polycations is a key factor for maximizing antimicrobial activity⁵⁸. Therefore the second mechanism as mentioned above seems to be more realistic.

Antibiotics bonded surface

Several antibiotics such as vancomycin and penicillin have been covalently linked onto biomaterial surface⁷⁴⁻⁷⁷. For instance, expanded polytetrafluoroethylene (ePTFE) functionalized with penicillin (PEN) has high anti-bacterial activity towards gram-positive *S.aureus* bacteria⁷⁶. In a study carried by Antoci et al., a titanium rod covalently derivative with vancomycin prevented bacteria association on the surface after: (1) exposure to high level of *S. aureus*; (2) long-term incubation in physiological buffers; and (3) repeated challenges of *S.aureus*⁷⁸. The same group further showed that osteoblast cells seeded on the vancomycin-titanium surface exhibited no change in viability, indicating the surface supported osteoblast adhesion⁷⁹. However, another study showed that the anti-bacterial activity of vancomycin-titanium surface quickly reduced to undetectable level after incubation of vancomycin-titanium surface with blood⁸⁰. In Urban's recently studies, they modified polypropylene surface with controllable mounts of covalently bonded penicillin (kill gram-positive bacteria) and gentamicin (gram-negative), attached through a molecular spacer. By controlling their molar ratios of these two antibiotics, this approach provides an opportunity to get the tuneable killing strength to gram-negative and gram-positive bacteria⁸¹.

Similarly, anti-microbial peptide is covalently coupled onto poly(ethylene) film with a spacer PEG and render the surface with inhibitory effect against *E. coli* growth⁸². Recently, lysozyme, a powerful natural antibacterial protein, was incorporated into a layer-by-layer multilayers composed of single wall carbon nanotube. The multilayer coating as mentioned above showed long term antibacterial property toward gram-positive *S.aureus*⁸³.

Silver coating

Silver has been reported to have the highest anti-bacterial activity and low cytotoxicity compared to other metals⁸⁴⁻⁸⁶. The antimicrobial activity of silver is probably caused by the release of Ag^+ . Other study has also suggested that silver functions as a contact active material without releasing Ag^+ ⁸⁷.

Silver coatings were found to be effective in reducing the occurrences of infection to medical device which was implanted or inserted into the body. Silver-coated urinary catheters showed remarkable reductions in bacterial adhesion in small-scale clinical trials⁸⁸⁻⁹⁰. The similar reduction in the adhesion of *E. coli*; *P. aeruginosa*; and *K. pneumoniae* is also detected on fixation pins coated with silver metal *in vitro*⁹¹⁻⁹². However, the silver coating didn't reduce the number of *S. aureus* colonies on the same pin⁹³. The silver film formed as mentioned above is minimally leachable and kills bacteria upon direct contact formation. Magnetron deposition and physical vapour deposition have been used for coating silver metal on different polymer sheet or dialysis catheter. Generally, these coating shows good anti-microbial efficacy and biocompatibility to human cells⁹⁴⁻⁹⁵.

Others

Other metal coatings, such as copper and copper alloy, have also been considered as a hygienic material with proven toxicity to microbes⁹⁶ and have attracted some interest⁹⁷⁻¹⁰⁰. Modifying a surface with bacteriophages is thought to be a possible way to produce an antimicrobial surface recently and this phage-modified surface is interesting that the antibiotics-resistant bacteria show no phage resistance^{97, 101-105}.

2.3.2 Microbe-repelling anti-adhesive coatings

One method to fight against bacterial contaminations is to prepare a surface that prevents bacteria adhesion onto the surface. If the bacteria can not adhere, the biofilm will not develop. As such, this method works as a preventative strategy and have attracted a lot of researcher attention. These kind of surface are usually known as anti-fouling surface¹⁰⁶.

Poly (ethylene glycol) coatings

Poly(ethylene glycol) (PEG) is the most effective macromolecule for modifying the surface in order to reduce bio-adhesion (protein adsorption, bacterial and cell adhesion)¹⁰⁷. The use of anti-adhesive coating for the modification of the biomedical materials aims to minimize bacteria adhesion. Poly(ethylene oxide) (PEO) alone and PEO-based materials are used as anti-adhesive coating for bacteria from 1990s on different materials¹⁰⁸⁻¹¹³, followed by more studies subsequently^{107, 114-115}.

PEG modified surfaces are antifouling because the highly hydrate PEG layers works like a physical barrier and prevents the adsorption of protein. Thus most micro-organism would establish firm adhesion on PEG modified surface. Second, the highly hydrophilic PEG doesn't induce hydrophobic interaction which is critical to microbial adhesion¹¹⁶⁻¹¹⁸. Due to these factors, it is more difficult for microbes to attach onto these PEG coated surface.

Some reports claimed that a surface with 'zero' bacteria adherence *in vitro* is impossible to be achieved by the use of anti-adhesive coating alone¹¹⁹⁻¹²⁰. However, Kingshott et al. (2003) have demonstrated negligible bacteria adhesion on hydrophilic PEG hydrogel layers which function as steric barrier coating¹²¹. Jiang et al. further found that linear PEG were more effective in inhibiting bacterial adhesion than branched PEG, and they believed that is due to the

differences in the magnitudes of steric repulsion of these two kinds of PEG¹²². Moreover, the surface coated with RGD functionalized PEG show certain bio-selectivity, which simultaneously repelled the bacteria and promoted adhesion of mammalian cells^{15, 123}. The dual functions of RGD-PEG are considered to be critical properties for novel coating in medical implants.

Zwitterionic polymers

Recently, it has been found that the polymer containing zwitterionic end groups, such as poly(phosphorylcholine), poly(sulfobetaine) and poly(carboxybetaine) polymers¹²⁴⁻¹²⁸, can be used to stop protein and bacterial adhesion, thus to inhibit biofouling of the surface. The reason behind the excellent anti-fouling property is that zwitterionic groups have the ability to associate with excess water and at the same time do not perturb the native water hydrogen network at the interface just like the neutral PEG¹²⁹⁻¹³¹.

The latest studies by Jiang et al. showed that this zwitterionic surface containing poly(sulfobetaine) and poly(carboxybetaine) brushes could reduce fibrinogen adsorption to a same level as that on PEG surface¹³² and also it can prevent both initial bacterial adhesion and long time biofilm formation of Gram-positive and Gram-negative bacteria^{127, 133}. Furthermore, the same group developed new dual-functional polymer coatings, which combines the advantages of both nonfouling and contact-killing properties. Over 99.9 % of the adhered *E.coli* in one hour was killed by the cationic derivatives on the surface. Then, the bodies of dead bacteria will be removed from the surface when these cationic derivatives are hydrolyzed to become nonfouling zwitterionic polymers¹³⁴⁻¹³⁵.

Others

Both approaches as mentioned above lead to strong hydration of a surface and thus reduced bacteria adhesion. Some other methods also use the same principle to make an anti-adhesive surface, such as graft several kinds of polyacrylate brushes¹³⁶ or binding specific proteins such as albumin onto the material surface¹³⁷⁻¹⁴⁰. Diamond-like carbon (DLC) films¹⁴¹⁻¹⁴³ or other easily cleanable surface also attracts some attention¹⁴⁴⁻¹⁴⁶.

2.3.3 Controlled release biocides coatings

Antimicrobial coating with controlled release capability can be engineered by incorporating biocides inside the coating matrix. When such coating is put into an aqueous environment, drugs will be released into the nearby area of the device. In comparison with other conventional methods of drug administration, a higher biocide concentration which exceeds the MIC and minimum bactericidal concentration (MBC) of susceptible bacteria is initially attained near the device surface¹⁴⁷⁻¹⁴⁸. A broad class of materials have been used in rendering the surface with the drug release-killing capacity, such as heavy metals ions¹⁴⁹⁻¹⁵², antibiotics and small molecule biocides¹⁵³⁻¹⁵⁷, and nitric oxide/halogen species¹⁵⁸⁻¹⁶⁰.

Ag⁺

Among all those anti-microbial agents, silver ions are of special interest because they exhibit excellent biocidal activity toward broad-spectrum pathogens including bacteria, fungi and viruses¹⁶¹⁻¹⁶², at the same time showed lower cytotoxicity in comparison with other heavy metal ions⁸⁴⁻⁸⁶ and didn't easily provoke drug resistance¹⁴⁸. Generally, silver ions are known to interact with electron-donating groups such as thiols, carboxylates, amides, imidazoles, indoles, hydroxyls, etc^{84, 163} on enzymes and DNA of bacteria, thus deactivate

them. Silver is also known to increase cell wall permeability and then death by the formation of pits in the bacterial cell walls¹⁶⁴.

With the recent advances in nanotechnology, silver nanoparticles with highly controlled physical properties were synthesized and eventually introduced into the anti-microbial coating. For instance, layer-by-layer (LBL) deposition of Ag nanoparticles allows fine control over thickness of the resulted films on a wide variety of surfaces. Silver ions have been effectively loaded into the negatively charged layers of polyelectrolyte multilayer film (PMF)¹⁶⁵⁻¹⁶⁹ by putting PMF into Ag⁺ solution, and also silver loading has been achieved by LBL assembly of silver containing polyelectrolyte complex with an oppositely charged polyelectrolyte^{165, 168, 170}. In both loading methods, Ag nanoparticles are subsequently formed in the PMFs through the reduction of silver ions.

Dai *et al.* first reported the synthesis of silver nanoparticles-containing films through LBL deposition of a polyelectrolyte-metal ion complex and a polyanion and final reduction of the metal ions. This method has several advantages over others and has been shown to inhibit *E.coli* growth. First, nanoparticles are well dispersed throughout the LBL film because the metal ions are well distributed along the polymer chains. Second, the polyelectrolyte adjacent to metal ions limited the aggregation of metal particle and thus yielded a smaller particle size. Third, the LBL process as mentioned above eliminated the synthesis and separation of colloidal particle of metals in solution¹⁷¹. Not only electrostatic polyelectrolyte multilayer can be used, Lee *et al.* constructed a hydrogen-bonded multilayer which contained Ag nanoparticles by *in situ* synthesis. Both Gram-positive strain (*S. epidermidis*) and Gram-negative strain (*E.coli*) bacteria were killed by the release of Ag⁺ from the antimicrobial coating¹⁶⁶.

Other than PMF, other coating materials also been used to incorporate Ag nanoparticles as antibacterial coatings. A hybrid coating of silver nanoparticles ranging from 1 to 2 nm in size, which is stabilized in between highly branched amphiphilic modified polyethyleneimine layer, reduced the growth of *E. coli* by 98%¹⁷². Wang *et al.* incorporated silver ions into an ultrathin film of titanium phosphate through an ion-exchange process and produced silver nanoparticles embedded in the film by *in situ* reduction with NaBH₄. The silver containing titanium phosphate films as mentioned above are effective in prohibiting the growth of *E. coli*¹⁷³. Chen *et al.* designed a multifunctional surface by co-sputtering the osteoblast conductive hydroxyapatite (HA) and antibacterial Ag¹⁷⁴. A significant reduction of *S. epidermidis* and *S. aureus* adhesion on Ag-HA coated surface comparing with that on bare titanium (Ti) and HA surfaces. At same time, *in vitro* cytotoxicity result show that there is no significant difference between HA and Ag-HA surfaces¹⁷⁴.

Antibiotics

Entrapping antibiotics into polymer coatings applied on device surfaces has shown promising results in several studies, which have examined the clinical efficacy of catheters coated with polymer.

For example, polyurethane catheters have been coated with both minocycline and ethylene-diamine-tetraacetate for combating against recurrent vascular catheter-related bacteraemia¹⁷⁵. The same group reported that catheters that had been coated with minocycline and rifampicin significantly reduced the degree of bacteria colonization and infections in the bloodstream¹⁷⁶ and maintained effective antimicrobial activity against *S. epidermidis* for at least two weeks¹⁷⁷.

Chloromelamine-based bioresponsive fibrous materials not only show excellent ability to kill bacteria, yeast, virus, and spores, and also effectively inhibited the formation of bacterial biofilms. The biocidal effects of material coating contribute to the release of chlorines and the bound chloromelamine molecules¹⁷⁸. Johnson, Delavari, and Azar described the *in vitro* antimicrobial activity of a nitrofurazone-containing urinary catheter and a silver hydrogel catheter against multi-drug resistance bacteria involved in catheter-associated urinary tract infection¹⁷⁹.

A biocompatible dextran-based hydrogel adsorbed with amphotericin B can effectively kill fungi in contact within 2 h and retain its activity for at least 53 days. At the same time, the hydrogel does not cause hemolysis in human blood¹⁸⁰.

Titanium oxide (TiO₂)

Titanium oxide (TiO₂) is another common anti-microbial coating which is believed to kill microbes by releasing light-induced production of hydroxyl radicals upon contact with bacteria. The antimicrobial efficacy of TiO₂ was first introduced in 1985 by Matsunaga and co-workers¹⁸¹. After that, research of TiO₂ as antimicrobial reagents has focused on three areas: First, how TiO₂ acts as a photocatalyst¹⁸²⁻¹⁸³. The hydroxyl radical is the principle reactive species. It is the product of redox reactions between photos excited TiO₂ and adsorbed H₂O, molecular oxygen and from hydroxide groups on the catalyst surface¹⁸⁴. Second, how are microbes killed by the TiO₂ surface^{181, 184-185}. The main reason is the cell wall damage and then cytoplasmic membrane damage¹⁸⁵⁻¹⁸⁷. Third, how can the surface be made to be more efficient for killing microbes^{105, 188-194}.

NO

Nitric oxide(NO) is a free radical that is naturally produced in mammals and known to participate in a wide variety of physiologically important processes^{158-159, 195}. Nitric oxide is also known as the wide-spectrum antimicrobial properties. Schoenfisch's group did a lot of work on NO-releasing xerogel polymers, formed via the sol-gel process, to both reduce microbial adhesion and treat biofilms¹⁹⁶⁻¹⁹⁹.

2.3.4 Multifunctional anti-bacterial coatings

All the three types of anti-microbial coatings as mentioned above have certain advantages and disadvantages. For instance, a drug-releasing coating will dramatically lose its anti-bacterial efficiency after the concentration of released drugs falls below the MIC²⁰⁰. Contact-killing antimicrobial coating exhibits long-term durability. At the same time, it reduced the development of drug-resistant mutation of bacteria and are regarded as more environmentally friendly²⁰¹. However, this method requires the use of intricate chemistry in surface modifications^{59, 66}. Another problem is that dead body of bacteria still stay on this type of anti-bacterial coatings, which may trigger immune response, and most importantly block its anti-microbial functional groups.

Efforts towards the development of new bacteria-resistant coating will take the major advantages of existing approaches while they should overcome the inherent shortcomings. Therefore it is desirable to combine several desirable properties usually found in different antimicrobial coatings in a single system, such as contact-killing and release killing together or release-killing and anti-adhesive together.

Some example has been shown as follows: Li *et al.* have recently constructed a thin film coating with two distinct functional layers: a reservoir for the loading and release of bactericidal chemicals and a nanoparticles-containing surface with

immobilized bactericides¹⁶⁷. The novel coating as mentioned above provides dual-functional roles for killing bacteria through drug release or contact formation. Shi *et al.* modified PET surface with viologen, N-hexyl-N'-(4-vinylbenzyl)-4,4'-bipyridinium dinitrate (HVVN, and photo-reduced silver nanoparticles²⁰². The pyridinium groups of the HVVN possess contact-active bactericidal effects on *Escherichia coli* which can be significantly enhanced by the incorporation of silver nanoparticles on the HVVN-PET film due to the release of Ag⁺²⁰². The author also functionalized stainless steel with a dual antibacterial polyelectrolyte multilayer film which contained contact-active quaternized-PEI and controlled release-killing silver nanoparticle¹⁶⁸. Grunlan developed a similar polyelectrolyte multilayer film, containing contact-active PEI and release-killing cetrimide and silver nanoparticles, which showed good anti-microbial capacity¹⁶⁵. It is believed that the highly negatively charged surface has led to higher deposition of positively charged anti-microbial agents. Sen and co-workers created dual-action antimicrobial coatings through spin coating of a two-component composites consisting of a poly(4-vinyl-N-hexylpyridinium bromide) (NPVP) matrix and embedded silver bromide nanoparticles²⁰³. This coating has both controlled release-killing and contact-killing properties. The result also showed that the combined layer with the releasing killing ability of AgBr, the composites exhibit a higher antibacterial activity than NPVP alone²⁰³. These dual-functional coatings can kill bacteria with high efficiency at initial period due to the release of Ag⁺ and maintain its strong antibacterial activity after using up of embedded Ag, due to the presence of immobilized quaternary ammonium salts^{167, 203}.

Fu *et al.* constructed a multilayer film via LBL assembly of anti-adhesive heparin and contact-killing chitosan²⁰⁴. The anti-adhesive heparin of the multilayer coating could keep its contact-killing longer before it is covered by the dead bacteria. DLC films may be doped with microbicidal species such as Ag or Cu, yielding contact-killing or releasing killing properties in addition to the anti-adhesive properties¹⁴². Moreover, silver nanoparticles with sizes around 10-40 nm were introduced into the chitosan/heparin multilayer, thus equipping this film with all three killing ability, anti-adhesive, contact-killing and release-killing properties²⁰⁵. Tiller and co-workers have developed novel anti-microbial coatings that can simultaneously repel and kill bacteria upon contact and through drug release²⁰⁶. The group applied bacteria-repelling PEO as a top layer of the coating, contact-active poly (ethylene imine) with embedded silver nanoparticles as the inner release-killing layer.

2.4 LBL assembly PEM for antibacterial purpose

The LBL method, initially developed by Decher, based on self-assemble and self-organize polyelectrolyte deposition, is powerful way to prepare PEM on the materials surface²⁰⁷⁻²⁰⁸. The basic preparation involves dipping a charged substrate (positive charge) into a negative charged polyelectrolyte solution for several minutes and reversing the charge of the substrate to be negative. Then the substrate is put into another positive charge polyelectrolyte solution, which adsorbs the positive charged polyelectrolyte and re-created a positively charged surface. By repeating adsorption of negative and positive charged polyelectrolyte, a PEM is finally fabricated.

Since the assembly is based on spontaneous adsorptions, it is applicable for preparing ordered multilayers on substrate with any size, shape, topography, or topology. The properties of PEM can be precisely controlled by using a broad range of polymers, nanoparticles, proteins, drugs and even cells as building block^{166, 209-212}. Thus, an antibacterial coating with controlled nanostructure polymeric systems can be made by the incorporation any of the above mentioned antibacterial agents into PEM systems.

2.4.1 Adhesive-resistant PEM coatings

In order to get a bacterial adhesion-resistant PEM coating, incorporating anti-adhesive polymers, such as PEG, heparin into the LBL systems is a good idea. By assembling poly(L-lysine) (PLL) with negatively charged polyacid biopolymer such as poly(L-glutamic acid) (PGA) attached with PEG, multilayers with one to three PGA-g-PEG bilayers on top significantly reduced *E. coli* attachment of even in the presence of nutrient containing media¹¹⁸. The anti-adhesive heparin with strong negatively charge can directly work as building block of multilayers with the positively charged chitosan. Fu et al. thus created antibacterial multilayers with both anti-adhesive and contact-kill properties²⁰⁴. Hyaluronan (HA), an anti-adhesive polysaccharide can also be used as multilayer building block²¹³⁻²¹⁴. Richert et al. manipulated ionic strength of the preparation solution, the reduction of *E. coli* adhesion can be controlled from 80% to 20-40%²¹⁴.

Recent studies have demonstrated that even without anti-adhesive polymer inside the PEM coatings as mentioned above, by controlling Young's elastic modulus E (i.e., low E , high mechanical compliance) of highly hydrated PEM assembled in certain conditions also can significantly inhibit bacterial

adhesion²¹⁵. They found that the adhesion efficiency of the *E. coli* and *S. epidermidis* was strongly correlated with elastic modulus E of these hydrated PEM systems²¹⁵.

2.4.2 Contact-killing PEM coatings

To build contact-killing PEM coatings, chitosan, a biocompatible cationic antibacterial polysaccharide, is often chosen as one of the main components^{204, 214}. PEMs with chitosan as the outermost layer show better antimicrobial activity, as positive charged chitosan are able to make bacterial have better contact with the chitosan containing coatings^{204, 214, 216}. PH and ionic strength strongly affect the antibacterial properties of chitosan containing PEM, by influencing the charge density and mobility of chitosan chain segments at the film surface^{204, 214}.

Similar results have been found for other PEM systems with cationic segment as antibacterial agent. PEMs comprising of poly(allylamine hydrochloride) (PAH) and sulfonated poly(styrene) (SBS) at high pH do not show contact-killing properties. While at low pH, the hydrophobic segments are get positive charged and showing potent antimicrobial capabilities²¹⁷.

In corporation of other antibacterial agents, such as lysozyme^{83, 209} or antimicrobial peptides into PEM is also effective method in enhancing antimicrobial activities⁶⁰⁻⁶¹. It is believed that multiple layers structure increase the peptide concentration, and the electrostatic nature of PEM make it possible for incorporating multiple peptides and at the same time also keep them from denature⁶⁰⁻⁶¹.

Etienne (2004) has developed a new strategy for incorporation of anti-microbial peptide (defensins from *Anopheles gambiae* mosquitoes) into PEM films⁶⁰. Based on the bacteria adhesion measurements with confocal or electron

microscopy, the anti-microbial activity of the defensins-functionalized films was not linked to defensins release into the culture medium. Instead, defensins retains its biological activity when it is embedded inside the multilayer film and interacts with the bacterial membrane upon adhesion of bacteria⁶⁰. Moreover, the same group inserted a chromogranin A-derived antifungal peptide (CGA 47-66, chromofungin) into a multilayer film⁶¹ and showed that the embedded peptide keeps its antifungal activity by penetrating into the fungal membrane. Recently, lysozyme, a powerful natural antibacterial protein, was incorporated into a single wall carbon nanotube PEM⁸³. The multilayer coating showed long term antibacterial property toward gram-positive *S. aureus* mainly due to the contact-killing property of lysozyme⁸³.

2.4.3 Releasing-killing PEM

Silver and silver ions are the most common biocide releasing material using PEM will with releasing-killing properties^{165-169, 205, 218-222}. Silver ions have been effectively loaded into the negatively charged layers of PMF¹⁶⁵⁻¹⁶⁹. Alternatively, silver loading has been achieved by LBL assembly of silver containing polyelectrolyte complex with an oppositely charged polyelectrolyte^{165, 168-170, 205, 222}. In both loading methods, Ag nanoparticles are often subsequently formed in the PMFs through the reduction of silver ions by a variety of methods including chemical reduction, heating, and UV-irradiation^{178, 219}. Recently, liposome loaded with silver ion is subsequently embedded inside PEM and by changing the temperature; silver ions were released from the temperature response liposome. Thus strong antibacterial effect is observed²¹⁸.

Antibiotics is another widely used material to be incorporated into PEM, such as gentamicin²²³, ciprofloxacin hydrochloride²²⁴⁻²²⁵ and triclosan^{210, 226}.

PEM loaded with centrimide, a small quaternary ammonium compounds, have been show have strong antibacterial property by releasing centrimide¹⁶⁵.

2.4.4 Multifunctional antibacterial PEM

PEM is also an excellent platform for preparing antibacterial coating with multiple strategies. For example, lysozyme and antibacterial peptide loaded PEMs can act as contact killers with them as the outermost layer before them releasing out from the PEM^{209, 227}. For application related to silver ion, Shi *et al.* functionalized stainless steel with a dual antibacterial PEM which contained contact-active quaternized-PEI and controlled release-killing silver nanoparticle¹⁶⁸. Li *et al.* have recently constructed a thin film coatings with two distinct functional layers: a reservoir for the loading and release of bactericidal chemicals and a nanoparticles-containing surface with immobilized contact-killing bactericides¹⁶⁷. Moreover, silver nanoparticles with sizes around 10-40 nm were introduced into the chitosan/heparin multilayer, thus equipping this film with all three killing ability, anti-adhesive, contact-killing and release-killing properties²⁰⁵.

2.5 Bioselectivity

In 1987, Anthony G. Gristina used “race for the surface” to describe the fate of biomedical implants²²⁸. After being putting into human body, the fate of a biomaterial implant was dependent on the race between tissue cell integration and microbial adhesion and growth. If host body’s cells win the battle, then the implant surface is covered by a viable tissue layer and the chance for bacteria

growth is decreased. On the other hand, if bacteria won the race, then a biofilm rapidly forms on the implant and bacterial virulence factors and toxins released from the biofilm will have negative effect on tissue cell function²²⁸⁻²³⁰.

Unfortunately, microorganisms usually reach an implant surface earlier than host body cells, because they can be introduced during surgery and *in vivo*, so microorganisms can start earlier in the race for the surface before tissue integration can happen²³⁰. Thus it is very useful to make a bio-selective device surface, which can lead to antimicrobial activity without cytotoxicity, in other words, simultaneously suppresses bacteria adhesion or growth but enhances host cells adhesion or growth.

Generally, there are two kinds of these bioselective surfaces, the first type is a selective-killing surface, which means both bacteria and cell could adhere onto the surface, but the bacteria will be killed after making contact on device surface or by the antibiotics release from the coating while the activity of mammalian cells will not be effected²³¹⁻²³².

For example, Agarwal et al. get a silver loaded PEM coating that the amount of silver nanoparticles and adhesive property of the PEM are in careful control manner by changing the pH of the LBL fabrication process. The resulted PEM coating show killing selectivity that can kill bacteria but support growth of mammalian cells²³⁹. Similar result was obtained by Vasilev et al., through controlling the amount of loaded silver nanoparticles through some methods and the release rate of silver ions through depositing another polymer layer, the coatings can be tuned to be acceptable to osteoblast adhesion and growth but inhibiting bacterial colonization²⁴⁰. But in Ramstedt's work, they questioned that if there is a concentration range of silver when bacteria will be killed while

mammalian cells will not be affected after comparing the antibacterial effects and cytotoxicity of a silver loaded polymer brush in a non-protein and protein rich environment²³³. Because silver ions are known to bind to proteins, such as serum albumin, the antibacterial property will be modulated. Thus the concentration ranges mentioned above is very small and in some cases does not exist²³³.

The second type with selective activity is selective-adhesion surface, which inhibits pathogenic bacteria adhesion but enhances host tissue cells adhesion and growth^{123, 234-235}. The specific cell adhesion is achieved by the introduction of adhesive ligands such as RGD sequences onto an anti-adhesive surface^{123, 234-235}. It has been demonstrated that osteoblast growth and proliferation are significantly enhanced on a RGD coupled hyaluronic acid and chitosan polyelectrolyte multilayers while the anti-adhesive property against bacteria of the PEM is retained^{123, 234-235}. Poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) coupled with RGD can retain the bacteria-repulsive property found in conventional and at the same time allows cell adhesion^{123, 235}, which will not happen on PLL-g-PEG. The choice of adhesive ligand would be critical to introduce selected biological activity onto device surfaces. Therefore, the development of novel device surface will be largely benefited from understanding both mammalian cells and bacterial behaviour upon their encounters with the implant surfaces with adhesive ligand such as RGD.

Chapter 3 Covalent Layer-By-Layer Assembly of Polyethyleneimine Multilayer for Antibacterial Applications

3.1 Introduction

The accumulation and growth of bacteria on the surface of medical device trigger chronic infection, inflammation, tissue necrosis and implant failure in patients through biofilm formation²³⁶⁻²³⁷. In particular, biofilms are very difficult to eradicate once they are formed on the material surfaces. Among common antimicrobial strategies, surface functionalization which directly modifies the interfacial properties of the medical device appears to be the best preventative measure against biofilm formation. For instance, surface functionalization on medical implants prevents biofilm associated infections *in vivo* where antibiotics cannot be effectively delivered.

Recently, surface functionalization of biomaterial with (a) antibacterial polymer or peptides⁵⁵⁻⁶³, (b) microbe-repelling or anti-adhesive polymer^{108-110, 121, 136-138}, (c) controlled release system of biocides^{156-158, 173-174} have been applied for combating device associated infections. Among various antimicrobial surfaces, silver containing systems are of special interest because they are proven biocides against a broad spectrum of bacterial strains, fungi and viruses^{161, 238}. So far, silver ions have not been reported with detectable toxicity in

human cells²³⁹. Most importantly, silver ions have not been associated with the development of antibiotics resistance in most bacteria²⁴⁰. Silver have also been applied in other commercial applications of bacteria treatments such as water sterilization²⁴¹.

Recently, PMF formed from layer-by-layer assembly of a pair of polyelectrolytes through electrostatic attractions has emerged as a promising approach of surface functionalization for antimicrobial applications. Moreover, PMF can be potentially incorporated with various antibacterial reagents such as fictional polymers, carbon nanotube, antibacterial peptides and drugs^{60-61, 83, 118, 225, 242-244}. For instance, silver ions have been effectively loaded into the negatively charged layers of PMF¹⁶⁵⁻¹⁶⁹. Alternatively, silver loading has been achieved by LBL assembly of silver containing polyelectrolyte complex with an oppositely charged polyelectrolyte^{165, 168, 170}. In both loading methods, Ag nanoparticles are subsequently formed in the PMFs through the reduction of silver ions. Among common polyelectrolytes, PEI with demonstrated antibacterial property has been used to build silver containing PMF^{55, 245}. Moreover, PEI contains amine groups for binding with a wide range of transition metal cations^{165, 168, 170}. On the other hand, conventional PEI based multilayer does not offer the structural stability against the change of physiological conditions due to the absence of chemical bonds between adjacent polyelectrolyte layer within the PMF.

In this study, a stable PMF composed of only single polyelectrolyte comparing with traditional two polyelectrolytes system has been developed by covalent LBL assembly of one type of polyelectrolyte PEI followed by *in situ* reduction of silver ions. First, the covalent build-up of PEI layers with the use

of chemical cross-linker, terephthalaldehyde (TA) was validated by UV-Vis absorption spectroscopy and atomic force microscopy. Second, a reducing agent, NaBH₄ was loaded into the PEI multilayer before the introduction of Ag⁺ ions. This method of loading Ag nanoparticles by pre-adsorbed NaBH₄ is unique compared to other reported methods¹⁶⁵⁻¹⁷⁰. Lastly, the antibacterial properties of PEI multilayer in the presence or absence of embedded Ag nanoparticles were measured by several standard assays.

3.2 Materials and Methods

3.2.1 Materials

1× phosphate buffered saline (PBS) (1× PBS: 8 g/l of NaCl, 0.2 g/l of KCl, 1.44 g/l of Na₂HPO₄ and 0.24 g/l of KH₂PO₄) was diluted from the original solution of 10× PBS (Invitrogen Inc., Singapore) with ultrapure water (Millipore Inc., Singapore). Sulfuric acid (H₂SO₄), hydrogen peroxide (H₂O₂), PEI (molecular weight: 750 000), terephthalaldehyde (TA), poly(acrylic acid) (PAA), silver nitrate (AgNO₃), sodium borohydrate (NaBH₄), and fluorescein isothiocyanate (FITC) were purchased from Sigma Aldrich Pte Ltd (Singapore). Ultrapure water with resistivity of 18.2 MΩ (DI water) was used in all solution preparations and washing. *E. coli* DH5 (ATCC#: 53868) and *S. aureus* (ATCC#: 25923) were obtained from American Type Culture Collection (ATCC, USA).

For monitoring the LBL assembly of PEI multilayer, PEI needs to be labelled by a fluorescence probe. PEI (100 mg) and FITC (10 mg) were dissolved in 10ml ultrapure water and then stirred for 24 hours. The solution was then thoroughly dialyzed against water for 4 weeks until there was no

fluorescence signal to be detectable in the water. Finally, the FITC labelled PEI was obtained after freeze-drying.

3.2.2 Bacteria Culture

Two bacteria strains including *E. coli* and *S. aureus* were cultured in LB broth (10 g of tryptone, 5 grams of yeast extract, and 10 g of NaCl) for 16 hours at 37°C before any further experiment. The bacteria-containing broth was centrifuged at 2700 rpm for 10 min. After the removal of the supernatant, the cells were washed twice with 1× PBS and then re-suspended in 1× PBS (containing 10% of LB broth). Different densities of bacteria in 1× PBS were used in different measurements.

3.2.3 Preparation of amino-modified glass

Glass slides were cleaned by Piranha solution ($\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2 = 70:30$ v/v) for 1 h at 70 °C and then rinsed extensively by ultrapure water before they were dried under a stream of nitrogen. 3-aminopropyl-trimethoxysilane (APSi, Aldrich) at a concentration of 1 vol. % was dissolved in an ethanol/water (95:5 v/v) mixture. The cleaned glass slides were then dipped into APSi solution for 0.5 h and rinsed with ethanol for three times followed by ultrapure water for three times. After drying by a stream of nitrogen, these silanized slides were heated in a vacuum oven at 120°C for 1 hour. Afterwards, these amino-modified glasses serve as the substrate for the LBL film assembly. Alternatively, amino-modified quartz slides were prepared with the same procedures.

3.2.4 LBL Deposition of TA/PEI multilayer on NH_2 -Glass

To build the 1st PEI layer of LBL film, amino-modified glass slides were dipped in TA solution (10mg/ml in ethanol) for 15 min, then rinsed with pure ethanol for 3 times before drying in a stream of nitrogen. Then TA-modified glasses were submerged in the PEI solution (5 mg/ml in ultrapure water) for 15 minutes in order to build 2nd PEI layer of the LBL film. The PEI modified glasses were rinsed three times with ultrapure water and dried under a stream of nitrogen. After the covalent depositions of PEI through TA cross linking, one layer of the PEI film was built. The procedures as mentioned were repeated for several times to fabricate the desirable number of PEI layers. FITC-PEI and amino-quartz slides were used for monitoring the covalent LBL deposition and testing stability of the multilayers by UV-Vis spectroscopy.

3.2.5 Loaded TA/PEI multilayer coatings with Ag Nanoparticles

The PEI multilayer supported on glass slides were immersed into 25 mM NaBH₄ in ethanol for 1 hour and then rinsed with ethanol for three times before drying under a stream of nitrogen. Afterwards, these NaBH₄ loaded PEI multilayer films were dipped into 10 mM AgNO₃ solution for 0.5 hour and then washed three times with ultrapure water followed by drying under a stream of air. Multiple cycles of Ag nanoparticles loading into PEI multilayer were carried by repeating the procedures as mentioned above. The number of PEI layer in any multilayer film was denoted by (PEI)_x herein.

3.2.6 Viabilities of Bacteria in Contact of Coating Surface

Live/dead *Baclight* bacteria viability kit (molecular probes, L13152) was used for measuring the viability of the bacteria upon forming contacts with material surfaces. The kit, which contains Propidium Iodide (PI) and SYTO 9,

diffuse into the bacteria and produce fluorescence signal upon binding to nucleic acids. In the presence of PI, SYTO 9 specifically stains live bacteria and results in green fluorescence emission. On the contrary, PI is excluded from viable cells and specifically diffuses into dead cells. PI has high affinity for nucleic acids which results in red fluorescence signal. The viable and dead cells can be distinguished by fluorescence microscopy since the viable cells appear green and dead or non-viable cells (with membrane compromised) appear red.

Various types of PEI multilayer were immersed in a 0.5 ml of bacterial suspension, with 10^8 *E.coli* cells/ml or 10^7 *S.aureus* cells/ml at 37°C for 1 h. The samples were then washed with ultrapure water for 3 times and stained with 50 µL of the dye mixture for 0.5 hour. These samples were subsequently imaged with a confocal microscope (Carl Zeiss, Germany). The live or dead bacterial number is got by count the green or red dots in the images. The dead bacterial ratio we use in our description equals to

$$100\% \times \text{dead bacterial number} / \text{total number of live and dead bacteria}.$$

3.2.7 Antibacterial Activity of the Coating in solution

Various types of PEI multilayer were immersed into 10 ml of LB broth with 10^5 *E. coli* inside a beaker and were shaken at 225 rpm under 37 °C. After shaking, 1 ml of the medium was extracted from the sample for optical density measurement (at 600nm) at different time points. After each measurement, the 1 ml LB broth was transferred back to the beaker.

3.2.8 Kirby-Bauer test (Disk-diffusion method)

The Kirby-Bauer test was performed to determine the release-killing property of biomaterial. First, an agar gel plate was swabbed with 50 µl of

bacteria containing medium at a concentration of 10^6 cells/ml. Various types of PEI multilayer supported on glass slides were put onto the agar gel plate. The agar gel plate was fully hydrated to induce the contact formation between the agar gel plate and PEI multilayer. The sample as mentioned above was then incubated for 24 h at 37 °C. The zone of inhibition (ZoI) around the multilayer sample, where there is no bacterial colony formation was detected by optical microscope. The size of ZoI would be highly sensitive to the antimicrobial properties of the sample.

3.2.9 Surface Characterizations

Static contact angles of water on different PEI multilayers were measured by the sessile drop method at 50% relative humidity and 25°C. In brief, 3µL water droplet was loaded onto the sample surface inside a telescopic goniometric stage (FTA 200 dynamic contact angle analyzer). All samples were put in the contact angle chamber for 20 minutes before measurement. At least ten measurements from different regions of a sample were taken.

PEI multilayer supported on glass substrate were also examined with an AFM (Asylum Research, model MFP-3D) under tapping mode. Each AFM topographic image was a representative view of at least three samples (with at least five regions of interest in each sample). The root-mean-square roughness (RMS) analysis was performed with the Asylum Research software. The UV-Vis absorption spectra of PEI multilayer with or without loaded Ag nanoparticles were recorded by a UV-Vis spectrophotometer (Nicolet Evolution 500, USA).

3.3 Results and Discussion

3.3.1 Covalent Layer-by-Layer Assembly of PEI Film

Figure 3.1 shows the schematic diagram of the covalent LBL assembly of PEI on amino-modified (NH_2 -) glass by chemical crosslinking with TA. NH_2 -glass was first dipped into TA solution to convert the amino group to aldehyde group via the formation of imine bonds²⁴⁶. These aldehyde groups on TA then reacted with the amino group of PEI via the formation of the same imine ($\text{C}=\text{N}$) bond and led to the immobilization of 1st PEI layer onto the surface. The PEI coated substrate was again dipped into TA solution in order to introduce additional aldehyde groups into the immobilized PEI chain for the immobilization of the 2nd PEI layer. The procedures as mentioned above were repeated several times until the desired number of PEI layers would be reached.

Figure 3.1 The schematic diagram for the covalent LBL assembly of PEI on amino-modified (NH_2 -) glass by chemical crosslinking with TA.

In order to validate the chemical immobilization of PEI on TA modified surface, FITC-PEI, which was detectable by UV-Vis spectroscopy, was utilized as a fluorescence reporter for monitoring the deposition cycle. In this study, a chemical crosslinker substituted the polyanion which was conventionally used for physical immobilization of PEI in LBL assembly.

Figure 3.2A shows the UV-Vis absorption spectra of PEI multilayer with different PEI layer numbers (from bottom to top: 2nd, 4th, 6th, 8th, 10th layer of PEI) at different stages of covalent LBL assembly. The result indicated that there was one main absorbance peak at 515 nm which was originated from FITC-PEI on all spectra. Moreover, the absorbance intensity at 515 nm was positively correlated with the increase in the number of PEI layer (Figure 3.2A, inset). Generally, the new PEI layer was successfully linked to the preceding PEI layer through the chemical crosslinker, TA. The use of FITC-PEI instead of PEI should not affect the properties of the resulted film since PEI contains excess amines for simultaneous coupling to both FITC and TA. It is also crucial to monitor the modification of amino-modified glass and PEI with chemical crosslinker during covalent LBL assembly.

It is shown in Figure 3.2B that the UV-Vis absorption spectra of TA modified PEI layers (from bottom to top: 0, 2nd, 4th, 6th, 8th, 10th layer of PEI). The absorbance peak at around 265 nm emerged from TA, which was chemically coupled to the amino-modified glass or PEI through imine bond formation between the primary amines on glass and aldehyde groups of TA. As a consequence, the free aldehyde group of TA molecule was exposed on the substrate surface. Moreover, the absorbance peak value of TA, which was successively coupled to the PEI layer, increased linearly against the

Figure 3.2 A

Figure 3.2B

Figure 3.2 (A) The UV-VIS absorption spectra of PEI multilayer with different PEI layer numbers (from bottom to top: 2nd, 4th, 6th, 8th, 10th layer of PEI) at different stages of covalent LBL assembly. The inset is the peak value at 515 nm. (B) The UV-Vis absorption spectra of TA modified PEI surface (from

bottom to top: 2nd, 4th, 6th, 8th, 10th layer of PEI). The inset is the peak value at around 265 nm.

increase of the number of PEI layers (Figure 3.2B, Insert). The trend as mentioned above confirmed the effective coupling of a TA onto the PEI layer.

By repeating the covalent LBL assembly, a PMF composed of only one polyelectrolyte was produced. Our approach of making PMF composed of one polyelectrolyte may provide certain competitive advantages overall conventional PMF. It is because the presence of two types of polyelectrolytes in conventional PMF leads to several potential shortcomings including interlayer diffusion, film irregularity and structural instability²⁴⁷⁻²⁴⁹. The reaction between PEI and TA was completed within 15 min since the absorbance of both TA and FITC-PEI peaks were independent of the reaction time beyond 15 min.

Stability of the PEI multilayer was assessed by comparing the UV-Vis absorbance before and after incubation in PBS or DI water for different time. Fig 3.3 shows the relative absorbance of (PEI)₁₅ multilayer after incubation for 24 or 72 hours. The relative absorbance represented the percentages of FITC-PEI remained on the glass support, which is equal to the absorbance after incubation over that before incubation. It was shown that around 99% and 95% of PEI remained on the film after incubation for 24 hours and 72 hours, respectively, in DI water. Similar result was found in PBS in which 96% and 92% of PEI remained on the film after incubation for 24 hours and 72 hours, respectively. These results indicated that the PEI multilayers at least demonstrated short-term stability under physiological conditions. In our current work, covalent bonds were used in the LBL build-up of polyelectrolyte layers while conventional polyelectrolyte films are constructed by electrostatic

attraction or hydrogen bonding of two oppositely charged polyelectrolytes. Therefore the stability test validated that our multilayer is stable.

Figure 3.3 Relative absorbance of (PEI)₁₅ multilayer after incubation in PBS and DI water for different time. The relative absorbance represents the percents of PEI remaining on the surface, which equals to the absorbance value after incubation for 34 and 72 hours divided by the absorbance value before incubation at 0 hour.

Since plain PEI and TA modified PEI surfaces had different hydrophobicity, the covalent LBL assembly would likely alter the wetting properties of the PMF surface. Figure 3.4 shows the contact angle of water during covalent LBL assembly TA/PEI multilayers. The odd number points represent TA layers and the even number points represent PEI layers. First, the high contact angle of 80° (layer #0) indicates that the hydrophilic glass surface with contact angle of 15° was turned hydrophobic following the silanization with APSi. After the chemical coupling of TA onto primary amine on glass

(layer #0), the contact angle was slightly reduced to 76° (layer #1). Subsequently, the contact angle of the outmost layer rose and fell after TA modification and PEI deposition, respectively. The result was caused by the difference in hydrophobicity between plain PEI and TA modified PEI. For instance, TA modified PEI was significantly more hydrophobic than the positively charged PEI surface. The alternative switching of contact angle as mentioned above confirmed the change in surface chemistry during LBL deposition.

Figure 3.4 The water contact angle during covalent LBL assembly of TA/PEI multilayers. The odd number points represent TA layers and the even number points represent PEI layers.

Because the covalent PMF described herein is a relatively new material system, AFM and contact angle measurements were necessary for monitoring the covalent coupling of PEI layer and characterizing the resulted film quality, respectively. Figure 3.5 shows the AFM topographic images of (a) amino-

modified glass (b) one PEI layer (c) five PEI layers (d) ten PEI layers during the assembly of PEI multilayer. Before PEI deposition, the root-mean-square (RMS) roughness of amino-modified glass was determined as 0.2 nm. After the deposition of one PEI layer on amino-modified glass, the RMS roughness of the film was significantly increased by ten times (from 0.2 to 2 nm). After the deposition of five PEI layers, the RMS roughness of the film rose from 2 nm to 12 nm due to the formation of numerous particles with an averaged diameter of 65 nm. Further build-up from five to ten PEI layers led to further increase of RMS roughness and particulate diameter from 12 to 30 nm and 65 to 107 nm, respectively.

Figure 3.5 AFM topographic images of (a) amino-modified glass (b) one PEI layer (c) five PEI layers (d) ten PEI layers during the assembly of PEI multilayer.

It has been recently reported that the RMS roughness was reduced against the increase of the layer number in conventional PMF composed of a pair of oppositely charged polyelectrolytes²⁵⁰⁻²⁵¹. The trend observed herein deviated from that as mentioned above due to the use of one polycation instead of a pair of opponent charged polyelectrolytes.

In this study, PEI deposition and TA modification was carried in ultrapure water and ethanol, respectively. The surface was dipped into TA dissolved in ethanol after the coupling of 1st PEI layer in water. However, ethanol was a poor solvent for PEI in comparison with water. As a result, the immobilized PEI chains tended to collapse and self-entangle in ethanol during its reaction with TA. Also, TA cross-linked the PEI molecules to form small particulates. At the same time, these cross-linked PEI particles contain excess non-reacted aldehyde groups for binding to next PEI layer through TA as linker. The change in reaction conditions during covalent LBL as mentioned above caused the increases in both particle size as well as the RMS roughness of multilayer against the increase of layer number. Moreover, the nanotopology of the (PEI)₁₀ multilayer resembled that of certain nonlinear LBL assembled PMF such as PAA/PEI/Ag⁺²⁵².

3.3.2 Loading Ag Nanoparticles into TA/PEI Multilayer

In order to introduce Ag nanoparticles into PMF, silver ions were first incorporated into PMF films by either direct immersion in silver salt solution¹⁶⁶⁻¹⁶⁷ or LBL deposition of one Ag⁺ containing polyelectrolyte with another oppositely charged polyelectrolyte^{165, 168, 170}. In both cases, silver ions were loaded into the matrix of PMF before they were reduced to the silver nanoparticles *in situ*. In our current work, NaBH₄, the reducing agent was first

introduced into the PEI multilayer before Ag^+ loading. It was because the negatively charged BH_4^- strongly bound with the positively charged backbone of PEI. In hypothesis, Ag^+ ions which came into contact with the immobilized BH_4^- within the PEI matrix would be reduced to Ag nanoparticles.

Figure 3.6A shows the UV-Vis spectrum of $(\text{PEI})_{10}$ multilayer (a) before NaBH_4 loading, (b) after NaBH_4 loading, (c) after 1 cycle of NaBH_4 loading/ Ag^+ reduction and (d) after 2 cycles of NaBH_4 loading/ Ag^+ reduction. The immobilization of NaBH_4 didn't cause any detectable change in the UV-Vis spectra of $(\text{PEI})_{10}$ multilayer (by comparing spectrum b and a). After AgNO_3 solution added for 0.5 hour, a new peak at 420 nm appeared on the spectrum of NaBH_4 loaded $(\text{PEI})_{10}$ multilayer (spectrum c on Fig. 5A). The absorbance peak at 420 nm was originated from Ag nanoparticles within the multilayer which generated surface plasmon from the collective oscillations of conducting electrons after excitation by visible light²⁵³. It would be beneficial in controlling the amount of Ag nanoparticles within PMF for tailored antimicrobial applications. By performing two rounds of $\text{NaBH}_4/\text{AgNO}_3$ reduction, the absorbance at 420 nm was shown to be increased by more than two times from 0.8 to 0.17 compared to that with one round of loading (spectrum c vs. d). The enhancement in Ag nanoparticles loading as mentioned above was likely caused by the excess primary amines within the ten PEI layers for binding with additional BH_4^- ions during the second round of Ag nanoparticles loading.

Figure 3.6A

Figure 3.6B

Figure 3.6 (A) The UV-Vis spectrum of (PEI)₁₀ multilayer (a) before NaBH₄ loading, (b) after NaBH₄ loading, (c) after 1 cycle of NaBH₄ loading/Ag⁺ reduction and (d) after 2 cycles of NaBH₄ loading/Ag⁺ reduction. (B) UV-Vis spectrum of (e) (PEI)₁₀ and (f) (PEI)₂₀ multilayer both with one round of silver nanoparticles loading.

Alternatively, the number of free amines on PEI multilayer film can be engineered by the number of PEI layers. Fig 3.6B shows the UV-Vis spectrum of (PEI)₁₀ multilayer with one round of silver nanoparticles loading and (PEI)₂₀ multilayer with one round of silver nanoparticles loading. The result indicated that the absorbance peak at 420 nm which represented silver nanoparticles inside (PEI)₂₀ multilayer was two times higher than that inside (PEI)₁₀ multilayer. The amount of Ag-nanoparticles generated within the PMF increased with the number of PEI layer because the excess amines of (PEI)₂₀ multilayer provided larger capacity for binding with more BH₄⁻ ions than (PEI)₁₀.

The total silver releasing time is strongly dependent on the total amount of Ag nanoparticles as well as the size of Ag nanoparticles inside the multilayer coatings¹⁶⁶. The size of fresh prepared silver nanoparticles in our study is probable ranging from 5-10 nm, due to the similar characteristic peak around 420nm in other's research^{166-167, 171, 254}. Ag ions diffused very fast in the multilayer coatings so the oxidation of zero-valent Ag to Ag⁺ on the nanoparticles surface is the rate-limiting step. The smaller Ag nanoparticle has larger surface area, therefore, resulting in faster Ag ionization process, thus short total Ag releasing time¹⁶⁶.

Although our method of Ag nanoparticles loading (immobilization of NaBH₄ onto PEI before Ag⁺ ion loading) has not been directly compared with other conventional methods (e.g., loading Ag⁺ ions into PEI before addition of reducing agents), the amount of loaded Ag nanoparticles in PMF as described herein was conveniently controlled compared to other reported methods^{166-167,}

²⁵⁴. Most importantly, our new method provided an accurate way for introducing various amounts of metal nanoparticles into PMF formed from covalent LBL assembly.

3.3.3 Antibacterial Activity Measurement

Conventional antimicrobial PMF provided two main actions for killing bacteria. For instance, bacteria were either killed by direct contact formation with PMF or by the controlled release of the antimicrobial agents from the PMF into the biological fluids. A standard way for quantifying the contact induced killing of bacteria on PMF was by the use of “Live and Dead” assay.

Figure 3.7 is the fluorescence images of “Live” and “Dead” *E. coli* on amine-modified glass as control, (PEI)₁₀ multilayer and (PEI)₁₀ multilayer loaded with Ag nanoparticles after bacteria seeding for one hour. First of all, the result indicated that most *E. coli* remain intact (large number of green cells on Figure 3.7) while only a smaller number of *E. coli* was found dead (red cells) on amine-modified glass. On the other hand, around 89% of *E. coli* were found dead and only 11% of cells remained intact on the (PEI)₁₀ surface. The result supported that just (PEI)₁₀ multilayer alone possessed significant antimicrobial properties against *E. coli*. Interestingly, over 99% of *E. coli* were killed while only a small number of cells was found to be viable on the (PEI)₁₀ multilayer with embedded Ag nanoparticles.

Figure 3.7 The fluorescence images of “Live” and “Dead” *E. coli* on amine-modified glass, (PEI)₁₀ multilayer and (PEI)₁₀ multilayer loaded with Ag nanoparticles after seeding for one hour.

It is critical to examine the antimicrobial properties of the PMF against various types of bacteria. Figure 3.8 is the fluorescence images of “Live” and “Dead” *S. aureus* on amine-modified glass, (PEI)₁₀ multilayer and (PEI)₁₀ multilayer loaded with Ag nanoparticles after bacteria seeding for one hour. Again, the lowest number of dead cells detected on amine-modified glass implied the minimal antimicrobial activities of amine-modified glass against *S. aureus* among the three samples used herein. In resemblance to the result of *E. coli*, (PEI)₁₀ surface presented certain degree of contact induced killing of *S. aureus* although it failed to kill the majority of bacteria (around 52% of *S. aureus* killed) after one hour of incubation. In contrast, nearly all (over 99.9%) *S. aureus* cells were killed by (PEI)₁₀ multilayer embedded with Ag nanoparticles.

Figure 3.8 The fluorescence images of “Live” and “Dead” *S. aureus* on amine-modified glass, (PEI)₁₀ multilayer and (PEI)₁₀ multilayer loaded with Ag nanoparticles after seeding for one hour.

Generally, covalent PEI multilayer had a net positive charge because it is only composed of a single polycation, which is crosslinked by TA which carries zero net charge. When bacteria approaches a cationic surface, the bacteria membrane which mainly composes of divalent cations was significantly perturbed through the ion exchange between the bacteria membrane and cationic surface⁷³. The depletion of these positively charged components as mentioned above in bacteria resulted in the reduction of membrane integrity and finally led to bacteria death⁷³. The significant antibacterial activities of (PEI)₁₀ multilayer as observed herein agreed well with the previously reported activities of cationic surfaces⁷³. The difference in the extent of killing against the two bacterial strains (89% for *E.coli* and 52% for *S.aureus*) was likely

attributed to the considerable differences in membrane composition between Gram positive and Gram negative bacteria²⁵⁵.

In the past, PEI itself was mainly used as carrier for Ag⁺ or other antibacterial agents^{61, 165-166}. In our case, PEI multilayer also showed considerable contact-killing property against bacteria. Actually, the contact-killing property of our covalent PEI multilayer was compared with widely used PAA/PEI multilayer both with PEI as outmost layer. The PAA/PEI multilayers do not show any contact-killing property, while the PEI multilayer demonstrated better kill ability especially to *E.coli* (data not shown).

Most importantly, (PEI)₁₀ with embedded Ag nanoparticles showed highest efficiencies in killing both bacterial strains (over 99%) used herein. It has been recently shown that Ag and Ag⁺ killed a broad spectrum of bacterial strains, fungi and viruses^{161, 238}. For instance, Ag⁺ was known to deactivate the enzymes and DNA within the bacteria cytoplasm via the binding with the electron-donating groups. It also caused the formation of pits in bacterial cell wall and led to the increase of membrane permeability and ultimate cell death^{84, 164}. Overall, it was concluded that our new method of Ag nanoparticles incorporation into covalent PEI multilayer resulted in excellent contact-killing activities against both Gram-positive and Gram-negative bacteria strains.

To assess the control release properties of the (PEI)₁₀ multilayer, *E. coli* in suspended culture was incubated with different types of multilayer and the optical density (OD, at 600nm) of the media was measured at different time points. Figure 3.9 shows OD of *E. coli* suspension (at 600 nm) against time in the growth medium which contains amine-modified glass as control, (PEI)₁₀ multilayer or (PEI)₁₀ multilayer with loaded Ag nanoparticles.

Figure 3.9 OD of *E. coli* suspension (at 600 nm) after different time in the growth medium which contains either amine-modified glass, (PEI)₁₀ multilayer or (PEI)₁₀ multilayer with loaded Ag nanoparticles.

As expected, amine-modified glass imposed minimal antibacterial activities towards the *E. coli* suspended in solution. The result was consistent with the negligible contact induced killing of *E. coli* on amine-modified glass. Interestingly, the bacterial growth curve for *E. coli* in medium containing (PEI)₁₀ film was similar to that for amine-modified glass as shown by the similar trend of OD against time. Although the (PEI)₁₀ film demonstrated effective contact-induced killing of around 90% of *E. coli*, it failed to inhibit the growth of the bacteria in growth medium. It was because the chemically cross-linked (PEI)₁₀ multilayer itself doesn't release any antimicrobial reagents to the exceedingly large number of bacteria in the medium. In contrast, (PEI)₁₀ embedded with Ag nanoparticles effectively inhibited the growth of *E. coli* in the culture medium as the OD remains at low level even after 12 hours of

culture. The result strongly supported that Ag^+ ions were effectively released into the culture medium for killing a large number of bacteria suspending in medium.

To validate the prolonged release of Ag nanoparticles, a standard diffusion assay for measuring antimicrobial activities was carried for amine-modified glass, $(\text{PEI})_{10}$ or $(\text{PEI})_{10}$ with loaded Ag nanoparticles on *E. coli* cultured on gel (Figure 3.10). First, the result validated that both amine-modified glass (A) and $(\text{PEI})_{10}$ multilayer (B) surfaces didn't release any antimicrobial reagent into the surrounding gel medium, since bacteria had remained at the edge of the two samples. In contrast, a clear ring which indicated the absence of bacteria was detected at region adjacent to $(\text{PEI})_{10}$ substrate embedded with Ag nanoparticles (C). The result confirmed the effective release of Ag^+ from the Ag nanoparticles loaded multilayer into the surrounding gel medium.

Figure 3.10 A Kirby-Bauer (Disk-diffusion) test plate, (a) amine-modified glass as control, (b) $(\text{PEI})_{10}$ multilayer and (c) $(\text{PEI})_{10}$ multilayer with loaded Ag nanoparticles.

It was worthy to mention that the (PEI)₁₀ embedded with Ag nanoparticles film contained both silver ion and silver nanoparticles. In our Ag loading method, the last step was to immerse the TA/PEI multilayer preloaded with reducing agents into Ag⁺ ion containing solution. As a result, certain Ag⁺ ions was reduced to Ag nanoparticles with zero charge while other Ag⁺ ions just bound with the primary, secondary and tertiary amines of PEI through electrostatic attractions^{256, 165, 168, 170}. Moreover, our covalent PEI film likely provided an initial burst release of Ag⁺ which was followed by the sustained release of Ag⁺ from the prolonged oxidation of Ag nanoparticles. The mechanism as mentioned above was supported by the fact that the oxidation rate of Ag to Ag⁺ is significantly slower than the diffusion rate of Ag⁺ from the film interior to the surrounding liquid medium¹⁶⁶.

3.4 Conclusions

A novel PMF consisting of a single polyelectrolyte, PEI was constructed by covalent LBL deposition with the use of TA as the chemical cross-linker. First of all, it was shown that TA acted as an effective anchor for successive PEI immobilization during all stages of LBL. Moreover, Ag nanoparticles were uniquely incorporated into the PMF through *in situ* reduction of Ag⁺ by the pre-adsorbed NaBH₄ within the film matrix. It was also shown that the mass of embedded Ag nanoparticles was controlled by altering the film thickness or loading cycles.

Interestingly, live/dead bacterial assay showed that our PMF not only worked as a carrier of Ag nanoparticles but also possessed considerable

antimicrobial property against bacteria in contact. For instance, (PEI)₁₀ killed around 89% *E. coli* and 52% *S. aureus* through direct contact. Interestingly, the contact killing efficiency of the resulted film was increased to over 99% against both bacterial strains upon the loading of Ag nanoparticles in (PEI)₁₀. Disk diffusion test and solution test supported that Ag nanoparticles loading enhanced the contact-killing property. Most importantly, good release-killing property against the two bacteria strains was introduced into covalent (PEI)₁₀ multilayers by the embedded Ag nanoparticles. In the future, an anti-adhesive layer will be added onto this Ag nanoparticles loaded PEI multilayer to further inhibit the initial bacterial adhesion.

Chapter 4 Effect of adhesive ligands on bacterial and fibroblast adhesions

4.1 Introduction

The use of implantable medical devices, such as prosthetic heart valves, cardiac pacemakers, orthopaedic joint replacement, etc., has become an indispensable day-to-day practice in modern health care ^{1, 257}. Despite the increasing application of medical devices, biofilm formation which is triggered by the adhesion of bacteria onto material surfaces often leads to the failure of medical devices ¹.

Biofilm is extremely difficult to eliminate from device surface compared to the planktonic counterparts due to the development of antibiotic/antimicrobial resistances within the biofilm⁶. Currently, the prevention of bacterial adhesion onto the device surface preceding the biofilm formation remains an attractive strategy for combating device-associated infection. Surface functionalization is a relatively straight-forward method for modifying the interfacial properties of medical device without disrupting the bulk property properties of device material. Another advantage is that it can prevent device-associated infections *in vivo* at the implantation sites where antibiotics cannot reach. Several surface functionalization methods have been used for rendering biomaterial/device surface anti-microbial: (a) grafting the surface with antibacterial polymer^{58, 83},

(b) coating the surface with microbe-repelling or anti-adhesive polymer^{136, 138}, (c) using polymeric system with controlled release of biocides^{157, 174}, (d) multifunctional surface with two or three properties as mentioned above²⁵⁸.

More recently, research efforts have been devoted to exploit device surface with selected biological activity, i.e. enhancing the integration of biomedical device with host tissues while inhibiting bacterial adhesion^{123, 234-235}. If the host cells adhere on the device surface before bacterial adhesion, the membranes and glycocalyx of adherent host cells/tissues would prevent the growth of the bacteria on the device^{228, 259}.

One strategy is to promote the adhesion of host cells onto an antibacterial surface through the introduction of extracellular matrix (ECM) proteins or adhesive peptide containing RGD sequences²³⁴. Our group have demonstrated that the chemical coupling of RGD on PEM composed of hyaluronic acid and chitosan significantly enhances osteoblast proliferation while the antimicrobial activity of the PEM is retained. PLL-g-PEG functionalized with RGD has also been shown to have both bacteria-repulsive and cell-adhesive properties^{123, 235}.

The choice of adhesive ligand would be critical to introduce selected biological activity onto device surfaces. It is rationalized that the development of device surface will be largely benefited from understanding the biophysics of both bacteria and mammalian cells during their encounters with the device surface. But there is still a lack of quantitative design approach for correlating the nature of adhesive ligand, bacterial adhesion and host cell integration. Therefore, in order to get more quantitative correlation between the adhesion strength of bacteria or mammalian cells and adhesive ligand, in Chapter 4, the biophysical responses of bacteria (*E.coli* and *S. aureus*) and mammalian cells

(fibroblast) towards two common adhesive ligand (RGD and collagen) on model device surfaces are elucidated.

In this work, for the first time, bioanalytical techniques were applied to compare the biophysical responses of both bacteria and mammalian cells on to a surface incorporated with different adhesive ligand. The effects of different surface modifications, especially RGD and collagen bonding on the kinetics of 3T3 fibroblasts cell deformation, average adhesion energy and adhesion energy per cell of fibroblast during initial seeding, as well as on the detachment force *E. coli* and *S. aureus* were elucidated. The results herein strongly suggest that there is a highly intricate interplay between the type of adhesive ligand, bacteria strain, adhesion strength of bacteria and adhesion kinetics of mammalian cells which would be helpful for the design of advanced device surface.

4.2 Material and methods

4.2.1 Materials

High glucose Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), streptomycin, penicillin, PBS (8g/l of NaCl, 0.2g/l of KCl, 1.44g/l of Na₂HPO₄ and 0.24g/l of KH₂PO₄) originally in the form of 10×PBS were purchased from Invitrogen Inc. (Singapore). Dopamine, RGD, N-hydroxysulfosuccinimide sodium salt (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), Ethylenediaminetetraacetic acid(EDTA), formaldehyde was purchased from Sigma Pte. Ltd. (Singapore). *E. coli* DH5 (ATCC#: 53868) and *S. aureus* (ATTC#: 25923) were obtained from American Type Culture Collection (U.S.A.). Fluorescein isothiocyanate (FITC)-labelled RGD were purchased from Ana Spec Inc. (U.S.A.).

4.2.2 3T3 Fibroblast Culture

3T3 fibroblasts (3T3-Swiss albino, ATCC) were cultured in high glucose DMEM medium supplemented with 10% fetal bovine serum (FBS), 5mg/ml penicillin and 5mg/ml streptomycin (Invitrogen Inc., Singapore). Cell cultures were maintained at 37°C under a humidified atmosphere of 5% CO₂ in air. The medium was changed every 2 days, and the cells were passage at least two times a week. Cells were detached from the culture flask by the addition of a 5.3 mM trypsin-EDTA solution in PBS. The cell suspension was transferred to a 15 ml Falcon tube and was centrifuged at 1200 rpm for 3 min. After the removal of trypsin, the remaining cell pellets were re-suspended in the DMEM medium supplemented with 10% FBS.

4.2.3 Bacterial Culture

The two bacterial strains used including *E. coli* and *S. aureus* were cultivated in LB broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl) at 37°C. The bacteria were cultured for about 16 h before harvest. The bacteria-containing broth was centrifuged at 1300g for 10 min, and after the removal of the supernatant, the cells were washed twice with sterile PBS solution and then re-suspended in PBS. Different densities of bacteria in PBS were used in various measurements.

4.2.4 Surface Preparations and Characterizations

Glass coverslips were cleaned in ‘piranha’ solution composed of 30% (v/v) of hydrogen peroxide and 70% (v/v) of concentrated sulfuric acid for 1 h, rinsed thoroughly with double distilled water (with resistance of 18.2 MΩ) and dried in N₂ stream. The cleaned glass samples were immersed in water to keep the

surface clean and hydrophilic before further use. The clean glass samples were put into 1mg/ml dopamine in water for 24 hours and then rinsed with water. After drying in a stream of N₂, the dopamine-glass samples were put in a vacuum oven at 37°C for 12h and were kept in the 4 °C refrigerator. RGD peptide (10µg/ml in PBS) solution or collagen solution (10 µg/ml in H₂O) was mixed with EDC (20mM) and NHS (50mM) solution at volume ratio of 1:1. Afterwards, dopamine-glass samples were immersed in the above-mentioned RGD solution or collagen solution for 72 h at 4°C in order to carry out the coupling reactions. Subsequently, RGD-dopamine-glass and collagen-dopamine-glass were dried in N₂ stream and then put in vacuum oven at 37°C for 30 min. The samples were kept in 4 °C refrigerator before further experiments.

The chemical composition of the surfaces was analyzed by X-ray photoelectron spectroscopy (XPS) on an AXIS HSi spectrometer (Kratos Analytical) with an AlK α X-ray source (1486.6 eV photons). In order to validate the conjugation of RGD and collagen onto the dopamine-glass, a solution droplet composed of FITC-labelled RGD or FITC-labelled collagen and other coupling reagents (NHS/EDC) was dispensed onto a selective region of the substrate at 4 °C for 72 hours. After that, the sample was washed with water for 3 times. Fluorescence image of the boundary region of RGD/collagen modified area was taken by using an Olympus IX71 fluorescence microscope (Japan) equipped with an Olympus DP70n CCD camera.

4.2.5 Contact Angle Measurement

Static contact angles of the four different surfaces were measured at 25°C and 50% relative humidity by the sessile drop method by using a 3µl water droplet inside a telescopic goniometer (FTA 200 dynamic contact angle analyzer). The glass samples were taken out from water and after drying in the N₂ stream, it was put in the contact angle equipment room for 20 minutes. The other sample were taken out from the refrigerator and then put at room temperature for 20 minutes before measurement. For each sample, at least ten measurements from different regions of a surface were taken.

4.2.6 Atomic Force Microscopy

Plain glass, dopamine-glass, RGD-dopamine-glass and collagen-dopamine-glass were examined with an atomic force microscope (Asylum Research, model MFP-3D) under tapping mode in air. All data analysis was performed with the Asylum Research software.

4.2.7 Confocal Reflectance Interference Contrast Microscopy (C-RICM)

For our biophysical measurement of cell adhesion, the cells were seeded on the substrates inside a temperature control chamber (Carl Zeiss, Germany) attached to the confocal microscope at 37°C and 5% CO₂ atmosphere. A series of phase contrast and C-RICM images on a selected region was taken as a function of time at 37°C. The image analysis was carried with software ZSM 5 (Carl Zeiss, Germany). The average radius of contact area (a) and projected cell area (R) of each cell were determined by C-RICM and phase contrast microscopy, respectively. At each time point, the average value of a and R was

measured from fifty or more cells on at least three identical samples. The ratio a/R represented the degree of deformation for each cell.

4.2.8 Detachment Force Measurement of Bacteria

The method used was described in detail elsewhere^{138, 260}. After incubation of the model surfaces with bacterial suspension for 12h, the samples were washed three times with PBS, followed by drying for 12h in a humid environment. The sample was immersed in deionised water for 0.5h. The force measurement was carried out using an AFM (Asylum Research, model MFP-3D) under contact mode in deionised water. The spring constant of each AFM cantilever used in this study was recalibrated with the build-in thermal noise method provided by the MFP-3D software. And a scanning rate of 0.5 Hz was used in all the experiments. The force of AFM tip exerted on the sample was then calculated as the product of tip spring constant (obtained from the AFM software), the cantilever sensitivity (nm/V) and the tip deflection distance. At the start of each experiment, areas of the sample (scan size of $20 \times 20 \mu\text{m}^2$) were scanned at the minimum force (1 nN). Once an individual cell was selected, the scan size was adjusted to encompass the size of the cell (scan size $4 \times 4 \mu\text{m}^2$, scan speed $5 \mu\text{m/s}$). The detaching force was taken to be the minimum value of applied tip force when cell detachment occurred. For each type of substrate, 2–3 samples were tested and on each sample, at least 5 cells were measured.

4.2.9 Scanning Electron Microscope (SEM)

After incubation with bacterial suspension for 1h, the samples were washed three times with PBS before fixing with 3% glutaraldehyde for at least 4 h at 4 °C. The films were then subjected to step dehydration with 25, 50, 70, 100%

ethanol for 10 min each. SEM (JEOL, model JSM-6390) was used for imaging the surfaces of four samples. Bacteria on the sample surfaces were directly counted from various regions each with an area of $10 \times 10 \mu\text{m}^2$ on the SEM images. The bacterial count for each sample was averaged from at least five different SEM images.

4.2.10 Determination of Cell Adhesion Energy

In this study, confocal Reflection Interference Contrast Microscope (C-RICM), which actually is a high-resolution (HR) RICM, is used. In order to measure the cell-substrates interaction in bio-adhesion, With C-RICM or HR-RICM, the spatial resolution of the cell's adhesion profiles was improved in comparison with that in conventional RICM, which is based on a conventional epi-fluorescence microscope. The major improvements of the current optical system include a coherent laser light-source used for sample illumination and the confocal optics that minimize the amount of stray light commonly found in the conventional RICM system; the dynamics range of in measuring membrane–substrate separation is improved from 0.05-1 μm to 0.05-4.5 μm ; the details are reported elsewhere²⁶¹⁻²⁶².

The details of the contact mechanics model of adherent cells have been previously described²⁶³. The equilibrium geometry of a cell adhering on a non-deformable substrate was modeled as a truncated sphere with a mid-plane radius R . The degree of deformation, $\sin \theta = (a/R) = \alpha$ is an experimentally measurable parameter where R and a are measured by C-RICM and phase contrast microscopy, respectively. The cell wall is under a uniform equi-biaxial stress, $\sigma = C\varepsilon$, where C is the stress equivalent and is equal to $Eh/(1-\nu)$ in a

linear system under small strain. E , h and ν represents the elastic modulus, membrane thickness and Poisson's ratio, respectively. The average biaxial strain, ε , was directly calculated as follows:

$$\varepsilon = \frac{1}{2} \left[\frac{2 + 2(1 - \alpha^2)^{1/2}}{4 - \alpha^2} - 1 \right] \quad (4-1)$$

In the absence of external force, it has been shown earlier that the adhesion energy, W , is as follows: $W = (1 - \cos\theta)C\varepsilon + C\varepsilon^2$ (4-2)

Based on the mid-plane diameter R (from phase contrast microscope) and the radius of contact zone, a (from C-RICM), W can be calculated from equation (4-1) and (4-2). The elastic modulus E of 3T3 fibroblast cells has been reported to 12 k Pa. h is 5 nm and ν is 0.5 in this study.

4.3 Results

4.3.1 Surface modification

Figure 4.1 shows the basic scheme for the immobilization of dopamine, RGD peptide and collagen on glass substrate. First, dopamine in solution was allowed to undergo chemisorptions on the unmodified glass surface through dehydration similar to the surface modification of Ti and TiO_2 ²⁶⁴ and Fe_2O_3 ²⁶⁵.

Figure 4.1 Scheme for the immobilization of dopamine, RGD peptide and collagen on glass substrate.

Figure 4.2 shows the wide scan spectra of XPS for both unmodified glass and dopamine-glass. There was an obvious increase in the N1s peak at 400 eV for dopamine-glass compared to that of plain glass. The N/Si ratio increased from 0.03 on unmodified glass to 0.26 on the dopamine-glass due to the amino groups in the dopamine molecule. The result indicated that the dopamine was effectively coupled onto the clean glass surface.

Figure 4.2 XPS wide scan spectra of unmodified glass and dopamine-glass.

Figure 4.3 shows the fluorescence images of a selected area of dopamine-modified glass which has been covered by the FITC-labelled RGD (a) and FITC-labelled collagen (b) after chemical coupling. The bright green region on the image corresponds to the region originally occupied by either FITC-labelled RGD (Figure 4.3a) or FITC-labelled collagen (Figure 4.3b) during chemical coupling while the dark region represents the area which is adjacent to the original location of the FITC- labelled RGD or FITC-labelled collagen containing solution droplet. The high contrast between the bright and dark regions indicates that RGD and collagen were successfully coupled onto dopamine-glass through the reaction between the primary amine groups of dopamine-glass and carboxyl

groups of RGD or collagen. The RGD and collagen conjugated to the dopamine glass surface remained stable after multiple washing steps.

Figure 4.3 Fluorescence images of a selected area of dopamine-modified glass which has been covered by the FITC-labelled RGD (a) and FITC-labelled collagen (b) after chemical coupling. The scale bar is 50 μm .

AFM is a highly versatile tool for probing nanotopology of biomaterial/biodevice surfaces. The RMS roughness of unmodified glass determined from AFM is 0.30 ± 0.03 nm (Table 1) and this low value indicates that the glass was rather smooth after the cleaning procedure. Following dopamine modification, the RMS roughness of dopamine-glass increased by 251% compared to that of unmodified glass to 0.75 ± 0.08 nm. The RMS roughness of RGD-dopamine-glass (0.68 ± 0.05 nm) was similar to that of dopamine-glass. Upon the chemical coupling of collagen, collagen fibres can be found on the surface (image not shown here). The RMS value of collagen-dopamine-glass was 0.95 ± 0.1 nm, which was significantly higher than those of dopamine-glass and RGD-dopamine-glass due to the formation of these fibres.

Table 4.1 Contact angle and surface roughness of different surfaces.

	Water contact angle °	RMS (nm)
Clean glass	11.1°±1.1°	0.30±0.03
Dopamine-glass	44° ±1.8 °	0.75±0.08
RGD-Dopamine-glass	46.9 ° ±2.4 °	0.68±0.05
Collagen-Dopamine-glass	39.3°±1.5°	0.95±0.1

Surface wettability has been known to dictate the cell-substrate interaction on biomaterial/biodevice surfaces. Table 1 shows the static water contact angle of unmodified glass, dopamine-glass, RGD-dopamine-glass and collagen-dopamine-glass. The contact angle on the unmodified glass is 11.1±1.1°. After the chemisorptions of dopamine on glass, the static contact angle increased significantly to 44 ± 1.8°. The result further supported the successful immobilization of dopamine on the glass surface. The chemical coupling of RGD peptides on dopamine-glass did not change the static contact angle (46.9±5.4°). However, the bonding of collagen onto the dopamine-glass reduced the contact angle to 39.3°±1.5. This difference in surface hydrophobicity may trigger different responses of cell and bacteria upon contact with the anti-microbial coating.

4.3.2 Bacterial adhesion

SEM has been commonly applied for determining the cell density and the morphology of adherent bacteria on the surfaces. In Table 4.2, density of adherent bacteria on different surface can be found.

Table 4.2 Density of adherent bacteria and bacteria detachment force on different sample.

	<i>E. coli</i>		<i>S. aureus</i>	
	Number of adherent cells	Cell detachment	Number of adherent cells	Cell detachment
	10 ⁶ Cells/cm ²	force (nN)	10 ⁶ Cells/cm ²	force (nN)
Clean glass	0.1±0.1	<1	1.5±1.0	3.3±2.1
Dopamine-glass	2.7±0.8	21.8±4.3	19.7±3.4	44.4±10.7
RGD-dopamine-glass	3±1.2	17.6±6.3	19.7±2.7	41.3±6.7
Collagen-dopamine-glass	0	<1	64±3	>80

The density of *S. aureus* on the unmodified glass was $(1.5 \pm 1.0) \times 10^6$ cells/cm². The modification of glass with dopamine resulted in an increase of adherent bacteria density by 13 times ($19.7 \pm 3.4 \times 10^6$ cells/cm²). The coupling of RGD on dopamine-glass had no apparent effect on the adhesion of *S. aureus* as shown by the similar cell density of $19.7 \pm 2.7 \times 10^6$ cells/cm² compared with that on dopamine-glass surface. On the other hand, the density of adherent *S. aureus* on collagen-dopamine-glass surface ($64 \pm 3 \times 10^6$ cells/cm²) was three times higher than that on dopamine-glass. *E. coli* has weak affinity for unmodified glass as shown by the low density of adherent bacteria of $0.1 \pm 0.2 \times 10^6$ cells/cm². The density of *E. coli* on either dopamine-glass ($2.7 \pm 0.8 \times 10^6$ cells/cm²) or RGD-dopamine-glass ($3 \pm 1.2 \times 10^6$ cells/cm²) was around 30 times higher than that on unmodified glass. This trend was similar to that of *S. aureus*. In contrast, the density of *E. coli* on the collagen-dopamine-glass surface was the lowest (0 cells/cm²) among all surfaces whereas this type of surface had the highest density of *S. aureus*. These results strongly indicated that the macroscopic parameter of bacterial adhesion was both strain and ligand dependent.

Although SEM probes the extrinsic and macroscopic parameters of bacterial adhesion, it does not provide any quantitative information on the physical driving force behind bacterial adhesion onto different surfaces. Recently, our group has applied AFM to quantify the adhesion between single bacteria and biomaterial surface through the determination of lateral detachment force¹³⁸.

Figure 4.4 shows a series of AFM images of a typical *S. aureus* cell against the increase of lateral force during cell detachment from dopamine-glass. A *S. aureus* cell strongly adhered to the dopamine-glass surface of 1 nN. After scanning with a force of 23 nN, the total area of adherent cell decreased. The cell was finally detached with a lateral force of 40 nN. The bacterium disappeared from half of the 40 nN image (top part) because it was actually removed from the original position by AFM tip during the scanning of the first half of the bacterium (bottom part).

Figure 4.4 A series of AFM images of a typical *S. aureus* cell during cell detachment with increasing lateral force from dopamine-glass.

In this study, the average lateral force required for detaching individual bacteria was dependent on the substrate properties (Table 4.2). The average detachment force of *S. aureus* (3.3 ± 2.1 nN) on unmodified glass was lowest among the four surfaces. The average detachment force of *S. aureus* upon dopamine modification of the glass was increased by 13.5 times to 44.4 ± 10.7 nN (the standard deviation was based on measurements of at least five cells of three samples). On RGD-dopamine-glass, the average detachment force of *S. aureus* was 41.3 ± 6.7 nN which was not statistically different from that on dopamine-glass ($t < 0.05$). However, the detachment force of *S. aureus* on collagen-dopamine-glass was larger than 80 nN and approached the upper limit of this measurement.

The adhesion strength of *E. coli* on different surfaces was also quantified with the same biophysical technique. The detachment force of *E. coli* also varies with the surface chemistry of model substrates (Table 4.2). Similar to the *S. aureus*, the weakest *E. coli* adhesion was on the unmodified glass with the lowest average detachment force (less than 1nN) among the four surfaces. *E. coli* was detached by a larger force of 21.8 ± 4.3 nN from dopamine-glass than that from unmodified glass. However, the detachment force of *E. coli* on RGD-dopamine-glass (17.6 ± 6.3 nN) was not statistically different from that on dopamine-glass ($t < 0.05$). Interestingly, the *E. coli* detachment force on the collagen-dopamine-glass was the lowest (<1 nN) and was significantly lower than that on dopamine-glass or RGD-dopamine-glass. This trend was contrary with that of *S. aureus* on the same surface which had the highest detachment force. The detachment force of a single *E. coli* positively correlated with the density of adherent *E. coli* on each substrate (Table 4.2).

4.3.3 3T3 Fibroblast Adhesion

After examining the biophysics of bacterial adhesion on our model surfaces, C-RICM coupled with phase contrast microscopy was used to probe the spreading and adhesion contact dynamics of mammalian cells on the same substrates in order to elucidate the effect of surface chemistry, especially RGD and collagen coupling.

The change in cell morphology is a phenotypic indicator of an activated cellular response upon cell adhesion. Figure 4.5 shows a series of phase contrast images of a typical 3T3 fibroblast at 10 min (top panel) and 120 min (bottom panel) on the four surfaces. All fibroblasts showed a round shape on all four surfaces during the first 10 min of cell seeding. After 2 hours culture, the fibroblasts had different degrees of spreading in response to different surface chemistry. It is clear that the cells spread least on unmodified glass while they spread most on RGD-dopamine-glass.

Figure 4.5 The series of phase contrast images of a typical 3T3 fibroblast at 10 min (top panel) and 120 min (bottom panel) after cell seeding on unmodified glass, dopamine-glass, RGD-dopamine-glass and collagen-dopamine-glass (scale bar represents 20 μm).

To quantify the change of cell morphology, the change in projected spread area of 3T3 fibroblasts during the 2 hour seeding was measured. Figure 4.6 shows the percentage change in the projected area of the 3T3 fibroblasts (calculated as the projected area after two hours of cell seeding divided by that after 10 min seeding) on different samples. On unmodified glass surface, no change was found in the projected area ($114\% \pm 12\%$) of adherent fibroblasts due to the lack of specific cue on glass for adhesion. For dopamine-glass, the projected area of the cells increased by 2.33 times ($233\% \pm 63\%$), while for the collagen-dopamine-glass and RGD-dopamine-glass surfaces, the projected area of adherent cells increased by 4.5 times ($450\% \pm 109\%$) and 9.53 times ($953\% \pm 153\%$), respectively.

Figure 4.6 Percentage change in the projected area of 3T3 fibroblasts (equal to the projected area after two hours of cell seeding, divided by that after 10 min seeding) on unmodified glass, dopamine-glass, RGD-dopamine-glass and collagen-dopamine-glass. Each error bar in the figure represents the standard deviation from experiments with at least 50 cells on three different samples.

Since phase contrast microscopy did not provide any information at the cell-substrate interface, C-RICM was used for probing the adhesion contact dynamics between the cell and model substrate. Figure 4.7 shows a series of C-RICM images of a typical 3T3 fibroblast during seeding on the four surfaces. On unmodified glass, the cell was slowest in adhesion contact evolution and took ~40 min to develop a significant adhesion contact with an area of $96 \mu\text{m}^2$ (projected area of cell was $200 \mu\text{m}^2$). On the dopamine-glass, the fibroblast took ~20 minutes to develop a strong adhesion contact with an area of $108 \mu\text{m}^2$ (projected area $294 \mu\text{m}^2$). On RGD-dopamine-glass, the adhesion contact

evolution was significantly faster than that on unmodified glass and dopamine-glass, and the cell with an initial projected area of $300 \mu\text{m}^2$ (from phase contrast image) took ~ 10 min to develop a strong adhesion patch with an area of $\sim 263 \mu\text{m}^2$. Moreover, the adhesion contact area on RGD-dopamine-glass after 120min seeding was larger than that on dopamine-glass. Interestingly, the rate of adhesion contact evolution of fibroblast during the initial 10 min of seeding on collagen-dopamine-glass was similar to that on RGD-dopamine-glass but the adhesion contact area was smaller than that on RGD-dopamine-glass after 120 min.

Figure 4.7 A series of C-RICM images of typical 3T3 fibroblasts within a cell population from 10 to 120 min after cell seeding on the unmodified glass, dopamine-glass, RGD-dopamine-glass and collagen-dopamine-glass (scale bar represents $20 \mu\text{m}$).

Figure 4.8 shows the average adhesion contact area of fibroblast against seeding time on the four surfaces. The initial rate for increase in adhesion contact area on collagen-dopamine-glass ($41.2\mu\text{m}^2/\text{min}$) and RGD-dopamine-glass ($34.0\mu\text{m}^2/\text{min}$) were significantly larger than that on dopamine-glass surface ($2.6\mu\text{m}^2/\text{min}$) during the initial 10 min of seeding. The unmodified glass surface had the lowest rate of increase in adhesion contact area of $1.4\mu\text{m}^2/\text{min}$ and reached steady state after $\sim 80\text{-}100$ min. The second slowest was the dopamine-glass surface where the increase in adhesion contact area of fibroblast was an approximate linear trend. On RGD-dopamine-glass and collagen-dopamine-glass surface, the adhesion contact area reached a steady state at $\sim 40\text{-}60$ min. After seeding for 120 min, the cells on RGD-dopamine-glass reached the largest average adhesion contact area of $2650\mu\text{m}^2$ while cells on collagen-dopamine-glass had the second largest average adhesion contact area of $1700\mu\text{m}^2$. The average adhesion contact areas were $570\mu\text{m}^2$ and $230\mu\text{m}^2$ on dopamine-glass and unmodified glass, respectively.

Figure 4.8 Average adhesion contact area of fibroblast against seeding time on the four different surfaces (averaged from at least 50 cells on each sample). Each error bar in the figure represents the standard error from experiments with at least 50 cells on at least three different samples.

From the combination of C-RICM and phase contrast microscopy, the key geometric parameter of adherent cell on the planar substrate can be determined. Figure 4.9 (a) shows the degree of deformation (a/R) against seeding time for 3T3 fibroblasts on the four surfaces. Generally, a/R rapidly increased during the initial time of cell seeding and finally reached a steady state. The initial rates of cell deformation (the change of a/R against time) on the RGD-dopamine-glass (0.089 min^{-1}) and collagen-dopamine-glass (0.094 min^{-1}) were three times higher than that on unmodified glass (0.024 min^{-1}) or dopamine-glass (0.028 min^{-1}). Moreover, it took ~ 20 min and 30 min for a/R to reach the steady state level of 0.99 on collagen-dopamine-glass and RGD-dopamine-glass surfaces,

respectively, whereas the corresponding time to reach the steady state a/R level of around 0.9 on both unmodified glass and dopamine-glass were ~ 80 min. The adhesion energy of 3T3 fibroblasts on various model surfaces was calculated from our contact mechanics model (Equation 1-1 and 1-2) after the determination of a/R .

Figure 4.9 (a)

Figure 4.9 (b)

Figure 4.9 (c)

Figure 4.9 (a) Degree of deformation (a/R) against seeding time for 3T3 fibroblasts on the four sample surface. (b) Average adhesion energy of 3T3 fibroblasts against seeding time on the same four surfaces. (c) Adhesion energy per cell for 3T3 fibroblasts against time during incubation on the four surfaces. The error bars represent the standard error from experiments with at least 50 cells on three identical samples.

Figure 4.9 (b) shows the average adhesion energy of 3T3 fibroblasts against seeding time on the four surfaces. Average adhesion energy was positively correlated with the degree of deformation. The result shows that the average adhesion energy first increases and then reaches a steady state. From 5 min to 120 min after cell seeding, the adhesion energy of cells on unmodified glass or dopamine-glass spanned four orders of magnitude and takes longest time, 60 min (compared to 10 min on RGD-dopamine-glass and collagen-dopamine-glass), to reach steady state. Interestingly, the steady state adhesion energy attained by the cells was around 10^{-6} J/m² on all four surfaces. Our average adhesion energy reported herein compared well with the value of 10^{-7}

J/m^2 to 10^{-6} J/m^2 reported by Goennenwein *et al.*²⁶⁶ for the free energy per unit area of vesicles bearing reconstituted integrin receptors from blood platelet on cellulose surface.

Figure 4.9 (c) shows the adhesion energy per cell against time during incubation on the four surfaces. The curves had the same trend as shown that in Figure 4.9 (b). However, the adhesion energy per cell at steady state was strongly dependent on the surface chemistry because it took the change of adhesion contact area against time into consideration. The steady state values of adhesion energy per cell were 2.0×10^{-16} , 5.0×10^{-16} , 1.6×10^{-15} and 2.4×10^{-15} J on unmodified glass, dopamine-glass, collagen-dopamine-glass and RGD-dopamine-glass, respectively. The steady state values of adhesion energy per cell on these two surfaces were 3-4 times higher than that on dopamine-glass surface. In the absence of adhesive ligand, the adhesion energy attained by individual fibroblast on unmodified glass was ten times lower than that on the RGD and collagen modified surfaces.

4.4. Discussion

Dopamine or phenethylamine is a hormone and neurotransmitter and is found in both vertebrates and invertebrates. Dopamine containing primary amine has been applied as the base layer for surface modifications²⁶⁴⁻²⁶⁵. Interestingly, dopamine has been shown to possess moderate antimicrobial activities against antibiotic resistance bacterial strains such as *E. coli* and *S. aureus*²⁶⁷. Thus, dopamine was chosen as a cross-linker for RGD and collagen for our biophysical study herein.

Surface wettability plays an important role in cell adhesion, spreading and proliferation on surfaces and in the bacterial adhesion. Generally, hydrophilic materials are more resistant to bacteria and cell adhesion than hydrophobic materials²⁶⁸⁻²⁷⁰. Moreover, the charge of device surface is another important physical factor for cell and bacterial adhesion. For instance, bacteria and mammalian cells are negatively charged and they would like to adhere onto a positively charged surface. Therefore the high hydrophilicity and negative charge on unmodified glass inhibits both bacteria adhesion, e.g., lowest detachment force and smallest density of adherent bacteria. The adhesion kinetics and adhesion strength of fibroblasts on unmodified glass are also the lowest among all samples herein. After dopamine modification, the surface became more hydrophobic than the unmodified glass and acquires slightly positive charges as dopamine is cationic²⁷¹. Therefore the bacterial adhesion and cell adhesion dynamics were enhanced on dopamine-glass, as shown by the significantly enhanced bacterial detachment force and bacteria density on dopamine-glass, together with the increased fibroblast spreading, adhesion contact evolution, cell deformation rate and adhesion energy. For instance, the adhesion energy attained by individual fibroblast on dopamine-glass was two times higher than that on unmodified glass surfaces. Most importantly, the increase on the lateral detachment forces of the two bacteria strains against the reduction of surface hydrophilicity and reverse of surface charge directly coincides with the trend of biophysical dynamics of mammalian cells.

The further enhancements in biophysical responses of fibroblasts by the immobilization of RGD and collagen on the dopamine-glass were not caused by the change of surface wettability as both surfaces have similar water contact

angle as the dopamine-glass. The result was not likely caused by change of surface charge as both RGD and collagen surfaces should have nearly zero net charge at neutral pH. It is because the isoelectric points of RGD and collagen are around 7²⁷²⁻²⁷³. The specific RGD sequence found in ECM proteins like collagen, fibronectin and gelatine has been shown to mediate receptor-mediated cell adhesion. Moreover, collagen is one of the major ECM proteins containing a lot of cell binding sites such as RGD sequences. Our results basically indicated that an adhesive ligand was necessary to trigger effective cell-substrate interaction on model device surface. RGD and collagen coupling significantly enhanced the rate in adhesion contact formation and the final value of the adhesion contact area. The trend of a/R on collagen-dopamine-glass or RGD-dopamine-glass implied that the adhesion contact area approached the cell projected area in a shorter time interval than that on dopamine-glass. Average adhesion energy was positively correlated with the degree of deformation. RGD and collagen coupling increased the average adhesion strength of fibroblasts comparing with dopamine-glass. The steady state values of adhesion energy per cell on RGD and collagen coupling surfaces were 3-4 times higher than that on dopamine-glass surface. All results further support that the integrin-RGD binding between cell and substrate are major driving force for the adhesion enhancement of mammalian cells on our model surfaces.

The RGD motif is well known to interact specifically with a number of integrin receptors of mammalian cells. Thus it has been widely used to induce specific attachment of fibroblasts to biomaterial/biodevice surfaces. On the other hand, few bacteria have been identified to bind directly to the RGD-motif of proteins²⁷⁴⁻²⁷⁵. For instance, Harris *et al.* demonstrated that that RGD

modification did not affect the anti-adhesive property of PEG coating as non-functionalized PLL-g-PEG and RGD-peptide functionalized PLL-g-PEG reduced the bacterial adhesion to the same extent ¹²³. The past findings as mentioned above are consistent with the intrinsic adhesion phenomena revealed in the present work, i.e., RGD has no specific binding against the membrane proteins on either *E. coli* or *S. aureus* as there are no significant differences between the average detachment force and bacteria density of both bacteria strains on RGD-dopamine-glass and those on dopamine-glass.

Our results herein provided new insights into the biophysical origin of the bacteria-ECM protein interaction. In comparison with dopamine-glass surface, RGD bonding only promoted fibroblast adhesion but had no apparent effect on both *E. coli* and *S. aureus* adhesions (similar detachment force). Collagen bonding enhanced the fibroblast adhesion. Moreover, ECM proteins such as collagen and fibronectin which were often bonded onto biomaterial surface to promote cell adhesion also contain specific bacterial binding sites ²⁷⁶. Our results show that the collagen coating enhanced the *S. aureus* adhesion which is a major human pathogen behind several infectious diseases including bacterial arthritis, osteomyelitis, and acute infectious endocarditis. Interestingly, collagen significantly reduced *E. coli* adhesion. Although there are some reports on *E. coli* binding to collagen I ²⁷⁷⁻²⁷⁸, our unique biophysical study showed that the *E. coli* adhesion was completely inhibited on the collagen-dopamine-glass which was an ideal model system.

The enhancement of *S. aureus* adhesion induced by collagen was further supported by other related studies on *S. aureus*-ECM protein interactions. For instance, *S. aureus* has been shown to utilize the FnBPA on their membrane

surface to bind to fibronectin, which then acted as a bridging molecule between the bacteria and adhesion receptors of mammalian cells²⁷⁹. *S. aureus* has also been shown to bind to collagen through specific binding²⁸⁰⁻²⁸¹. However, single RGD sequence does not act as the molecular bridges like complete ECM proteins but only promotes mammalian cell adhesion via ligand-receptor interaction.

The results herein will be useful in developing antibacterial coating. Ideally, device coating on medical device will promote adhesion of specific mammalian cells while not increasing bacterial adhesion. The interesting aspect of this finding is that one can introduce specific biological cues for mammalian cell adhesion on model device coating through the functionalization with RGD but not with collagen. Thus, it is possible to make a surface just inductive to cells such as fibroblast, osteoblast and endothelial cells, but totally repellent to bacteria, if RGD is bound onto an anti-adhesive polymers surface such as those modified by PEG. Another point worthy to be mentioned is that the lateral detachment forces of *E. coli* from all four model surfaces were lower than that of *S. aureus*. Our results are supported by a related study showing that PEG-derived polymers were generally more effective in repelling *E. coli* than other bacteria²⁸². The exact reason behind the differences in the adhesion strength and affinity between *S. aureus* and *E. coli* is still unknown. A possible explanation is the inherent differences in surface properties of the Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria.

4.5 Conclusions

In summary, we have successfully applied dopamine-glass as a model substrate for examining the effect of RGD and collagen modification on the biophysics of bacteria and fibroblast adhesions. From a comparison of the detachment force and bacteria density on unmodified glass and dopamine-glass, it can be seen that the increased hydrophobicity and positive charge on our model substrate significantly promote bacterial adhesion. Our results further demonstrate that immobilized RGD sequence only promotes fibroblast adhesion but has no apparent effect on *E. coli* and *S. aureus* adhesion, while immobilized collagen promotes fibroblast and *S. aureus* adhesions at the same time. Our findings as shown herein provide new insight into the design of multi-functional coating for device and tissue engineering applications through the introduction of highly integrated biophysical measurements. Specifically, one can make a device surface to induce specific adhesion of mammalian cells while minimizing bacterial adhesion through the choice of the right adhesive ligand.

Chapter 5 Developing Smart Antibacterial Coatings

with Bioselectivity Based on an Anti-adhesive Multilayer and Specific Adhesive Ligand

5.1 Introduction

Among different methods of surface functionalization, of medical implant, recent efforts has been focused on the modification of device surface with selected biological activities such as combine antibacterial properties with good host cell integration ability. As discussed in Chapter 4.1, the fate of the medical implant is dependent on the race between tissue cell integration and microbial adhesion and growth. Thus it is very important to make a device surface, which simultaneously enhances and suppresses host cells adhesion and bacteria adhesion, respectively.

Generally, there are two kinds of antimicrobial surface meeting the requirements. The first type is a selective-killing surface. Both bacteria and cell could adhere onto the surface, but the bacteria will be killed after contact the surface or by the antibiotics release from the coating^{136, 138, 283} while the activity of mammalian cells will not be effected²³¹⁻²³². The second type is selective-adhesion surface, which inhibits pathogenic bacteria adhesion but enhances host tissue cells adhesion and growth^{123, 234-235}. The specific cell adhesion is achieved by the introduction of adhesive ligands such as RGD sequences onto an anti-adhesive surface^{123, 234-235}. For example, PLL-g-PEG coupled with RGD can

retain the bacteria-repulsive property found in conventional and at the same time allows cell adhesion^{123, 235}, which would not happen on PLL-g-PEG.

The choice of adhesive ligand would be critical to introduce selected biological activity onto device surfaces. In Chapter 4, the biophysical responses of both bacteria and mammalian cells towards ligand modified surfaces were investigated²⁸⁴. Along the way, the differences in the role of immobilized RGD and collagen (CL) in tailoring the adhesion strength of both mammalian cells and bacteria on model biomaterial were elucidated²⁸⁴. The result indicated that RGD only enhances mammalian cell adhesion while collagen enhances both mammalian cells and bacteria on fused glass substrate²⁸⁴. It is reasonable to say that RGD is a good candidate for making a smart coating surface with bioselective activity.

Based on the result of Chapter 4, smart antibacterial coatings with bioselectivity were developed based on an anti-adhesive multilayer and specific adhesive ligand RGD. The difference on RGD and collagen (CL) on bacterial and cell adhesion was compared again on this anti-adhesive surface. Among the way, different densities of RGD peptide and CL were chemically coupled to an anti-adhesive PEM surface composed of DS/CS. Then the adhesion of cell and bacteria on the ligand modified PEM surface was probed in order to elucidate the role of RGD in the performance of anti-adhesive coating commonly used by the other researchers in medical devices^{123, 234-235}. At the same time, a smart bioselective surface based on bind RGD onto an anti-adhesive PEM system herein was developed. Moreover, the effects of different ligand density on fibroblast adhesion, as well as on the adhesion of *E. coli* and *S. aureus* were determined. In addition to macroscopic adhesion parameters of bacteria adhesion

such as cell density, the biophysical parameters-the AFM detachment forces of the two bacteria from the PEM surface were measured.

5.2 Material and methods

5.2.1 Materials

Dextran sulfate (D8906), Chitosan (medium molecular weight) and acetic acid were purchased from Sigma Aldrich Pte Ltd (Singapore).

The rest are the same as list in 4.2.1.

5.2.2 3T3 Fibroblast Culture

Method is the same as 4.2.2

5.2.3 Bacteria Culture

Method is the same as 4.2.3.

5.2.4 Surface Preparations

Glass cover slips were cleaned in 'Piranha' solution composed of 30% (v/v) of hydrogen peroxide and 70% (v/v) of concentrated sulfuric acid for 1 h, rinsed thoroughly with distilled (DI) water (with resistance of 18.2 MΩ) and dried in a stream of N₂. After drying in oven at 37 °C for 30 minutes, the glass slides was dipped into 1% PEI solution for 30 minutes in order to introduce high density of positive charges for the subsequent layer-by-layer assembly of DS and CS. Then the glass was rinsed with deionised water and dried in a stream of compressed air. This prepared glass as mentioned above was called PEI-coated glass, used as control in all experiment throughout this manuscript.

PEI-coated glass was stacked onto a Petri dish. Then 0.3 ml of 5mg/ml DS solution was added for 30 minutes. Afterwards, the glass was washed with DI water and dried under a stream of compressed air. Following DS adsorption, the glasses were dipped into 0.3 ml of 5 mg/ml CS for 15 minutes before they were rinsed with DI water, dried by compressed air. These steps formed the first DS/CS bilayer. The subsequent bilayers were formed following the same procedures as mentioned above, but with the glass immersing in each solution for 15 minutes instead. Moreover, only the first layer of dextran and last layer of chitosan require 30 minutes of immersion. In all experiment, 10 bilayers of DS and CS are used unless mentioned otherwise.

5 mg/ml of dextran sulfate solution was made by dissolving dextran sulfate into 0.1 M NaCl solution. 5 mg/ml of chitosan solution was made by adding chitosan into solution containing 0.1 M NaCl and 0.3% CH₃COOH solution. The chitosan solution was filtered and stored at a temperature of 4 °C.

RGD solution (10 µg/ml, 100 µg/ml, 1mg/ml in DI water) or collagen solution (1 µg/ml, 10 µg/ml, 100 µg/ml in DI water) with added EDC (20 mM) and NHS (50 mM) was loaded onto the DS/CS multilayer inside a Petri dish and reacted for 72 h at 4°C. Subsequently, RGD or collagen coated PEM were washed with DI water and dried in stream of compressed air and then put in vacuum oven at 37°C for 30 min. The glass slides modified by PEM were kept in 4 °C refrigerator before further experiments.

5.2.5 Bacteria and cell image and counting

1 ml suspension of fibroblast cells with a density of 8×10^4 cells/ml was added onto each PEM sample. After culturing for 1 hour or 24 hours, the

samples were washed with PBS before imaging with a fluorescence microscope (Olympus). The cell count on each surface was determined from at least 15 images from 3 samples. 0.5 ml *S.aureus* with a density of 10^7 cells/ml or *E.coli* with a density of 10^8 cells/ml was added onto each sample. After incubation with bacteria suspension for 1h, the sample was washed three times with 1X PBS before imaging with a confocal microscope (Carl Zeiss, Germany). The bacteria count for each surface was averaged from at least 15 images from 3 samples.

5.2.6 Detachment Force Measurement of Bacteria

The method used herein was slightly modified from that in our previous work^{138, 260}. In this study, the AFM scan area is increased so that multiple bacteria instead of one single cell will be probed during one measurement. For *S. aureus*, after the incubation of bacteria suspension for 15 minutes, the samples were washed three times with PBS before AFM measurement. For *E. coli*, after 1hour of incubation, it is further incubated for 12 h in a humid environment. Then the sample was immersed in DI water for 0.5 h before AFM measurement.

The force measurement was carried out with an AFM (Asylum Research, model: MFP-3D) under contact mode in DI water. The spring constant of each AFM cantilever used in this study was recalibrated with the build-in thermal noise method provided by the MFP 3D software and a scanning rate of 1 Hz was used in all experiments. The force of AFM tip exerted on the sample was then calculated as the products of spring constant of AFM tip (obtained from the AFM software), the cantilever sensitivity (nm/volt) and the tip deflection distance. At the start of each experiment, different areas of the entire sample

(scan size of $40 \times 40 \mu\text{m}^2$) were scanned at the minimum force (1 nN). Once bacteria were found, the scan size was reduced to $10 \times 10 \mu\text{m}^2$ (scan speed is $25 \mu\text{m/s}$). In our previous work, we scan an individual cell one at a time under the scan size of $4 \times 4 \mu\text{m}^2$. By increasing the scan size, we can probe more bacteria at one measurement. The AFM measurement can quantify the subtle difference on bacteria-substrate interaction. Since the AFM tip would have made contact with single bacteria at anytime during the scanning of the entire region, the use of slightly larger area during AFM imaging should not affect the accuracy in the measurement of detachment force of bacteria. The size of AFM scan area was kept the same for all experiments. The detachment force was taken to be the value of applied tip force when cell detachment occurred. For each type of substrate, 3 samples were tested and on each sample, at least 30 cells were measured.

5.3 Results

Figure 5.1 shows the general scheme for the preparation of DS/CS multilayer functionalized with adhesive ligands. After PEI deposition, the surface was modified with excessive positive charges for performing layer-by-layer assembly of DS and CS. By repeating several cycles of DS/CS deposition, a PEM containing ten bilayers of DS and CS with CS as outmost layer was produced. RGD and collagen were subsequently coupled onto the PEM through the reaction between the primary amine groups of CS and carboxyl groups of RGD or collagen. The PEI-coated surface acted as the

control for all study if not otherwise mentioned. RGD or collagen solution at different concentrations was used for the functionalization of the PEM film.

Figure 5.1 Schematic sketches on the procedures for surface preparation

5.3.1 Fibroblast Adhesion

The adhesion of fibroblasts onto different PEM was investigated after 1 and 24 hours of cell seeding. Figure 5.2 shows the relative cell numbers adhering on the different surfaces after 1 hour of seeding. The number of adhered cells on DS/CS multilayers (10 bilayers) was only $0.5\% \pm 0.2\%$ of that on PEI surface which served as the control of this study. After the functionalization of DS/CS multilayers with the lowest concentration of RGD or collagen (CL), the relative cell numbers on DS/CS-1mg/ml RGD and DS/CS-10mg/ml CL were $0.9\% \pm 0.6\%$ and $0.8\% \pm 0.6\%$, respectively.

Generally, there was no significant change in the number of adherent fibroblasts compared to that on DS/CS multilayer.

Figure 5.2 Relative fibroblast cell numbers on different surface after 1 hour culture. PEI-coated surface acted as the control for all study if not mentioned. DS/CS represents the surface with 10 bilayers of DS/CS fabricated on PEI-coated surfaces with CS as outmost layer. DS/CS-1mg/ml RGD means the above DS/CS surface treated with 1mg/ml RGD solution.

At higher RGD and collagen concentration, the relative cell number were increased to $48.4\% \pm 12.9\%$ and $61.6\% \pm 16.9\%$, on DS/CS-100mg/ml RGD and DS/CS-10mg/ml CL, respectively. Further increase in the concentration of RGD significantly enhanced fibroblast adhesion, as shown by $90.2\% \pm 5.7\%$ on RGD and $100.7\% \pm 11.3\%$ relative cell density on DS/CS-1mg/ml RGD and DS/CS-100 mg/ml CL, respectively. Obviously, the number of adherent cells was positively correlated with the concentration of immobilized ligand.

Figure 5.3 shows the phase contrast images of fibroblasts after 24 hours culture on various surfaces. The change of cell morphology after 24 hours was dependent on ligand concentrations. On PEI surface, all cells spread out and demonstrate the elongated morphology of typical fibroblasts. After coating DS/CS multilayer on PEI surface, the adhesion of fibroblast was abolished. At the lowest ligand concentration, the inhibition of cell adhesion remained obvious as shown by the absence of adherent cells on DS/CS-10mg/ml RGD and DS/CS-1mg/ml CL (Figure 5.3). Upon the increase of ligand concentration, fibroblasts tended to adhere and form aggregate on both DS/CS-100mg/ml RGD and DS/CS-10mg/ml CL. On DS/CS-100 μ g/ml RGD, the cell aggregate was larger than that on DS/CS-10 μ g/ml CL coated surface. At the highest ligand concentration, cell growth was significantly improved as shown by the formation of cell sheet on DS/CS-1mg/ml RGD and DS/CS-100mg/ml CL. In contrast, PEI surface failed to form cell sheet in the absence of adhesive ligands.

Figure 5.3 Phase contrast image of the fibroblast cell after 24 hours. The scale bar (in control) is 50 µm.

5.3.2 Bacteria Adhesion

The representative Gram-positive bacteria, *S. aureus* and Gram-negative bacteria *E. coli* were used herein in the bacteria adhesion assay. Figure 5.4 shows the relative number of *S. aureus* on various surfaces which was determined by normalizing the cell density on one sample against that on the PEI control after 1 hour of bacteria seeding. The relative cell number of *S. aureus* was only $1.9\% \pm 1\%$ on DS/CS multilayer compared with that on the control surface. The relative numbers of *S. aureus* were $2.0\% \pm 0.4\%$, $1.8\% \pm 0.7\%$ and $2.3\% \pm 0.6\%$, on DS/CS-10mg/ml RGD, DS/CS-100mg/ml RGD and DS/CS-1mg/ml RGD surfaces, respectively. Thus RGD bonding did not affect the anti-adhesive property of DS/CS multilayer towards *S. aureus* at all RGD concentrations used herein. In contrast, the relative number of *S. aureus* increased against the increase of collagen concentration on DS/CS multilayer. For instance, the relative numbers of *S. aureus* on DS/CS-1 μ g/ml CL, DS/CS-10 μ g/ml CL and DS/CS-100 μ g/ml CL surface were $25.4\% \pm 5.4\%$, $69.3\% \pm 10.1\%$ and $99.3\% \pm 13.9\%$, respectively. Although DS/CS-100 μ g/ml CL and DS/CS-1mg/ml RGD demonstrated similar adhesive affinity towards fibroblasts, the adhesion of *S. aureus* triggered by collagen was significantly higher than that by RGD.

Figure 5.4 The relative *S. aureus* number after 1 hour of bacterial seeding on different sample.

Figure 5.5 shows the relative number of *E. coli* on various surfaces which was determined by normalizing the cell density on one sample against that on the PEI control after 1 hour of bacteria seeding. Similar to the result of *S. aureus* adhesion, DS/CS multilayer significantly reduced *E. coli* adhesion. For instance, the number of *E. coli* adhered on DS/CS multilayer was reduced by 97.5% (Relative number: $2.5\% \pm 0.8\%$) compared with that on control surface. It is clear that both RGD and collagen did not significantly alter the affinity of DS/CS multilayer towards *E. coli*. The relative number of *E. coli* was between 1% and 2%, on DS/CS-10mg/ml RGD, DS/CS-100mg/ml RGD and DS/CS-1mg/ml RGD surfaces.

Figure 5.5 The relative *E. coli* number after 1 hour of bacterial seeding on different surface.

Although light microscopy probes the extrinsic parameters of bacterial adhesion like cell number and morphology, it doesn't provide any quantitative information on the adhesion strength of bacteria on different surfaces. Recently, we have used AFM to quantify the adhesion strength of single bacteria on biomaterial surface by measuring the lateral detachment forces²⁸⁴. In this study, the detachment force of the two types of bacteria on DS/CS-100 µg/ml CL and DS/CS-1mg/ml RGD surfaces was probed. The two surfaces as mentioned above were chosen herein because they have similar affinity for fibroblast adhesion and demonstrate distinct properties for bacteria adhesion.

Figure 5.6 shows a series of AFM images of a typical *E. coli* against the increase of lateral force applied from AFM cantilever during the cell detachment assay from PEI surface that serves a control. All *E. coli* strongly adhered on PEI surface under a lateral force of 5 nN. After increasing the lateral force to 25 nN,

some scratch marks appeared on the bacteria, which remained to be firmly adherent on the surface. When the lateral force reaches 45 nN, two *E. coli* cells were on the verge of detachment from the PEI surface (marked by the two black circles). At 65 nN, all cells were removed except one cell remained on the control surface.

Figure 5.6 A series of AFM images of a typical *E. coli* against the increase of lateral force applied from AFM cantilever during the cell detachment assay from PEI surface that serves a control.

In this study, the averaged lateral detachment forces of *E. coli* and *S. aureus* were highly dependent on the substrate properties (Table 5.1). The average detachment force of *S. aureus* (> 100 nN) on DS/CS-100 μ g/ml CL surface was highest among the four surfaces tested herein. The average detachment force of *S. aureus* upon control PEI surface was 29 ± 12.4 nN (the standard deviation was

based on measurements of at least 30 cells of 3 samples). In contrast, the average detachment forces of *S. aureus* on DS/CS-1mg/ml RGD and DS/CS multilayer were both lower than 5 nN. The detachment force of *E. coli* on the four types of surfaces was also quantified with the same AFM method. The detachment force of *E. coli* also varied against the change of surface chemistry of model substrates (Table 1). Interestingly, the weakest *E. coli* adhesion was found on DS/CS-100 µg/ml CL with the lowest average detachment force of smaller than 5nN among the four surfaces. This trend as mentioned above contrasts with *S. aureus* on the same surface which had the highest detachment force in all four surfaces. *E. coli* was detached by a largest force of 51.4±10.8 nN from control PEI surface. However, the detachment force of *E. coli* on DS/CS-1mg/ml RGD (12.4±4 nN) was not statistically different from that on DS/CS multilayer surface 13.9±7.4 nN ($t < 0.05$).

Table 5.1 AFM bacteria detachment force on different samples

	AFM bacterial detaching force (nN)	
	<i>S.aureus</i>	<i>E.coli</i>
Control	29±12.4	51.4±10.8
DS/CS	< 5	13.9±7.4
DS/CS-1 mg/ml RGD	< 5	12.4±4
DS/CS-100 µg/ml collagen	> 100	< 5

5.4 Discussion

A bio-selective surface, which promotes mammalian cell adhesion but inhibits bacteria adhesion, was usually attained by immobilizing adhesive ligand containing RGD sequences onto an anti-adhesive surface such as a PEG or multilayer^{123, 234-235}. Recently, PEG and hyaluronic acid/chitosan polyelectrolyte multilayers has been chosen as the anti-adhesive coating for subsequent RGD binding^{123, 234-235}. Polysaccharide multilayers containing hyaluronan, chitosan or heprin have been applied to reduce *E. coli* adhesion^{204, 214} as they can usually form highly hydrated gel-like layer. Lichter et al. has demonstrated that polyacrylic acid (PAA)/poly-(allylamine hydrochloride)(PAH) multilayer significantly inhibits bacterial attachment due to the formation of fully hydrated surfaces with low Young's elastic modulus²¹⁵. In comparison with PEG, PEM composed of PAH and PAA provides more reaction sites such as amino groups and carboxyl groups along the entire polymer backbone, while PEG only has two reaction sites at the terminals of polymer chain. As a result, PEM is likely a better choice for collagen and RGD coupling.

In our study, DS/CS multilayer was chosen because of the intrinsic anticoagulant activity of negatively charged DS²⁸⁵⁻²⁸⁶. Our result provided new evidence that DS/CS multilayer had excellent anti-adhesive properties against both mammalian cell and bacteria. Less than 2% of fibroblast cells or bacteria were found on the DS/CS multilayer with 10 bilayers in comparison with control surface. The anti-adhesive property of DS/CS multilayer may be originated from two physical properties including, 1) highly hydrated gel-like

surface with low Young's elastic modulus; 2) the excellent anti-adhesive properties of the building blocks in DS. With CS as the outmost layers, its amino groups could react with carboxyl group on RGD and collagen, thus binding them onto the surface. One of the main objectives of this work is to compare the effect of RGD and collagen on mammalian cell and bacteria adhesion. Thus different densities of RGD and collagen were coupled to the DS/CS bilayers surface for this purpose.

RGD and collagen coupling could significantly enhance fibroblast adhesion and the number of adherent cell was highly dependent on the ligand density. Also *S.aureus* number increased on all collagen coated surface. These results indicated that adhesive ligands are effective promoting mammalian cell or bacterial adhesion.

Surface wettability is a key factor in bacterial and mammalian cell adhesion. Generally, bacteria and cell prefer hydrophobic surface rather than hydrophilic surface²⁶⁸⁻²⁷⁰. Surface charge is another crucial factor for bacterial and cell adhesion. Also, bacteria and cells are slightly negatively charged and thus they could adhere onto a positively charged surface easier than a negatively charged surface. First, the enhancements of fibroblasts or *S.aureus* adhesion on RGD and collagen surface is not caused by the change of surface wettability as both RGD ($46.8^{\circ} \pm 4.3^{\circ}$ on DS/CS-1 mg/ml RGD) and collagen ($37.6^{\circ} \pm 6.7^{\circ}$ on DS/CS-100 μ g/ml CL) coated surfaces have lower water contact angle than DS/CS surface ($64.8^{\circ} \pm 3.9^{\circ}$). Second, it is also not caused by the change of surface charge as both surfaces should have zero net charge at neutral pH, because isoelectric point of RGD, collagen and pKa of chitosan are

all around 7^{272-273, 287}. Therefore specific binding between the adhesive ligand and cell and bacterial must be taken into consideration.

RGD sequence which is widely found in ECM proteins such as fibronectin, collagen and gelatin plays important role in receptor-mediated cell adhesion²⁸⁸. Collagen is one of major ECM proteins, which has numerous cell binding sites including RGD sequences. Our results herein further supports the adhesion enhancement of fibroblasts is caused by the specific binding such as the RGD-integrin interaction between PEM substrate and cell.

On the other hand, RGD may not bind specifically to bacterial surface²⁷⁴⁻²⁷⁵. Our result agrees with other reports, which show that RGD has no specific affinity towards either *E. coli* or *S. aureus*. The density of adherent bacteria strains on various DS/CS surfaces with RGD is not changed. While looking at the detachment force of *S. aureus* and *E.coli* on DS/CS-1 mg/ml RGD surface, there was not significantly different from that DS/CS surface. The negligible specific binding between RGD and *S. aureus* and *E.coli* as determined herein is consistent with others' finding that no bacteria have been identified to bind directly to the RGD-motif of adhesion proteins²⁷⁴⁻²⁷⁵. Although there are reports suggesting that bacteria may invade cells via RGD containing protein such as fibronectin, it has been proven that bacteria does not directly recognize RGD^{279, 289-290}. Shinji et al. showed that the adhesion of *S. aureus* onto macrophages can be blocked when fibronectin-coated *S. aureus* are exposed to soluble RGD, thus indicating that the RGD sequence of fibronectin is in charge of specific mammalian cell binding but not bacteria binding²⁹¹

Collagen coating on DS/CS enhances the adhesion of *S. aureus*, a major human pathogen which causes several infectious diseases such as osteomyelitis, bacterial arthritis and acute infectious endocarditis²⁹²⁻²⁹⁴. The important role of collagen in triggering bacterial adhesion is supported by trend of the increase of relative number of *S. aureus* against the increase collagen concentration. Another surprising finding is that, on DS/CS-1 µg/ml CL surface, there was almost no fibroblast cell adhesion detected, while *S. aureus* adhesion has been strongly enhanced compared to CS/DS surface. This result indicated that *S. aureus* adheres onto PEM surface even at low collagen density when fibroblasts could not adhere. In other words, *S. aureus* is more responsive to very low density of collagen on device coating and would eventually triggers bacterial infection in the medical device.

AFM detachment force can provide quantitative information on the strength of bacteria-surface interaction. The AFM detachment force of *S. aureus* on DS/CS-100 µg/ml CL (> 100 nN) was significantly larger than that on control surface (29±12.4 nN) under the same bacterial density. The result furthermore supports that there was specific binding between collagen and *S. aureus* which is absent from the interaction of *S. aureus* on control surface. As discussed before, the control surface was slightly more hydrophobic (44.8°±5.1° on control surface, while 37.6±6.7° on DS/CS-100 µg/ml CL surface) and was more positively charged, if there was no specific binding, control surface should have larger detachment force. This result is similar to our previous results, which found that *S. aureus* detachment force on collagen coated surface was significantly larger than that on control surface²⁸⁴. The enhancement of *S. aureus* adhesion induced by collagen functionalized on PEM was further

supported by other related studies on *S. aureus*-ECM protein interactions²⁷⁹. For instance, fibronectin has been shown to act as a bridging molecule between the bacteria and mammalian cells. *S. aureus* can utilize the FnBPA on their membrane surface to bind with fibronectin, which contains other binding sites for mammalian cells²⁷⁹. *S. aureus* has also been shown to bind to collagen through specific binding²⁸⁰⁻²⁸¹.

Interestingly, different from the result of *S. aureus*, collagen seems to reduce *E. coli* adhesion strength as the detachment force (less than 5 nN) was even lower than that on DS/CS surface (13.9 ± 7.4 nN). However, the density of adherent *E. coli* on DS/CS-100 $\mu\text{g/ml}$ CL was not significantly lower than that on DS/CS surface. It is possible DS/CS surface already had excellent anti-adhesive property to *E. coli* ($2.5\% \pm 0.8\%$) and the difference in adhesion strength was not significant to lead to further reduction. Although there are some reports of *E. coli* binding to collagen I²⁷⁷⁻²⁷⁸, our study showed that the *E. coli* adhesion was not enhanced on collagen modified PEM.

Another thing worth to mention is that the lateral detachment forces of *E. coli* from all surfaces were lower than that of *S. aureus* at the same experimental conditions. If we culture *E. coli* for only 15 min, which was the same condition as *S. aureus*, detachment force on all surfaces would be lower than 1 nN. Thus, we slightly changed our experiment condition in order to show the difference on *E. coli* adhesion to different sample surface. Our finding are supported by a related study showing that PEG-derived polymers were generally more effective in repelling *E. coli* than *S. epidermidis*²⁸². Also, the result is supported by our previous study, which showed that *E. coli* has lower detachment force comparing with *S. aureus* at the same condition. The exact reason behind the different

adhesion strength and affinity between *S. aureus* and *E. coli* on same surface is still not clear. A possible explanation is the different surface structure and properties of Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria.

Our results herein not only provided new insights into the interaction of both cell-ECM protein and bacteria-ECM protein interactions but also were helpful in developing antibacterial coatings for application as bio-selective surface. Through the functionalization of device surface with RGD not collagen onto an anti-adhesive surface, specific biological cues only for mammalian cell adhesion can be introduced while no affect its anti-adhesive property to bacteria. Thus, it is a logical choice to couple RGD onto PEG by previous researchers to make a surface just inductive to cells such as fibroblast, osteoblast and endothelial cells, but totally repellent to bacteria^{123, 235}.

In our experiment, DS/CS-1mg/ml RGD surface induced bio-selectivity to mammalian cells and bacteria, which promoted only fibroblast adhesion but inhibit both gram-positive *S. aureus* and gram-negative *E. coli* adhesion. Such a bacteria-repellent but cell-adhesive structure may be helpful in keeping the interface away from bacterial infection during the early, clinically critical stage of the implant implementations. In particular, the method helps the host cells to win the so called race-for-surface battle up to the point in time when host cells form a confluent layer on the implant surface which protects the surface from microbial adhesion and colonization. Comparing with selective-killing surface on which bacteria and cell could adhere but bacteria were killed while mammalian cells were not effected^{136, 138, 231-232, 283}, our bioselective system have some advantages. For example, for silver incorporated selective-killing coatings, the total amount and the release rate of silver have to been well controlled

because too high density of silver ions would kill mammalian cells²³⁹⁻²⁴⁰. Also some others have questioned about the concentration range where silver could be used to kill bacterial without harmful effects on mammalian cells²³³. While our bioselective coatings do not have such problem as the materials used are nontoxic. Also, the preparation of the bioselective coating is simple LBL process use water as solvent, while selective-killing coatings usually come with complicate chemical process^{136, 138, 231-232, 283}.

While trying to coat collagen onto an anti-adhesive surface in order to get a bio-selective surface, is not a good choice, not only because it enhanced the adhesion of infectious bacteria such as *S. aureus* but also it was much easier for *S. aureus* to adhere. The interesting finding was that, on DS/CS-1 $\mu\text{g/ml}$ CL surface, on which almost no fibroblast adhesion happened, while adhesion of *S. aureus* has been already strongly enhanced. The relative *S. aureus* number on DS/CS-1 $\mu\text{g/ml}$ CL ($25.8\% \pm 5.4\%$) was 10 times more than that on DS/CS ($1.9\% \pm 1\%$), while on both surface there was no fibroblast cell adhesion. It is clear that at this low collagen concentration, on DS/CS-1 $\mu\text{g/ml}$ CL in our case, *S. aureus* adhesion was enhanced but fibroblast adhesion was not improved. The result is opposite to the desirable effect. In this situation, *S. aureus* had more chance to win race-for-surface battle simply because it had more chance to adhere on this surface; as a result there was more chance for biomedical implant infection to happen. But the collagen coated system is still helpful if the bacteria strain involved in the particular application has no specific binding with collagen, such as only *E. coli*. In our study, on DS/CS-100 $\mu\text{g/ml}$ CL surface, *E. coli* adhesion was completely inhibited while mammalian cell adhesion was promoted. Such an *E. coli*-repulsive but cell-adhesive coating may

be useful in protecting implant from *E.coli* related infection but also have good mammalian cell adhesion property.

5.5 Conclusions

In summary, we have elucidated the effect RGD and collagen on bacteria and fibroblast adhesion onto the otherwise anti-adhesive DS/CS multilayer. By comparing the relative fibroblast cell density and morphology on each surface, it can be seen that both RGD and collagen promote fibroblast adhesion. Interestingly, the immobilized RGD sequence only promotes fibroblast adhesion but has no apparent effect on *E. coli* and *S. aureus* adhesion, the RGD coated DS/CS film maintains the anti-adhesive property to both bacterial strains, while collagen coupling simultaneously promotes fibroblast and *S. aureus* adhesions. The result also indicates that *S. aureus* are more sensitive to lower collagen concentration than fibroblast. AFM detachment force measurement shows that collagen imposes specific-binding with *S. aureus* as the detachment force on collagen is significantly larger than that on control under similar *S. aureus* density. Finally, we can make a device surface with tailored bio-selectivity, that will induce specific adhesion of mammalian cells while minimize bacterial adhesion, through the choice of the right adhesive ligand RGD onto an anti-adhesive substrates. In our case, 1mg/ml RGD coated DS/CS multilayer meet this requirement and get this selective activity. Such a bacteria-repulsive but cell-adhesive coating may be useful in protecting the medical device interface from bacterial infection.

Chapter 6 Conclusions and Future work

6.1 Conclusions

In this work, two different kinds of antibacterial surface coating were developed. They are made from different materials including synthetic polymer, inorganic materials and natural polysaccharide, and thus display different antibacterial properties.

6.1.1 Antibacterial coating based on synthetic Polyethyleneimine and silver

The first kind of coating is based on synthetic PEI and inorganic silver. Silver is a biocide towards a broad spectrum of bacterial strains, fungi and viruses^{161, 238}. It has low toxicity²³⁹ and is unlikely to induce the development of antibiotics resistance in most bacteria. PEI is demonstrated to have antibacterial property^{55, 245}. Moreover, PEI contains amine groups with the ability to bind with a wide range of transition metal cations^{165, 168, 170}.

In Chapter 3, an antibacterial coating made up of PMF incorporating silver nanoparticles was developed. The multilayer film was build up by layer-by-layer deposition of PEI and a small cross-linker TA. We have shown that TA acted as an effective anchor for successive PEI immobilization during all stages of LBL. Ag nanoparticles were incorporated into the PMF through *in situ*

reduction of Ag^+ by the pre-adsorbed NaBH_4 within the film matrix. The mass of embedded Ag nanoparticles was well controlled by altering the film thickness or loading cycles.

Bacterial culture results showed that our PMF also possessed considerable antimicrobial property against bacteria in contact. For instance, (PEI)₁₀ killed around 89% *E. coli* and 52% *S. aureus* through direct contact. Ag nanoparticles loading enhanced the contact-killing property: the contact killing efficiency increased to over 99% against both bacterial strains upon the loading of Ag nanoparticles in (PEI)₁₀. Most importantly, good release-killing property against the two bacteria strains was introduced into covalent (PEI)₁₀ multilayers by the embedded Ag nanoparticles.

6.1.2 Multifunctional antibacterial coating based on natural polysaccharides and adhesive ligands

In Chapter 4 and 5, an anti-bacterial coating with bio-selectivity, which promotes host cell adhesion but inhibits bacterial adhesion, was developed mainly based on natural polysaccharide. This kind of bio-selective surface can be achieved by the introduction of adhesive ligands such as RGD sequences onto an anti-adhesive surface^{123, 234-235}. The choice of adhesive ligand would be critical to introduce selected biological activity onto device surfaces. Therefore, it is critical to get more quantitative correlation between the adhesion strength of bacteria or mammalian cells and adhesive ligand, in order to choose the optimal ligand type. In Chapter 4, the responses of bacteria include *E.coli* and *S. aureus* and mammalian cells (fibroblast) towards two common adhesive ligands (RGD and collagen) on model device surfaces are elucidated by biophysical methods.

The initial rate of deformation and adhesion energy determined from confocal-reflectance interference contrast microscopy (C-RICM) shows that both collagen and RGD significantly enhances fibroblast cell adhesion. By the determination of AFM detachment forces and the bacterial density, it is found that RGD has no apparent effect on *E. coli* and *S. aureus* adhesion, while collagen enhances *S. aureus* adhesion strength. These result indicated that, RGD may bring bio-selectivity on to a coating surface as it only has effect on mammalian cell adhesion.

To prove that, based on the results of RGD and collagen on a model surface without anti-adhesive property as mentioned above, in Chapter 5, different density of (10, 100, 1000 $\mu\text{g/ml}$) RGD and collagen (1, 10, 100 $\mu\text{g/ml}$) were chemically coupled to an anti-adhesive PEM surface composed of DS and CS by using water-soluble carbodiimide chemistry. The comparison of relative fibroblast cell density and morphology on each surface shows that either RGD or collagen effectively promotes fibroblast adhesion and proliferations in a concentration dependent manner.

However, different adhesive ligands impose distinct effects in bacterial adhesion. First, collagen significantly enhances *S. aureus* adhesion on DS/CS multilayers even at very low surface concentration (1 $\mu\text{g/ml}$) where no significant fibroblast adhesion occurs. The detachment forces of *S.aureus* on collagen coated (100 $\mu\text{g/ml}$) DS/CS measured by AFM is significantly larger than that on unmodified DS/CS multilayers, and more importantly, also higher than that on control with similar fibroblast cell density. Thus it is confirmed that there is specific interaction between *S. aureus* and collagen. This result confirmed that collagen as an adhesive ligand can promote both fibroblast cell

and *S. aureus* adhesion, in other words no selectivity towards bacteria adhesion. Second, RGD does not disturb multilayer's anti-adhesive property toward *S. aureus* and *E. coli*. In contrast, RGD brings bio-selectivity. After RGD binding, DS/CS multilayers are only adhesive to fibroblasts but retain its anti-adhesive property to both *E.coli* and *S. aureus*. The lowest detachment force of both bacterial strain was determined on both RGD (1000 µg/ml) modified DS/CS and unmodified DS/CS multilayer.

By binding RGD (1mg/ml) onto an anti-adhesive DS/CS multilayer, we finally develop multifunctional anti-bacterial surface with bio-selectivity, which enhances host cell integration but reduces the bacterial adhesion, thus reduces the chance for biofilm related infection. Our work will pave the way for designing functional coating with tailored bio-selectivity that will induce specific adhesion of mammalian cells while minimizing bacterial adhesion.

6.2 Future work

Although silver ion is generally non-toxic to human beings, it still has a dose-related toxicity which demands special attention^{81, 231-232}. The polyelectrolyte multilayers loaded silver nanoparticle developed herein has good antibacterial property. Due to the potential dose-related toxicity, the total amount of silver nanoparticles should be precisely controlled. It is proved that the amount of silver inside the coating should be less than 0.4 µg/cm²²³⁹. At this silver loading concentration, polyelectrolyte multilayers show no toxicity to mammalian cells and still exhibited antimicrobial activity²³⁹. In our work, we have shown that altering the film thickness or loading cycles can achieve the

control of silver concentration. But a lot of work still needs to be done. Controlling the release rate of silver ion is another work that needs to be done. Similar to a recent study²⁴⁰, we may apply another PEM layer on top of our silver containing system. The additional layers work as diffusion barrier for silver ion and also stop the direct contact of mammalian cell onto the silver coating. Thus we make this silver containing single polyelectrolyte multilayer to kill the bacterial but also support mammalian cell growth. This property is critical for the implant used in human body.

Although some researchers show that they can make silver containing antimicrobial coating without cytotoxicity, some others have questioned about the concentration range where silver could be used to kill bacterial without harmful effects on mammalian cells²³³. The antibacterial effect of silver will be directly influenced in the presence of serum, because complexes can be formed between silver ions and proteins in serum. Based on their results, they indicate that the concentration range of silver ion, where it is anti-bacteria while not harmful to mammalian cells, is very small or even does not exist in some cases. Thus, we may consider not to use silver in the PEM we developed to avoid this problem. Actually, our PEM composed of only PEI is able to incorporate other antibiotics, especially those with negative charges since the PEM itself is strongly positive charged. Also, by changing PEM layer number, the total charge of PEM as well as the amount of antibiotics can be controlled.

All three types of anti-microbial coatings as mentioned in our literature review have certain advantages and disadvantages. Efforts towards the development of new bacteria-resistant coating will take the major advantages of existing approaches while they should overcome the involved shortcomings.

Therefore it is desirable to combine the several desirable properties from several anti-microbial coating in a single system, such as contact-killing and release killing together or release-killing and anti-adhesive together. It will be beneficial if the two different coatings that we developed are combined together. If bio-selective coatings were put onto the silver containing PEM, then the combined surface will have two different anti-bacterial approaches: releasing-killing by silver and anti-adhesive by the multilayer systems. At the same time, the bio-selective systems work as diffusion barrier to control the release rate of the antibiotics and to enhance the host mammalian cell integration.

PEM made by LBL method is a easy but powerful way to prepare an anti-bacterial coatings on the materials surface²⁰⁷⁻²⁰⁸. It is already shown in this study that an anti-adhesive coating can be fabricated by this method. By the incorporation of different antimicrobial agents into the PEM systems, it can make tailored antimicrobial coating for different purposes. Antimicrobial peptides are important components of host defence system of animals and have attracted a lot of attentions. The main limitation in the use of antimicrobial peptides as therapeutics is the requirement of high dosage for achieving reasonable antimicrobial activity. The likely solution to the problem as mentioned above is to get local delivery of the peptides at the interface between tissues and material surface. So the covalent bond or adsorption of these antimicrobial peptides onto the PEM coating is a promising ways for surface fictionalization.

It is found that different bacterial strains showed different responses to the same anti-microbial surface. Thus for real application, the anti-bacterial test should use the same bacterial stain directly involved in the infection, although the result get by testing with representative strains, such as *E. coli* and *S. aureus*, is

helpful. Furthermore, the present antibacterial assay involves several bacterial strain used in the laboratory, which may lack many surface structures and properties that would make them survive in a hostile wild environment. Moreover, biofilms in natural environments always contains several populations of different microorganisms. Because of that, the wide type bacteria also change their function. Therefore, for real application, it is necessary for an antibacterial material to be test in terms of the condition of wild-type biofilm formation.

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Appendix: Publication

- [1] **Tao He**, Zhilong Shi, Ning Fang, K.G. Neoh, E.T. Kang and Vincent Chan, “The effect of adhesive ligands on bacterial and fibroblast adhesions to surfaces”, *Biomaterials* 30(2009),317-326.
- [2] **Tao He** and Vincent Chan, “Covalent Layer-By-Layer Assembly of Polyethyleneimine Multilayer for Antibacterial Applications”, *Journal of Biomedical Materials Research. Part A*, 95A(2010),454-464,
- [3] **Tao He** and Vincent Chan, “The Effect of Adhesive Ligands on Bacterial and Fibroblast Adhesions to an Anti-adhesive Multilayer Surfaces”, submitted.
- [4] **Tao He** and Vincent Chan, “Comparing anti-adhesive property of covalent assembly Dextran/chitosan multilayer toward bacteria and fibroblast cells”, in writing.