# Plasma deposited polymeric films for antimicrobial applications

**by**


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# List of Abbreviations

| | |
|---|---|
| AA | Allylamine |
| AFM | Atomic Force Microscopy |
| AMP | Ampicillin |
| ATCC | American Type Culture Collection |
| BE | Binding Energy |
| C | Carbon |
| CFU | Colony Forming Units |
| CW | Continuous Wave |
| DC | Direct Current |
| DMEM | Dulbecco's Modified Eagles Medium |
| FBS | Foetal Bovine Serum |
| GTAC | Glycidyltrimethyl Ammonium Chloride |
| HDF | Human Dermal Fibroblasts |
| LPS | Lipopolysaccharide |
| MBC | Minimum Bactericidal Concentration |
| Mg | Magnesium |
| MIC | Minimum Inhibitory Concentration |
| N | Nitrogen |
| O | Oxygen |
| OD | 1,7-Octadiene |
| Ox | Polyoxazoline |
| PBS | Phosphate Buffered Saline |
| PI | Propidium iodine |
| PMX | Polymyxin |
| QAC | Quaternary Ammonium Compound |
| RF | Radio Frequency |
| sccm | standard cubic centimetres per minute |
| Si | Silicon |
| TSB | Tryptone Soya Broth |
| UV | Ultra Violet |
| XPS | X-ray Photoelectron Spectroscopy |

# Summary

Undesired microbial adhesion to surface has attracted substantial attention since it is longstanding and unresolved problem in wide range of technological applications in the medical, environmental and industrial fields. For example; indwelling biomedical devices are highly vulnerable to biological fouling, especially from potentially toxic micro-organisms, such as bacteria. Generally speaking, antimicrobial surfaces can be employed to decrease the risk of microbiological fouling surfaces. This thesis describes versatile methods of developing surface coatings directly aimed at decreasing microbiological contamination on surfaces for the use in the biomedical field.

Firstly, a novel two-step approach to the immobilisation of a controlled density of positively charged quaternary ammonium groups on a surface was explored. Plasma deposition of a functional amine rich film was conducted in a custom designed plasma reactor. This allowed for a facile and substrate independent method to bind quaternary amines through amine-epoxy chemistry. Using a precise dip coating method, the charge density caused by the addition of quaternary amine was linearly controlled over a 13 mm range. This was confirmed by careful characterisation by X-ray Photoelectron Spectroscopy (XPS) and Atomic Force Microscopy (AFM). Such surfaces provided an excellent way of determining the minimum surface potential required to induce antimicrobial effects on *Escherichia Coli*. It was confirmed by use that a surface potential of approximately 120 mV is required to induce membrane permeability to the bacteria. Furthermore the complexity of interactions between different bacteria and different environmental conditions and quaternary amine surfaces was shown.

Secondly, the ability to bind polymyxin, a peptide-like antibiotic, to surfaces as a contact killing antimicrobial was investigated. A plasma deposited propionaldehyde was utilised as a base coating to which the polymyxin was attached. This was confirmed by high resolution XPS. The interaction of *E. coli* and lipopolysaccharides (LPS) with the surface immobilised polymyxin was determined. It was determined that this surface is low fouling and has the ability to bind 52ngcm$^{-2}$ of LPS. This has the potential to be used as an intracorporeal endotoxin removal\isolation system.

Thirdly, oxazoline based thin films were developed via the plasma deposition of 2-methyl-2-oxazoline and 2-ethyl-2-oxazoline. Surface characterisation of these films via XPS and ellipsometry revealed vast changes in chemistry, chemical functionality and deposition rates depending on deposition conditions. When deposited under specific conditions a 77% reduction in strongly adhered *Staphylococcus epidermidis* biofilm mass was obtained. Additionally, the unique and highly tuneable properties of these oxazoline surface coatings and their high antibiofouling ability make them attractive candidates for other medical applications.

Finally, the ability to translate simple plasma deposited films directly on the surfaces of pharmaceuticals was investigated. Ampicillin and vancomycin were added to the plasma reactor on a custom designed agitator with controllable speed. The random motion of the powders allowed for complete coverage of the surface of the powder in the polymer. This provided a facile solvent-free technique for encapsulation of drug particles and was utilised to sustain ampicillin and vancomycin release for 5 days.

**Declaration**

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.



Alex Cavallaro 27-11-2015

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# 1 Introduction

## 1.1 General introduction

Antimicrobial surfaces have instilled interest from the medical field for use on devices such as implants[1-3] and wound dressings.[4, 5] However, the application of antimicrobial surfaces is not limited to the medical field and have potential application within a variety of other fields; ranging from the food industry,[6, 7] waste treatment[8] and purification.[9] The addition of an antimicrobial onto a surface as a coating, or into a surface coating is of particular interest. [10-17] The use of plasma deposited coatings allows for a substrate independent approach to generating antimicrobial surfaces.

### 1.1.1 Infections

Infectious diseases are still the second highest cause of mortality, after heart diseases, of which a majority (approximately two-thirds) are caused by bacteria.[18] It is common to think of bacteria as pathogenic or "non-pathogenic" as most bacteria are harmless, and many play beneficial roles. However, even some of these bacteria display opportunistic pathogenic behaviours.[19] Although many pathogenic bacteria exist some appear more frequently and appear more harmful than others. For example, *Staphylococcus aureus* is found on human skin however, it is a major cause of chronic infection in hospitals[20, 21] and *E. coli* is commonly associated with catheter infections.[22] Furthermore, there is evidence that suggest that bacteria can play a role in the development of cancer, obesity and increase the risk of heart disease.[23-25]

The incorrect and general overuse of antibiotics is a major factor in the appearance of multidrug resistant pathogenic bacteria. Antibiotic resistance in bacteria is known to

be derived through sequential accumulation of resistance plasmids and genes through bacterial populations. An individual resistance plasmid tends to code for one mechanism of resistance which can lead to resistance against a single or multiple antibiotics. This is usually achieved through four major methods; antibiotic inactivation, target site modification, antibiotic efflux or activation of alternate metabolic pathways.[26-28] Instances of bacteria with the ability to resist one, or several antibiotics, is increasing. The prevalence of multidrug resistant pathogenic bacteria is a serious problem in the healthcare industry. [11, 26, 29-31]

## 1.2 Device related infections

Medical devices are an integral supplement within modern healthcare systems. General hospital visits involve the use of medical devices to support medical interventions. Medical devices, such as joint replacements, heart stents, intravenous lines and catheters, can be colonised by bacteria before implantation. This has become increasingly relevant in the last few decades, as the increase in population size has led to an increase in dependence on the use of various medical devices.

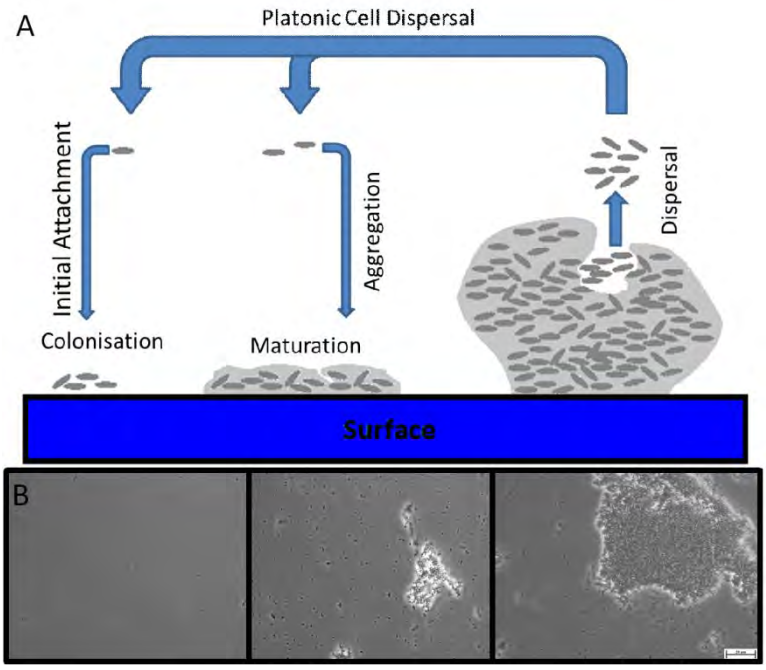
Infection strikes in approximately four percent of cardiovascular implants, two percent of joint prostheses and five percent of fracture fixation devices.[32] In each case the cost of intervention is upwards of fifty thousand dollars ($US). Non-permanent implants, such as catheters, are also often colonised by bacteria which can cause severe infections and dramatically increase treatment costs.[29, 33, 34] 8-10% of all hospitalised patients experience some form of Hospital Acquired Infection (HAI) and the average hospitalisation time for these patients is thirteen days.[35] In Australia alone HAI attribute in excess of one billion dollars per annum due to the prolonged hospital stays

of patients.[36] Furthermore, the percentage and severity of HAI tends to be higher in developing nations.

Initial infectious bacterial colonisation of medical device surfaces can occur during device storage or during their use, when they come into contact with air, are being handled by hospital staff or by patients themselves. These infectious bacteria can be transferred directly from the surface of these infected medical devices and inserted directly into the body of patients subcutaneously. Despite current aseptic techniques and sterilisation, infections are still a major cause of implant failure. In such cases of infected implants or other medical devices, devices often need to be replaced, which not only adds these exceptional costs to medical treatment but, adds considerable pain and suffering to patients.

## 1.3 Biofilms

A major contributor to the pathogenicity of bacteria is their ability to form highly structured and dynamic communities called biofilms. Biofilms are generally defined as any matrix-enclosed microorganisms adhered to each other or surfaces.[37] It is estimated that approximately 99% of bacteria live within a biofilm.[38] After the initial colonisation of microbes to a material, surfaces can be conditioned to allow for irreversible attachment through the production of extracellular materials to generate a stable microcolony. Such microcolonies mature and grow through increased cell proliferation and recruitment and production of a complicated extracellular matrix as seen in Scheme 1.1. Although the major contributor to the extracellular matrix is extracellular polysaccharides, the importance of proteins and self-sacrificial bacteria contributing other extracellular components has been identified.[39, 40]




Scheme 1.1 A) General stages of biofilm formation; showing initial attachment of bacteria, maturation and aggregation with release of extracellular polysaccharides and proteins, and dispersal of bacteria from within the biofilm. B) *Staphlococcus Epidermidi*s surface colonisation over time.

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Virulence factors, such as bacterial adhesions not only allow for increased colonisation of surfaces but also contribute to the immune evasion. As opposed to their planktonic form, bacteria within biofilms exhibit up to 1000 times greater resistance to conventional antimicrobial treatments due to the condition and microstructures within biofilms. Planktonic bacteria that exhibit antimicrobial resistance usually have individual genetic or phenotypic method of resistance. Such resistance methods include drug inactivation or modification, alteration of the target site or metabolic pathways or reducing drug accumulation through efflux. Within a biofilm the complex structures and environmental conditions control antimicrobial diffusion throughout the matrix. This can cause chemical and physical alteration or isolation of the

antibiotic. Furthermore, having different structures within the biofilm ensures that the individual cells are at different stages of the cell cycle.[37]

## 1.4 Antimicrobial surface coatings

As discussed in the methods of this thesis there are three main categories in which surfaces can exhibit antimicrobial action. Here, specific examples of surfaces with attached antimicrobials, namely quaternary ammonium compounds (QAC) and antibiotics, from the literature are discussed.

### 1.4.1 Quaternary ammonium compounds

The general structure of QAC, '$NR^1R^2R^3R^4 + X^-$', as shown in Figure 1.1, allows for many different chemical structures of QAC and thus possible functionalities of various QAC. The antimicrobial properties of QAC were first noted in 1916 and since have been studied in one form or another for various applications.[41-44] The generally accepted hypothesis as to why QAC exhibit antimicrobial properties is that the long charged C-chains penetrate the cell membrane and hence kill the cells.[10] It has been previously shown that the antimicrobial action will vary depending on the length and types of the compound's side chains and the temperature of exposure.[15, 16, 41] However, QAC with short side chains have also been shown to exhibit antimicrobial activity. Thus, another proposed method of the antimicrobial effect was attributed to the net surface charge prompting ion exchange with the surface preventing membrane charge neutralisation and inducing a loss of membrane integrity.[45-47]

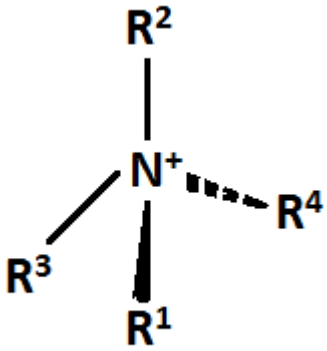


Figure 1.1 Representation of basic QAC structure; where nitrogen atom has four bonds.

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The bactericidal effect of QAC on methicillin resistant *S. Aureus* was demonstrated by Ioannau *et al.* 2006. It was observed that the addition of QAC to the bacterial culture induced autolysis. The QACs studied were membrane active agents and had the ability to interact with the cytoplasmic membrane of the bacteria. This interaction caused the membrane of the bacteria to immediately leak.[15] It was later shown using scanning electron microscopy that the effect on *Pseudomonas fluorescens* was the same.[41] This research all backs up the previous research suggesting the bactericidal properties of QAC.[42, 43]

### 1.4.2 Antibiotics

Antibiotic loaded surface coatings have been widely studied.[48] Creating implants or other medical devices with antibiotics integrated within their surfaces will decrease the reliance on conventional antibiotic usage, and associated techniques of usage, by ensuring a release of broad spectrum antibiotics at the site of potential infection.[48, 49]

An example antibiotic surface coating was the immobilization of vancomycin on calcium aluminium oxide as published by Palchesko et al. 2011. Two methods were used to load vancomycin onto calcium aluminium oxide discs; *Adsorption* and *Covalent*

*Attachment.*[50] The adsorption method simply involved immersing the calcium aluminium oxide discs in a vancomycin-methanol solution. The covalent attachment method involved the use of a 1,12-dodecanedicarboxylic acid treatment followed by a 'N'-dicyclohexylcarbodiimide and 2mM N-hydroxysuccinimide treatment. This is followed by the vancomycin-methanol dipping giving a final covalently bound vancomycin.[50] Antibacterial studies based on turbidity carried out on both of these types calcium 'aluminium oxide + vancomycin' discs demonstrated a clear decrease in bacterial survival compared to the control and showed that the covalent linkage didn't affect the properties of the antibiotic.[50] Inhibition zone studies showed that both methods inhibited bacterial growth. It is understood that the adsorbed vancomycin will leach into the agar to form an inhibition zone.[50] It is clear that the antibiotic leeches out from the coating as the bacterial growth is not only prevented on the surface of the disc.

More recently, Bastari *et al*[51] developed a ceramic-polymer hybrid for drug release. Poly(lactic-***co***-glycolic acid) particles with a calcium phosphate over coating were prepared via an emulsion technique with a subsequent adsorption-nucleation coating method. These hybrid particles were loaded with antibiotics, namely levofloxacin and nafcillin. The addition of the calcium phosphate over coating slowed the release of the antibiotics. Due to the release of the antibiotics, it was demonstrated that biofilm formation was inhibited.

### 1.4.3 Outlook

Surface coatings have been identified as having the potential to decrease surface fouling caused by bacteria. With a dramatic increase in the need to invest in new materials, research has sought alternate methods of generating new and exciting

antimicrobial surfaces and coatings. However, it must be remembered that all antimicrobial assays only provide answers to the specific scientific questions proposed; the strictly defined and controlled conditions tested. Antimicrobial tests should be chosen to accurately reflect the expected mechanism of action and the intended purpose of the surface. Knowing how the surface will interact with the test microbes, will allow for specific tailoring of assays. However, if the mechanism is not known, utilising the aforementioned techniques, one could determine the mechanism of action i.e. by looking at any diffusion into solid or liquid media or, looking at the viability of attached cells. A summary of assay appropriateness based on function and interaction is given in Table 1.1. Although there are many possible variables within a single assay, controlling specific parameters to relate to, and reflect the intended application is an important factor to consider. A single assay may show the existence of antimicrobial capabilities of a surface, but the mechanism in which it works should always be confirmed using an alternate test.

## 1.5 Plasma and surface modification

Plasma is defined as an ionised gas, with the presence of electrons, charged ions and natural species, with a net zero charge. Plasma occurs naturally at low pressures, as seen in the upper atmosphere, and\or at high temperatures, as seen in stars. By utilising an energy input to ionise gases, plasma can be induced. This is the basis of fluorescent lamp functionality and nuclear fusion reactors. However, early research conducted in the field revealed the possibility to deposit films using plasma for use as barrier coatings.[52, 53] Since this point similar deposition techniques have been utilised for surface modification.[54, 55]

Surface modification technologies have been proposed as a method for regulation of biological interaction with substrates. Plasma deposition is a surface modification technique used for the modification of surface chemistry and properties.[11, 55] Surface interactions, based on chemical functionality, can be changed by the application of a functional plasma deposited organic film. Plasma deposition boasts as a single-step, dry process that does not require the use of solvents or other potentially toxic reagents. Furthermore, it can be used to generate uniform functional films of controllable thicknesses within the nanometre scale and the organic films deposited via plasma can be tailored to produce precise film functionalities or properties by regulating the deposition conditions.

In general, when dealing with low pressure plasma deposition, a precursor is added to the system and excited with an external energy source to excite and fractionate molecules. Essentially, the deposition process involves initial grafting of these fractions directly onto a substrate. As the precursor continues to deposit, the films grow. Generally speaking, the plasma used within this work is ignited using a 13.56 MHz radio frequency (RF), as it is reserved for use in biomedical and research applications. This induces a plasma glow due to the presence of electrons, ions and photons within the plasma field.[53, 56] The chemistry of the precursor, precursor flow rates, excitation powers and deposition duration all affect film properties.[54, 57] Interestingly, after initial deposition, after a few nanometres, film properties remain constant, regardless of the initial properties of the underlying surface, making the technique completely substrate independent. However, due to the variations between different deposition systems,

such as dimensions and configuration, relative precursor retention and electrode configurations films will show differences between different reactors.

Due to the variety of potential plasma deposition precursors, the variety of surface chemistries is almost limitless. Various organic films have been made using plasma deposition including pure hydrocarbon films, halogenated films, amine-rich films, alcohol and organic acid films. This can provide a basis for immobilisation of antimicrobial agents.[58]

## 1.6 Overview of antimicrobial surfaces: testing and approaches

Due to the variety of potential applications and in what form their antimicrobial action is required, such as their ability to release antimicrobials or prevent fouling, different microbiological testing is required. This is complicated by the fact that although various industry standards exist, none can distinctly classify an antimicrobial surface as a specific efficacy for its intended application to a standard requirement. Within the literature a variety of methods are used to determine the antimicrobial activity of surfaces. Although there are industry standards relating to such assays,[59-61] these simply provide recommendations of test protocols. These protocols allow the freedom to change the test concentration and strains of bacteria, the time points the bacteria are assayed and other conditions. As a simple example, an antibacterial surface that releases a payload, such as antibiotics[62,63] or nanoparticles,[64,65] will kill all bacteria in the surrounding medium and no viable bacteria should be seen on the surface. A surface that is purely antifouling will prevent bacterial adhesion to the surface but will not have a bactericidal effect. If the test method purely quantifies the viability of bacteria that have adhered to the surface both surface types will appear highly

effective as no viable adhered bacteria will be seen. However, all the planktonic bacteria in the test media for the antifouling surface will still remain viable. Although this surface can still be declared antimicrobial it would clearly behaves differently and would have different intended applications compared to the payload releasing surface.

The level of uncertainty in choosing individual antimicrobial assays, and the many possible variations that can be implemented in each test, present a problem for those developing different antimicrobial surfaces. As in the example above, using an inappropriate test, i.e. testing purely for total adhered bacteria to the sample, may provide potentially misleading antimicrobial observations. It needs to be understood that each antimicrobial test has its own advantages and disadvantages for testing certain surface types. With this in mind, the choice of assay should reflect the mechanism of action of the surfaces.

### 1.6.1 Assay Appropriateness

#### *1.6.1.1 Mechanisms of antimicrobial functionality*

The most fundamental property that should be considered before attempting any antimicrobial testing on a surface is the mechanism by which the surface is expected to work. With some prior knowledge of the surface, it can easily be categorised into one or more of the following three groups: antifouling, contact killing or releasing surfaces (Figure 1.2). In order to accurately test for antimicrobial properties, how the surface would interact with microorganisms should be known.[66]

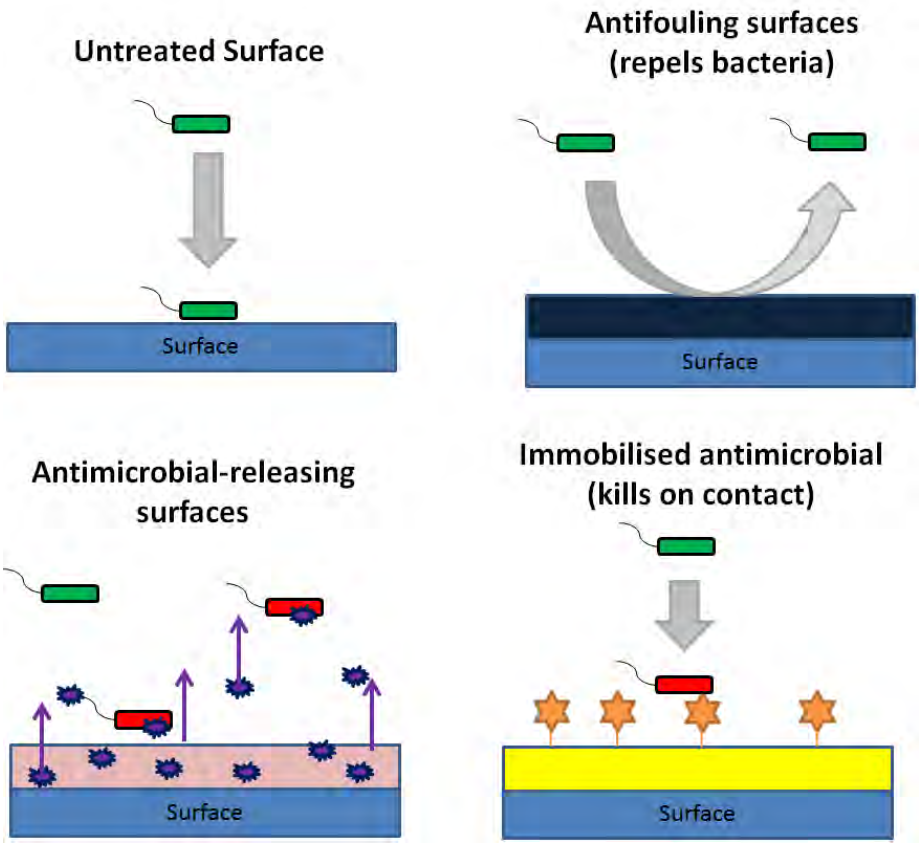


Figure 1.2 Differing surface mechanism of antimicrobial action based on surface modification. Where green represents living bacteria and red represents dead bacteria.

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As illustrated above different surfaces would need different microbiological testing to demonstrate their effectiveness based on the mechanisms by which it works; i.e. it is expected that microorganisms which can live in either planktonic or adherent states will just remain in a planktonic state if not able to adhere to a surface.

When performing antimicrobial assays it is possible to fall into the trap of "confirmation bias." An example can be illustrated by assuming a surface is contact killing and only analysing microorganisms on the surface itself. If the microorganisms on the surface appear to be dead one could easily assume that the decrease in viability was due to the direct contact of those microorganisms with the surface. However, you

cannot rule out the possibility that the surface released an antimicrobial without looking at the microorganisms in the surrounding environment. Multiple assays are usually needed to confirm the hypotheses. If the mechanism of functionality is not known, designing assays for antimicrobial ability of surfaces may be difficult.

Many hydrophilic and zwitterionic surfaces, such as polyethylene glycol based surfaces[67,68] or polyoxazoline surfaces,[69,70] fall under the category of antifouling. Simply put, antifouling surfaces prevent the attachment of microorganisms, proteins or other biological agents. In a medical sense, such surfaces will prevent initial colonisation of surfaces and any microorganisms can be easily removed. In turn, this will prevent biofilm formation. Although many "antifouling" products prevent fouling by releasing biocides to kill microorganisms before they can adhere, in the context of this thesis antifouling refers simply to those surfaces that prevent microorganism attachment *without* releasing antimicrobial agents.

Another mechanism of action of common antimicrobial surfaces is those which can induce direct killing. Examples of such surfaces include those with immobilised antimicrobial peptides,[71] polycationic surfaces,[72] including those with immobilised quaternary ammonium compounds,[73] and some polymers.[74-77] It is commonly accepted that the immobilised antimicrobial still has a direct interaction with cell membranes or cell walls.[78, 79] In the case of antimicrobial peptides this works as these peptides directly induce membrane damage.[71] It should be noted surfaces that rely purely on contact with cells and do not leach any antimicrobial agents into the surrounding medium, tissue or environment.

Antimicrobial releasing surfaces are all those which unload a payload. Various antimicrobial agents, such as antibiotics[80, 81] or antifungals,[82] can be used as the payload. Releasing surfaces also include the release of antimicrobial metal ions from particles, alloys and pure metals.[83, 84] In general, if the released agent is effective against the test microorganism, the surface will exhibit some antimicrobial action. However, specific release rates and the volume in which the antimicrobial is released should be taken into account when looking at antimicrobial efficacy.

#### *1.6.1.2 Key interactions*

There are two fundamental interactions that antimicrobial tests for surfaces look at; the effect on microorganisms that have adhered on or directly come into contact with the surface and, the effect on microorganisms in the surrounding environment.

Antifouling and contact killing surfaces will only have a direct effect on microorganisms that come in contact with the surface. Thus, it is logical that the assay to test the efficacy of antifouling and contact killing surfaces will be based on the analysis of cells adhered to the surface. This can be achieved by either directly analysing cells whilst on the surface or removing cells from the surface and analysing solutions containing the removed cells.

Payload releasing surfaces will tend to have a large effect on microorganisms in the surrounding environment. Subsequently, this will decrease the quantity of viable cells adhering to the surfaces as there are not as many viable cells. Thus, designing experiments to look at the number of viable cells in the surrounding media and on the surface may be of use. It should be noted that in many cases it is possible to analyse

the microorganisms in the original surrounding media and those on the surface can be carried out sequentially on the same sample.

In this work, plasma polymer derived coatings were used to generate surfaces that are either contact killing, antifouling or used to control drug release via an encapsulation method.

#### *1.6.1.3 Other considerations*

The variable factors of experimental setup within individual tests will need consideration based on the intended purpose of the test substrate. The usefulness of any technique and results is directly related to how the surface will be used. Thus, the eventual fate of the surface should be taken into account during experimental design. For example, influences of surrounding media and environment on the surface may play a role and the determination of this should be considered.[85, 86]

- Would the media influence the surface? (e.g. influence the charge or payload release rate)
- Would constituents within a broth lead to increased fouling?
- Does the test broth relate to the real environment? (e.g. a wound environment when testing antibacterial bandages)
- Is the use of nutrient containing media or saline warranted or reflective of natural environments?

The type or strain of a microbe to be tested and its inoculation concentration may need consideration.

- Is the microbe used reflective of what is seen in the intended field?

- Is the concentration of inoculum representative of what occurs naturally?

Other environmental or experimental factors, such as temperature, incubation times or media removal, will also influence perceived antimicrobial activity.

A special note should be taken when dealing with stimuli-responsive surfaces, although they tend to fall in one or more of the categories for mechanism of action, they require another stimulus to be activated.[87, 89] When testing the efficacy of a temperature, pH or electrically stimulated surfaces, the ability for the microorganism to withstand the "stimulated environment" must be considered or biological changes caused by the stimulus. An example of this would be an electrical stimulus causing an electroporetic or a bioelectric effect which may increase antimicrobial susceptibility.[90-92] Biologically stimulated antimicrobial surface coatings may require specific protein or enzymes to be activated, thus care should be taken when selecting microorganisms. Logically, using an organism that is able to activate the bio-stimulated surface is important. For example, when using a sample trigged by bacterial virulence factors, such as endotoxins or lipases, bacteria that express these should be used.[93]

Although lists of such considerations could be infinite, modifying the experimental design to that which best reflects the natural state of the intended application will allow for accurate assessment of antimicrobial ability. With this being said the following methods described are those that seem commonly used and can be used to determine specific properties of surfaces.

### 1.6.2 Antimicrobial Assays

#### *1.6.2.1 Cell quantification*

Various methods for directly quantifying the total number of cells and counting cells within an aqueous sample or adhered to a surface are available. Individual methods have their own benefits and limitations. Some common methods and their limitations are discussed below.

A simple method for quantification of microorganisms is the use of optical density (OD) a.k.a turbidity of samples.[94-96] This makes the assumption that the same number of microorganisms from different samples will scatter the same amount of visible light and each individual cell will scatter the same amount of light. Typically scattering of visible light, of wavelengths of 590-600nm, can be measured using simple spectroscopy. Turbidity readings will allow for a quick determination of concentration percentages relative to an undiluted (or fully grown or untreated) sample. However, direct comparison between the turbidity and a microbe specific calibration curve or a standard, such as the McFarland standard for bacteria, will allow for a quick estimation of total microbe number as shown in Figure 1.3; usually expressed as Colony Forming Units per millilitre ($CFU.ml^{-1}$). However, the size, distribution and any pigmentation of the microbes will influence the readings. Thus, each individual spectrophotometer should be independently calibrated for use in enumerating specific microorganisms.

Turbidity based methods are commonly used for determining minimum inhibitory concentrations (MIC) of current and potential antimicrobial agents.[98] However, even though they have been used extensively, variability in the way the assay is performed, such as the microbe inoculation concentrations, total volumes, assay lengths and media types, tend to make direct comparisons difficult. Thus, these factors need to be considered when using turbidity assays to investigate antimicrobial surfaces.

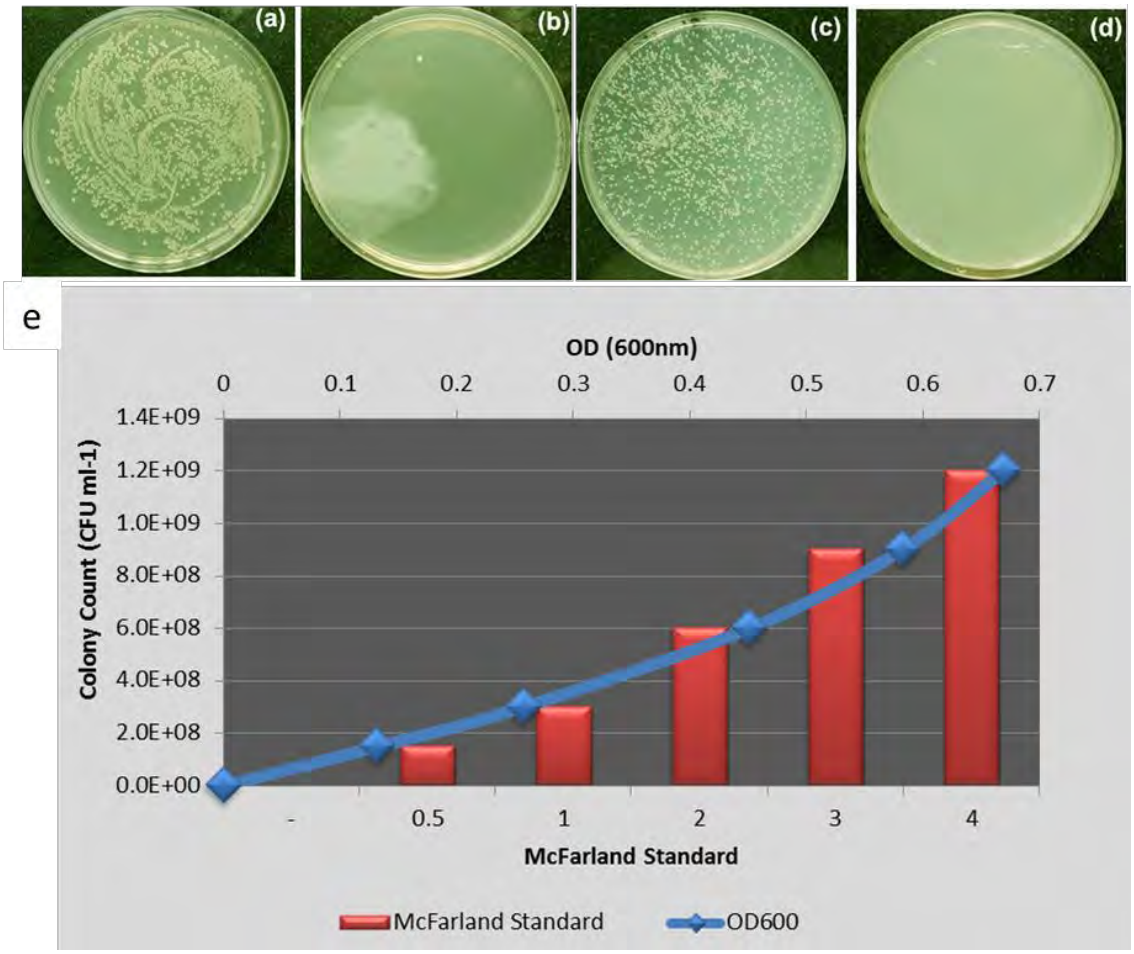


Figure 1.3 a) Images of agar plates counts for control (a), (b) E. coli and (c), (d) S. aureus: (a), (c) control groups, (b), (d) treated with silver nanoparticles/halloysite nanotubes/graphene nanocomposites (Ag/HNTs/rGO), showing a decrease in total bacteria on the Ag/HNTs/rGO treated samples. Reprinted with permission from <Yu, L., Y. Zhang, et al. (2014). "Enhanced Antibacterial Activity of Silver Nanoparticles/Halloysite Nanotubes/Graphene Nanocomposites with Sandwich-Like Structure." Sci. Rep. 4.>[97] under Creative Commons Licence. Copyright Nature Publishing 2014. e) Turbidity standard curves for bacterial enumeration based on McFarland Standard (red columns; bottom horizontal axis) and optical density at 600nm wavelength (blue diamonds; top horizontal axis).

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Turbidity measurements can be used to readily observe the effect of antimicrobial releasing surfaces by quantifying the cells in the surrounding media of an antimicrobial

sample. In the case of an antimicrobial releasing surface, a distinct difference in the total number of cells within the surrounding medium should be seen. However, when faced with surfaces that release antimicrobials that may discolour or change the turbidity of the surrounding medium or absorb visible light, an uninoculated turbidity control should be added. This technique can also be used to quickly quantify the total number of microorganisms on a surface by analysing solutions containing cells removed from the surface.

Yu *et al.* [97] used turbidity to determine the MIC of Silver Nanoparticle/Halloysite nanotube/graphene composites (Ag/HNTs/rGO). Serial dilutions of the materials in broth were made and bacteria added. Post incubation turbidity was used to determine cell growth. The lowest concentration where no obvious turbidly was observed was determined to be the MIC of the Ag/HNTs/rGO. Furthermore, OD was used to determine the relative antibacterial efficacy of the composites by comparing turbidity of bacteria treated with the composites and other samples after 5h and 12h.[97] Unnithan *et al.*[99] used turbidity to quantify the bactericidal ability of nanoceria doped nanofibers. OD was measured after 12h contact incubation with the nanofibers and showed a clear decrease in bacterial growth.

Although this technique provides a quick basis for analysis there are some limitations for its use. Spectroscopy has a minimum detection limit, where extremely low concentrations may not be detected. Thus slow growing microorganisms or those that are in a static stage, for example bacteriostatic cells, may appear as though they are dead when not. This is further complicated by the fact that we cannot directly confirm viability of the cells within the sample and cellular debris, extracellular components or

other contamination may interfere with the assay. With such, as in these cases above,[97, 99] turbidity was not used as the only endpoint for investigating antimicrobial action. The results were confirmed by other methods.

Another method of direct quantification of cells is spread plating\colony counting. The underlying principal of this is that when an aqueous sample is adequately diluted and spread or poured evenly on high nutrient solid media, usually agar plates, individual colonies will form after incubation. Counting individual colonies that have grown on the solid media will give a direct indication of the total number of CFU in that sample.[100, 101] Using simple calculations based on the dilution factor (*d*), plated volume (*V*) and colony count ($x$), the CFU ml$^{-1}$ (*N*) can be found (see Equation 1.1).

$$N = \frac{x}{V.d}$$

Equation 1.1 Calculation of total viable bacteria (CFU ml$^{-1}$ (N)) from colony counts ($x$) and dilution factors (d) and total plated volume (V) used during spread or pour plating.

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The observable growth range is quite considerable. Due to the nature of the test cell numbers from as little as 10 CFU ml$^{-1}$ to greater than $10^{10}$ CFU ml$^{-1}$ can be quantified by varying the dilution factors.

Generally, various dilutions of microorganisms would be plated out. A sample will be considered valid if the colony count is between 30-300 with only individual colonies seen. This can make the process quite labour intensive as a single sample may require many dilutions to be plated out. Although it is possible to enumerate clusters of colonies, this may cause inaccuracies in the measurement. Having direct visualisation of the grown colonies will also allow the user to quickly see if there is any

contamination from other microorganisms within the sample. The major benefit of this method is that it a relatively simple method and can be used to directly quantify the total number of viable cells within the sample. However, the time for colonies to fully form usually takes upwards of 18h.

Spread plating for quantification of viable cells in solution post treatment with surface immobilised antimicrobial agents is the basis for the ASTM E2149 – 10.[102] This international standard aims at utilising dynamic conditions, achieved by shaking, to quantify the antimicrobial ability of surface-bound antimicrobials surfaces after 1h of contact. The samples are added to low-nutrient solutions of E. coli and the number of viable microbes in solution is compared to the control. This standard also acknowledges that the leaching of antimicrobials from the surface must be confirmed.

The antimicrobial efficacy of antimicrobial silver foam dressings were confirmed by Payne & Ambrosio 2009, using methods as per ASTM E2149, tested at 0.5, 1, 2, 6, 24, 48 and 72h.[102, 103] The antibacterial capacity of the dressings against S. Aureus and P. aeruginosa was confirmed. Post incubation with the dressing bacteria, spread plate counts were used to determine the number of viable bacteria remaining in solution. Wei *et al*.[104] utilised spread plating to quantify the antimicrobial activity of Cu and $CuCl_2$ embedded polymers against *E. coli* and *B. subtilis*. The time taken to eliminate $10^6$ bacteria in nutrient broth was determined. Wei et al. confirmed that the increased Cu ion release from the polymers containing $CuCl_2$ increased antimicrobial action. Yu *et al.*[97] used a simple spread plating dilution methods to confirm the antimicrobial activity of Ag/HNTs/rGO composites.

The effectiveness of contact killing an alkylammonuim polyurethane surface coating was evaluated using colony counts of bacteria retrieved from the surface. Kurt *et al*.[105] simulated an aerosol deposition of pathogenic bacteria and the bacteria were allowed to interact with the surfaces in a humid environment. To quantify the viable bacteria after incubation, samples were vortexed to remove the bacteria from the surface. This now allows for quantification of the bacteria from solution.

Spread plating methods are commonly used to isolate antimicrobial resistant strains of microorganisms by adding the antimicrobial in question to the solid media. Thus, when dealing with antimicrobial releasing surfaces the ability for the microorganisms to resist the antimicrobial can also be tested. Furthermore, individual colonies may be isolated and further propagated to help determine any genealogical or phenotypical changes the microorganisms may incur. Such an assay could also be utilised to quantify the number of viable cells adhered to a surface by first removing the cells from the surface and analysing them in solution.

Counting cells via microscopy is an inexpensive way to make a direct comparison between samples by looking at the total number of cells. Differences in depths of aqueous solution will change the cellular counts and make this quantification method more difficult. The use of counting chambers (i.e. cytometers, Helber bacteria chambers and etc.) will not only eliminate this factor but allow for direct calculation by controlling the sample size. This is a convenient method and can be performed quickly as there is no need to add an extra incubation step as you would have to when preforming colony counts. Depending on the assay and the test sample, number of cells adhered on a sample can be calculated.

Microscopy counts can also be used for quantifying the number of cells in solution, either directly post treatment or removed from the surface, or counting the total number of cells attached to a surface post treatment where individual cells can be seen. Observing cells that are still attached to the surface can be difficult when cells are clustered and have grown on one another, such is the case for an established biofilm. In many cases, observing visible cells per field of view can give a qualitative measure for adhesion. This can either be counted directly or with algorithmic assistance from microscopy images. This may be of use especially when dealing with an antifouling surface. The use of biological stains, for example crystal violet or safranin stains (those used in Gram-staining which will be discussed later) may assist in direct visualisation of individual cells within the test sample. It must be noted that distinguishing between live and dead cells is difficult via this method. However, the use of fluorescent dyes does make this possible. An overview of fluorescent assays is given later in this article.

### 1.6.3 Agar diffusion tests

Agar diffusion tests, which include a variety of zone of inhibition assays, disc diffusion assays, Kirby-Bauer tests or direct contact assays, are used to determine the relative efficacy of antimicrobial agents to prevent growth on a solid medium (agar). A typical agar diffusion test involves spread-plating the test organism and placing the test sample directly on the agar. A positive result in the assay is observed when the inhibition of microbe growth within a confluent “lawn” of microorganisms is caused by the diffusion of an antimicrobial agent.[106-111] The relative effectiveness of the antimicrobial against the test strain can be semi-quantified by measuring the zone of inhibited. Although Kirby-Bauer antibiotic testing is standardised, ensuring that the

same agar is used (Mueller-Hinton agar), the agar is poured to the same depth (4 mm) and antibiotics are loaded in specific discs, in other applications of the agar diffusion tests there is still much variation in the assays.

Agar diffusion assays seem quite rudimentary but variation in the assay, such as using different nutrient agar (e.g. Mueller-Hinton agar, Blood agar or Tryptone soya agar), the thickness of the agar, the level and nutrient content in the initial inoculum, humidity and moisture levels and, possibly most importantly, the solubility of the antimicrobial, will change the perceived inhibition zones.[111]

A common approach to generation of antimicrobial surfaces utilises controlled release of antibiotics or other antimicrobial agent.[112-114] Antibiotics have previously been loaded into surface coatings through methods of absorption or covalently linked to porous coatings,[80, 104] creating antibiotic gel-films or alcohol solutions and dipping.[114, 115] Intuitively, agar diffusion assays can quickly determine the effectiveness of antimicrobial releasing surfaces. After inoculation of the agar plates the test surface can be place directly onto the agar and its payload will release. Controlled release studies can be conducted via this method by looking at the varying inhibition zones over time or between samples. It should also be noted that the size of the inhibition zone will also be affected by the antimicrobial release rate of the surface. Using this test for surfaces that are deemed purely contact killing or antifouling should show no inhibition zones as they should not leach any antimicrobial agents into the surrounding agar. Figure 1.4 shows a clear example of an inhibition zone around inoculated diffusion discs.

Stigter *et al.* 2004 utilised agar diffusion to test the release of various antibiotics from titanium plates and discs.[81] Titanium plates (and discs) were sandblasted and coated with a layer of amorphous calcium phosphate and this was recoated using "biomimetic carbonated hydroxyapatite" using a supersaturated salt solution containing antibiotics.[81, 116] This research showed the antibiotics did not lose functionality after the attachment process. It was shown that surface thickness did not affect the release rates of antibiotics, but different antibiotics have different release rates (ranging from 1h – 16h).[19]

The same problems mentioned above will still apply and have to be considered. However, assaying released products may overcome this. Swanson *et al.* 2011 modified titanium by sandblasting and etching the surface.[117] The surface was enriched with hydroxide groups and an aminopropyltriethoxysilane (APTES) reaction was used to exchange the surface hydroxide groups to functional amines which were later changed to aldehyde groups using a glutaraldehyde reaction. A 1% chitosan (a polysaccharide) solution was used to coat the surface, to which a chitosan-vancomycin coating surface was loaded onto.[117] The chitosan-vancomycin coatings showed that 95% of the drug was released within 5 minutes of PBS exposure. In this case the leached vancomycin was loaded directly to diffusion discs and placed onto agar plates. The bonding of the vancomycin did not seem to lessen its antimicrobial ability.[117]

Braceras *et al.* 2011 also used inhibition zones created by disks loaded with the release products of a polymer to quantify its activity, effectively testing the release produced. Braceras *et al.* also demonstrated that ciprofloxacin can be chemically altered to a silylated ciprofloxacin and, using argon plasma, coated polymerised onto surfaces.[118] It

was demonstrated that the modification and polymerisation of the ciprofloxacin did not influence its antibacterial properties.

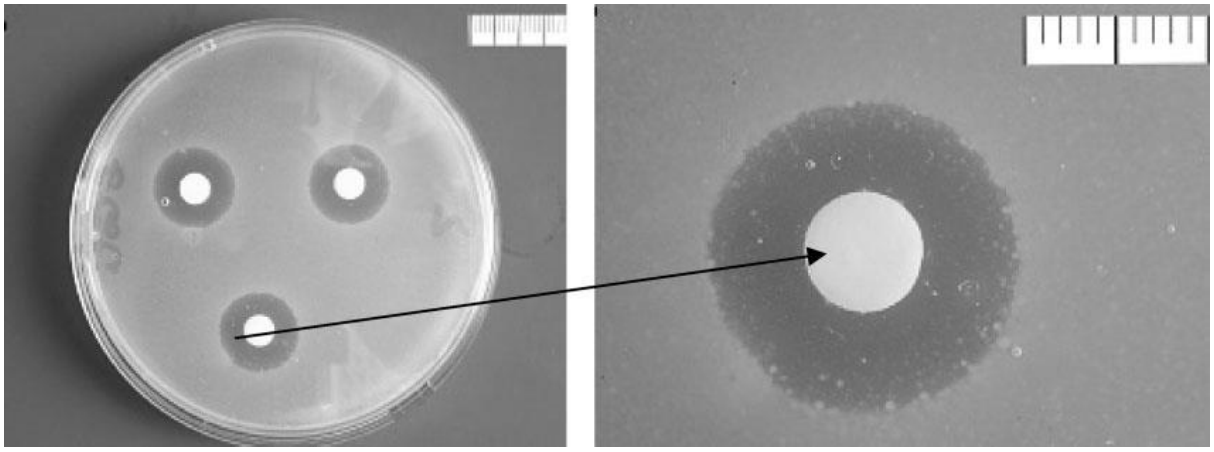

Figure 1.4 Agar diffusion from disks soaked in released products from plasma polymerised coatings formed silylciprofloxacin against E. coli. Reprinted with permission from <Braceras, I., P. Azpiroz, et al. (2011). "Plasma Polymerized Silylated Ciprofloxacin as an Antibiotic Coating." Plasma Processes and Polymers 8(7): 599-606.> Copyright 2011 John Wiley and Sons[118]

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### 1.6.4 Histological staining

Histological staining refers to the staining for biological structures and\or molecules. This is done with intent to facilitate microscopic analysis of samples. One example of use of histological staining for testing antimicrobial surfaces is the use of safranin or crystal violet stains;[119] the stains used in Gram-staining. Although it is possible to use the stains to stain a variety of microorganisms,[120, 121] the use of Gram stains in antibacterial surface testing is very common. Thus the following is based mostly on use as an antibacterial assays.

Bacteria are commonly referred to by their Gram type, i.e. Gram-positive or Gram-negative, which is based on the physical properties and chemical composition of cell

walls. Gram-staining is traditionally used for determination of Gram type. A typical Gram-stain assay involves the following:

- Application of the primary stain (usually crystal violet) to a (heat) fixed sample of bacteria
- Adding iodine to help entrap the stain in the cells
- Decolourising the sample by rinsing with a solvent (usually ethanol or acetone)
- Applying the counterstain (usually safranin) to stain any decolourised bacteria.

Due to the cell membrane composition of the different Gram types, Gram-staining will stain Gram-positive bacteria purple\blue and Gram-negative bacteria red\pink. In the case of testing antimicrobial surfaces, Gram-staining can be utilised to help visualise, and eventually quantify, using microscopy.

The ability for crystal violet and safranin stain to bind to any bacteria, and other microorganisms[120, 121] or cultured cells,[123] can be utilised to indirectly quantify total bioburden on surfaces. All cells can be easily stained and the sample washed to remove any free stain. Although this can be quantified as mentioned above, the stain in the cells could also be released into solution, using an organic acid or other solvent, and total released stain quantified via spectroscopy.[124] This provides a method for quantification of the stained biofilms as in Figure 1.5.

Such a technique assumes that each individual cell uptakes the same amount of stain. This is extremely useful as an indirect quantification of the number of attached bacteria, or total bioburden, on specific test substrates; especially antifouling and possibly antimicrobial releasing surfaces. In the case of contact killing surfaces, all non-

viable bacteria will also be stained, thus its usefulness is directly dependant on whether non-viable bacteria can be removed from the surface. In many cases this may not be possible. Thus using such a technique on contact killing surfaces will be of little use. A combination of crystal violet staining and turbidity measurements can be used to quantify total biofilm biomass and planktonic biomass.[125, 126,]

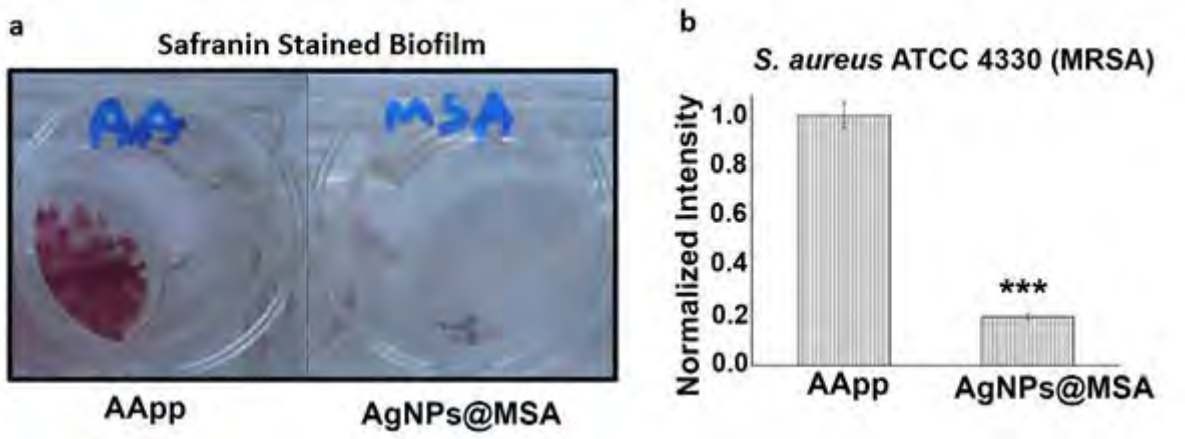


Figure 1.5 a) Safranin stained biofilms on plasma polymerised allylamine (left) silver releasing surface (right) and b) total biofilm on surface as quantified via eluting safranin stain and spectroscopy from plasma polymerised allylamine (left) and silver releasing surface (right). Reprinted with permission from <Taheri, S., A. Cavallaro, et al. (2014). "Substrate independent silver nanoparticle based antibacterial coatings." Biomaterials 35(16): 4601-4609>.[127] Copyright 2014 Elsevier.

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### 1.6.5 Fluorescence

Commonly, fluorescently labelled cells or auto fluorescent cells can be detected via either fluorescent microscopy, spectroscopically or via flow cytometry. By utilising specific fluorophores or fluorescent labels, cell presence and, in many cases, viability can be determined.[128]

Viability can be determined via the use fluorescent stains which only stain non-viable cells. The main premise of this is the use stains can only enter cells with permeable

membranes. Propidium iodine (PI) and 7-Aminoactinomycin D (7-AAD) may be used for this purpose; however PI is said to be more effective when using bacteria.[129] The general approach to microbe viability determination via fluorescence is the combination of one of these stains with a general stain. Combination stains, i.e. “Live\Dead” or “fluorescent viability” stains, are commercially available. The use of Syto9 and PI for such is commonly used. Syto 9 is a fluorescent nucleic acid stain and stains all DNA. When PI enters a cell it will tend to displace Syto 9.[130] This will allow for a clear distinction between viable and non-viable cells based on the fluorescence of the stain as seen in Figure 1.6. Quantifying the total number of Syto 9 and PI cells microscopically or via fluorescent spectroscopy will give an indication of total number of viable and non-viable cells. Bioluminescence can also be used for quantification of total numbers of on a surface or in solution.[131, 132] Assuming that only viable cells bioluminate, viability may be determined by this also.

Although it is usually difficult to determine the mechanism of action from a single assay, Green *et al.*[133] developed a Surface-Separated Live–Dead Staining (SSLDS) assay which is outlined in Figure 1.6. Utilising a closed environment, the number of live and dead cells in different key interaction areas can be quantified. This assay takes advantage of the fact that contact killing surfaces will only affect cells on its surface and anti-microbial releasing surfaces affect the entire environment.

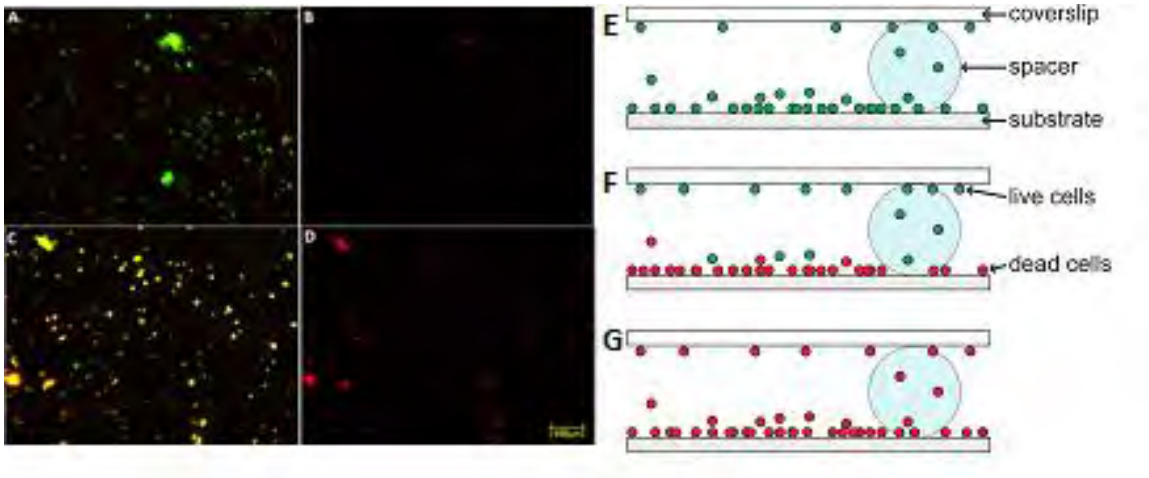


Figure 1.6 BacLight LIVE\DEAD images obtained via Nikon B-2A and Texas Red filters showing total E. coli (live and, membrane permeable) and membrane permeable only E. coli, after 4h incubation at 37°C. A) Fluorescent microscope image of total E. coli on ppAA sample. B) Fluorescent microscope image of membrane-permeable E. coli on ppAA sample. C) Fluorescent microscope image of total E. coli on ppAA+GTAC sample. D) Fluorescent microscope image of membrane permeable E. coli on ppAA+GTAC sample. (Used with permission from ref [14]) Schematic of Surface-Separated Live–Dead Staining (SSLDS) assay where E) control or non-antimicrobial substrates F) contact killing and G) antimicrobial releasing surfaces can be distinguished depending on where live (green) or dead (red) cells can be visualised Reprinted with permission from <Green, J.-B. D., S. Bickner, et al. (2011). "Antimicrobial testing for surface-immobilized agents with a surface-separated live–dead staining method." Biotechnology and Bioengineering 108(1): 231-236.> [133]. Copyright 2010 Elsevier.

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### 1.6.6 Other assays

The metabolic reduction of compounds, such as tetrazolium salts or resazurin, can change their absorbance or fluorescent properties. This is the basis for metabolic activity assays. Such tests are used as an indirect indicator of viability as only metabolically active, i.e. viable cells, will be able to metabolise the compounds.[134-136-] A greater amount of metabolic activity directly relates to more cells. Such assays can be standardised by assuming that each individual cell has the same metabolic activity. Thus, a standard curve depicting total reduction as a number of cells can be used.

Repropagation of treated microorganisms can be used to determine viability.[74, 137] Agar printing methods have been utilised to determine antimicrobial properties of surfaces. The basic principle being that any attached microorganisms will grow onto the agar to which it was printed. With respect to antifouling surfaces, no microorganisms would grow on the agar as no microorganisms will be transferred from the surface and subsequently no growth would be seen on the agar. Similar results can be achieved by transferring the test sample into fresh media: no microorganisms would inoculate the fresh environment.

### 1.6.7 Supplementary assays

#### *1.6.7.1 Sample specific properties*

Although it may seem intuitive, knowing some basic properties of the test sample prior to attempting antimicrobial assaying will help choose an appropriate assay. Outlined below are some sample specific properties that will influence antimicrobial activity.

##### 1.6.7.1.1 **Release rates**

When dealing with releasing surfaces the release rate of the antimicrobial agent will seem to change antimicrobial function. When looking at agar diffusion, if the antimicrobial does not release until after the test microorganism has fully grown, no inhibition zone will be seen.[111] An alternate method to observe antimicrobial release would allow for the full release of the antimicrobial agent into the test medium prior to inoculation.

##### 1.6.7.1.2 **Antimicrobial concentration**

The concentration of antimicrobial agent used plays a major role in releasing surfaces. If only sub-MIC, taking into account release times and unit volume, is seen it may not

be possible to see any antimicrobial action. The concentration of active agent on a contact killing surface is also of high importance. For example, a contact killing surface, based on a polycationic coating, tends to require a specific charge for optimal function.[78]

#### 1.6.7.1.3 **Surface stability**

A surface that is not stable may not be suitable for long-term applications. An example of this would be a releasing surface that releases its payload too quickly in a fluid environment. If the antimicrobial is fully released and washed away the surface may be completely ineffective. Another example of this is an antifouling surface which is only stable in the short term would not be suited for application in long-term water treatment and filtration.

### 1.6.8 Toxicity

Although not strictly antimicrobial testing, and somewhat outside the scope of this article, testing the effect of antimicrobial surfaces on the viability of mammalian cells, marine environment or other relatable entity is important when conducting research into antimicrobial surfaces.[138, 139,] Having an antimicrobial surface coating for a medical device or environmental use which is toxic is not particularly useful. Utilising additional assays to test this will show whether the antimicrobial surface is safe for its intended purpose. It should be noted, when specifically looking at antimicrobial surfaces for biomedical applications, mammalian cytotoxicity studies may have similar limitations as those discussed above.

Common practices to ensure the most human-relevant representation of toxicological effects involve the use of human cell lines in culture. By studying the effect of a

treatment on a human cell line *in vitro*, observations can be translated to predict probable effects that may occur to similar cells *in vivo.*[140, 141] From a toxicological standpoint, mammalian cell culture can be used to determine the effects of a substrate on cells.

Table 1.1 Summary of testing techniques and appropriateness for area of interaction and mechanism of function

| | Mechanism of Function | | | Key Interaction | | | | |
|---|---|---|---|---|---|---|---|---|
| **ASSAY** | **Antifouling** | **Contact Killing** | **Releasing** | **Solution** | **Surface** | **Cells removed from surface** | **No additional bacterial growth** | **Determine Viability** |
| **Turbidity** | P | N | Y | Y | N | Y | Y | N |
| **Microscopy** | Y | N | Y | Y | Y | Y | Y | N |
| **Spread plating** | P | P | Y | Y | N | Y | N | Y |
| **Histochemical** | Y | N | Y | Y | Y | Y | Y | N |
| **Fluorescence** | Y | Y | Y | Y | Y | Y | Y | Y |
| **Auto-fluorescence** | Y | Y | Y | Y | Y | Y | Y | Y |
| **Disc Diffusion** | N | N | Y | Y | N | N | N | Y |
| **Metabolic activity** | P | Y | Y | Y | Y | Y | Y | Y |

Y = Appropriate
N= Not appropriate
P= Potentially appropriate (needs isolation of cells specific interaction area)

## 1.7 Overarching aim of thesis work

The aim of this thesis is to design and implement novel coatings derived by plasma deposition that lead to new fundamental knowledge of bacteria\surface interactions and facilitate development of new antibacterial technologies. The coatings targeted in this work are those consisting of immobilised antimicrobial agents and intrinsic low-fouling properties. Furthermore, this thesis targets development of a process for translating plasma deposited coatings for the encapsulation of antibiotics to achieve controlled and sustained release.

## 1.8 Specific Aims

1. Develop novel coatings with tailored density of immobilised quaternary ammonium compounds that allow for:
    a. Determination of the minimum quaternary ammonium group surface concentration required to achieve bactericidal properties
    b. Determination the influence of quaternary ammonium group surface concentration on biocompatibility and cytotoxicity to primary human cells.
    c. Understanding the mechanisms governing the bacterial colonisation and eukaryotic cell growth on this type of coating.
2. Develop a substrate independent method for the immobilisation of polymyxin to generate an antimicrobial surface with the ability to remove endotoxins useful in the removable biomedical devices.
3. Generate novel low fouling plasma deposited coatings of oxazolines and evaluate their chemical and antimicrobial potential.

4. Demonstrate the translation of plasma deposition for rapid encapsulation of antibiotics as a potential drug release strategy to combat infection

## 1.9 References


1. Qureshi, A. T.; Terrell, L.; Monroe, W. T.; Dasa, V.; Janes, M. E.; Gimble, J. M.; Hayes, D. J., Antimicrobial biocompatible bioscaffolds for orthopaedic implants. *Journal of Tissue Engineering and Regenerative Medicine* 2012, n/a-n/a.

2. Jones, D. S.; Muldoon, B. C. O.; Woolfson, A. D.; Andrews, G. P.; Sanderson, F. D., Physicochemical Characterization of Bioactive Polyacrylic Acid Organogels as Potential Antimicrobial Implants for the Buccal Cavity. *Biomacromolecules* 2008, 9, 624-633.

3. Kargupta, R.; Bok, S.; Darr, C. M.; Crist, B. D.; Gangopadhyay, K.; Gangopadhyay, S.; Sengupta, S., Coatings and surface modifications imparting antimicrobial activity to orthopedic implants. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* 2014, 6, 475-495.

4. Maneerung, T.; Tokura, S.; Rujiravanit, R., Impregnation of silver nanoparticles into bacterial cellulose for antimicrobial wound dressing. *Carbohydrate Polymers* 2008, 72, 43-51.

5. Madhumathi, K.; Sudheesh Kumar, P. T.; Abhilash, S.; Sreeja, V.; Tamura, H.; Manzoor, K.; Nair, S. V.; Jayakumar, R., Development of novel chitin/nanosilver composite scaffolds for wound dressing applications. *Journal of Materials Science: Materials in Medicine* 2010, 21, 807-813.

6. Quintavalla, S.; Vicini, L., Antimicrobial food packaging in meat industry. *Meat Science* 2002, 62, 373-380.

7. Appendini, P.; Hotchkiss, J. H., Review of antimicrobial food packaging. *Innovative Food Science & Emerging Technologies* 2002, 3, 113-126.

8. Yu, H.-Y.; Xie, Y.-J.; Hu, M.-X.; Wang, J.-L.; Wang, S.-Y.; Xu, Z.-K., Surface modification of polypropylene microporous membrane to improve its antifouling property in MBR: CO2 plasma treatment. *Journal of Membrane Science* 2005, 254, 219-227.

9. Li, Q.; Mahendra, S.; Lyon, D. Y.; Brunet, L.; Liga, M. V.; Li, D.; Alvarez, P. J. J., Antimicrobial nanomaterials for water disinfection and microbial control: Potential applications and implications. *Water Research* 2008, 42, 4591-4602.

10. Tiller, J. C.; Liao, C.-J.; Lewis, K.; Klibanov, A. M., Designing surfaces that kill bacteria on contact. *Proceedings of the National Academy of Sciences* 2001, 98, 5981-5985.

11. Vasilev, K.; Cook, J.; Griesser, H. J., Antibacterial surfaces for biomedical devices. *Expert Rev Med Devices* 2009, 6, 553-67.

12. Banerjee, I.; Pangule, R. C.; Kane, R. S., Antifouling Coatings: Recent Developments in the Design of Surfaces That Prevent Fouling by Proteins, Bacteria, and Marine Organisms. *Advanced Materials* 2011, 23, 690-718.

13. Danese, P. N., Antibiofilm Approaches: Prevention of Catheter Colonization. *Chemistry & Biology* 2002, 9, 873-880.

14. Harris, L. G.; Richards, R. G., Staphylococci and implant surfaces: a review. *Injury* 2006, 37, S3-S14.

15. Ioannou, C. J.; Hanlon, G. W.; Denyer, S. P., Action of disinfectant quaternary ammonium compounds against Staphylococcus aureus. *Antimicrob Agents Chemother* 2007, 51, 296-306.

16. McCubbin, P. J.; Forbes, E.; Gow, M. M.; Gorham, S. D., Covalent attachment of quaternary ammonium compounds to a polyethylene surface via a hydrolyzable ester linkage: Basis for a controlled-release system of antiseptics from an inert surface. *Journal of Applied Polymer Science* 2006, 100, 538-545.

17. Water, J. J.; Bohr, A.; Boetker, J.; Aho, J.; Sandler, N.; Nielsen, H. M.; Rantanen, J., Three-Dimensional Printing of Drug-Eluting Implants: Preparation of an Antimicrobial Polylactide Feedstock Material. *Journal of Pharmaceutical Sciences* 2015, 104, 1099-1107.

18. World Health Organization, Annex Table 2: Deaths by cause, sex and mortality stratum in WHO regions, estimates for 2002. *The world health report* 2004.

19. Poindexter, H.; Washington, T., Microbial opportunism in clinical medicine. *Journal of the National Medical Association* 1974, 66, 284.

20. Lowy, F. D., Staphylococcus aureus infections. *New England Journal of Medicine* 1998, 339, 520-532.

21. Serra, R.; Grande, R.; Butrico, L.; Rossi, A.; Settimio, U. F.; Caroleo, B.; Amato, B.; Gallelli, L.; de Franciscis, S., Chronic wound infections: the role of Pseudomonas aeruginosa and Staphylococcus aureus. *Expert Review of Anti-infective Therapy* 2015, 13, 605-613.

22. Maki, D. G.; Tambyah, P. A., Engineering out the risk for infection with urinary catheters. *Emerging Infectious Diseases* 2001, 7, 342-347.

23. Parsonnet, J.; Friedman, G. D.; Vandersteen, D. P.; Chang, Y.; Vogelman, J. H.; Orentreich, N.; Sibley, R. K., Helicobacter-pylori infection and the risk ofgastric-carcinoma. *New England Journal of Medicine* 1991, 325, 1127-1131.

24. Turnbaugh, P. J.; Ley, R. E.; Mahowald, M. A.; Magrini, V.; Mardis, E. R.; Gordon, J. I., An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006, 444, 1027-131.

25. Progulske-Fox, A.; Kozarov, E.; Dorn, B.; Dunn, W.; Burks, J.; Wu, Y., Porphyromonas gingivalis virulence factors and invasion of cells of the cardiovascular system. *Journal of Periodontal Research* 1999, 34, 393-399.

26. Nikaido, H., Multidrug resistance in bacteria. *Annu Rev Biochem* 2009, 78, 119-46.

27. Mah, T.-F.; Pitts, B.; Pellock, B.; Walker, G. C.; Stewart, P. S.; O'Toole, G. A., A genetic basis for Pseudomonas aeruginosa biofilm antibiotic resistance. *Nature* 2003, 426, 306-310.

28. Fisher, J. F.; Meroueh, S. O.; Mobashery, S., Bacterial resistance to β-lactam antibiotics: compelling opportunism, compelling opportunity. *Chemical Reviews* 2005, 105, 395-424.

29. Noimark, S.; Dunnill, C. W.; Wilson, M.; Parkin, I. P., The role of surfaces in catheter-associated infections. *Chemical Society Reviews* 2009, 38, 3435-3448.

30. Gould, I. M., Antibiotics, skin and soft tissue infection and meticillin-resistant Staphylococcus aureus: cause and effect. *International Journal of Antimicrobial Agents* 2009, 34, S8-S11.

31. Men, T. Y.; Wang, J. N.; Li, H.; Gu, Y.; Xing, T. H.; Peng, Z. H.; Zhong, L., Prevalence of multidrug-resistant gram-negative bacilli producing extended-spectrum β-lactamases (ESBLs) and ESBL genes in solid organ transplant recipients. *Transplant Infectious Disease* 2013, 15, 14-21.

32. Darouiche, R. O., Current concepts - Treatment of infections associated with surgical implants. *New England Journal of Medicine* 2004, 350, 1422-1429.

33. Trautner, B. W.; Darouiche, R. O., Catheter-associated infections - Pathogenesis affects prevention. *Archives of Internal Medicine* 2004, 164, 842-850.

34. Halton, K.; Graves, N., Economic evaluation and catheter-related bloodstream infections. *Emerging Infectious Diseases* 2007, 13, 815-823.

35. Bjarnsholt, T.; Kirketerp-Møller, K.; Jensen, P. Ø.; Madsen, K. G.; Phipps, R.; Krogfelt, K.; Høiby, N.; Givskov, M., Why chronic wounds will not heal: a novel hypothesis. *Wound Repair and Regeneration* 2008, 16, 1. Qureshi, A. T.; Terrell, L.; Monroe, W. T.; Dasa, V.; Janes, M. E.; Gimble, J. M.; Hayes, D. J., Antimicrobial biocompatible bioscaffolds for orthopaedic implants. Journal of Tissue Engineering and Regenerative Medicine 2012, n/a-n/a.

36. Graves, N.; Halton, K.; Paterson, D.; Whitby, M., Economic rationale for infection control in Australian hospitals. Healthcare Infection 2009, 14, 81–88.

37. Costerton, J. W.; Lewandowski, Z.; Caldwell, D. E.; Korber, D. R.; Lappin-Scott, H. M., Microbial biofilms. Annual Reviews in Microbiology 1995, 49, 711-745.

38. Potera, C., Studying slime. Environmental health perspectives 1998, 106, A604.

39. Bayles, K. W., The biological role of death and lysis in biofilm development. Nature Reviews Microbiology 2007, 5, 721-726.

40. Decker, R.; Burdelski, C.; Zobiak, M.; Büttner, H.; Franke, G.; Christner, M.; Saß, K.; Zobiak, B.; Henke, H. A.; Horswill, A. R.; Bischoff, M.; Bur, S.; Hartmann, T.; Schaeffer, C. R.; Fey, P. D.; Rohde, H., An 18 kDa Scaffold Protein Is Critical for *Staphylococcus epidermidis.* Biofilm Formation. PLoS Pathog 2015, 11, e1004735.

41. Ferreira, C.; Pereira, A. M.; Pereira, M. C.; Melo, L. F.; Simões, M., Physiological changes induced by the quaternary ammonium compound benzyldimethyldodecylammonium chloride on Pseudomonas fluorescens. Journal of Antimicrobial Chemotherapy 2011.

42. Isquith, A. J.; Abbott, E. A.; Walters, P. A., Surface-bonded antimicrobial activity of an organosilicon quaternary ammonium chloride. Appl Microbiol 1972, 24, 859-63.

43. Rahn, O.; Van Eseltine, W. P., Quaternary Ammonium Compounds. Annual Review of Microbiology 1947, 1, 173-192.

44. Chaidez, C.; Lopez, J.; Castro-del Campo, N., Quaternary ammonium compounds: an alternative disinfection method for fresh produce wash water. J Water Health 2007, 5, 329-33.

45. Murata, H.; Koepsel, R. R.; Matyjaszewski, K.; Russell, A. J., Permanent, non-leaching antibacterial surfaces--2: How high density cationic surfaces kill bacterial cells. Biomaterials 2007, 28, 4870-4879.

46. Kügler, R.; Bouloussa, O.; Rondelez, F., Evidence of a charge-density threshold for optimum efficiency of biocidal cationic surfaces. Microbiology 2005, 151, 1341-1348.

47. Terada, A.; Yuasa, A.; Kushimoto, T.; Tsuneda, S.; Katakai, A.; Tamada, M., Bacterial adhesion to and viability on positively charged polymer surfaces. Microbiology 2006, 152, 3575-3583.

48. Simchi, A.; Tamjid, E.; Pishbin, F.; Boccaccini, A. R., Recent progress in inorganic and composite coatings with bactericidal capability for orthopaedic applications. Nanomedicine: Nanotechnology, Biology and Medicine 2011, 7, 22-39.

49. Wu, P.; Grainger, D. W., Drug/device combinations for local drug therapies and infection prophylaxis. Biomaterials 2006, 27, 2450-2467.

50. Palchesko, R. N.; McGowan, K. A.; Gawalt, E. S., Surface immobilization of active vancomycin on calcium aluminum oxide. Materials Science and Engineering: C 2011, 31, 637-642.

51. Bastari, K.; Arshath, M.; Ng, Z.; Chia, J.; Yow, Z.; Sana, B.; Tan, M.; Lim, S.; Loo, S., A controlled release of antibiotics from calcium phosphate-coated poly(lactic-co-glycolic acid) particles and their in vitro efficacy against Staphylococcus aureus biofilm. Journal of Materials Science: Materials in Medicine 2014, 25, 747-757.

52. Yasuda, H., Plasma polymerization. Academic press: 1985.

53. Yasuda, H.; Gazicki, M., Biomedical applications of plasma polymerization and plasma treatment of polymer surfaces. Biomaterials 1982, 3, 68-77.

54. von Keudell, A.; Benedikt, J., A Physicist's Perspective on “Views on Macroscopic Kinetics of Plasma Polymerisation”. Plasma Processes and Polymers 2010, 7, 376-379.

55. Vasilev, K.; Griesser, S. S.; Griesser, H. J., Antibacterial Surfaces and Coatings Produced by Plasma Techniques. Plasma Processes and Polymers 2011, 8, 1010-1023.

56. Yasuda, H.; Hirotsu, T.; Olf, H. G., Polymerization of organic compounds in an electrodeless glow discharge. X. Internal stress in plasma polymers. Journal of Applied Polymer Science 1977, 21, 3179-3184.

57. Gleason, K. K., A Chemical Engineering Perspective on "Views on Macroscopic Kinetics of Plasma Polymerisation". Plasma Processes and Polymers 2010, 7, 380-381.

58. Taheri, S.; Cavallaro, A.; Christo, S. N.; Smith, L. E.; Majewski, P.; Barton, M.; Hayball, J. D.; Vasilev, K., Substrate independent silver nanoparticle based antibacterial coatings. Biomaterials 2014, 35, 4601-4609.

59. ISO 22196:2007 Measurement of antibacterial activity on plastics surfaces. International Organisation for Standardisation: ISO 22196:2007.

60. Test For Antimicrobial Activity and Efficacy of Textile Products. Japanese Industry Standards Committee: JIS L 1902:2008.

61. Standard Test Method for Determining the Antimicrobial Activity of Immobilized Antimicrobial Agents Under Dynamic Contact Conditions. American Society for Testing and Materials: ASTM E2149-10.

62. Schierholz, J.; Jansen, B.; Jaenicke, L.; Pulverer, G., In-vitro efficacy of an antibiotic releasing silicone ventricle catheter to prevent shunt infection. *Biomaterials* 1994, 15, 996-1000.

63. van de Belt, H.; Neut, D.; Uges, D. R. A.; Schenk, W.; van Horn, J. R.; van der Mei, H. C.; Busscher, H. J., Surface roughness, porosity and wettability of gentamicin-loaded bone cements and their antibiotic release. *Biomaterials* 2000, 21, 1981-1987.

64. Wang, R.; Neoh, K. G.; Kang, E.-T.; Tambyah, P. A.; Chiong, E., Antifouling coating with controllable and sustained silver release for long-term inhibition of infection and encrustation in urinary catheters. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 2015, 103, 519-528.

65. Roe, D.; Karandikar, B.; Bonn-Savage, N.; Gibbins, B.; Roullet, J.-B., Antimicrobial surface functionalization of plastic catheters by silver nanoparticles. *Journal of Antimicrobial Chemotherapy* 2008, 61, 869-876.

66. Bazaka, K.; Jacob, M. V.; Chrzanowski, W.; Ostrikov, K., Anti-bacterial surfaces: natural agents, mechanisms of action, and plasma surface modification. *Rsc Advances* 2015, 5, 48739-48759.

67. Zanini, S.; Müller, M.; Riccardi, C.; Orlandi, M., Polyethylene Glycol Grafting on Polypropylene Membranes for Anti-fouling Properties. *Plasma Chem Plasma Process* 2007, 27, 446-457.

68. Dong, B.; Manolache, S.; Somers, E. B.; Lee Wong, A. C.; Denes, F. S., Generation of antifouling layers on stainless steel surfaces by plasma-enhanced

crosslinking of polyethylene glycol. *Journal of Applied Polymer Science* 2005, 97, 485-497.

69. Konradi, R.; Pidhatika, B.; Mühlebach, A.; Textor, M., Poly-2-methyl-2-oxazoline: A Peptide-like Polymer for Protein-Repellent Surfaces. *Langmuir* 2008, 24, 613-616.

70. Ramiasa, M.; Cavallaro, A.; Mierczynska-Vasilev, A.; Christo, S.; Gleadle, J.; Hayball, J. D.; Vasilev, K., Plasma Polymerised PolyOxazoline Thin Films for Biomedical Applications. *Chemical Communications* 2015, 51, 4297-4282.

71. Costa, F.; Carvalho, I. F.; Montelaro, R. C.; Gomes, P.; Martins, M. C. L., Covalent immobilization of antimicrobial peptides (AMPs) onto biomaterial surfaces. *Acta Biomaterialia* 2011, 7, 1431-1440.

72. Murata, H.; Koepsel, R. R.; Matyjaszewski, K.; Russell, A. J., Permanent, non-leaching antibacterial surfaces--2: How high density cationic surfaces kill bacterial cells. *Biomaterials* 2007, 28, 4870-4879.

73. Cavallaro, A.; Majewski, P.; Barton, M.; Vasilev, K., Substrate independent approach for immobilisation of quaternary ammonium compounds to surfaces to reduce bio-burden. *Materials Science Forum* 2014, 783-786, 1389-1395.

74. Tiller, J. C.; Liao, C.-J.; Lewis, K.; Klibanov, A. M., Designing surfaces that kill bacteria on contact. *Proceedings of the National Academy of Sciences* 2001, 98, 5981-5985.

75. Antimicrobial material for applying to surface of hospital buildings and medical equipments, contains hardened composition containing mixture of cement, and polymer emulsion dispersed with ferrous oxide. JP2005120040-A, 2005.

76. Antibacterial and scratch-proof surface coating for goods that frequently or constantly come in contact with human skin, contains anti-bacterial, germ-killing, blood-coagulating nano-particles and hard nano-particles. DE202007002036-U1, 2007.

77. Bromberg, L. E.; Chen, L.; Hatton, T. A.; Rutledge, G. C. Antimicrobial fiber having diameter useful for making article e.g. nanocomposite, sensor and filtration membrane, comprises electroprocessed blend of polymer, antimicrobial agent and cross-linker. WO2009064767-A2; WO2009064767-A3; US2010285081-A1, 2009.

78. Kügler, R.; Bouloussa, O.; Rondelez, F., Evidence of a charge-density threshold for optimum efficiency of biocidal cationic surfaces. *Microbiology* 2005, 151, 1341-1348.

79. Haynie, S. L.; Crum, G. A.; Doele, B. A., Antimicrobial activities of amphiphilic peptides covalently bonded to a water-insoluble resin. *Antimicrobial Agents and Chemotherapy* 1995, 39, 301-307.

80. Palchesko, R. N.; McGowan, K. A.; Gawalt, E. S., Surface immobilization of active vancomycin on calcium aluminum oxide. *Materials Science and Engineering: C* 2011, 31, 637-642.

81. Stigter, M.; Bezemer, J.; de Groot, K.; Layrolle, P., Incorporation of different antibiotics into carbonated hydroxyapatite coatings on titanium implants, release and antibiotic efficacy. *Journal of Controlled Release* 2004, 99, 127-137.

82. Şen, M.; Yakar, A., Controlled release of antifungal drug terbinafine hydrochloride from poly(N-vinyl 2-pyrrolidone/itaconic acid) hydrogels. *International Journal of Pharmaceutics* 2001, 228, 33-41.

83. Jiang, S. Q.; Newton, E.; Yuen, C. W. M.; Kan, C. W., Chemical silver plating and its application to textile fabric design. *Journal of Applied Polymer Science* 2005, 96, 919-926.

84. Carrillo-Carrión, C.; Nazarenus, M.; Paradinas, S. S.; Carregal-Romero, S.; Almendral, M. J.; Fuentes, M.; Pelaz, B.; del Pino, P.; Hussain, I.; Clift, M. J. D.; Rothen-Rutishauser, B.; Liang, X.-J.; Parak, W. J., Metal ions in the context of nanoparticles toward biological applications. *Current Opinion in Chemical Engineering* 2014, 4, 88-96.

85. Mceldowney, S.; Fletcher, M., Effect of Growth Conditions and Surface Characteristics of Aquatic Bacteria on Their Attachment to Solid Surfaces. *Journal of General Microbiology* 1986, 132, 513-523.

86. Stepanović, S.; Vuković, D.; Ježek, P.; Pavlović, M.; Švabic-Vlahović, M., Influence of Dynamic Conditions on Biofilm Formation by Staphylococci. *EJCMID* 2001, 20, 502-504.

87. Cavallaro, A.; Taheri, S.; Vasilev, K., Responsive and “smart” antibacterial surfaces: Common approaches and new developments. *Biointerphases* 2014, 9, 029005.

88. Komnatnyy, V. V.; Chiang, W.-C.; Tolker-Nielsen, T.; Givskov, M.; Nielsen, T. E., Bacteria-Triggered Release of Antimicrobial Agents. *Angewandte Chemie* 2014, 126, 449-451.

89. Chen, J.-K.; Chang, C.-J., Fabrications and Applications of Stimulus-Responsive Polymer Films and Patterns on Surfaces: A Review. *Materials* 2014, 7, 805-875.

90. Caubet, R.; Pedarros-Caubet, F.; Chu, M.; Freye, E.; de Belém Rodrigues, M.; Moreau, J. M.; Ellison, W. J., A Radio Frequency Electric Current Enhances Antibiotic Efficacy against Bacterial Biofilms. *Antimicrobial Agents and Chemotherapy* 2004, 48, 4662-4664.

91. Costerton, J. W.; Ellis, B.; Lam, K.; Johnson, F.; Khoury, A. E., Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria. *Antimicrobial Agents and Chemotherapy* 1994, 38, 2803-2809.

92. Delcour, A. H., Outer membrane permeability and antibiotic resistance. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 2009, 1794, 808-816.

93. Zhou, J.; Loftus, A. L.; Mulley, G.; Jenkins, A. T. A., A Thin Film Detection/Response System for Pathogenic Bacteria. *Journal of the American Chemical Society* 2010, 132, 6566-6570.

94. Naparstek, L.; Carmeli, Y.; Navon-Venezia, S.; Banin, E., Biofilm formation and susceptibility to gentamicin and colistin of extremely drug-resistant KPC-producing Klebsiella pneumoniae. *Journal of Antimicrobial Chemotherapy* 2014.

95. Nicklin, J.; Graeme-Cook, K.; Killington, R., *Microbiology*. 2nd ed.; BIOS Scientific: 2002.

97. Yu, L.; Zhang, Y.; Zhang, B.; Liu, J., Enhanced Antibacterial Activity of Silver Nanoparticles/Halloysite Nanotubes/Graphene Nanocomposites with Sandwich-Like Structure. *Sci. Rep.* 2014, 4.

98. Andrews, J. M., Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 2001, 48 Suppl 1, 5-16.

99. Unnithan, A. R.; Ramachandra Kurup Sasikala, A.; Sathishkumar, Y.; Lee, Y. S.; Park, C. H.; Kim, C. S., Nanoceria doped electrospun antibacterial composite mats for potential biomedical applications. *Ceramics International* 2014.

100. Hoben, H. J.; Somasegaran, P., Comparison of the Pour, Spread, and Drop Plate Methods for Enumeration of Rhizobium spp. in Inoculants Made from Presterilized Peat. *Applied and Environmental Microbiology* 1982, 44, 1246-1247.

101. Buck, J. D.; Cleverdon, R. C., The spread plate as a method for the enumeration of marine bacteria. *Limnol. Oceanogr* 1960, 5, 78-80.

102. American Society for Testing and Materials, ASTM E2149-10 Standard Test Method for Determining the Antimicrobial Activity of Immobilized Antimicrobial Agents Under Dynamic Contact Conditions. 2010.

103. Payne, J. L.; Ambrosio, A. M., Evaluation of an antimicrobial silver foam dressing for use with V.A.C.® therapy: Morphological, mechanical, and antimicrobial properties. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 2009, 89B, 217-222.

104. Wei, X.; Yang, Z.; Wang, Y.; Tay, S.; Gao, W., Polymer antimicrobial coatings with embedded fine Cu and Cu salt particles. *Applied Microbiology and Biotechnology* 2014, 1-10.

105. Kurt, P.; Wood, L.; Ohman, D. E.; Wynne, K. J., Highly Effective Contact Antimicrobial Surfaces via Polymer Surface Modifiers. *Langmuir* 2007, 23, 4719-4723.

106. Borchardt, K. A.; Andrews, D. H., Comparison of tube dilution and disc (agar diffusion) methods for testing microbial susceptibility to antibiotics. *Antibiotics & chemotherapy* 1959, 9, 596-9.

107. Cooper, K. E.; Woodman, D., The diffusion of antiseptics through agar gels, with special reference to the agar cup assay method of estimating the activity of penicillin. *The Journal of pathology and bacteriology* 1946, 58, 75-84.

108. Dye, W. E., An agar diffusion method for studying the bacteriostatic action of combinations of antimicrobial agents. *Antibiotics annual* 1955, 3, 374-82.

109. Stansly, P. G.; Schlosser, M. E., Studies on Polymyxin: An Agar Diffusion Method of Assay. *Journal of bacteriology* 1947, 54, 585-97.

110. Taber, W. A., An agar diffusion assay method for the antifungal polyene, candidin. *Canadian journal of microbiology* 1956, 2, 45-55.

111. Green, J.-B. D.; Fulghum, T.; Nordhaus, M. A., Review of immobilized antimicrobial agents and methods for testing. *Biointerphases* 2011, 6, MR13-MR28.

112. Kenawy, E.-R.; Worley, S. D.; Broughton, R., The Chemistry and Applications of Antimicrobial Polymers: A State-of-the-Art Review. *Biomacromolecules* 2007, 8, 1359-1384.

113. Lin, C.-C.; Metters, A. T., Hydrogels in controlled release formulations: Network design and mathematical modeling. *Advanced Drug Delivery Reviews* 2006, 58, 1379-1408.

114. Zhao, L.; Chu, P. K.; Zhang, Y.; Wu, Z., Antibacterial coatings on titanium implants. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 2009, 91B, 470-480.

115. Kittinger, C.; Marth, E.; Windhager, R.; Weinberg, A.; Zarfel, G.; Baumert, R.; Felisch, S.; Kuehn, K.-D., Antimicrobial activity of gentamicin palmitate against high concentrations of Staphylococcus aureus. *Journal of Materials Science: Materials in Medicine* 2011, 22, 1447-1453.

116. Stigter, M.; de Groot, K.; Layrolle, P., Incorporation of tobramycin into biomimetic hydroxyapatite coating on titanium. *Biomaterials* 2002, 23, 4143-4153.

117. Swanson, T. E.; Cheng, X.; Friedrich, C., Development of chitosan–vancomycin antimicrobial coatings on titanium implants. *Journal of Biomedical Materials Research Part A* 2011, 97A, 167-176.

118. Braceras, I.; Azpiroz, P.; Briz, N.; Fratila, R. M.; Oyarbide, J.; Ipiñazar, E.; Álvarez, N.; Atorrasagasti, G.; Aizpurua, J. M., Plasma Polymerized Silylated Ciprofloxacin as an Antibiotic Coating. *Plasma Processes and Polymers* 2011, 8, 599-606.

119. O'Toole, G.; Kaplan, H. B.; Kolter, R., Biofilm formation as microbial development. *Annual Review of Microbiology* 2000, 54, 49-79.

120. Seidler, M.; Salvenmoser, S.; Müller, F.-M. C., In vitro effects of micafungin against Candida biofilms on polystyrene and central venous catheter sections. *International Journal of Antimicrobial Agents* 2006, 28, 568-573.

121. Reynolds, T. B.; Fink, G. R., Bakers' Yeast, a Model for Fungal Biofilm Formation. *Science* 2001, 291, 878-881.

122. Bonnekoh, B.; Wevers, A.; Jugert, F.; Merk, H.; Mahrle, G., Colorimetric growth assay for epidermal cell cultures by their crystal violet binding capacity. *Arch Dermatol Res* 1989, 281, 487-490.

124. Stepanović, S.; Vuković, D.; Dakić, I.; Savić, B.; Švabić-Vlahović, M., A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods* 2000, 40, 175-179.

125. Jackson, D. W.; Suzuki, K.; Oakford, L.; Simecka, J. W.; Hart, M. E.; Romeo, T., Biofilm Formation and Dispersal under the Influence of the Global Regulator CsrA of Escherichia coli. *Journal of bacteriology* 2002, 184, 290-301.

126. Alexander, S.-A.; Rouse, E. M.; White, J. M.; Tse, N.; Kyi, C.; Schiesser, C. H., Controlling biofilms on cultural materials: the role of 3-(dodecane-1-thiyl)-4-(hydroxymethyl)-2,2,5,5-tetramethyl-1-pyrrolinoxyl. *Chemical Communications* 2015, 51, 3355-3358.

127. Taheri, S.; Cavallaro, A.; Christo, S. N.; Smith, L. E.; Majewski, P.; Barton, M.; Hayball, J. D.; Vasilev, K., Substrate independent silver nanoparticle based antibacterial coatings. *Biomaterials* 2014, 35, 4601-4609.

128. Ferreira, C.; Pereira, A. M.; Pereira, M. C.; Melo, L. F.; Simões, M., Physiological changes induced by the quaternary ammonium compound benzyldimethyldodecylammonium chloride on Pseudomonas fluorescens. *Journal of Antimicrobial Chemotherapy* 2011.

129. Khan, M. M. T.; Pyle, B. H.; Camper, A. K., Specific and Rapid Enumeration of Viable but Nonculturable and Viable-Culturable Gram-Negative Bacteria by Using Flow Cytometry. *Applied and Environmental Microbiology* 2010, 76, 5088-5096.

130. Stocks, S. M., Mechanism and use of the commercially available viability stain, BacLight. *Cytometry Part A* 2004, 61A, 189-195.

131. Virta, M.; Lineri, S.; Kankaanpää, P.; Karp, M.; Peltonen, K.; Nuutila, J.; Lilius, E.-M., Determination of Complement-Mediated Killing of Bacteria by Viability Staining and Bioluminescence. *Applied and Environmental Microbiology* 1998, 64, 515-519.

132. Squirrell, D. J.; Price, R. L.; Murphy, M. J., Rapid and specific detection of bacteria using bioluminescence. *Analytica Chimica Acta* 2002, 457, 109-114.

133. Green, J.-B. D.; Bickner, S.; Carter, P. W.; Fulghum, T.; Luebke, M.; Nordhaus, M. A.; Strathmann, S., Antimicrobial testing for surface-immobilized agents with a surface-separated live–dead staining method. *Biotechnology and Bioengineering* 2011, 108, 231-236.

134. Ramage, G.; Vande Walle, K.; Wickes, B. L.; López-Ribot, J. L., Standardized Method for In Vitro Antifungal Susceptibility Testing of Candida albicansBiofilms. *Antimicrobial Agents and Chemotherapy* 2001, 45, 2475-2479.

135. Vanitha, J. D.; Paramasivan, C. N., Evaluation of microplate Alamar blue assay for drug susceptibility testing of Mycobacterium avium complex isolates. *Diagnostic Microbiology and Infectious Disease* 2004, 49, 179-182.

136. Palomino, J.-C.; Martin, A.; Camacho, M.; Guerra, H.; Swings, J.; Portaels, F., Resazurin Microtiter Assay Plate: Simple and Inexpensive Method for Detection of

Drug Resistance in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy* 2002, 46, 2720-2722.

137. Maki, D. G.; Weise, C. E.; Sarafin, H. W., A Semiquantitative Culture Method for Identifying Intravenous-Catheter-Related Infection. *New England Journal of Medicine* 1977, 296, 1305-1309.

138. Jones, C. F.; Grainger, D. W., In vitro assessments of nanomaterial toxicity. *Advanced Drug Delivery Reviews* 2009, 61, 438-456.

139. Landsiedel, R.; Kapp, M. D.; Schulz, M.; Wiench, K.; Oesch, F., Genotoxicity investigations on nanomaterials: Methods, preparation and characterization of test material, potential artifacts and limitations--Many questions, some answers. *Mutation Research/Reviews in Mutation Research* 2009, 681, 241-258.

140. Freshney, R. I., *Culture of animal cells : a manual of basic technique; fifth edition*. 5 ed.; New York : Wiley-Liss: 2005.

141. Zucco, F.; De Angelis, I.; Testai, E.; Stammati, A., Toxicology investigations with cell culture systems: 20 years after. *Toxicology in Vitro* 2004, 18, 153-163.

# 2 Materials and Methods

## 2.1 Materials

Silicon wafers were used for thickness measurements and surface characterisation. 1mm thick 13mm diameter glass coverslips and 13 mm Thermanox coverslips (Nunc) were used as sample substrates for antimicrobial testing. Allylamine (98%), 1,7-octadiene, propionaldehyde, 2-methyl-2-oxazoline, 2-ethyl-2-oxazoline, carbonyl iron, LPS (from Escherichia coli 0111:B4), Polymyxin B solution, ampicillin, vancomycin and Glycidyltrimethylammonium chloride (GTAC; technical grade ≥90%) were purchased from Sigma-Aldrich Austrlia. *Escherichia coli* (ATCC 25922 and SCC1), *Staphylococcus Epidermidis* (ATCC 35984) were used as model organisms of infection. These bacteria were chosen as they are representative of both a Gram-positive and Gram-negative bacteria and were clinically isolated. Nutrient agar, Cold Filterable Tryptocase Soy Broth (TSB), Lysogeny broth (LB) and Lysogeny agar (LA) were purchased from Oxoid Australia and prepared as per manufacturer's instructions.

M63 media was used as a slow growth medium for confocal studies and was prepared to standard protocols: 13.6g $KH_2PO_4$, 2g $(NH_4)_2SO_4$, 0.5mL 0.1% $FeSO_4$ and 0.2g $MgSO_4$ were dissolved per 1L of water and the solution was raised to a pH 6.8 with KOH. The autoclaved solution was supplemented with vitamin B1 and glucose to promote bacterial growth.

Complete fibroblast culture media was prepared by supplementing Dulbecco's Modified Eagle Medium (DMEM; purchased from Life Technologies) with 10% (v/v) foetal bovine serum (Atlas Biologicals), 100 $IU.ml^{-1}$ penicillin, 100 $mg.ml^{-1}$

streptomycin, 0.625 $mg.ml^{-1}$ amphotericin B (Sigma-Aldrich Australia). Resazurin sodium salt was purchased from Sigma-Aldrich Australia.

## 2.2 Methods

### 2.2.1 Sample Preparation

#### *2.2.1.1 Plasma Deposition*

The plasma deposition process allows for the formation of polymeric films of desired chemistry as discussed in Section 1.5. Current plasma deposition technologies can provide a convenient single-step method to deposit films of controllable thicknesses (usually in the nm range) on any substrate regardless of underlying chemistry.[1, 2] Plasma polymerisation was performed in a purpose built deposition chamber, or plasma reactor, based on a previous design.[3, 4] As depicted in Figure 2.1, this plasma reactor consists of a glass vacuum chamber (body) with an acrylic top (head) and bottom (base). The reactor is designed with a "U-Shaped" RF cathode and flat plate anode. The base of the reactor has a single outlet connected to a vacuum pump and an optional inlet for additional accessories such as the attachment of a DC line for the shaking unit (Figure 2.1b).[5] A Pirani gauge is used for measuring pressure and calculating flow rate measurements. The head has a single precursor inlet where flow is controlled via a needle valve. Specific deposition chambers were used for different precursors to ensure reproducibility of deposited surfaces.

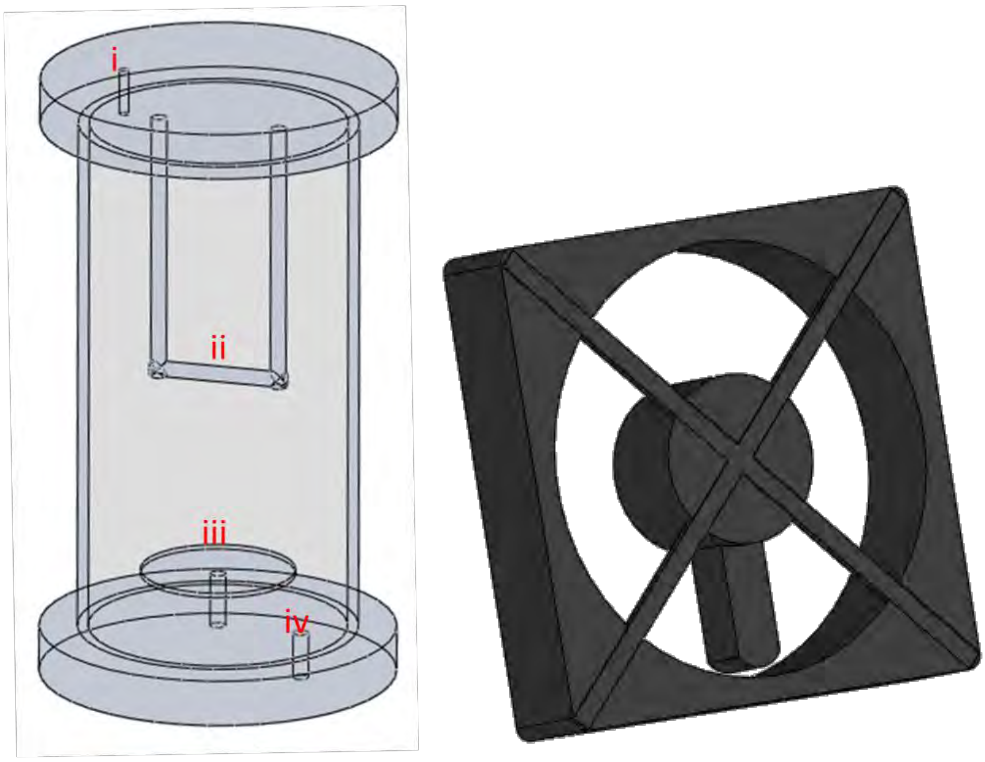


Figure 2.1 a) Schematic of plasma reactor setup i) monomer inlet ii) U-shaped cathode iii) circular plate (anode) iv) vacuum port. b) Schematic of shaking platform consisting of mount and off-centre weighted motor.

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Precursor flow into the chamber is controlled via the use of a valve. Gas within the chamber is ionised using a controlled radio frequency (RF) from a Coaxial Power Systems 13.56MHz RF generator. All impedance was matched using an automatic matching network to control energy input into the system. All planar samples were washed with acetone and ethanol and dried with nitrogen sequentially and further cleaned with air plasma ($1.0x10^{-2}$ mbar 50W) within the reactor for 3 minutes prior to deposition. The different precursors used in this work are outlined in Figure 2.2.

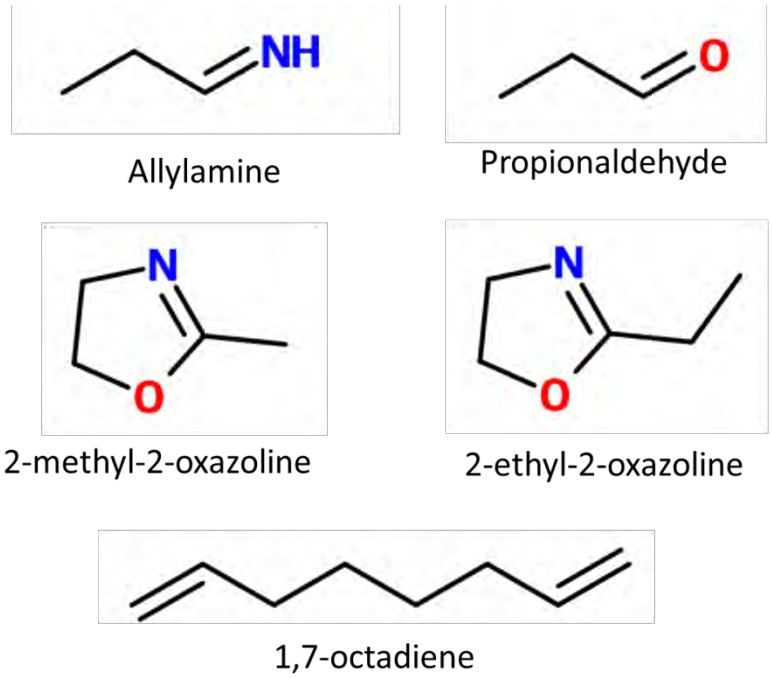


Figure 2.2 Selected precursors for plasma deposition including allylamine, propionaldehyde, 2.methyl-2-oxazoline, 2-ethyl-2-oxazoline and 1,7-octadiene.

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**Plasma Polymerised Allylamine (ppAA)**

ppAA was chosen as a model polymer due to its high primary amine content. For deposition of allylamine, the chamber was brought to a vacuum ($2x10^{-2}$ mbar) and allylamine vapour introduced to the chamber until a steady pressure of $2.1x10^{-1}$ mbar was achieved. A 40W RF was introduced to the chamber for 2 minutes.

**Plasma Polymerised Oxazolines (ppOx)**

ppOx was chosen as a model polymer due to its specific chemistry and ability to bind carboxylic acids. When the chamber reached appropriate base pressure ($3.5x10^{-2}$ mbar) the oxazoline monomer (2-methyl-2-oxazoline or 2-ethyl-2-oxazoline) was introduced via a needle valve until steady working flow rates were achieved. Plasma was ignited using various radio frequency (RF) powers in both continuous wave (CW) and pulsed plasma modes for selected times.

**Plasma Polymerised Aldehyde (ppA)**

ppA was chosen as a model polymer due to its high primary aldehyde content. To deposit propionaldehdye the plasma chamber was pumped down to a base vacuum pressure of $2x10^{-2}$ mbar and the liquid precursor (propionaldehyde) was introduced into the chamber until a steady pressure of $2.1x10^{-1}$ mbar was achieved. A 40W RF was used to induce plasma and total deposition time was limited to 2 minutes.

**Plasma Polymerised Octadiene (ppOD)**

ppAA was chosen as a model polymer for drug release as due to its hydrophobicity. To deposit octadiene the chamber was brought to a base pressure of $3x10^{-2}$ mbar and 1,7-octadiene flowed into the chamber until a steady pressure of $2.1x10^{-1}$ mbar was obtained. Plasma was induced using a 50W for 5, 10 and 20 minutes.

#### *2.2.1.2 Deposition on powdered substrates*

Plasma deposition directly onto the surface of powders has been performed for various applications.[6, 7] In this work a secondary reactor configuration containing a custom designed shaking unit mounted to the anode was used to coat powders. This configuration utilises an additional port as a direct current (DC) power supply. Dry powders were coated by using a shaking platform composed of a centre motor with DC voltage speed dependence and an offset weight (Figure 2.1b) to induce a random shaking motion.[5] All powders were used as purchased, brought to room temperature and placed on a Petri-dish attached to the platform, without any prior modification to the powder.

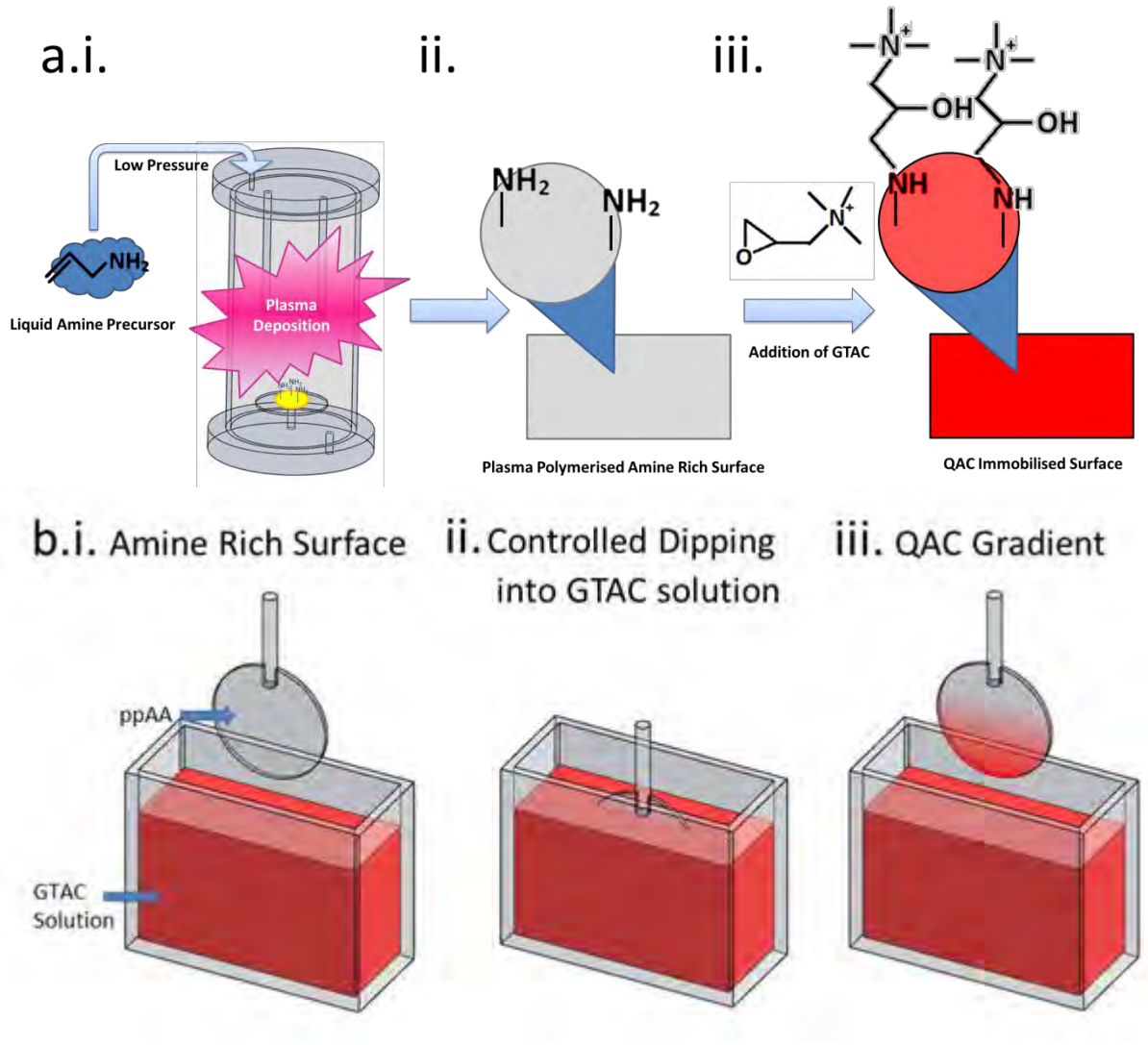
To deposit octadiene the chamber was brought to a base pressure of $3x10^{-2}$ mbar and 1,7-octadiene added to the chamber until a steady pressure of $2.1x10^{-1}$ mbar was obtained. Plasma was induced using a 50W for various times.

After deposition precursor flows were stopped and the chamber returned to base pressure to remove any unreacted precursor. The chamber was vented and Petri-dish containing coated powders removed.

#### *2.2.1.3 Chemical modification of plasma polymers*

**Covalent grafting of Glycidyltrimethylammonium chloride (GTAC)**

Surface immobilisation of GTAC was carried out as depicted in Scheme 2.1. Briefly, GTAC was immobilised to plasma polymerised allylamine (ppAA). All substrates were cleaned sequentially with acetone and ethanol, and dried with nitrogen prior to plasma deposition. Allylamine was deposited at a pressure of $2.1x10^{-1}$ mbar and a power of 40W for 2 minutes in the aforementioned plasma reactor. GTAC was covalently bound to the plasma deposited amine rich coatings from a 1 w% GTAC aqueous solution. The solution pH was adjusted to 10 by KOH. Samples were vigorously washed in MilliQ water to remove any unattached GTAC and excess KOH and dried with a stream of nitrogen.




Scheme 2.1 a) Schematic representation of plasma deposition of allylamine (i.) for generation of ppAA coated surfaces (ii.), immoiblisation of GTAC to primary amine groups for generation of homogenous ppAA+GTAC surfaces (iii). b) Amine rich surfaces (i.) are dip coated into GTAC solution (ii.) to generate QAC concentraiton gradients (iii.).

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Concentration gradients of GTAC were created based on controlling the duration of solution\surface contact.[8] A Zaber T-LSR linear motion drive was utilised to electronically control the dip rate of ppAA modified substrate into a GTAC solution as

prepared above. Samples were dipped at a continuous speed of 1 mm.h$^{-1}$ along the z-axis (Scheme 2.1b).

### Covalent binding of Acetic acid

Organic acids are known to bind covalently to oxazoline groups.[9] To mask any retained oxazoline groups on the surfaces, the plasma deposited oxazoline samples were soaked in 0.1M acetic acid for 18h. This allows for the covalent binding of the acid to the available oxazoline rings as a means to mask the surface.[10]

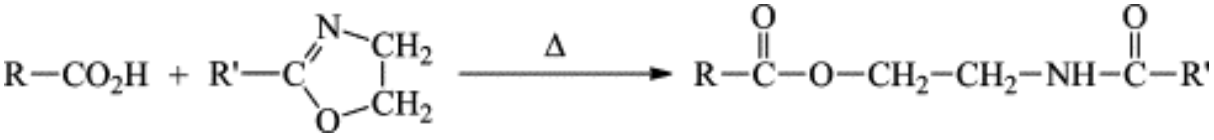

Figure 2.3 Reaction of carboxylic acids and oxazolines. Reproduced with permission from <Tillet, G., B. Boutevin, et al. (2011). "Chemical reactions of polymer crosslinking and post-crosslinking at room and medium temperature." Progress in Polymer Science 36(2): 191-217.> [9] Copyright 2011 Elsevier.

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### Attachment of Polymyxin

PMX was diluted to 20ug\mL in MilliQ filtered water and ppA surfaces submerged in the solution for various times. Samples were washed vigorously with MilliQ filtered water to remove any unattached PMX and dried with a stream of nitrogen.

## 2.2.2 Surface Characterisation

Here, surfaces were characterised in terms of chemistry, via X-ray Photoelectron Spectroscopy (XPS), surface charge, via Atomic Force Microscopy (AFM), and\or

hydrophobicity, by determining the water contact angle (WCA) of the films, as complementary techniques. This was used to give an indication of the relationships between the properties of the surfaces and their antimicrobial activity.

#### *2.2.2.1 XPS*

XPS is a powerful surface characterisation technique that utilises X-rays of 10-15 keV to irradiate a sample and a detector to measure the flux of electrons leaving the surface. Typically monochomatic X-rays, generated from an Aluminium or Magnesium anode, bombard a test sample and based on the kinetic energy of the X-ray and the binding energy of the electrons, energy is transferred to core-level electrons within the atoms of the substrate. A plot of the count of electrons emitted from the surface and the binding energies of these generates a XPS spectrum. Peaks within this spectrum correlate to electron configurations within atoms e.g., 1*s*, 2*s*, 2*p*, 3*s*, etc.

As elements produce specific XPS peaks at certain binding energies the spectrum of binding energies and number of electrons detected can be used to determine the atomic composition of materials. However, in order to calculate atomic percentages of elements, the signal (total number of electrons detected) must be normalised, by dividing by the Relative Sensitivity Factor (RSF) of each element, for the individual instrument. High resolution data of this type can also be used to characterise the oxidation states of elements as binding energies of electrons increase as the oxidation state increases. XPS has been commonly used for analysis of plasma deposited films.[10] A typical high resolution C1s spectrum for plasma deposited 2-methyl oxazoline is shown in Figure 2.4.

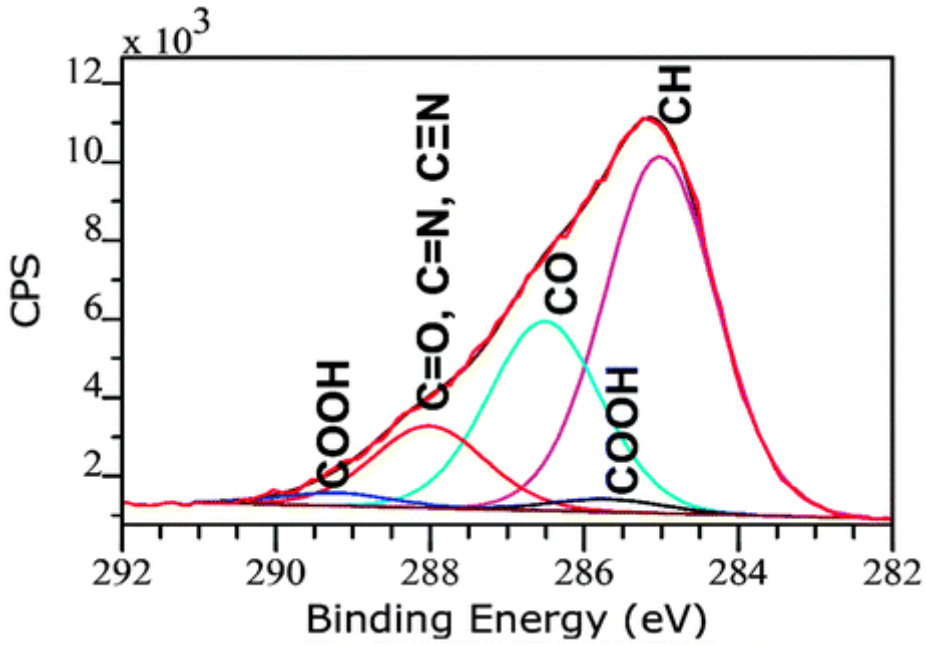


Figure 2.4 High resolution C1s spectrum of 2-methyl-2-oxazoline deposited at 50 W for 5 minutes showing different oxidation states of Carbon within the polymer film. Reproduced from Ref [11] with permission from the Royal Society of Chemistry.

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A SPECS Sage XPS equipped with a 200 kW monochromatic Mg source was used for XPS acquisition in this work with a slit size of 7 mm and focal point of 3 mm for general samples and 1 mm for gradients. Survey spectra were obtained using a 120 eV pass energy and used to confirm the presence of plasma deposited films on the surfaces. High resolution spectra were recorded for pertinent photoelectron peaks at a pass energy of 20 eV. This was used to determine the binding states of elements or the presence of attached compounds as follows:

- The presence of the QACs on the surface was confirmed via analysis of changes in the N1s peak (399.5 eV). Changes in NR4+ peak along the QAC concentration gradient were used to quantify minimum threshold limits.

- The retention of and modification of oxazoline structures were demonstrated by high quality C1s (285 eV) spectra.
- The appearance of N1s (399.5 eV) on PMX treated ppA was used to confirm the attachment of PMX.
- The decrease in intensity of the N1s (399.5 eV) and S2p (163.0 eV) peaks after coating ampicillin with octadiene.

XPS data were analysed using CasaXPS V2.3.14 (Casa Software Ltd.) and all binding energies were charge corrected relative to C-C at 285.0 eV.

#### *2.2.2.2 Ellipsometry*

Ellipsometry is a non-destructive method for measuring surface properties based on the reflectance of polarised light. [12, 13] Changes in the reflected polarised light is directly dependant on the optical properties and thickness of individual samples. In this work ellipsometry was used to determine film thicknesses. In all cases plasma polymer thicknesses were determined using A J.A Woolam Co. Variable Angle Spectroscopic Ellipsometer (VASE) with all data acquired and analysed with WVASE32 software. Polymers were deposited directly onto silicon wafers and mounted to the ellipsometer stage using a vacuum. Samples were measured at 5° angles between 65° and 75° and, set wavelengths at 10nm intervals between 250 and 1100 nm. Inbuilt Cauchy algorithms, assuming a 2nm oxidised silicon layer, were used to determine the thickness of the deposited films.

#### *2.2.2.3 Water Contact Angle*

The relative wettability of a surface can be a sensitive measure of the chemical properties of a surface as water contact angles can be sensitive to small changes on a

surface.[14-16] Measuring contact angles of a liquid on a surface gives indication of hydrophobicity or hydrophilicity of surfaces.

A simple way to measure contact angle is to use a sessile drop technique. This utilises an optical imaging system, a microscope or a high resolution camera, to directly visualise the contact between a water drop and a surface. If an image of an individual droplet on the surface can be taken and analysed to determine the tangent angle starting at the phase boundary (Figure 2.5a).

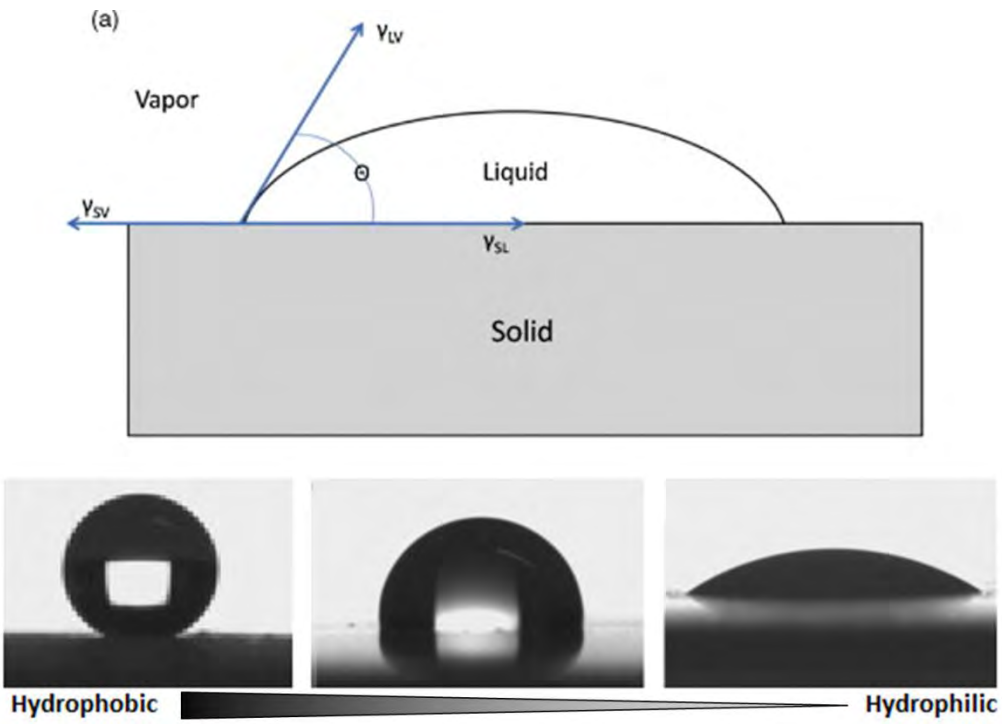


Figure 2.5 a) measurements of contact angles between a liquid and a solid where Θ is the contact angle b) examples of water drops on i) super-hydrophobic, ii) hydrophobic and iii) hydrophilic surfaces Reprinted with permission from <Vandencasteele, N. and F. Reniers (2010). "Plasma-modified polymer surfaces: Characterization using XPS." Journal of Electron Spectroscopy and Related Phenomena **178–179**(0): 394-408. Copyright 2010 Elsevier>[10]

A water contact angle of above 90° is indicative of low water\surface attraction i.e. a hydrophobic surface. As a surface becomes more hydrophilic, the water and surface attraction will increase and water contact angles will decrease towards complete wetting, where contact angle is 0°. Figure 2.5 shows examples of the interaction of water and super-hydrophobic, hydrophobic and hydrophilic surfaces.

It is known that the relative wettability of a surface can influence bacterial and mammalian cell interaction with the surfaces.[17,18] Although the effect of wettability on bacterial adhesion is not as great as that of surface charge or topography,[19] knowing the wettability gives important insight into the properties of a surface.

In this thesis, water contact angles of the ppAA, ppAA+GTAC, ppOx and ppOD were determined via a sessile drop method. Static water contact angles (WCA) were measured directly from captured images of the drops using the Drop-Snake (v2.1) ImageJ plug-in.[20]

#### *2.2.2.4 AFM*

A colloid probe AFM technique was used to determine the surface potentials at different points along the gradients.[21] A "NT-MDT NTEGRA SPM" AFM was used for all measurements with samples submerged in $1x10^{-2}$ M KCl at pH 7. The deflection upon approach with a curved hydrophilic Si AFM tip was measured and surface potential calculated.

#### *2.2.2.5 QCM*

A quartz crystal microbalance (QCM) measures variations in mass by measuring changes in resonance frequency of a quartz crystal based resonator. Materials can be

coated directly onto the sensors. As the total mass of the material is increased, usually due to attachment of compounds or chemicals, the resonance frequency lowers. This change in frequency (Δ*f*) is directly related to the change in mass (Δm).[22]

$$\Delta m = -C \frac{1}{n} \Delta f$$

Equation 2.2 Sauerbrey equation for determination of change in mass from change in frequency as determined via QCM. Where 'm' is mass, 'f' is frequency, 'n' is the vibrational overtone and 'C' is 17.7 ng.cm-2.

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**Effect of fouling on surface antimicrobial ability**

The capacity of constituents of the growth medium to adsorb to the GTAC modified surfaces was investigated using quartz crystal microbalance with dissipation monitoring (QCM-D). A Q-sense E4 QCM-D instrument was used in combination with 14 mm diameter quartz sensors with gold electrodes on either side of the sensor. All sensors were pre-washed with Milli-Q filtered water at a constant flow rate of 0.1 mL.min$^{-1}$ until stable. TSB was passed through the QCM chamber until both the dissipation and frequency of the sensors stabilised. Changes in resonance frequency (Δ*f*), post washing with Milli-Q filtered water are indicative changes in sample weight due to adsorption of molecules form the solution.

**Endotoxin absorbance**

The ability for ppA and PMX surfaces to attract proteins and LPS was demonstrated via the use of QCM-D. 10µg\mL LPS was pumped at 100µl per minute through the QCM chambers. Frequency and dissipation were measured and total mass of adhered molecules calculated as above (Equation 2.1).

### 2.2.3 Determination of drug release rates

Octadiene encapsulated ampicillin (5mg) was immersed in MilliQ filtered water or phosphate buffered solution (PBS) to a precise concentration of 400µg\mL and placed on a mechanical shaker for predetermined time periods of time. Absorbance was measured via UV-Vis spectroscopy (268nm) to determine the concentration of the leached ampicillin. A calibration curve was constructed by measuring the absorbance of neat ampicillin dissolved in MilliQ filtered water at concentrations of 1000µg\mL to 15.625 µg\mL. Octadiene coated silica was used as a control.

## 2.3 Testing surfaces for biological interactions

#### *2.3.1.1 Bacterial viability on surfaces*

*E. coli* (ATCC 25922) and *S. epidermidis* (ATCC 35984) were used as model organisms. Bacteria were grown from single colonies incubated at 37$^{o}$C overnight. 400 µl of 1x10$^{6}$ CFU.mL$^{-1}$ bacteria, based on the McFarland standard, was allowed to interact with the treated substrates for 4 hours. Samples were washed with PBS to remove any non-adhered bacteria. Adhered bacteria were stained using LIVE\DEAD stain and quantified by counting the number of live (green) and dead (red) bacteria per field of view. Fluorophores were excited using 470 nm CoolLED light source. A Nikon B-2A fluorescence filter cube set was used for imaging. The influence of environmental factors on the bacteria\surface interaction was made by testing homogenous QAC surfaces with samples in low nutrient (PBS) and high nutrient environments (TSB). Experiments were conducted ensuring that all surfaces remained completely submerged in liquid throughout the washing and staining procedures so as to not allow the bacteria to dry out.

#### *2.3.1.2 Quantification of total biofilm*

*S. epidermidis* was used as a model organism for biofilm formation. *S. epidermidis* was streak plated onto nutrient agar plates and grown overnight at 37$^{o}$C. Single colonies were picked and incubated in TSB overnight. *S. epidermidis* was diluted to 1x10$^{6}$ CFU\mL based on turbidity ($OD_{600nm}$). Substrates were added to a 24 well plate and 400µL *S. epidermidis* was added to each well. The bacteria were incubated overnight at 37$^{o}$C and allowed to form biofilms. After incubation all samples were washed twice with MilliQ water and 200µL of safranin stain was added so that biofilms could be visualised. Excess safranin stain was washed off of the samples before the stain was eluted with 33% glacial acetic acid in ethanol and quantified using a plate reader ($ABS_{490nm}$).

#### *2.3.1.3 Testing for leached compounds (Zone of Inhibition)*

A bacterial zone of inhibition was used as a means to test polymyxin leaching from the samples. *E. coli* (ATCC 25922) was streak plated grown from frozen stock and incubated overnight at 37°C on L-agar. S. aureus was streak plated from frozen and grown overnight on nutrient agar. Individual colonies were picked and added to 10ml of fresh media LB and incubated overnight 37°C. Bacterial lawns were grown on agar plates by spread plating 100µl of 1x10$^{7}$ CFU\mL bacteria, as determined by turbidity. Samples were placed face down onto the agar plate. An untreated glass coverslip was used as a control. Any inhibition of growth (zone of inhibition) was observed and measured.

#### *2.3.1.4 Total adhered bacteria: Autofluorescence*

*E. Coli* (SCC1) were grown overnight in LB at 30$^{o}$C. Saline was used to dilute the overnight culture 1:10 and the sample was incubated for another 4h to reach log phase. Samples were centrifuged for 20 minutes at 3500xg and pellets were resuspended in isotonic saline or M63G media to a concentration of 1x10$^{7}$ CFU\mL based on turbidity ($OD_{600nm}$). Samples were inoculated with bacteria and incubated for 2h at 30$^{o}$C. After incubation samples were washed with saline to remove any bacteria that was not attached and confocal microscopy was performed on a Zeiss LSM 700, Axio Imager 2 with a LD EC Epiplan-Neofluar 50x/0.55 DIC M27 objective. Individual bacterial cells were counted using ImageJ V1.46r.

### 2.3.2 Testing efficacy of leached antibiotics

Ampicillin efficacy was determined by looking at any changes in MIC against *S. epidermidis* (ATCC 35984) and *E. coli* (ATCC 25922) post coating with ppOD. The encapsulated ampicillin was allowed to leach for 24h in MilliQ filtered water before MIC was performed. 25µL of serial diluted leached ampicillin solution in filtered water was brought to a final working volume of 200µL with TSB and an end concentration of 1x10$^{6}$ CFU\mL bacteria. The MIC was determined by looking at wells with no turbidity. Results were confirmed by measuring optical density at 600nm using a Biotek ELx800.

### 2.3.3 Mammalian cell culture

Primary human dermal fibroblast cells were cultured in the complete culture media prepared as above. 20,000 cells were added into each well of a 24 well plate containing coated 13 mm glass coverslips and incubated for 48 or 72h. A resazurin reduction assay was used to measure relative metabolic activity. Resazurin was prepared in PBS (110mg.mL$^{-1}$) and diluted 1:10 in complete culture media. 500 µl was added to each sample and plates incubated for 4h. 200µl of each well was transferred

into a 96 well plate and fluorescence $544_{ex}$/ $590_{em}$ nm measured using a FLUOstar Optima plate reader.

### 2.3.4 Statistical analysis

All statistical analysis was performed on GraphPad Prism 5 for Windows using an unpaired ANOVA with a Dunnet post-hoc test to compare results to the control samples where appropriate, unless otherwise stated.

## 2.4 References


1. Vasilev, K.; Michelmore, A.; Griesser, H. J.; Short, R. D., Substrate influence on the initial growth phase of plasma-deposited polymer films. *Chemical Communications* 2009, 3600-3602.

2. Michelmore, A.; Steele, D. A.; Whittle, J. D.; Bradley, J. W.; Short, R. D., Nanoscale deposition of chemically functionalised films via plasma polymerisation. *Rsc Advances* 2013, 3, 13540-13557.

3. Vasilev, K.; Michelmore, A.; Griesser, H. J.; Short, R. D., Substrate Influence on the Initial Growth Phase of Plasma-Deposited Polymer Films. *Chemical Communications* 2009, 3600 - 3602.

4. Vasilev, K.; Michelmore, A.; Martinek, P.; Chan, J.; Sah, V.; Griesser, H. J.; Short, R. D., Early Stages of Growth of Plasma Polymer Coatings Deposited from Nitrogen- and Oxygen-Containing Monomers. *Plasma Processes and Polymers* 2010, 7, 824-835.

5. Cavallaro, A.; Vasilev, K., Controlled and sustained release of pharmaceuticals via single step solvent-free encapsulation. *Chemical Communications* 2015, 51, 1838-1841.

6. Akhavan, B.; Jarvis, K.; Majewski, P., Hydrophobic plasma polymer coated silica particles for petroleum hydrocarbon removal. *ACS Applied Materials & Interfaces* 2013, 5, 8563-8571.

7. Michl, T. D.; Coad, B. R.; Hüsler, A.; Vasilev, K.; Griesser, H. J., Laboratory Scale Systems for the Plasma Treatment and Coating of Particles. *Plasma Processes and Polymers* 2014.

8. Goreham, R. V.; Mierczynska, A.; Pierce, M.; Short, R. D.; Taheri, S.; Bachhuka, A.; Cavallaro, A.; Smith, L. E.; Vasilev, K., A substrate independent approach for generation of surface gradients. *Thin Solid Films* 2013, 528, 106-110.

9. Tillet, G.; Boutevin, B.; Ameduri, B., Chemical reactions of polymer crosslinking and post-crosslinking at room and medium temperature. *Progress in Polymer Science* 2011, 36, 191-217.

10. Vandencasteele, N.; Reniers, F., Plasma-modified polymer surfaces: Characterization using XPS. *Journal of Electron Spectroscopy and Related Phenomena* 2010, 178–179, 394-408.

11. Macgregor-Ramiasa, M. N.; Cavallaro, A. A.; Vasilev, K., Properties and reactivity of polyoxazoline plasma polymer films. *Journal of Materials Chemistry B* 2015, 3, 6327-6337.

12. McCrackin, F. L.; Passaglia, E.; Stromberg, R. R.; Steinberg, H. L., Measurement of the thickness and refractive index of very thin films and the optical properties of surfaces by ellipsometry. *Journal of research of the national bureau of standards—A. physics and chemistry A* 1963, 67.

13. Ulman, A., *An Introduction to Ultrathin Organic Films: From Langmuir--Blodgett to Self--Assembly*. Academic press: 2013.

14. Decker, E. L.; Frank, B.; Suo, Y.; Garoff, S., Physics of contact angle measurement. *Colloids and surfaces A: physicochemical and engineering aspects* 1999, 156, 177-189.

15. Janssen, D.; De Palma, R.; Verlaak, S.; Heremans, P.; Dehaen, W., Static solvent contact angle measurements, surface free energy and wettability determination of various self-assembled monolayers on silicon dioxide. *Thin Solid Films* 2006, 515, 1433-1438.

16. De Gennes, P.-G., Wetting: statics and dynamics. *Reviews of modern physics* 1985, 57, 827.

17. An, Y. H.; Friedman, R. J., Concise review of mechanisms of bacterial adhesion to biomaterial surfaces. *Journal of Biomedical Materials Research* 1998, 43, 338-348.

18. Choee, J.-H.; Lee, S. J.; Lee, Y. M.; Rhee, J. M.; Lee, H. B.; Khang, G., Proliferation rate of fibroblast cells on polyethylene surfaces with wettability gradient. *Journal of Applied Polymer Science* 2004, 92, 599-606.

19. Katsikogianni, M.; Missirlis, Y. F., Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. *Eur Cell Mater* 2004, 8, 37-57.

20. Stalder, A.; Kulik, G.; Sage, D.; Barbieri, L.; Hoffmann, P., A snake-based approach to accurate determination of both contact points and contact angles. *Colloids and surfaces A: physicochemical and engineering aspects* 2006, 286, 92-103.

21. Borkovec, M.; Szilagyi, I.; Popa, I.; Finessi, M.; Sinha, P.; Maroni, P.; Papastavrou, G., Investigating forces between charged particles in the presence of oppositely charged polyelectrolytes with the multi-particle colloidal probe technique. *Advances in Colloid and Interface Science* 2012, 179–182, 85-98.

22. Hemmersam, A. G.; Foss, M.; Chevallier, J.; Besenbacher, F., Adsorption of fibrinogen on tantalum oxide, titanium oxide and gold studied by the QCM-D technique. *Colloids and Surfaces B: Biointerfaces* 2005, 43, 208-215.

# 3 Substrate independent approach for immobilisation of quaternary ammonium compounds to surfaces to reduce bio-burden*

## 3.1 Preamble

Bacterial contamination of biomedical devices is an ongoing problem. One method to alleviate such contamination is the introduction of surface compounds onto devices which can kill bacteria on contact. Polymers containing quaternary ammonium groups are known for their antimicrobial properties. Here, a substrate-independent two-step method for the immobilisation of quaternary ammonium groups onto any type of surface is reported. To achieve this glycidlytrimethylammonium chloride was covalently bound to plasma polymerised allylamine interlayer. Changes in the membrane permeability of *Escherichia coli* were observed by BacLight LIVE\DEAD staining. 30% of *E. coli* grown on the treated surfaces showed high levels of membrane permeability within 4 hours. Importantly, there was no observable cytotoxic effect on human dermal fibroblasts.

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## 3.2 Introduction

Since the introduction of aseptic technique into the medical field infection rates have dramatically declined. However, infections continue to threaten human health and are the second most common cause of death, after heart related diseases.[1] The majority of these infections are caused by bacteria. After the initial colonisation of surfaces, bacteria continue to proliferate and produce extracellular polysaccharides, and mature into dynamic communities called biofilms.[2] Biofilms allow for increased intercellular signalling and communication, and also helps protect microorganisms within the community from antibiotics and other potential toxins.[3]

Infections caused by indwelling medical implants are harder to treat and tend to be detrimental, if not fatal, to patients due to the difficulties associated with traditional treatments and accessing the infected area.[4, 5] The impact is further complicated by the ability of bacteria within a biofilm to display increased antibiotic resistance compared to their planktonic counterparts. Since infections associated with medical devices often begin with the adhesion of bacteria to the surface of the device, antibacterial coatings have captured attention. One method to achieve that is the introduction of coatings that are capable of killing pathogens upon contact such as quaternary ammonium compounds (QACs).[5, 6]

Most methods for attachment of QACs require a specific surface chemistry which limits their applications to a particular substrate material, such as silica surfaces when salinised QACs compounds are used.[7, 8] Medical devices are made of various types of materials. Hence, an approach that allows immobilisation to surfaces independent of the substrate material would be highly beneficial. Plasma polymerisation has been established as a technique that is capable of depositing thin functional films on any

type of material. It has been demonstrated that after a thin film of a few nanometres thickness has been deposited, the film growth becomes substrate independent.[9, 10] Thus, the goal of this work is to establish a method for surface immobilisation of QACs on plasma modified surfaces.

Gram-negative bacilli, such as *Pseudomonas aeruginosa* and *E. coli*, have been associated with hospital acquired surgical site infection, urinary tract infections and bloodstream infections.[11] Herein a substrate independent method for addition of QAC groups to substrates based on plasma polymerised polymer films and its effect on *E. coli* as a medically relevant model organism is reported.

## 3.3 Results and Discussion

**Sample Preparation**

The allylamine plasma polymer (ppAA) film was deposited as mentioned above. These conditions produced a film of approximately 30 nm as determined via spectroscopic ellipsometry. The nitrogen content within the films was confirmed via XPS (Figure 3.1). XPS analysis revealed that amine plasma polymers contained atomic concentrations of 74.78% carbon (C), 15.22 % nitrogen (N) and 9.99% oxygen (O). At these conditions the films are stable as no measurable difference in film thickness was seen after soaking samples in water for 24 hours and drying with nitrogen. XPS analysis of the samples soaked revealed the N:C ratios remain constant but a small increase in O is observed within the films. There are two possible mechanisms for this; increased oxidation of the films due to the water treatment or excess water being irreversibly absorbed into the films.

Table 3.1 %NR4+ of total nitrogen as determined by XPS in MilliQ filtered water (H2O) and various pHs set via addition of KOH to water.

| | **$H_2O$** | **$H_2O$ +GTAC** | **pH 9** | **pH 9 +GTAC** | **pH 10** | **pH 10 +GTAC** | **pH 11** | **pH 11 +GTAC** |
|---|---|---|---|---|---|---|---|---|
| **$\%NR_4^+$** of total N | 0 ± 0.0 | 0 ± 0.0 | 0 ± 0.0 | 4.3 ± 0.6 | 0 ± 0.0 | 7.8 ± 1.7 | 0 ± 0.0 | 5.6 ± 0.7 |

ppAA films have been shown to contain a population of primary amines which have been used for various immobilisation reactions.[12, 13] A schematic of the proposed chemistry for the addition of quaternary ammonium groups to a substrate is shown in Scheme 2.1. Samples were treated with GTAC in KOH and washed twice in MilliQ filtered water.

Figure 3.1 shows XPS spectra from ppAA and ppAA+GTAC after processing with CasaXPS. All the binding energies were referenced to the C1s neutral carbon peak at 285 eV, to compensate for the effect of surface charging. A binding energy shift within high resolution N1s XPS was used to identify and quantify the presence of $NR_4^+$ groups.[14] It is known that in the N1s spectrum N-C bonds, from primary, secondary and tertiary amines, have typical binding energies of 399.0 - 400.2 eV. XPS results indicated a single amine peak on a typical ppAA surfaces at 399.5 eV representing N-C bonds. Alternatively, a typical immobilised-GTAC surface showed two peaks, one at 399.4 eV and another at 402.7 eV, representing N-C bonds and the quaternary ammonium nitrogen respectively. An increase in total oxygen was also observed which may be attributed to the oxygen group being transferred from the epoxide and increased

oxidation from the treatment. pH dependant attachment was determined by incubating samples with GTAC at various pH levels and looking for the presence of the $NR_4^+$ peak as seen in Figure 3.1. The $\%NR_4^+$ of total nitrogen content as determined via XPS is displayed in Table 3.1. All GTAC treatments in the presence of KOH revealed a peak in the XPS N1s spectra which could be assigned to $NR_4^+$.

This confirms the addition of the GTAC to the polymer films. Table 3.1 shows that attachment of GTAC was dependant on pH. These data demonstrated that the optimal pH for attachment of GTAC is pH 10. It was revealed that approximately 7.5 % ± 1.7 of the total nitrogen content in the treated films treated at pH 10 was $NR_4^+$.To determine the optimal time for surface immobilisation of GTAC on ppAA the surface grafting was conducted for 4, 6, 24, and 48h. Initial GTAC attachment appeared to be rapid and reached saturation at approximately 24h. Based on the pH and time studies samples were prepared at pH10 for 24h was used for all bacterial and mammalian cell culture studies reported below.

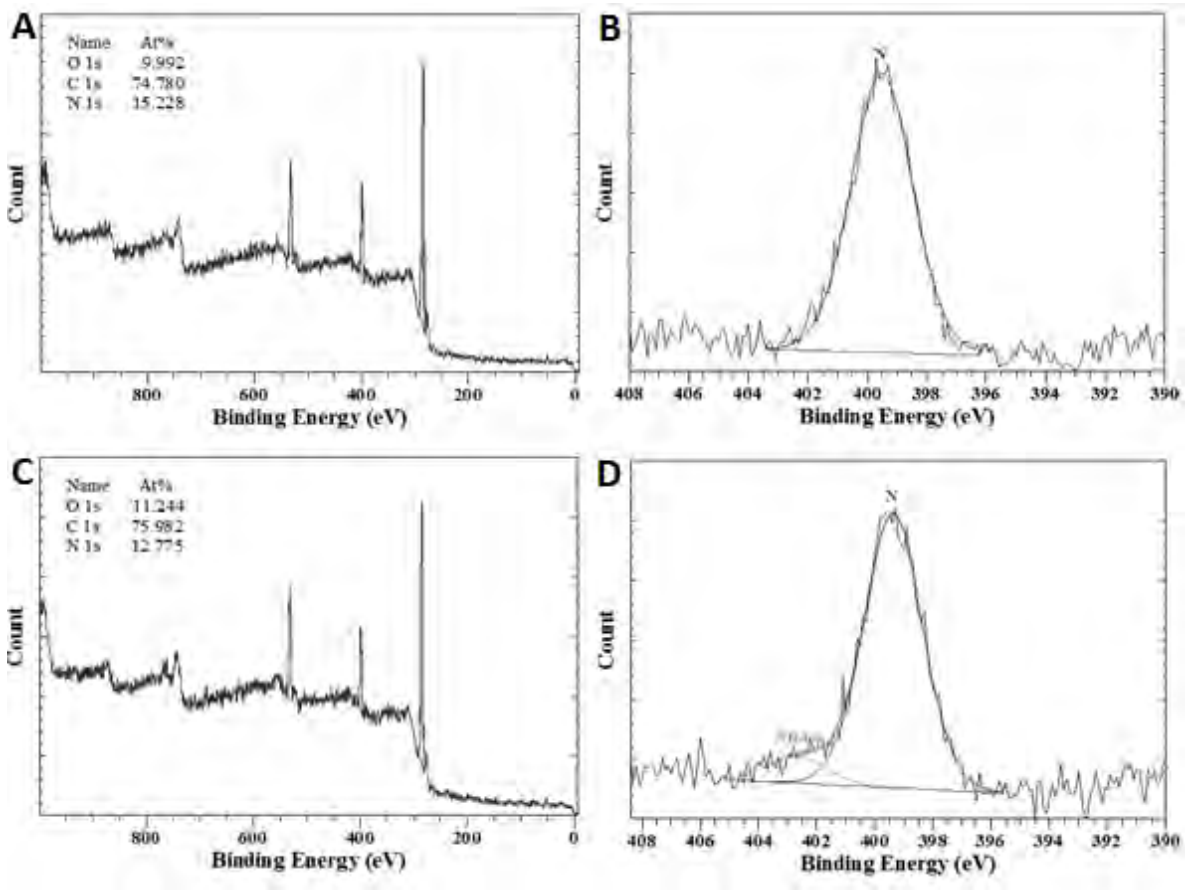


Figure 3.1 XPS survey and high-resolution N1s spectrum for ppAA (A and B respectively) and Survey and high-resolution N1s spectrum for ppAA+GTAC (C and D)

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Relative wettability of the samples was determined by static contact angles. ppAA+GTAC showed a decrease in water contact angle compared to the ppAA only control. ppAA films showed a static contact angle of 53.09° ± 0.29, similar to what was expected according to literature.[15] ppAA+GTAC surfaces showed a static contact angle of 45.99° ± 0.67. The change in water contact angle was attributed to the hydrophilicity of the $NR_4^+$.

**Biological Interaction**

The ability of the ppAA+GTAC surfaces to prevent viable bacteria colonisation was verified by fluorescent staining. BacLight LIVE\DEAD staining employs the use of two

fluorescent stains; SYTO9 and propidium iodide. The green-fluorescent SYTO9 stain stains both live and dead bacteria. The red-fluorescent PI penetrates cells with damaged membranes; thus dead cells, or cells with compromised membrane integrity, will fluoresce red.[16] Bacteria were added to each sample as described earlier and fluorescent microscopy images taken. Images were analysed and the number of membrane permeable cells as compared total number of cells.

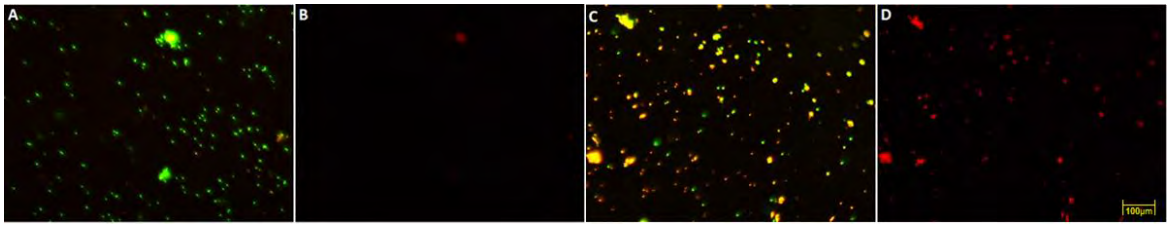


Figure 3.2 Baclight LIVE\DEAD images obtained via Nikon B-2A and Texas Red filters showing total E. coli (live and, membrane permeable) and membrane permeable only E. coli, after 4h incubation at 37°C. A) Fluorescent microscope image of total E. coli on ppAA sample. B) Fluorescent microscope image of membrane-permeable E. coli on ppAA sample. C) Fluorescent microscope image of total E. coli on ppAA+GTAC sample. D) Fluorescent microscope image of membrane permeable E. coli on ppAA+GTAC sample.

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Figure 3.2 shows bacterial viability post Baclight LIVE\DEAD staining. A low number of *E. coli* with permeable membranes are seen on the ppAA films (Fig. 3.2A and 3.2B) whereas a high number of *E. coli* with permeable membranes can be seen on the ppAA+GTAC films (Fig. 3.32 and 3.2D). After 4h 92.50% ± 1.50 of *E. coli* on ppAA films were deemed viable according to Baclight light staining and, viable *E. coli* on ppAA+GTAC at the same time point was significantly lower at 70.09% ± 4.30 ($P < 0.05$). The number of viable cells on ppAA+GTAC films decreased further to 44.71% ± 9.50 after 24h as seen in Figure 3.3. The overall trend of these data shows that the

membrane permeability of *E. coli* that is in contact with the ppAA+GTAC films is higher than that of the original ppAA films; this suggests an interaction of the QAC groups and the bacterial membrane.

It has generally been accepted that QACs relied on long C-chains to penetrate the cell membrane of microbes and kill the cells. However, it is shown that QACs with short C-chains exhibit antimicrobial activity when immobilised on surfaces. Thus, another mechanism of antimicrobial action was attributed to the charge of the QACs inducing unfavourable ion exchange with cell membranes.[17] In turn, this prevents membrane charge neutralisation and induces a loss of membrane integrity.[17-19] GTAC has short C-chains and although it can inhibit bacteria growth in solution (data not shown), the concentration required to see an effect is much higher than that of quaternary ammonium based surfactants.[16, 20]

Although not all bacteria on the surfaces were killed within the studied timeframe, the percentage of total bacteria with compromised membrane permeability increased. It should be noted that the outer membrane of gram-negative bacteria helps regulate the cells' intracellular environment by facilitating transport of materials in and out of the cell. This is also used by bacteria as a method of antibiotic resistance by preventing drug transport into the cell.[21] In a medical sense, an increase in membrane permeability, will allow for natural host defences or other contemporary treatments to be more effective. The permeability of the outer cell membranes of gram-negative bacteria, such as E. coli, influences the susceptibility of the bacteria to antibiotics[22] as many antibiotics target intracellular processes.[21]

Biocompatibility of biomedical devices is an important factor in determining the appropriateness of the device.[23] The coatings developed in this work were tested for any potential cytotoxicity in culture of human dermal fibroblasts because of the importance of these cells in wound healing.[24, 25] No difference in adhesion, proliferation and viability between fibroblasts grown on ppAA films and ppAA+GTAC surfaces was seen. This is discussed further in Chapter 4.

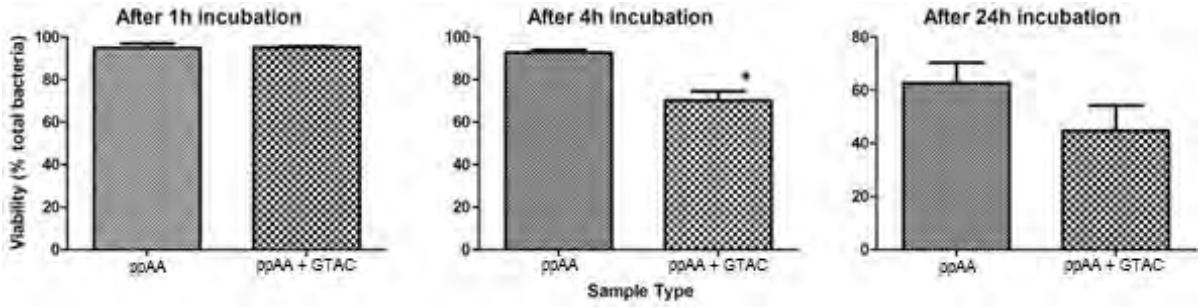


Figure 3.3 *E. coli* viability represented as Viability (% total bacteria) based on cell membrane permeability after 1, 4 and 24h incubation with treated substrates.

## 3.4 Conclusion

In summary, a substrate independent approach to generating a QAC based antimicrobial thin film was developed. This method is composed of two sequential steps: firstly, the generation of an amine functionalised thin film via plasma polymerisation allows for coating of any material and secondly, the covalent attachment of GTAC via epoxy-amine binding to the plasma polymer to add cationic properties to the surface. LIVE\DEAD staining showed an increase in cell membrane permeability of *E. coli* bacteria in the presence of the ppAA+GTAC surfaces. It is intended that studies will be extended to include other bacterial species such as *P. aeruginosa*, *S. epidermidis* and *S. aureus*. Current results provide an initial platform to

support investigation into potential use in the biomedical field for surface modification of infection prone devices.

## 3.5 References


1. World Health Organization, Annex Table 2: Deaths by cause, sex and mortality stratum in WHO regions, estimates for 2002. *The world health report* 2004.

2. McCann, M. T.; Gilmore, B. F.; Gorman, S. P., Staphylococcus epidermidis device-related infections: pathogenesis and clinical management. *Journal of Pharmacy and Pharmacology* 2008, 60, 1551-1571.

3. Fux, C. A.; Costerton, J. W.; Stewart, P. S.; Stoodley, P., Survival strategies of infectious biofilms. *Trends in Microbiology* 2005, 13, 34-40.

4. Vasilev, K.; Cook, J.; Griesser, H. J., Antibacterial surfaces for biomedical devices. *Expert Rev Med Devices* 2009, 6, 553-67.

5. Vasilev, K.; Griesser, S. S.; Griesser, H. J., Antibacterial Surfaces and Coatings Produced by Plasma Techniques. *Plasma Processes and Polymers* 2011, 8, 1010-1023.

6. Tiller, J. C.; Liao, C.-J.; Lewis, K.; Klibanov, A. M., Designing surfaces that kill bacteria on contact. *Proceedings of the National Academy of Sciences* 2001, 98, 5981-5985.

7. Gottenbos, B.; van der Mei, H. C.; Klatter, F.; Nieuwenhuis, P.; Busscher, H. J., In vitro and in vivo antimicrobial activity of covalently coupled quaternary ammonium silane coatings on silicone rubber. *Biomaterials* 2002, 23, 1417-1423.

8. Isquith, A. J.; Abbott, E. A.; Walters, P. A., Surface-bonded antimicrobial activity of an organosilicon quaternary ammonium chloride. *Appl Microbiol* 1972, 24, 859-63.

9. Vasilev, K.; Michelmore, A.; Griesser, H. J.; Short, R. D., Substrate Influence on the Initial Growth Phase of Plasma-Deposited Polymer Films. *Chemical Communications* 2009, 3600 - 3602.

10. Vasilev, K.; Michelmore, A.; Martinek, P.; Chan, J.; Sah, V.; Griesser, H. J.; Short, R. D., Early Stages of Growth of Plasma Polymer Coatings Deposited from Nitrogen- and Oxygen-Containing Monomers. *Plasma Processes and Polymers* 2010, 7, 824-835.

11. Weinstein, R. A.; Gaynes, R.; Edwards, J. R.; System, N. N. I. S., Overview of Nosocomial Infections Caused by Gram-Negative Bacilli. *Clinical Infectious Diseases* 2005, 41, 848-854.

12. Vasilev, K.; Mierczynska, A.; Hook, A. L.; Chan, J.; Voelcker, N. H.; Short, R. D., Creating gradients of two proteins by differential passive adsorption onto a PEG-density gradient. *Biomaterials* 2010, 31, 392-7.

13. Goreham, R. V.; Short, R. D.; Vasilev, K., Method for the Generation of Surface-Bound Nanoparticle Density Gradients. *Journal of Physical Chemistry C* 2011, 115, 3429-3433.

14. Kistamah, N.; Carr, C. M.; Rosunee, S., X-ray photoelectron spectroscopic study of Tencel treated with a cationic β-cyclodextrin derivative. *Surface and Interface Analysis* 2009, 41, 710-713.

15. Mierczynska, A.; Michelmore, A.; Tripathi, A.; Goreham, R. V.; Sedev, R.; Vasilev, K., pH-tunable gradients of wettability and surface potential. *Soft Matter* 2012, 8, 8399-8404.

16. Ferreira, C.; Pereira, A. M.; Pereira, M. C.; Melo, L. F.; Simões, M., Physiological changes induced by the quaternary ammonium compound benzyldimethyldodecylammonium chloride on Pseudomonas fluorescens. *Journal of Antimicrobial Chemotherapy* 2011.

17. Kügler, R.; Bouloussa, O.; Rondelez, F., Evidence of a charge-density threshold for optimum efficiency of biocidal cationic surfaces. *Microbiology* 2005, 151, 1341-1348.

18. Murata, H.; Koepsel, R. R.; Matyjaszewski, K.; Russell, A. J., Permanent, non-leaching antibacterial surfaces--2: How high density cationic surfaces kill bacterial cells. *Biomaterials* 2007, 28, 4870-4879.

19. Terada, A.; Yuasa, A.; Kushimoto, T.; Tsuneda, S.; Katakai, A.; Tamada, M., Bacterial adhesion to and viability on positively charged polymer surfaces. *Microbiology* 2006, 152, 3575-3583.

20. Ioannou, C. J.; Hanlon, G. W.; Denyer, S. P., Action of disinfectant quaternary ammonium compounds against Staphylococcus aureus. *Antimicrob Agents Chemother* 2007, 51, 296-306.

21. Delcour, A. H., Outer membrane permeability and antibiotic resistance. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 2009, 1794, 808-816.

22. Hancock, R. E. W., The bacterial outer membrane as a drug barrier. *Trends in Microbiology* 1997, 5, 37-42.

23. Abdull Rasad, M. S. B.; Halim, A. S.; Hashim, K.; Rashid, A. H. A.; Yusof, N.; Shamsuddin, S., In vitro evaluation of novel chitosan derivatives sheet and paste cytocompatibility on human dermal fibroblasts. *Carbohydrate Polymers* 2010, 79, 1094-1100.

24. Werner, S.; Krieg, T.; Smola, H., Keratinocyte-Fibroblast Interactions in Wound Healing. *Journal of Investigative Dermatology* 2007, 127, 998-1008.

25. Wang, A.; Pu, K.; Dong, B.; Liu, Y.; Zhang, L.; Zhang, Z.; Duan, W.; Zhu, Y., Role of surface charge and oxidative stress in cytotoxicity and genotoxicity of graphene oxide towards human lung fibroblast cells. *Journal of applied toxicology : JAT* 2013, 33, 1156-64.

# 4 Influence of Immobilised Quaternary Amine Density, Surface Charge and Environmental Factors on the Coating Antimicrobial Efficacy and Cytotoxicity

## 4.1 Preamble

Bacterial colonisation of medical devices causes infections and is a significant problem in healthcare. The use of antibacterial coatings is considered as one of the potential solutions to this problem and has attracted a great deal of attention. Through rationally designed concentration density gradients of immobilised quaternary ammonium compounds a specific threshold of quaternary ammonium surface concentration is required to induce significant bacterial death. It was determined that this threshold was 4.18 % $NH_4^+$ bound nitrogen and a surface potential of +120.4 mV. Furthermore, for the first time the adhesion of constituents of the culture medium to the QACs modified surface eliminates any cytotoxicity towards eukaryotic cells such as primary human fibroblasts was demonstrated. The implications of this type of surface fouling on the coating's antimicrobial efficacy are also elucidated.

## 4.2 Introduction

Increases in population and average life expectancy and advances in healthcare have led to a greater demand for medical devices. However, bacterial adhesion and colonisation of these devices has become progressively more relevant as up to 70% of Hospital Acquired Infections (HAIs) are associated with medical devices and are complex and costly to treat [1] Combined with the increased prevalence of multi-drug resistant bacteria, infections are a major concern within the medical community.[1-4] Widespread antibiotic use has effectively treated countless cases of infection and has had a profound positive impact on public health,[5] however, the overuse of antibiotics, within both the medical and agricultural fields, has been associated with increased number of multi-drug resistant bacteria.[3, 5, 6] Infections caused by multidrug resistant bacteria are becoming an increasingly common problem. Although their initial appearance seemed isolated to hospitals, evidence suggests that multi-drug resistant strains of bacteria are prevalent within the general community.[7] It should also be noted that even innocuous bacteria, such as *Staphylococcus epidermidis*, cause nosocomial sepsis.[1, 8]

Short term medical devices, such as catheters, wound dressings and medical ventilators, are frequently replaced in order to remove the devices before bacteria can successfully colonise them but this adds considerable costs to the healthcare system and inconvenience to patients.[3] The situation with permanent implants such as heart valves, hip or knee implants is much more serious since these devices cannot be easily replaced. Most inversions with such devices can be life threatening and require a substantial medical intervention.

Surface modification techniques have been proposed as a strategy to add intrinsic antimicrobial activity to medical device to reduce the risk of infection. The addition of quaternary ammonium compounds (QACs) within a coating, in both non-leaching and leaching forms, has been shown to effectively inhibit bacterial growth[9-11] QACs have been long known for their antibacterial properties.[12] The capacity of QACs to kill bacteria was first noted in 1916 and has been studied and applied to various cleaning applications since. It has been shown that the antimicrobial activity of QACs varies depending on the length and types of the compound's side chains and the conditions of exposure. It is generally accepted that, in solution, long C-chains combined with positively charged $NH_4^+$ groups penetrate cell membranes, and hence interfere with bacterial membrane integrity and ultimately kill the cells.[2, 13, 14]

QACs with short C-chains have been shown to be effective when immobilised on a surface.[15] Tiller *et al*.[9] studied the effect of chain length on immobilised QACs. It was concluded that if the C-chain was too long no antimicrobial action was seen. This is contrary to the effects seen with QACs in solution where a longer side chain tends to promote antimicrobial activity. Thus, a different mechanism of action was attributed to the QACs when immobilised to surfaces: the antimicrobial effect can be attributed to the net surface charge caused by the addition of QACs prompting unfavourable ion exchange with the surface.[16] This prevents membrane charge neutralisation and can induce a loss of membrane integrity.[15-17]

This work aims to address two important issues associated with the potential application of immobilised QACs as antibacterial coatings. Firstly the minimal QACs surface immobilisation density that is required to inhibit bacterial colonisation needs

to be determined. To address this unique and controlled surface gradients of QACs density were developed. It was hypothesised that there is a threshold of QACs surface density and a minimum positive surface potential that is required for a surface to become bactericidal. The second issue tackled in this work relates to the cytotoxicity of QACs-based antibacterial coatings. In the same way that QACs are lethal to bacteria in solution, they can be toxic to eukaryotic cells.[18] However, many polycationic antimicrobial agents, including quaternary ammonium based polymers, display minimal cytotoxicity [19-22] when placed on surfaces. Yet, the reason for this apparent biocompatibility has not been directly addressed. Here, the first explanation as to why immobilised QACs show limited observable cytotoxicity and how this reflects the antimicrobial efficacy of these surfaces is presented.

## 4.3 Results and Discussion

### Sample Preparation

QAC modified surfaces were generated via the addition of a 1 w% solution of GTAC to ppAA coated substrates.[23] The binding of GTAC was confirmed via high resolution XPS scans in the binding energies range of the N1s region. Quantification of the surface concentration of QAC was determined by deconvolution of the N1s spectra. Figure 4.1 shows high resolution XPS N1s spectra of ppAA and ppAA+GTAC. ppAA N1s spectrum (Figure 4.1a) can be fitted with a single component at 399.8 eV which is typically done with plasma polymer films due to the very small differences in binding energies of N-C bonded nitrogen species[24, 25] which makes fitting uncertain. After addition of GTAC (Figure 4.1b) a new peak at 402.4 eV appears, which indicates the presence of the

quaternary ammonium cation.[26] The atomic percentage of quaternary ammonium groups on these uniformly modified surfaces was 6.89% of total nitrogen.

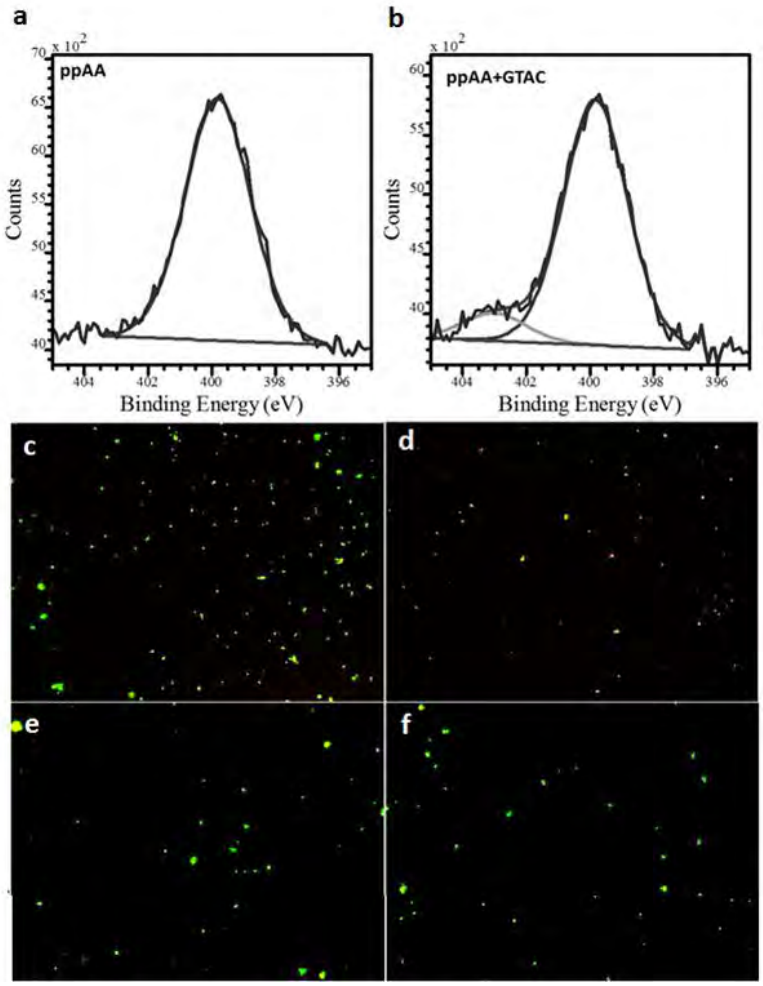


Figure 4.1. a) XPS N1s high resolution spectra of ppAAand b) ppAA+GTAC. c) Fluorescent images after LIVE\DEAD staining of bacteria incubation with the corresponding surfaces showing *E. coli* grown on ppAA and d) ppAA+GTAC e) *S. epidermidis* incubated on ppAA and F) ppAA+GTAC.

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**Bacteria**

To determine the antimicrobial efficacy of these uniformly modified ppAA+GTAC towards bacteria, cultures of *E. coli* and *S. epidermidis* were allowed to interact with the surface for 24 hours. *E. coli* and *S. epidermidis* were chosen as test organisms due

to their medical relevance and different cell wall structures, *S. epidermidis* being Gram-positive and *E. coli* Gram-negative.[27, 28]

The LIVE\DEAD stain assay was used to determine the viability of the individual bacterial cells adhered to the surface. All bacteria are stained with Syto9 (green), whereas propidium iodine (red) only enters cells with permeable membranes.[29] Figure 4.2 shows the effect of the immobilised QACs on adhering bacteria viability. *E. coli* adhered to (Figure 4.1c) ppAA surfaces appear viable as seen by the green stained cells. A clear reduction in the number of viable *E. coli* adhered to the ppAA+QAC surface is observed as shown by the large number of propidium iodine (red) stained cells (Figure 4.1d). Figure 4.1e and 4.1f show the interaction of *S. epidermidis* with ppAA and ppAA+GTAC modifies surfaces. Interestingly, there is no effect of the ppAA+GTAC coated substrates on the bacteria as the *S. epidermidis* appears viable regardless of surface type.

The ability for the GTAC modified surface to induce membrane permeability in *E. coli* and not in *S. epidermidis* was attributed to differences in the respected bacterial cell walls.[30, 31] Lipopolysaccharide (LPS) is the major component of the outer cell wall of Gram-negative bacteria, such as *E. coli*, contributing to its structural integrity. The positively charged groups of the ppAA+GTAC can interact with the outer membrane in two ways. Firstly the charged groups may repel ions, such as $Mg^{2+}$, used for charge stabilization.[9, 32] Removal of such ions from the cell membrane will result in its destabilization and lead to an increase in cell membrane permeability. Secondly, the charge may enhance the adhesion forces between the bacteria and the surfaces by attracting the LPS. This interaction will alter the structure of, or potentially remove, the

LPS, which can disrupt membrane integrity and lead to cell death. Although Gram-positive and Gram-negative bacteria tend to have net negative charges, Gram-positive bacteria, such as *S. epidermidis*, have thicker cell wall consisting of high levels of peptidoglycan.[30, 33] High levels of peptidoglycan gives the bacterial cell walls greater rigidity and greater thickness. This could be one of the reasons why *S. epidermidis* might be less susceptible to cell wall destabilization caused by the surface grafted QACs.[34, 35] Here it must be noted that compared to other immobilised QACs these surfaces do not induce as much membrane destabilisation. This may be due to the maximum achievable $NR_4^+$ concentration on the plasma polymer.[15] Due to the lack of susceptibility of the *S. epidermidis* to the immobilised QACs, only *E. coli* was used for further investigation.

**Concentration Gradients**

Next, the role of QACs surface immobilization density on bacterial viability was examined. To achieve this, surface density gradients of QACs were designed and implemented. These gradients allowed the use of a single substrate to screen the entire achievable concentration range of surface bound GTAC. Such gradients will allow for high throughput screening of antimicrobial surfaces. To create such gradients the time dependent nature of the epoxide-amine reaction was exploited. A precise dip-coating technique was used to control the times of contact between the amine modified surface and the solution of GTAC. The method is schematically depicted in Scheme 2.1b and used previously to create nanoparticle density gradients.[36] For the studies reported here, a 13 mm round coverslip is immersed with a rate of 1 $mm.h^{-1}$. This rate of immersion allowed a maximum concentration of quaternary ammonium

functionalities on the side of the substrate that remained in the solution for longest time (13 hours) while the opposite side of the substrate had no GTAC at all.

Figure 4.2a shows the surface concentration of $NR_4^+$ groups along the gradient determined deconvolution of the N1s high resolution XPS spectra. Position 13 mm remained in the solution of GTAC for longest time of 13 hours. At this position the $NR_4^+$ group concentration was 6.17% of the total nitrogen functionalities. With reducing the time of immersion towards position 0 mm, the $NR_4^+$ group density gradually decreased. At position 1.5 mm, only 1% of total nitrogen was in the form of quaternary ammonium ion. The presence of small amounts of quaternary ammonium functionalities on the bare plasma polymer film is not surprising since the process is known to deliver complex surface chemistries different to these produced by conventional polymerisation techniques.[24]

The surface potential along the gradient was evaluated by AFM based surface force measurements and is presented in Figure 4.2b. The surface charge was calculated from the approach curves using the DLVO model. The surface potential at ppAA side of the surfaces (position 1 mm) was in the range of 70 mV which is consistent with previously published studies.[37] With increasing GTAC surface grafting density, the positive surface charge gradually increased to above 150 mV at the highest $NR_4^+$ group concentration. This increase of positive surface charge is expected since the $NR_4^+$ has a greater protonation than the amine group.[26] As one could anticipate, the increase of surface potential follows the same trend as the density increase in $NR_4^+$ group surface density determined by XPS (Figure 4.2a).

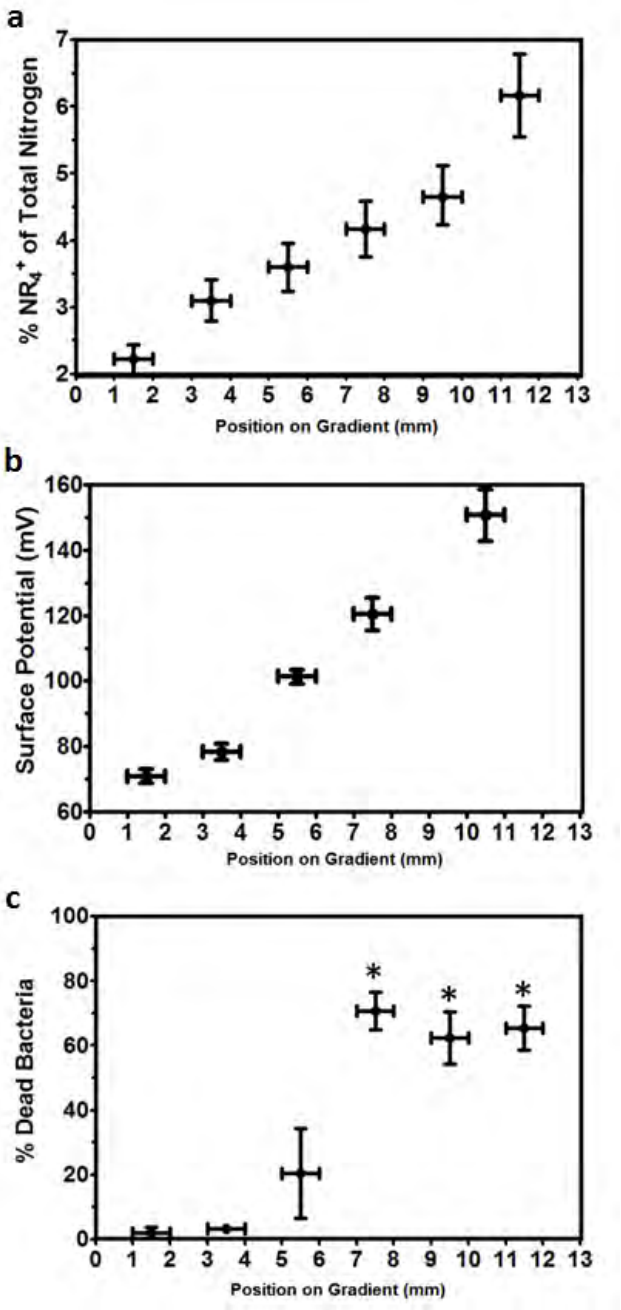


Figure 4.2 Characterisation of the concentration density gradient of immobilised GTAC: a) Atomic percentage of $NR_4^+$ bonded nitrogen along; b) Surface potentials (mV) determined by force approach using AFM based force measurements; c) Percentage of dead bacteria quantified from the LIVE\DEAD stained images along *p<0.01 compared to ppAA only end (1.5 mm). Vertical error bars represent standard error of mean. Horizontal error bars assume ±0.5 mm variation in sampling position.

**Minimum Bactericidal Concentration**

*E. coli* was allowed to interact with the QAC surface concentration density gradient following the same procedure as described for uniformly coated surfaces. Figure 4.3 shows images of LIVE\DEAD stained *E. coli* cells attached to the surface from six evenly spaced points along the gradient, corresponding to positions 1.5, 3.5, 5.5, 7.5, 9.5 and 11.5 mm. The first observation is that the total number of cells that adhered to the surface is roughly the same on each position along the gradient. This is to be expected since bacteria are cultured in low nutrient and slow growth conditions. However, the viability of the attached bacteria is different along the gradient. At low $NR_4^+$ group concentration e.g. positions 1.5 and 3.5 mm, mostly viable, Syto9 (green) stained cells are observed. When the $NR_4^+$ group surface density increases, position 5.5 mm and above, a high proportion of propidium iodine stained (red) dead cells can be seen.

Quantification of the percentage of dead (red stained) cells derived via ImageJ software analysis and is plotted in Figure 4.2c. Consistent with the images in Figure 4.3, the surface had no antibacterial properties on the bare ppAA end of the gradient (position 1.5 mm) and low $NR_4^+$ groups concentration (position 3.5 mm). As the concentration of $NR_4^+$ groups along the gradient increased the percentage of dead bacteria also increased. Dead bacteria (ca 20%) start appearing on the surface at position 5.5 mm. At position 7.5 mm and above nearly 70% of attached bacteria were non-viable. Interestingly, there was no significant difference between positions at 7.5, 9.5 and 11.5 mm when compared to each other ($p>0.05$). This indicates that there is a threshold of surface concentration density of quaternary ammonium groups that is

required to kill bacteria. This threshold in the case of surface immobilised GTAC is 4.18% (of total nitrogen) and surface charge of +120.4 mV. This result correlates with published studies which claim that a specific surface charge density must be achieved to induce significant antimicrobial effects.[16]

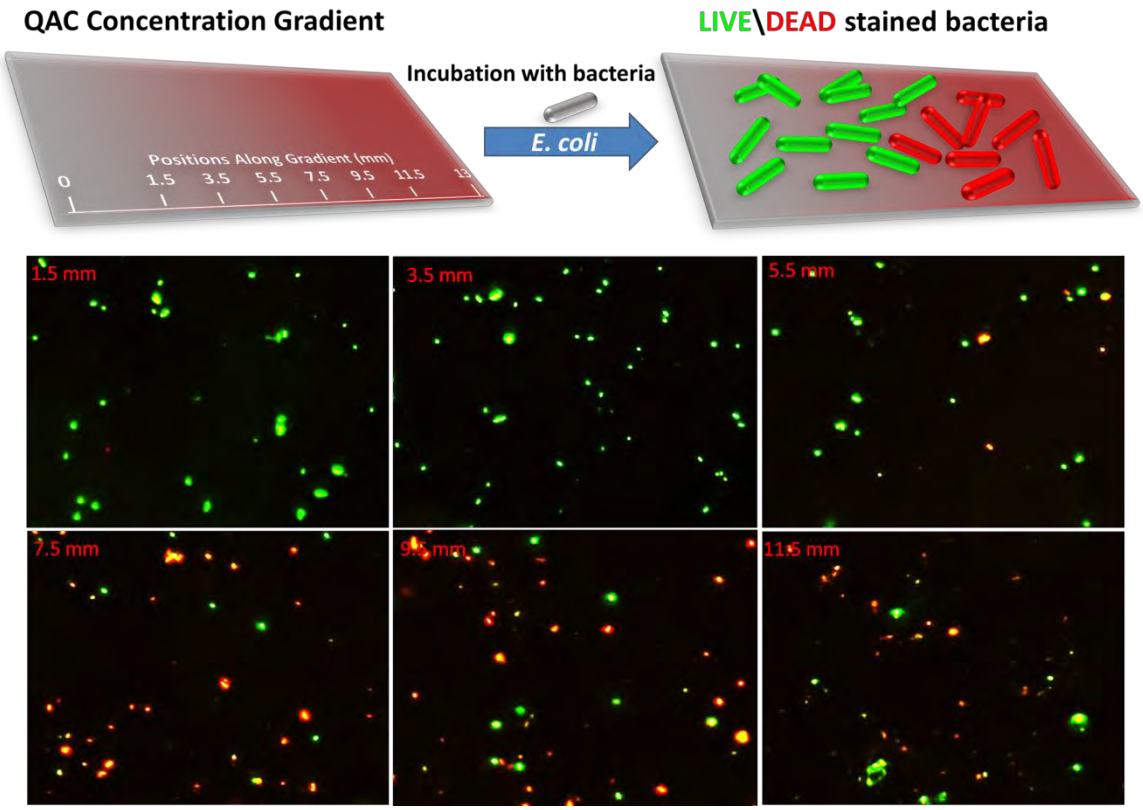


Figure 4.3 Fluorescent images after LIVE\DEAD staining of *E. coli* post incubation with the QAC concentration gradient showing a decrease in viable bacteria as the GTAC density increases from 1.5 mm (ppAA only end) to 11.5 mm ppAA+GTAC).

**Biocompatibility**

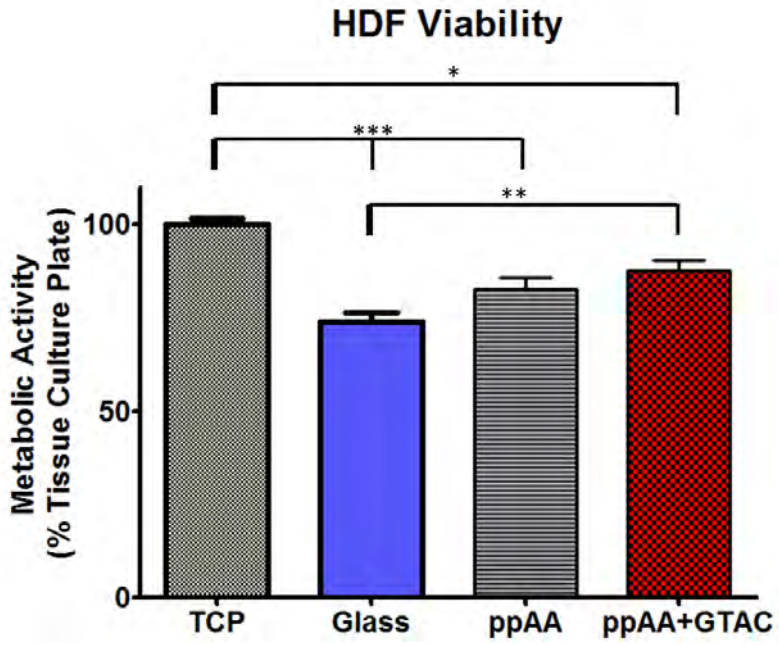


Figure 4.4 Relative metabolic activities of human dermal fibroblasts grown on ppAA and ppAA+GTAC as percentage of activity on tissue culutre plates showing no statistical difference between ppAA and ppAA+GTAC ($p>0.05$). *$p<0.05$; **$p<0.01$, ***$p<0.001$.

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The biocompatibility of biomedical devices is certainly one of the most important factors that determine the usefulness of a certain device.[38] To test the biocompatibility of the GTAC modified surfaces primary human dermal fibroblasts (HDFs) were used. It should be noted that these cells are primary cells and not immortalised cell lines Fibroblasts are a key player in wound healing and are an established model for assessing device biocompatibility and potential cytotoxicity.[39-43]

Figure 4.4 shows the metabolic activity (assessed using the resazurin assay) of HDFs cultured on ppAA+GTAC of highest surface density and controls of ppAA, glass slides and tissue culutre plates (TCP). There was no statistically significant difference between cells grown on ppAA and ppAA+GTAC ($p>0.05$). The metabolic activity of the cells cultured on ppAA+GTAC is also very similar to these on TCP. This result indicates

that the GTAC modified surface, even at the highest surface concentration, does not have any adverse effect on HDFs.

The fact that the GTAC modified surfaces were lethal to bacteria but were not toxic to HDFs was initially surprising. However, a careful consideration of these observations pointed towards differences in culture conditions. Bacterial culture studies were carried out in nutrient deficient conditions (i.e. PBS) while HDFs were grown in a complete cell culture medium. It was possible that proteins and other nutrients from the cell culture medium adsorb to the surface almost immediately after contact and in this way mask the immobilised GTAC molecules responsible for bacterial cell death. Hypothetically, this scenario is depicted in Figure 4.5a.

To test this hypothesis, *in situ* QCM-D measurements were used and are presented in Figure 4.5b. Sensors coated with ppAA and ppAA+GTAC were incubated in TSB. TSB is a bacterial culture medium containing various nutrients such as proteins and sugars. As seen from the seismogram, almost immediately after introduction of TSB, significant mass from the medium adsorbs to the sensor surface resulting in negative frequency change. Some of this mass is so strongly attached that it does not desorb after an exhaustive rinse with water. This strongly attached mass to the ppAA+GTAC modified sensor is estimated at 21.4±1.1 ng and sufficient to form a thin masking layer on top of the quaternary ammonium groups.

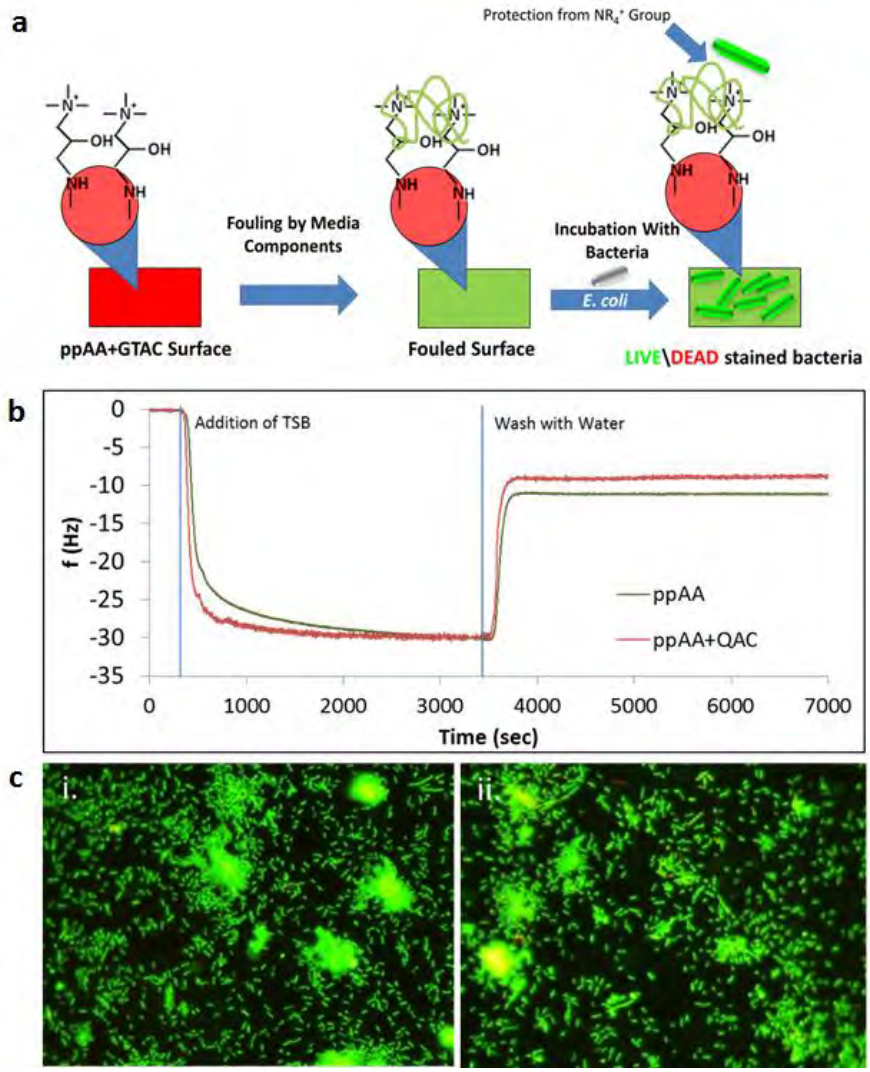


Figure 4.5 a) Schematic of fouling of surface from media constituents. b) QCM-D seismogram showing a negative change in frequency (Hz) after treatment with TSB. c.i) Surface attachment and growth of *E. coli* in TBS on ppAA and (ii) ppAA+GTAC showing no observable change in viability as determined via LIVE\DEAD staining.

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To evaluate how the adsorbed constituents of the culture medium affect the capacity of surface immobilised QACs to kill bacteria, *E. coli* was grown in nutrient-rich culture broth (TSB). Figure 4.5c shows images of *E. coli* grown on ppAA and ppAA+GTAC for 4 hours at 37$^{o}$C and stained with the LIVE/DEAD assay. It appears that in nutrient rich medium the presence of the QAC on the surface has no effect on *E. coli* growth and

viability. This can be attributed to two effects and the interplay between them. Firstly, the adsorption to the surface of nutrients from the medium forms a thin layer on top of the immobilised GTAC molecules which masks any antibacterial properties of the $NH_4^+$ groups. Secondly, the continuous proliferation of the bacteria in nutrient rich medium not only leads to production of extracellular matrix components which further masks the QAC groups, but also allows for repair of any damaged cell surfaces.[44, 45]

This study makes contributions to elucidating the use of surface grafted QACs for fabrication of antibacterial surfaces. Through surface density gradients of QACs, engineered at the molecular level, it is demonstrated that a specific threshold of GTAC density that is required to achieve antibacterial properties. This specific threshold relates to $NH_4^+$ groups concentration of 4.18% of total nitrogen and surface charge of 120.4 mV. These studies were conducted in low nutrient environment similar to these are seen repeatedly throughout the literature on antibacterial assessment of QACs based coatings.[19, 22, 46-49] However, when full growth medium is used (TSB) the surface completely loses its antibacterial properties.

The effect of the growth medium on the efficacy of QACs based coatings is not reported in the literature but elucidation of this phenomenon is important for understanding when and how such antibacterial coatings can be used. Using QCM-D measurements, it was revealed that the loss of surface activity in due to the adsorption of constituents from the growth medium. It can be hypothesised that the same phenomenon will be observed with other types of antibacterial coatings based of grafted compounds, except the compounds that had intrinsic low fouling properties. These findings do not necessary render the QACs based coatings and other

antibacterial surfaces achieved through surface grafting bactericidal compounds ineffective in medical device application: these coatings will be capable of protecting the device surface before the device is implanted into the body. This could be beneficial since it will ensure that viable bacteria will not be introduced with the device itself. Once placed into the body, the surface of the device will foul from the constituents of the body fluids and lose its antibacterial properties. However, with this any potential cytotoxicity to body cells and tissue will also be lost, which would render the device safe.

## 4.4 Conclusions

Collectively, concentration gradients of immobilised QACs were used to understand the critical surface concentration of $NH_4^+$ groups required to kill attaching pathogens. It was demonstrated that there is a specific threshold of $NH_4^+$ groups concentration of 4.18% and surface charge of 120.4 mV to induce death in the case of *E. coli*. It was also demonstrate that any antibacterial properties of the surface are lost in nutrient rich environments. This loss of activity is caused by the adsorption of constituents of the surface which form a thin layer on top of the quaternary ammonium groups. It can be speculated that antibacterial coatings based on other types of grafted compounds may have the same fate, thus the implications of the findings reported here are much broader. In terms of application on medical devices these coatings would retain their antimicrobial efficacy until they comes in contact with the complex fluids within the body. The latter might be beneficial as this rapid fouling would mask any potential toxic effects of the surface after entering the body decreasing the risk of any bio-incompatibility. Herein the biocompatibility of the coatings in culture of HDFs were

demonstrated, however, any effect on immunological responses or other secondary effects need to be assessed before the use of ppAA+GTAC on implantable medical devices.

## 4.5 References


1. Bryers, J. D., Medical biofilms. *Biotechnology and Bioengineering* 2008, 100, 1-18.

2. Ioannou, C. J.; Hanlon, G. W.; Denyer, S. P., Action of disinfectant quaternary ammonium compounds against Staphylococcus aureus. *Antimicrob Agents Chemother* 2007, 51, 296-306.

3. Noimark, S.; Dunnill, C. W.; Wilson, M.; Parkin, I. P., The role of surfaces in catheter-associated infections. *Chemical Society Reviews* 2009, 38, 3435-3448.

4. Vasilev, K.; Cook, J.; Griesser, H. J., Antibacterial surfaces for biomedical devices. *Expert Rev Med Devices* 2009, 6, 553-67.

5. Nikaido, H., Multidrug resistance in bacteria. *Annu Rev Biochem* 2009, 78, 119-46.

6. Gould, I. M., Antibiotics, skin and soft tissue infection and meticillin-resistant Staphylococcus aureus: cause and effect. *International Journal of Antimicrobial Agents* 2009, 34, S8-S11.

7. de Lencastre, H.; Oliveira, D.; Tomasz, A., Antibiotic resistant Staphylococcus aureus: a paradigm of adaptive power. *Curr Opin Microbiol* 2007, 10, 428-35.

8. McCann, M. T.; Gilmore, B. F.; Gorman, S. P., Staphylococcus epidermidis device-related infections: pathogenesis and clinical management. *Journal of Pharmacy and Pharmacology* 2008, 60, 1551-1571.

9. Tiller, J. C.; Liao, C.-J.; Lewis, K.; Klibanov, A. M., Designing surfaces that kill bacteria on contact. *Proceedings of the National Academy of Sciences* 2001, 98, 5981-5985.

10. Lee, S. B.; Koepsel, R. R.; Morley, S. W.; Matyjaszewski, K.; Sun, Y.; Russell, A. J., Permanent, Nonleaching Antibacterial Surfaces. 1. Synthesis by Atom Transfer Radical Polymerization. *Biomacromolecules* 2004, 5, 877-882.

11. Isquith, A. J.; Abbott, E. A.; Walters, P. A., Surface-bonded antimicrobial activity of an organosilicon quaternary ammonium chloride. *Appl Microbiol* 1972, 24, 859-63.

12. Hegstad, K.; Langsrud, S.; Lunestad, B. T.; Scheie, A. A.; Sunde, M.; Yazdankhah, S. P., Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health? *Microb Drug Resist* 2010, 16, 91-104.

13. McCubbin, P. J.; Forbes, E.; Gow, M. M.; Gorham, S. D., Covalent attachment of quaternary ammonium compounds to a polyethylene surface via a hydrolyzable ester linkage: Basis for a controlled-release system of antiseptics from an inert surface. *Journal of Applied Polymer Science* 2006, 100, 538-545.

14. Ferreira, C.; Pereira, A. M.; Pereira, M. C.; Melo, L. F.; Simões, M., Physiological changes induced by the quaternary ammonium compound benzyldimethyldodecylammonium chloride on Pseudomonas fluorescens. *Journal of Antimicrobial Chemotherapy* 2011.

15. Murata, H.; Koepsel, R. R.; Matyjaszewski, K.; Russell, A. J., Permanent, non-leaching antibacterial surfaces--2: How high density cationic surfaces kill bacterial cells. *Biomaterials* 2007, 28, 4870-4879.

16. Kügler, R.; Bouloussa, O.; Rondelez, F., Evidence of a charge-density threshold for optimum efficiency of biocidal cationic surfaces. *Microbiology* 2005, 151, 1341-1348.

17. Terada, A.; Yuasa, A.; Kushimoto, T.; Tsuneda, S.; Katakai, A.; Tamada, M., Bacterial adhesion to and viability on positively charged polymer surfaces. *Microbiology* 2006, 152, 3575-3583.

18. Ferk, F.; Mišík, M.; Hoelzl, C.; Uhl, M.; Fuerhacker, M.; Grillitsch, B.; Parzefall, W.; Nersesyan, A.; Mičieta, K.; Grummt, T., Benzalkonium chloride (BAC) and dimethyldioctadecyl-ammonium bromide (DDAB), two common quaternary ammonium compounds, cause genotoxic effects in mammalian and plant cells at environmentally relevant concentrations. *Mutagenesis* 2007, 22, 363-370.

19. Li, P.; Poon, Y. F.; Li, W.; Zhu, H.-Y.; Yeap, S. H.; Cao, Y.; Qi, X.; Zhou, C.; Lamrani, M.; Beuerman, R. W.; Kang, E.-T.; Mu, Y.; Li, C. M.; Chang, M. W.; Jan Leong, S. S.; Chan-Park, M. B., A polycationic antimicrobial and biocompatible hydrogel with microbe membrane suctioning ability. *Nat Mater* 2011, 10, 149-156.

20. Peng, Z.-X.; Wang, L.; Du, L.; Guo, S.-R.; Wang, X.-Q.; Tang, T.-T., Adjustment of the antibacterial activity and biocompatibility of hydroxypropyltrimethyl ammonium chloride chitosan by varying the degree of substitution of quaternary ammonium. *Carbohydrate Polymers* 2010, 81, 275-283.

21. Stratton, T. R.; Rickus, J. L.; Youngblood, J. P., In Vitro Biocompatibility Studies of Antibacterial Quaternary Polymers. *Biomacromolecules* 2009, 10, 2550-2555.

22. Milović, N. M.; Wang, J.; Lewis, K.; Klibanov, A. M., Immobilized N-alkylated polyethylenimine avidly kills bacteria by rupturing cell membranes with no resistance developed. *Biotechnology and Bioengineering* 2005, 90, 715-722.

23. Cavallaro, A.; Majewski, P.; Barton, M.; Vasilev, K., Substrate independent approach for immobilisation of quaternary ammonium compounds to surfaces to reduce bio-burden. *Materials Science Forum* 2014, 783-786, 1389-1395.

24. Vasilev, K.; Michelmore, A.; Martinek, P.; Chan, J.; Sah, V.; Griesser, H. J.; Short, R. D., Early Stages of Growth of Plasma Polymer Coatings Deposited from Nitrogen- and Oxygen-Containing Monomers. *Plasma Processes and Polymers* 2010, 7, 824-835.

25. Michelmore, A.; Martinek, P.; Sah, V.; Short, R. D.; Vasilev, K., Surface Morphology in the Early Stages of Plasma Polymer Film Growth from Amine-Containing Monomers. *Plasma Processes and Polymers* 2011, 8, 367-372.

26. Kistamah, N.; Carr, C. M.; Rosunee, S., X-ray photoelectron spectroscopic study of Tencel treated with a cationic β-cyclodextrin derivative. *Surface and Interface Analysis* 2009, 41, 710-713.

27. O'GARA, J. P.; HUMPHREYS, H., Staphylococcus epidermidis biofilms: importance and implications. *Journal of Medical Microbiology* 2001, 50, 582-587.

28. Russo, T. A.; Johnson, J. R., Medical and economic impact of extraintestinal infections due to Escherichia coli: focus on an increasingly important endemic problem. *Microbes and Infection* 2003, 5, 449-456.

29. Joux, F.; Lebaron, P., Use of fluorescent probes to assess physiological functions of bacteriaat single-cell level. *Microbes and Infection* 2000, 2, 1523-1535.

30. Silhavy, T. J.; Kahne, D.; Walker, S., The bacterial cell envelope. *Cold Spring Harb Perspect Biol* 2010, 2, a000414.

31. Willey, J., *Prescott, Harley, and Klein's Microbiology-7th international ed./Joanne M. Willey, Linda M. Sherwood, Christopher J. Woolverton*. New York [etc.]: McGraw-Hill Higher Education: 2008.

32. Groisman, E. A.; Hollands, K.; Kriner, M. A.; Lee, E.-J.; Park, S.-Y.; Pontes, M. H., Bacterial Mg2+ Homeostasis, Transport, and Virulence. *Annual Review of Genetics* 2013, 47, 625-646.

33. van der Wal, A.; Norde, W.; Zehnder, A. J. B.; Lyklema, J., Determination of the total charge in the cell walls of Gram-positive bacteria. *Colloids and Surfaces B: Biointerfaces* 1997, 9, 81-100.

34. Gottenbos, B.; Grijpma, D. W.; van der Mei, H. C.; Feijen, J.; Busscher, H. J., Antimicrobial effects of positively charged surfaces on adhering Gram-positive and Gram-negative bacteria. *Journal of Antimicrobial Chemotherapy* 2001, 48, 7-13.

35. Kenawy, E.-R.; Abdel-Hay, F. I.; El-Shanshoury, A. E.-R. R.; El-Newehy, M. H., Biologically active polymers: synthesis and antimicrobial activity of modified glycidyl methacrylate polymers having a quaternary ammonium and phosphonium groups. *Journal of Controlled Release* 1998, 50, 145-152.

36. Goreham, R. V.; Mierczynska, A.; Pierce, M.; Short, R. D.; Taheri, S.; Bachhuka, A.; Cavallaro, A.; Smith, L. E.; Vasilev, K., A substrate independent approach for generation of surface gradients. *Thin Solid Films* 2013, 528, 106-110.

37. Mierczynska, A.; Michelmore, A.; Tripathi, A.; Goreham, R. V.; Sedev, R.; Vasilev, K., pH-tunable gradients of wettability and surface potential. *Soft Matter* 2012, 8, 8399-8404.

38. Abdull Rasad, M. S. B.; Halim, A. S.; Hashim, K.; Rashid, A. H. A.; Yusof, N.; Shamsuddin, S., In vitro evaluation of novel chitosan derivatives sheet and paste cytocompatibility on human dermal fibroblasts. *Carbohydrate Polymers* 2010, 79, 1094-1100.

39. Taheri, S.; Cavallaro, A.; Christo, S. N.; Smith, L. E.; Majewski, P.; Barton, M.; Hayball, J. D.; Vasilev, K., Substrate independent silver nanoparticle based antibacterial coatings. *Biomaterials* 2014, 35, 4601-4609.

40. Werner, S.; Krieg, T.; Smola, H., Keratinocyte-Fibroblast Interactions in Wound Healing. *Journal of Investigative Dermatology* 2007, 127, 998-1008.

41. Bose, R. K.; Lau, K. K. S., Initiated CVD of Poly(2-Hydroxyethyl Methacrylate) Hydrogels: Synthesis, Characterization and In-vitro Biocompatibility. *Chemical Vapor Deposition* 2009, 15, 150-155.

42. Wang, A.; Pu, K.; Dong, B.; Liu, Y.; Zhang, L.; Zhang, Z.; Duan, W.; Zhu, Y., Role of surface charge and oxidative stress in cytotoxicity and genotoxicity of graphene oxide towards human lung fibroblast cells. *Journal of applied toxicology : JAT* 2013, 33, 1156-64.

43. Yang, A.; Cardona, D. L.; Barile, F. A., In vitro cytotoxicity testing with fluorescence-based assays in cultured human lung and dermal cells. *Cell Biology and Toxicology* 2002, 18, 97-108.

44. Lund, B.; Baird-Parker, T. C.; Gould, G. W., *Microbiological safety and quality of food*. Springer: 2000; Vol. 1.

45. Virto, R.; Mañas, P.; Álvarez, I.; Condon, S.; Raso, J., Membrane Damage and Microbial Inactivation by Chlorine in the Absence and Presence of a Chlorine-Demanding Substrate. *Applied and Environmental Microbiology* 2005, 71, 5022-5028.

46. American Society for Testing and Materials, ASTM E2149-10 Standard Test Method for Determining the Antimicrobial Activity of Immobilized Antimicrobial Agents Under Dynamic Contact Conditions. 2010.

47. Li, Z.; Lee, D.; Sheng, X.; Cohen, R. E.; Rubner, M. F., Two-Level Antibacterial Coating with Both Release-Killing and Contact-Killing Capabilities. *Langmuir* 2006, 22, 9820-9823.

48. Gottenbos, B.; van der Mei, H. C.; Klatter, F.; Nieuwenhuis, P.; Busscher, H. J., In vitro and in vivo antimicrobial activity of covalently coupled quaternary ammonium silane coatings on silicone rubber. *Biomaterials* 2002, 23, 1417-1423.

49. Lee, S. B.; Koepsel, R. R.; Morley, S. W.; Matyjaszewski, K.; Sun, Y.; Russell, A. J., Permanent, nonleaching antibacterial surfaces. 1. Synthesis by atom transfer radical polymerization. *Biomacromolecules* 2004, 5, 877-82.

# 5 Immobilised antibiotics on plasma polymers for dual mechanism antimicrobial coatings

## 5.1 Preamble

The use of antimicrobial coatings has been considered as a potential solution to the problems associated with medical device infection. Infections caused by Gram-negative bacteria are of particular concern in the medical field due to the presence of toxic lipopolysaccharides in their outer membranes. In this Chapter a substrate independent coating based on plasma polymerisation which not only reduces the initial adhesion of bacteria but can actively absorb lipopolysaccharides is designed. A hydrophilic, aldehyde-rich coating was generated via the plasma polymerisation of propionaldehyde which decreases initial bacterial adhesion. By exploiting the aldehyde chemistry, polymyxin, which can bind lipopolysaccharides, was covalently anchored to the coating. The coatings with the immobilised polymyxin can subsequently decrease bacterial attachment and can absorb up to 52 ng of LPS per $cm^2$. This may lead to the development of a surface coating for removable medical devices such as catheters that, not only decrease attachment of bacteria but also limit their toxicity by actively removing virulent endotoxins.

## 5.2 Introduction

Nosocomial infections, or Hospital Acquired Infections (HAIs), are becoming increasingly prevalent as population sizes and average life expectancy increase. They cause pain and suffering and have been associated with extremely high mortality rates, making them the second highest cause of death globally.[1] Although the introduction of antimicrobial compounds and aseptic technique has curved this, the general overuse of antibiotics is said to have led to an increase in multi-drug resistant bacterial strains. The appearance of multi-drug resistant bacteria has incited a need to revert back to the use of old antibiotics with minimal or no observed resistances.[2] Another strategy employed to help alleviate the risk of infections is the use of substrates with inherent antimicrobial ability. Such antimicrobial coatings have been investigated for use on medical devices such as implants [3, 4] and wound dressings.[5, 6]

Gram-negative bacteria are the predominate cause of hospital acquired urinary tract infections caused by catheters, and cause 30% of central vascular catheter related blood infections. Polymyxins were commonly used in the early 1980's to treat Gram-negative, but their use was abandoned due to their potential toxicity. However, the emergence of multi-drug resistant gram-negative bacteria, namely b-lactam and quinolone antibiotic resistant bacteria, prompted the return of polymyxin usage.[2] Polymyxins show rapid bactericidal effect on most Gram-negative aerobic bacilli, including *Escherichia Coli* and *Pseudomonas aeruginosa.*[2, 7] This effect is attributed to cationic regions of the polymyxins competing and displacing cations ($Mg^{2+}$ and $Ca^{2+}$) which the lipopolysaccharide-based (LPS) outer membrane of Gram-negative bacteria

require to remain stable. A secondary effect of polymyxins has been attributed to its ability to bind directly to LPS.[8]

It has been suggested that medical devices which have inherent antimicrobial properties will decrease the risk of infection and the reliance on antibiotics.[9, 10] The use of such coatings is not limited to the medical field and have potential application within a variety of other fields ranging from the food industry,[11, 12] waste treatment[13] and purification.[14] Previous study into the immobilisation of polymixin revealed a retention of its antimicrobial activity after binding to agorose or polystyrene fibres.[15, 16] Polymyxin B bound fibres have been used in the medical field as an antibacterial wound dressing.[16] It has clearly been demonstrated that immobilised PMX can be used for haemoperfusion treatments as proposed by Kodma *et al.*[17] and have been marketed as endotoxin removal cartridges.[15] Shepherd et al.[18] utilised a hyperbranched polymer with immobilised PMX and showed its ability to actively bind Gram-negative bacteria and remove them from a burn wound model.

Current methods of PMX immobilisation require the carrier fibre to have specific chemistry or the bulk polymer to be synthesised for this particular application, which can limit their application. Plasma deposition boasts as a substrate independent technique for surface modification.[19] Using plasma deposition, nano-thin films of specific chemistries can be easily applied to any substrates to allow for further modification.

Here, novel antibacterial coatings based on immobilised PMX were generated, such that deposition onto any substrate material was possible, for the main purpose of coating removable medical devices. This technique can not only be used for wound

dressings but can be applied directly onto other medical devices such as catheters, regardless of the underlying chemistry of the device. To achieve this goal, plasma polymerised propionaldehyde (ppA) was deposited and functionalised by the covalent anchoring of PMX (ppA+PMX). To evaluate the usefulness of these surface coatings, the effect on adhering Gram-negative *E. coli* was evaluated *in vitro*. Furthermore, the ability for the immobilised PMX to absorb LPS from solution was demonstrated. This provides the first basis for the use of PMX as an intracorporeal endotoxin isolation\removal system.

## 5.3 Results and Discussion

**Substrate preparation**

To generate surfaces of immobilised PMX, a two stage approach consisting of an initial plasma deposition followed by covalent binding of PMX was chosen due to ease and substrate independence of translation of the plasma deposition technique.[20, 21] Propionaldehyde was chosen as a plasma deposition precursor due to its common use and ability to produce aldehyde rich surface coatings. It has been previously shown that ppA films have the ability to covalently bind primary amine groups through imine binding. [22]

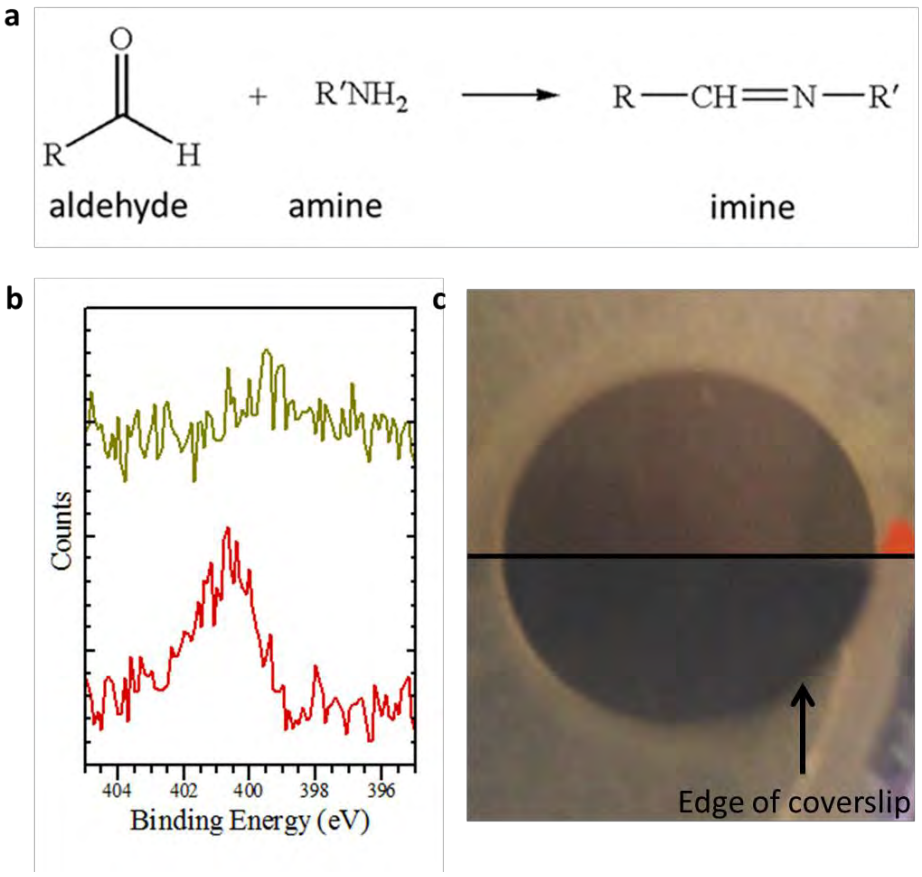


Figure 5.1 a) Proposed chemical reaction between aldehyde (on surface) and amines from the polymyxin. b) high resloution N1s XPS showing nitrogen on the ppA+PMX (bottom; red) and no nitrogen on the ppA control sample (top; green). c) Photograph of Zone of Inhibition study of ppA (top) and ppA+PMX (bottom) showing no observable zone of inhibition when placed face down on a bacterial lawn of E. coli (ATCC 25922) on LB-agar.

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The proposed mechanism of attachment of PMX to the ppA coated substrate utilises amine-aldehyde coupling to form imines (Figure 5.1a). To achieve this reaction, substrates containing ppA were submerged in an excess of 20ug\mL PMX in MilliQ filtered water and left to incubate for 24h. Samples were removed and washed vigorously with MilliQ filtered water to remove any unbound PMX. XPS revealed a change in total atomic concentrations and the appearance of nitrogen on the surface due to the PMX attachment. Figure 5.1b shows high resolution N1s regions of the ppA

(top) and the ppA+PMX (bottom). 1.3 At% nitrogen is clearly detectable in the high resolution XPS spectra of the coating.

To confirm that no PMX is leaching from the substrates, a Kirby–Bauer (agar diffusion) assay was conducted. It is generally accepted that PMX exhibits antimicrobial action by attaching to LPS on the outer surface of bacterial membranes and interacting with its negative charge. After attaching to the LPS, the hydrophobic tail of PMX inserts into the bacterial membrane causing a detergent-like lysis of the cells. However, it should be noted that changes in LPS can lead to decreased sensitivity towards PMX. [23] Due to this mechanism of action, only Gram-negative bacteria were used for determination of the antimicrobial effect of the ppA+PMX surfaces.

Figure 5.1c shows the results of the agar diffusion study using ppA+PMX. Both the ppA control (top) and ppA+PMX (bottom) exhibit no observable zone of inhibition when placed face down on a bacterial lawn of *E. coli* (ATCC 25922) cultured on LB-agar which is in line with routine methods performed regularly in our laboratories.[24] As there is no inhibition of growth observed, it is confirmed that no PMX or a quantity below the minimum inhibitory concentration of the bacteria, is leached from the samples. Thus, it can be assured that any effects on bacterial growth on the surfaces are not due to PMX leaching into the growth medium.

**Bacteria**

Next, to determine the effect of the ppA and the ppA+PMX on bacteria, *E. coli* was grown directly on the surfaces and the total number of adhered bacteria quantified. *E. coli* (SCC1) was chosen as a test organism for initial bacterial adhesion to produce

Green Florescent Protein (GFP) and be easily quantified using confocal microscopy. SCC1 were cultured in different mediums to control their growth to determine the interactions of the bacteria in different environments and metabolic conditions. SCC1 were grown in saline to ensure that the bacteria were in the static growth phase or full medium conditions to encourage controlled growth. As outlined in Figure 5.2a, bacteria were cultured and grown to log phase before the test substrates were inoculated.

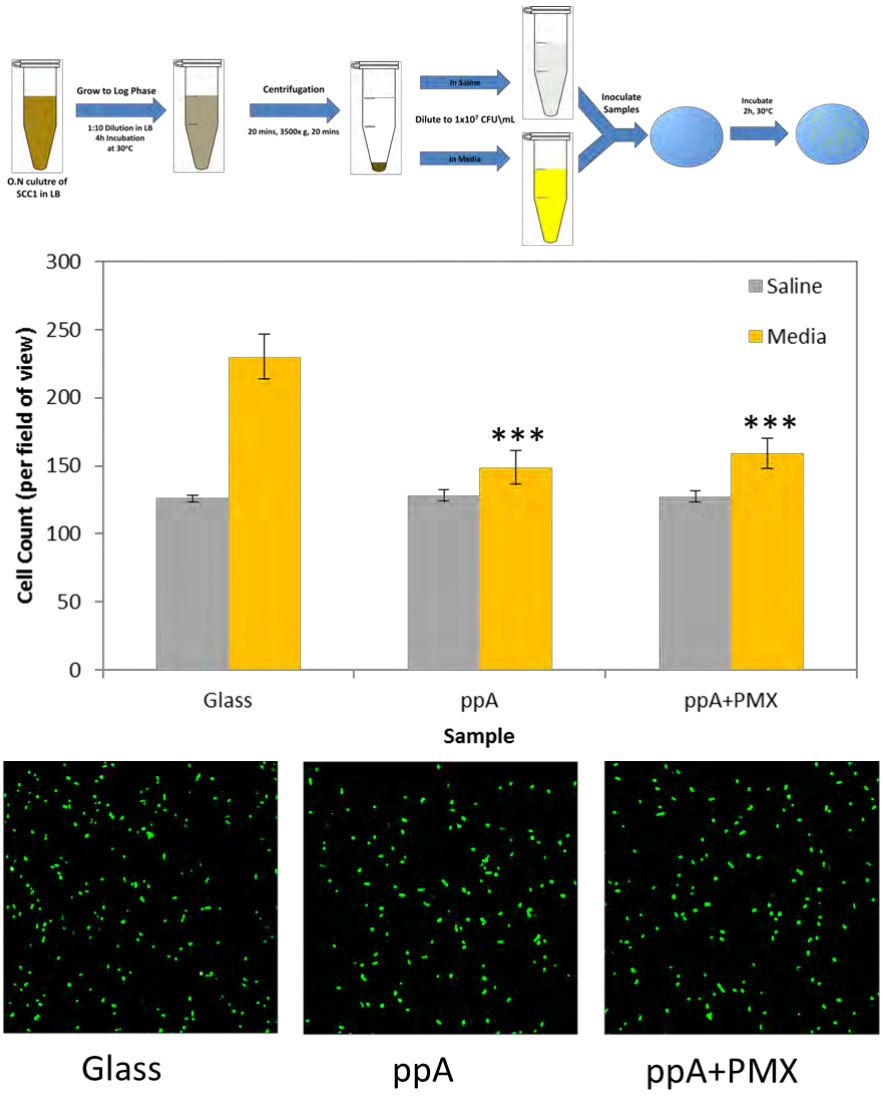


Figure 5.2a) Schemetic of experimental scheme for testing interaction of E. coli (SCC1) with substrates. b) The total number of attached cells (per field of view; 128x128 µm) on Glass, ppA and ppA+PMX with

standard error of mean shown. The total number of cells adhered to the ppA and ppA+PMX is significantly different ($p<0.001$) to the control glass when grown in full culture media. c) representative figures of average number of SCC1 grown in media on Glass, ppA and ppA+PMX per field of view (128x128 µm).

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Figure 5.2b shows the interaction of the SCC1 with the substrates by showing the total number of bacteria per 128x128 µm field of view. SCC1 were grown in saline (grey bars) and growth medium (yellow bars). There was no statistical difference ($p>0.05$) between the total number of cells on the surfaces when grown in saline. There was a statistically significant decrease ($p<0.001$) of approximately 30% in the number of adhered cells on the ppA and ppA+PMX samples when the bacteria were grown in full media.

By culturing the bacteria in full media they are able to grow and proliferate on the surface. Intuitively, this explains the increased number of adhered bacteria when comparing those grown in media and those in saline. However, there is a decrease in total bound bacteria on the ppA and ppA+PMX compared to the glass control. It is expected that initial adhesion to the highly oxygenated and hydrophilic (ppA) decreases initial adhesion rates of bacteria due to electrostatic interactions.[22, 25-28] It can be assumed that the same electrostatic forces would influence binding when the bacteria is cultured in saline and not in culture media. If the initial binding was directly induced by electrostatic interactions, the addition of the PMX to the surface would modify the bacterial binding. This indicates that adhesion to these surfaces is highly influenced by the bacteria in terms of phenotypic expression and metabolic changes

induced by the assay conditions and not only the physico-chemical properties of the surfaces.

That being said, initially, it was assumed that the presence of PMX on the surface would increase E. coli adhesion to the surface due to the PMX and *E. coli* interactions, or cause some antimicrobial effects as the immobilised PMX interacts with the bacterial cell membranes. However, there were no observable differences between the number of cells adhered to the ppA and ppA+PMX compared to the glass only control when grown under static (saline) conditions. This demonstrates that the adhesion forces between the surfaces and the bacteria are not altered and there is no enhancement of cellular capture relative to the control. Furthermore, it is evident that the ppA and ppA+PMX exhibit no antibacterial activity against the SCC1 compared to the untreated glass control.

In solution, the cationic ring structure of the PMX interacts directly with the LPS and due to its high affinity, displaces divalent cations in the bacterial membrane which destabilises it.[29, 30] After binding to the LPS the fatty acid tail of the PMX inserts into the membrane and disrupts the membrane. This relies on the direct interaction of the PMX and the bacteria. However, in the case of surface immobilised PMX, the addition of polymyxin does not seem to influence the interaction of the SCC1 when compared to the ppA only surfaces. Although there may be an initial binding of the bacteria to the ppA+PMX, the interaction between the immobilised PMX and the bacteria is strong enough to induce displacement of the cations. Furthermore, it is expected that due to the number of amines located on the polymyxin, there are multiple orientations in which it can bind to the ppA. No evidence of lysed cells could be seen on the confocal

images. This implies that the orientation of the fatty acid chain, or its lack of fluidity, if it has bound to the surface at multiple sites, does not allow interaction between the fatty acid and the bacteria.

**LPS absorption**

Extreme cases of infection can lead to sepsis. Sepsis caused by Gram-negative bacteria is directly related to the body's immunological response to LPS. PMX directly interacts with LPS and PMX immobilised to fibrous carriers have been used for extracorporeal endotoxin removal.[15, 17] This works by the direct interaction between the immobilised PMX and the LPS.

To show that the interaction between LPS and PMX immobilised on ppA has been retained, QCM was used to measure total attachment of LPS to the surface. QCM sensors were coated with ppA and PMX as above. PBS was used to wash the samples within the QCM chamber until the vibrational frequency had stabilised. LPS was pumped through the chamber until sample resonance frequencies stabilised. Samples were washed with PBS and changes in resonance frequency were used to quantify adhered LPS.

Figure 5.3a shows the resonance frequency of the ppA (blue line) and ppA+PMX (red line) coated QCM sensors after the absorption of LPS. After LPS was added to the QCM chamber the ppA+PMX sample showed a steady decrease in resonance frequency until the sensor stabilised after approximately 35 minutes. PBS was used to wash the samples to remove any unbound LPS. ppA samples attached a total of 15 ng of LPS whereas ppA+PMX samples attached 52 ng of LPS per $cm^2$.

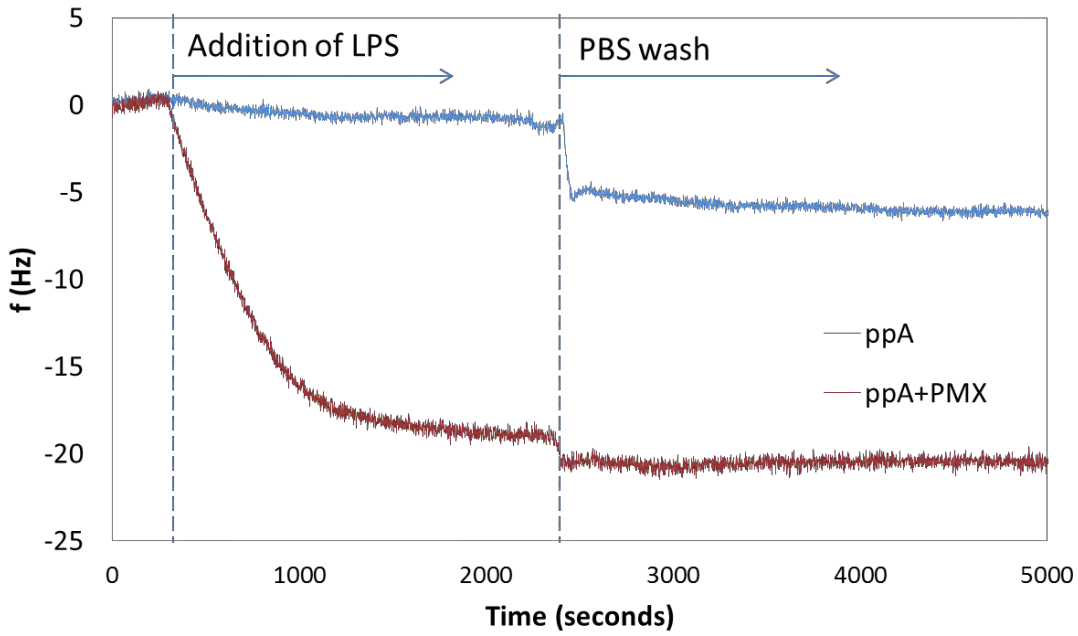


Figure 5.3 Real time quartz crystal microbalance trace demonstrating the decreae in resonance frequency of the sensors due to the mass gained from the attachment of LPS from solution onto ppA (top:blue) and ppA+PMX (bottom: red).

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It is evident that although the PMX is immobilised to a solid substrate it is still able to attach to LPS. The cationic nature of PMX allows it to interact specifically with the phosphate groups present on the Lipid A portion of LPS due to electrostatic interactions.[31] Lipid A is highly conserved and is responsible for the high toxicity of various LPS from an array of bacterial species. The work presented here builds on the ability for PMX to absorb LPS by combining it with a surface that seems to decrease initial bacterial adhesion under controlled growth conditions. Further to this the process is completely substrate independent which allows the coating to be transferred to any surface regardless of underlying chemistry.

It has been estimated that 70% of all hospital-acquired infections are a result of bacterially infected biomedical devices. In the case of Gram-negative bacteria, the lysis

of the bacterial cells or their general virulence causes the release of LPS.[32] When high levels of LPS enter the blood stream, a systemic overexpression of immunological responses known as septic shock are induced. Extracorporeal treatments to remove LPS are commonly used in the medical field to treat or prevent this. As the plasma polymerisation technique is substrate independent,[21] it is foreseeable that this coating could be used directly on removable biomedical devices such as catheters, to actively slow bacterial growth and remove any endotoxins. The use of a more hydrophilic plasma polymerised co-polymer could be used to achieve less bacterial binding, however, a lower number of active groups may decrease the attachment of polymyxin and subsequently decrease LPS absorption. It is also expected that this surface coating could be transferred to a variety of substrates; including retrievable magnetic micro-particles (Appendix A.1.1) for medical and water purification purposes.

## 5.4 Conclusion

In summary, a substrate independent method for the covalent anchoring of PMX to surfaces was developed. Firstly, it was revealed that ppA alone can decrease bacterial adhesion to surfaces by 30% compared with the glass only control. Next, it was demonstrated that ppA+PMX can also decrease bacterial binding comparable to the ppA. Furthermore, the ability for ppA+PMX to absorb up to 52 ng per $cm^2$ LPS from solution was confirmed. This is the first example of a substrate-immobilised PMX that may be useful for generating coatings for removable medical device that not only decrease the rate of bacterial colonisation but are able to be used for intracorporeal endotoxin isolation and removal.

## 5.5 References


1. World Health Organization, The world health report (2004).

2. R. Maviglia, R. Nestorini and M. A. Pennisi, Current Drug Targets **10** (9), 895-905 (2009).

3. A. T. Qureshi, L. Terrell, W. T. Monroe, V. Dasa, M. E. Janes, J. M. Gimble and D. J. Hayes, Journal of Tissue Engineering and Regenerative Medicine (2012).

4. D. S. Jones, B. C. O. Muldoon, A. D. Woolfson, G. P. Andrews and F. D. Sanderson, Biomacromolecules **9** (2), 624-633 (2008).

5. T. Maneerung, S. Tokura and R. Rujiravanit, Carbohydrate Polymers **72** (1), 43-51 (2008).

6. K. Madhumathi, P. T. Sudheesh Kumar, S. Abhilash, V. Sreeja, H. Tamura, K. Manzoor, S. V. Nair and R. Jayakumar, Journal of Materials Science: Materials in Medicine **21** (2), 807-813 (2010).

7. D. R. Storm, K. S. Rosenthal and P. E. Swanson, Annu Rev Biochem **46**, 723-763 (1977).

8. J. Mares, S. Kumaran, M. Gobbo and O. Zerbe, Journal of Biological Chemistry **284** (17), 11498-11506 (2009).

9. A. Simchi, E. Tamjid, F. Pishbin and A. R. Boccaccini, Nanomedicine: Nanotechnology, Biology and Medicine **7** (1), 22-39 (2011).

10. P. Wu and D. W. Grainger, Biomaterials **27** (11), 2450-2467 (2006).

11. S. Quintavalla and L. Vicini, Meat Science **62** (3), 373-380 (2002).

12. P. Appendini and J. H. Hotchkiss, Innovative Food Science & Emerging Technologies **3** (2), 113-126 (2002).

13. H.-Y. Yu, Y.-J. Xie, M.-X. Hu, J.-L. Wang, S.-Y. Wang and Z.-K. Xu, Journal of Membrane Science **254** (1–2), 219-227 (2005).

14. Q. Li, S. Mahendra, D. Y. Lyon, L. Brunet, M. V. Liga, D. Li and P. J. J. Alvarez, Water Research **42** (18), 4591-4602 (2008).

15. H. Shoji, T. Tani, K. Hanasawa and M. Kodama, Ther Apher **2** (1), 3-12 (1998).

16. Y. Yamamoto, S. Takahashi, M. Kawai, N. Ninomiya, K. Matsui, A. Tsujii, A. Kurokawa, T. Otsuka and H. Shoji, Ann. Medit. Burns Club **6** (3) (1993).

17. M. Kodama, K. Hanazawa, T. Oka, K. Teramoto and S. Nishiumi, Patent No. EP129786-A; JP60005166-A; US4661260-A; US4771104-A; EP129786-B; DE3481280-G; JP5329207-A; JP95049052-B2 (EP129786-A 02 Jan 1985 198502).

18. J. Shepherd, P. Sarker, S. Rimmer, L. Swanson, S. MacNeil and I. Douglas, Biomaterials **32** (1), 258-267 (2011).

19. K. Vasilev, A. Michelmore, H. J. Griesser and R. D. Short, Chemical Communications (24), 3600-3602 (2009).

20. K. Vasilev, J. Cook and H. J. Griesser, Expert Rev Med Devices **6** (5), 553-567 (2009).

21. K. Vasilev, S. S. Griesser and H. J. Griesser, Plasma Processes and Polymers **8** (11), 1010-1023 (2011).

22. B. R. Coad, K. Vasilev, K. R. Diener, J. D. Hayball, R. D. Short and H. J. Griesser, Langmuir **28** (5), 2710-2717 (2012).

23. A. X. Tran, M. E. Lester, C. M. Stead, C. R. H. Raetz, D. J. Maskell, S. C. McGrath, R. J. Cotter and M. S. Trent, Journal of Biological Chemistry **280** (31), 28186-28194 (2005).

24. S. Taheri, A. Cavallaro, S. N. Christo, L. E. Smith, P. Majewski, M. Barton, J. D. Hayball and K. Vasilev, Biomaterials **35** (16), 4601-4609 (2014).

25. B. Gottenbos, D. W. Grijpma, H. C. van der Mei, J. Feijen and H. J. Busscher, Journal of Antimicrobial Chemotherapy **48** (1), 7-13 (2001).

26. A. M. Sandstrom, M. Jasieniak, H. J. Griesser, L. Grøndahl and J. J. Cooper-White, Plasma Processes and Polymers **10** (1), 19-28 (2013).

27. Y. H. An and R. J. Friedman, Journal of Biomedical Materials Research **43** (3), 338-348 (1998).

28. M. Katsikogianni and Y. F. Missirlis, Eur Cell Mater **8**, 37-57 (2004).

29. R. E. W. Hancock, The lancet **349** (9049), 418-422 (1997).

30. A. K. Duwe, C. A. Rupar, G. B. Horsman and S. I. Vas, Antimicrobial Agents and Chemotherapy **30** (2), 340-341 (1986).

31. R. A. Moore, N. C. Bates and R. E. Hancock, Antimicrobial Agents and Chemotherapy **29** (3), 496-500 (1986).

32. A. Kulp and M. J. Kuehn, Annual Review of Microbiology **64** (1), 163-184 (2010).

# 6 Antibiofouling properties of plasma deposited oxazoline thin films[*†]

## 6.1 Preamble

Infections caused by bacteria adhesion and colonisation of medical devices are of increasing concern. Polymer coatings, such as biopassive polyoxazolines, are attracting attention in the biomedical field as a means to overcome this issue. Here oxazoline-based films of controllable thickness and surface properties were developed via the plasma deposition of 2-methyl-2-oxazoline and 2-ethyl-2-oxazoline which do not require the use of organic solvents and catalysts for antifouling applications. It is shown that varying and optimising the deposition conditions of the oxazoline precursor can create films with differing chemistries, as determined via XPS. Human derived fibroblasts were used to assess the biocompatibility of oxazoline polymers deposited at different powers. Furthermore, when deposited under certain conditions, these films can reduce total biofilm formation of *Staphylococcus epidermidis* on the surface by 63% and 77% for 2-methyl-2-oxazoline and 2-ethyl-2-oxazoline, respectively. These plasma deposited oxazoline surface coatings have customisable properties which make them attractive candidates for medical devices.

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## 6.2 Introduction

Undesirable bacterial adhesion to solid surfaces and subsequent biofilm formation affect the performance of many industrial processes, and pose serious problems to the public health. [1, 2] In the medical field, infections caused by the bacterial colonisation of medical devices constitute more than half of all hospital acquired infections (HAI) which are expensive and complex to treat.[3, 4] Each year HAI cause approximately 100,000 deaths in the USA and cost about $40 billion to the healthcare system.[5] It is also well-known that marine vessels have to be regularly cleaned from fouling caused largely by adhered microorganisms.[1, 2],. As a result, the US Navy spends $2.1 billion annually on problems related to biofouling.[6] For similar reasons, filtration membranes used in various industries need to be regularly replaced at great expense.[7, 8] Undesirable biological adhesion also has detrimental effects in food processing [9] and retailing industries,[8] heating and cooling systems,[10, 11] liquid storage and transport,[10] and more.[1]

To date, two main coating strategies are commonly used to prevent fouling by biological entities, namely biocidal and non-biocidal surfaces. Biocidal surface coatings are surfaces that kill bacteria and prevent them from adhering to the surface. These can be further divided into two sub-categories based on their mechanism of action. Contact killing surfaces rely on direct interaction between immobilised antimicrobial molecules and microorganisms (e.g. quartenised surfaces [12, 13]) while releasing surfaces leech a payload and such as antibiotics [14] or other biocidal agents [15] which directly interact with microorganisms. Non-biocidal approaches, also known as biopassive approaches, utilise surfaces that decrease adhesion of proteins or cells.[16, 17]

Biopassive surfaces have gathered attention within the medical and other fields. It is considered that such substrate will prevent initial bacterial attachment and and that any microorganisms that may have attached would be easily removed. Thus it will be possible to prevent biofilm formation which is the greatest concern in many industries. A commonly used strategy towards biopassive properties is the adsorption of poly(ethylene) glycol (PEG) and PEG-copolymers to a surface.[18, 19] However, a common downfall with PEG based surfaces coatings seems to be degradation.[20-22] For this reason, alternatives are actively being sought. One such alternative that recently gained considerable attention are polyoxazoline (POx) based polymers.

POx coatings are typically generated using living cationic ring-opening polymerisation and have been noted for their potential biomedical applications.[23, 24] Tauhardt *et. al*[25] showed that POx generated by either tetraether lipid or (3-glycidyloxypropyl) trimethoxysilane binding, significantly reduced biofilm formation regardless of the test organism. In a comparative review, Konradi *et al*.[21] stated that despite differences between PEG and POx coatings, both polymers exhibit equally effective antifouling ability. Besides, it was concluded that when generated using similar methods, POx surfaces tend to be more stable than their PEG counterparts.[21]

Traditionally, preparation of POx is a lengthy wet process conducted in organic solvents. POx organisation in thin films occurs in a subsequent step which needs to be tailored for particular types of substrates. [24, 26, 27] Other polymerisation techniques including photocoupling [28, 29] and grafting [30] have also been applied to generate POx but these tend to be limited to substrates with specific underlying chemistry. Overall, the formation of polyoxazoline coatings via conventional methods is a slow and

complex multistep procedure which creates organic waste and can only be conducted on a selected range of substrates.

These disadvantage have recently been overcome by using plasma deposition processes to produce nanometre thin polyoxazoline coatings.[31-33] A number of oxazoline ring containing compounds are perfectly suitable candidates for plasma polymerisation due to their low molecular weight liquid at room temperature (e.g. from 85g/mol for 2-methyloxazoline to 147g/mol for 2-phenyloxazoline).

In a pioneer study on the plasma deposition of 2-methyl-2-oxazoline, it was demonstrated that plasma deposited polyoxazolines (ppOx) offer a set of unique properties some not achievable by conventional means of polymerisations. The ppOx retained the chemical reactivity of the oxazoline group which allowed to covalently bind biomolecules.[31] Furthermore, initial testing revealed that the coatings had excellent biocompatibility and capacity to withstand deposition of strong bacterial biofilm. In view of the potential applications and impact that ppOx could have on the biomedical and other affected industries, the antifouling performance of ppOx and show how the variation in film chemistry resulting from changes in the plasma deposition parameters can be used to influence mammalian cell adhesion and bacteria growth on the surface are further evaluated.

Herein, the effect of plasma deposition time, power and flow rates on the bio-adhesive properties of plasma deposited 2-methyl-2-oxazoline is determined. Substrate independent films with controlled deposition were generated. The coatings were evaluated in terms of chemical composition, film thicknesses and stability. The high biocompatibility of various ppOx coatings and their properties on human dermal

fibroblasts attachment is shown. These films were further optimised to reduce biofilm formation by *S. epidermidis.* Next, the balance between film properties and antifouling properties of the 2-methyl-2-oxazoline films was investigated by inducing chemical changes on the surface. Finally, the antifouling of another plasma polymerisable oxazoline precursor (2-ethyl-2-oxazoline) was demonstrated.

## 6.3 Results and Discussion

**Substrate Preparation**

It is well known that in plasma polymerisation the deposition conditions allow for generation of coatings with a coatings vast variety of physical and chemical properties. [34, 35] Figure 6.1a shows the thicknesses of the methyl-ppOx coatings deposited using combinations of CW RF powers of 10, 30 and 50 W and deposition times of 1, 2 and 5 minutes at precursor flow rate of 8 sccm. It can be seen that in the investigated conditions film thickness increases as function of both plasma deposition power and deposition time.[36] Figure 6.1b shows the thicknesses of coatings generated at 50 W using flow rates of 4, 8 and 12 sccm.

The corresponding ppOx films thicknesses were 68, 76 and 111 nm respectively. As anticipated, increasing the flow rates and allowing more monomer into the chamber led to an increase in the ppOx film thickness. In all cases the results indicate that the oxazoline deposition conditions used constructive processes of film build up dominate over destructive processes. The stability of the films in water was tested by assessing changes in film thickness. Assessment of film behaviour in aqueous solvents is important for biologically related applications such as biofouling.

Figure 1c shows the percentage of film thickness loss for ppOx prepared using power of 10, 20 30 40 and 50 W, deposition time of 5 minutes and a set flow rate of 8 sccm. ppOx film stability varies greatly with plasma deposition power.[31] Films deposited at 10 W exhibit a 97.7% loss in film thickness when placed in water. As power increases to 20, 30 and 40 W coatings become more stable with a 85.0%, 62.7% and 38.7% loss in thickness, respectively. Samples produced at 50W were deemed stable as only 6.7% thickness loss was measured.

The loss of thickness at low deposition power upon immersion in water is not surprising and consistent with published studies on other plasma deposited coatings. This thickness loss has been often associated with dissolution of low molecular weight water soluble fractions of the films which have not been sufficiently cross-linked to other building blocks of the deposit or substrate.[37] It should be noted that there was no delamination of the coatings since floating sheets of the film were not observed.

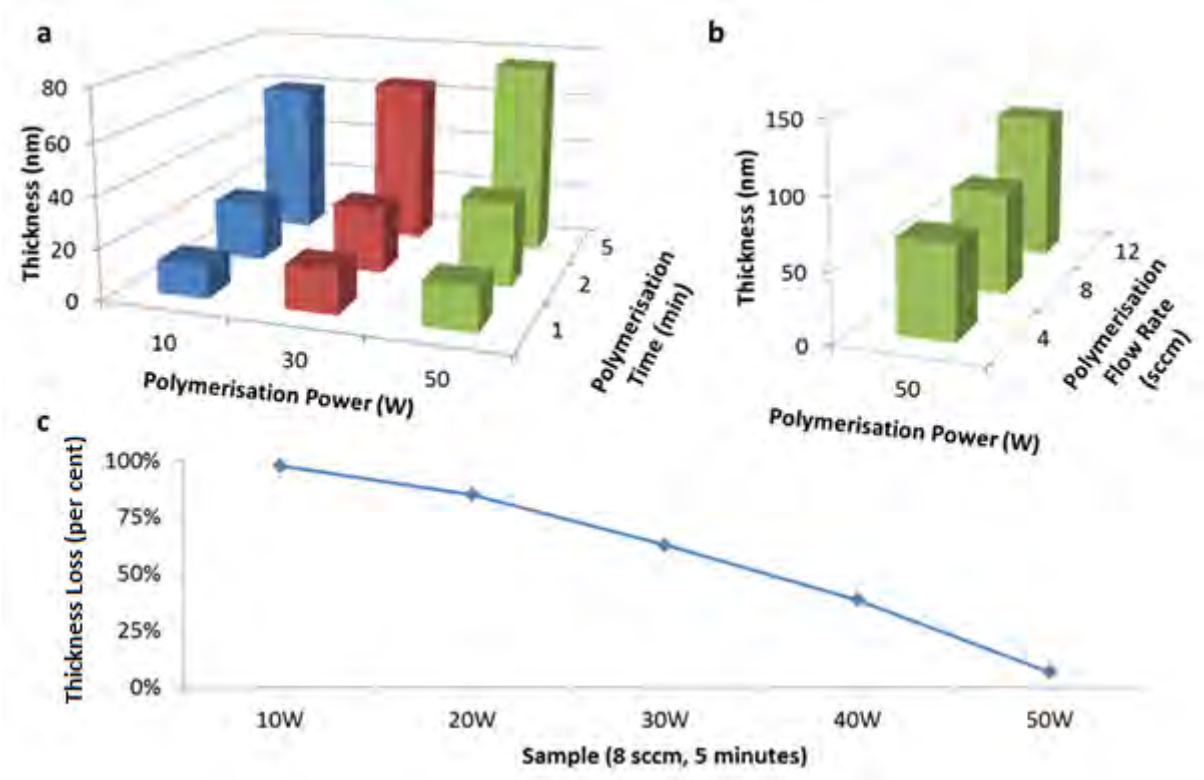


Figure 6.1 a) Film thickness as determined by ellipsometry for varying polymerisation deposition power (10, 30 and 50 W) and times at a set flow rate of 8 sccm. b) The effect of 4, 8 and 12 sccm plasma polymerisation flow rates on ppOx thickness when deposited at 50 W. c) Stability of ppOx films deposited at 10, 20, 30, 40 and 50 W at 8 sccm showing percentage of film thickness loss. All values show mean where n=2.

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**Biocompatibility**

Firstly, the biocompatibility of the newly developed ppOx coatings is examined. Good biocompatibility is an essential requirement for any coating which may be applied on medical devices.[38] HDFs are used as a model for assessing any potential cytotoxic effects of the ppOx. These cells were chosen as they are associated with wound healing and tissue repair and often used for evaluation of surface biocompatibility.[39-42] The metabolic activity of the adhered cells were used as a relative measure of the viability of HDFs on the surface. The metabolic activity of fibroblasts adhered to the

glass, plasma deposited allylamine (ppAA) and 10, 20, 40 and 50 W ppOx samples deposited at 8 sccm for 5 minutes was quantified in terms of florescence intensity. Resazurin is reduced by the cells and converted into a florescent state called resorufin. This assay assumes that each cell has the same metabolic activity.

The fibroblast metabolic activity relative to ppAA is shown in Figure 6.2a. ppAA was chosen as a reference as it is known for its good biocompatibility and is often used as a surface for cell culture,[15] as in Chapter 4. Samples produced at 10 and 20 W show a reduction in HDF adhesion, compared to the ppAA, as determined by the decrease in resorufin present after incubation. This can be directly attributed to the film stability. When deposited at low powers, the films are unstable and dissolve in aqueous environments as shown in Figure 6.1 and discussed above. An increase in the HDF viability is seen as the plasma deposition power increases. Coatings prepared using deposition power of 50W showed similar viabilities as the ppAA control surfaces which are well known, as other nitrogen rich surfaces, for their excellent biocompatibility.[43, 44] In these cases cell numbers were assumed to be equal as all grew to full confluence on all stable samples.

The potential inflammatory response to the ppOx coating deposited at 50W for 5 minutes was assessed by measuring cytokine secretion from bone marrow-derived primary macrophages (BMDM), *in vitro*. BMDM cells have the capacity to medicate early innate inflammatory responses. BMDM were directly incubated on the surfaces and pro-inflammatory responses stimulated using lipopolysaccharide. Expression of cytokines were quantified using ELISAs. The level of TFN-α, IL-6 secretion are shown in Figures 6.2.b. and 6.2.c. These studies showed an overall reduction in the expression of

TNF-α and IL-6 on the ppOx coating compared to the tissue culture plate control (TCP) and the ppAA.

Overall, these results indicate that that the biocompatibility of the coatings is strongly related to their stability in aqueous solvents. Overall, coatings having greatest stability had also the highest biocompatibility.

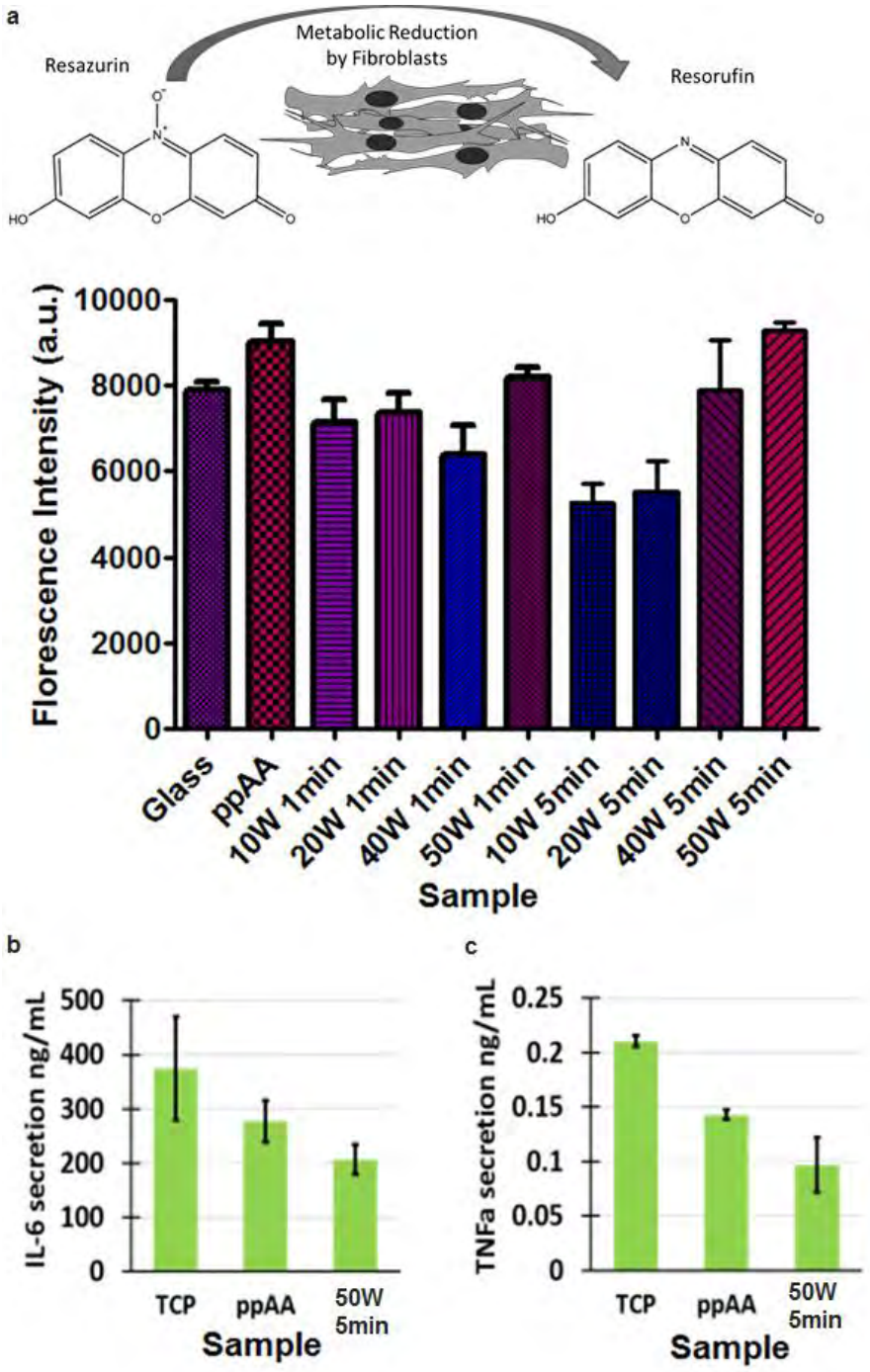


Figure 6.2a) Metabolic activity of HDF grown on ppAA and 10W, 20W, 40W and 50W ppOx deposited for 1 and 5 minutes as an indication of total HDFs adhered to the surface after 3 days as determined from florescence intensity (λex =544nm and λem =590nm) from metabolically reduced alamar blue.. b) IL-6 cytokine and (c) TNF-α cytokine expression of BMDM grown on POx, tissue culture plates and ppAA surfaces. All error bars show ±SEM. Reproduced in part, with permission from < M. Ramiasa, A. Cavallaro, A. Mierczynska-Vasilev, S. Christo, J. Gleadle, J. D. Hayball and K. Vasilev, Chemical

Communications, 2015, 51, 4297-4282.>[31] with permission from the Royal Society of Chemistry as "Author reusing their own work published by the Royal Society of Chemistry"

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**Antifouling Properties**

The ability for ppOx to be used as an antifouling surface was assessed using ppOx films deposited using high plasma RF powers. Surface chemistry is expected to be the same at set power levels for the same flow rates, [36, 45] i.e. all 50W 8 sccm samples should have the same chemistry regardless of polymerisation time, due to the constant level of precursor fractionation within the plasma field.[36, 45]

In order to generate films with different surface chemistry, precursors flow rates of 4, 8 and 12 sccm were used during the plasma deposition. High resolution XPS C1s spectrum reveals that changes in flow rates induced variations in the carbon atomic environment of the PPOx coatings, as evidenced from the differences in C1s peak shape (Figure 6.3a) Figure 6.3b shows C:N ratios of ppOx shows a trend towards decreasing with increased flow rates determined from the full XPS spectra. Deconvolution of the C1s revealed an increase in the peak located at 286.5 eV. This change correlates to an increase in C-N within the film when deposited at higher flow rates.

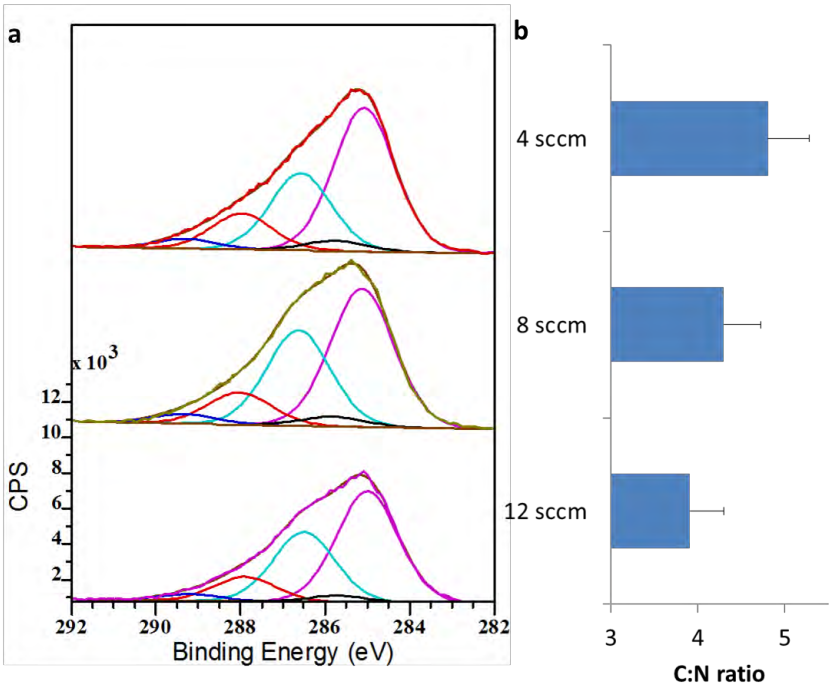


Figure 6.3 a) C1s spectra of ppOx showing differnces in surface chemistry of ppOx deposited at 4 sccm (top) 8 sccm (middle) and 12 sccm (bottom). b) C:N ratios as determined via XPS survey showing a downwards trend with increasing flow rates. Error bars assume 10% sampling error.

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*S. epidermidis* was used as a model organism to evaluate the ability of the ppOx coating to resist bacterial colonisation. This organism was used because it is a very proficient biofilm former and is medically relevant. *S. epidermidis* is the organism most often associated with indwelling medical device infections.[46, 47] $1x10^6$ CFU\mL of bacteria were allowed to interact with the ppOx coated substrates and form biofilms overnight. Samples were then washed twice to remove any loosely bound bacteria before staining the formed biofilms with safranin. Excess safranin stain was washed off of the samples to ensure that the only remaining stain was that absorbed by bacteria. During the routine washing stages the biofilm detached from ppOx.

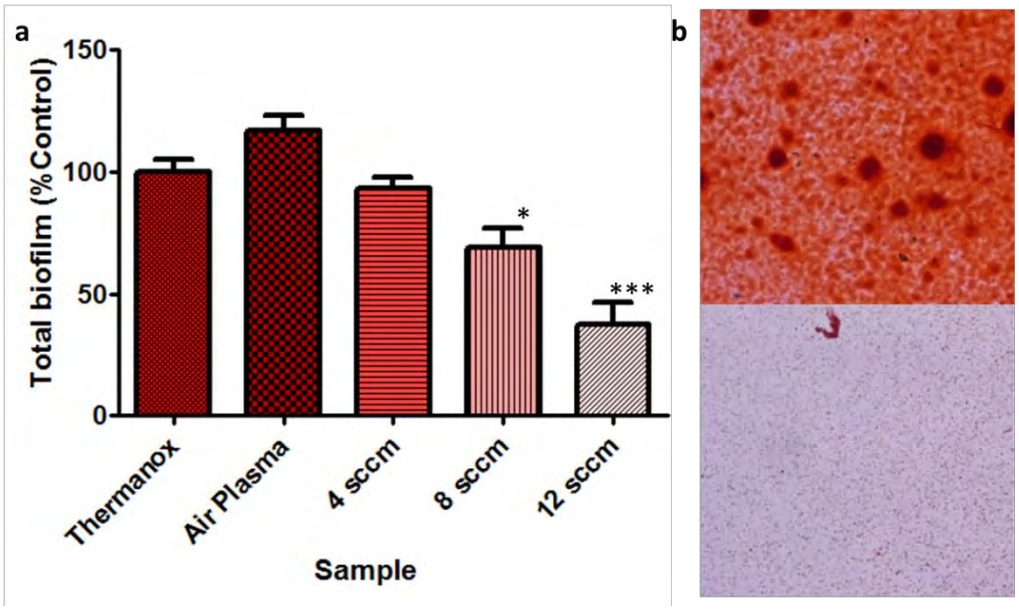


Figure 6.4 a) Eluted safranin as indication of total biofilm as precent biofilm formed of ppOx films. Error bars show ±SEM; n=3. b) Microscopy images of full biofilm grown of glass (top) and reduced\dislodged biofilm on plasma deposited ppOx (bottom) showing the difference between fouled and low-fouled samples.

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Figure 6.4a shows the level of biofilm formed on ppOx films deposited for 5 minutes at 4, 8 and 12 sccm compared to the total biofilm formed on uncoated Thermanox substrates. Themanox treated with Air Plasma was used as a second control substrate. All samples were blanked relative to an untreated coverslip. The total biofilm, as quantified by eluting safranin stain on the ppOx samples, decreased by 7%, 31% and 63% for the 4, 8 and 12 sccm samples, respectively. The total biofilm on the Air Plasma treated Thermanox showed a 17% increase. The results show an evident change in total adhered biofilm on ppOx samples prepared at the different flow rates.

The ppOx generated at 8 sccm and 12 sccm showed a statically significant decrease in the total adhered biofilm after washing compared to the control Thermanox ($p<0.05$ and $p<0.001$, respectively). This indicates a loose binding of the bacteria and

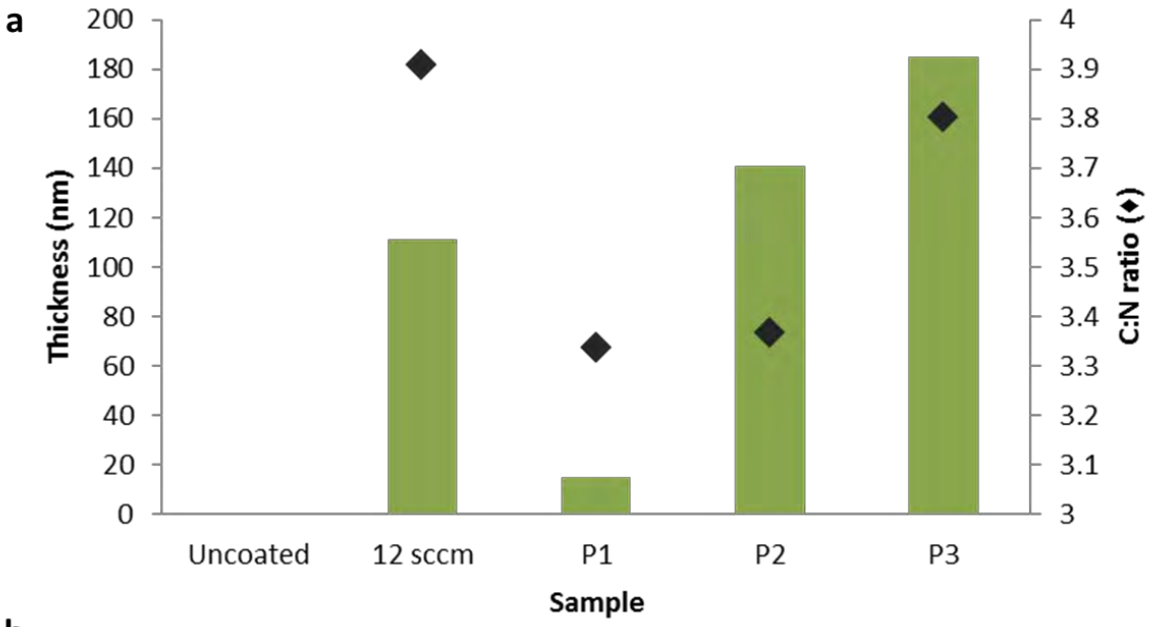
extracellular matrix. No statistical difference ($p>0.05$) between the control and the Air Plasma and the 4 sccm samples were observed. These results indicate that film chemistry plays a major role in the antifouling ability of the surfaces. As flow rates increase the precursor tends to fractionate less within the plasma field allowing for a greater retention of precursor chemistry. Here it appears that retention of oxazoline chemistry is important for antifouling.

Figure 6.4b shows the difference in adhered biofilm on Air Plasma treated Themanox coverslips (top) and 12 sccm ppOx on Thermanox after staining with safranin (bottom). The decrease in observable safranin stained biofilm (red) demonstrates the ability to decrease bacterial adhesion and biofilm formation. Changes in biofilm adhesion may be due to a combination of effects such as a decrease in extracellular matrix adhesion forces or, a disruption in intercellular signalling and communication which would in turn induce changes in the way that the bacteria interact with the surface. These mechanisms will be interrogated in follow-up studies.

The ability to deposit the antifouling ppOx coatings on a variety of surfaces is demonstrated by generating the same antifouling film on a 13mm glass coverslip. Figure 6.5 shows the addition of ppOx to glass coverslips. As expected there is a clear decrease in strongly adhered biofilm, as indicated by the lack of safranin (red) stained bacteria on the surface compared to the untreated glass control. This shows the capability of the plasma deposition technique to coat any surface without any specific pre-treatments. In practical terms having the ability to modify any surface is of high importance as different materials may need modification even in the same medical

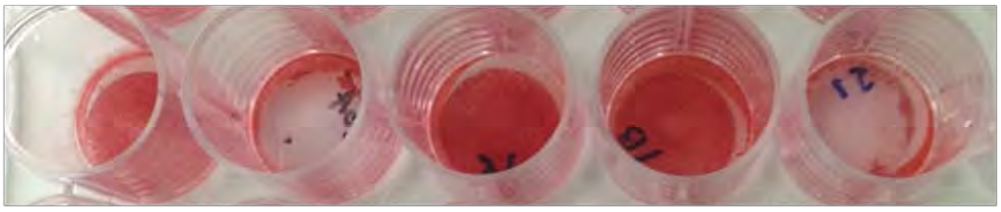
device. For example different components of a hip implant may be made from different materials such as metals, polymers, ceramics and various composites [48].

The use of a “pulsed” plasma polymerisation mode, as opposed to a continuous wave plasma, helps retain original precursor chemistry within the deposited organic layer as there is less fractionation of the monomer due to the “off” period.[49] A pulsed plasma technique was used to increase the retention of oxazoline chemistry within the ppOx films by varying on\off times of 50 W RF samples with flow rates of 12 sccm.. These surfaces were assessed for antibiofilm properties. Furthermore, by increasing the polymerisation time to 10 minutes for the pulsed plasma polymerisation experimentation, any additional effects of surface thickness and underlying substrates can be explored. These changes in plasma polymerisation method were utilised to further understand the influence of ppOx on the adhering bacteria. Pulsed plasma polymerisation of ppOx was performed with “on” times of 1 ms, 10.5 ms and 20 ms respectively, with a total cycle of 21ms to make samples P1, P2 and P3. Untreated glass coverslips were used as controls.




b

Stained Biofilm



c

Polymerisation Conditions

| Uncoated | 12 sccm<br>50 W<br>5 min | P1_Pulsed<br>On: 1ms<br>Off: 20ms<br>12 sccm<br>50 W<br>10 minutes | P2_Pulsed<br>On: 10.5ms<br>Off: 10.5ms<br>12 sccm<br>50 W<br>10 minutes | P3_Pulsed<br>On: 20ms<br>Off: 1ms<br>12 sccm<br>50 W<br>10 minutes |
|---|---|---|---|---|

Figure 6.5 a) Thickness of ppOx produced with at pulsed plasma polymermisation conditions of uncoated glass, 12 sccm ppOx samples, P1, P2 and P3 (left axis) and C:N ratio as determined from XPS survey spectrum (right axis). b) photograph of stained *S. epidermidis* on glass, 12 sccm ppOx samples, P1, P2 and P3 (left to right) with pulsed plasma conditions listed below.

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Figure 6.5a shows the thicknesses of the pulsed plasma deposited samples for comparison against the original samples. Plasma deposition time was increased to 10 minutes in order to match the thickness of sample P2 to that of the 12 sccm 5 minute ppOx as shown in Figure 6.5a Samples P1, P2 and P3 had thicknesses of 15, 141 and 185 nm respectively as seen on the left axis. The changes in the C:N ratio on the

surface show changes in surface chemistry depending on the deposition conditions. ppOx has a theoretical C:N ratio of 4. The ppOx deposited under CW and sample P3 have C:N ratios of 3.9 and 3.8 respectively. Samples P1 and P2 have C:N ratios below this, 3.3 and 3.4 respectively. Figure 6.5b shows the antifouling efficacy of the ppOx deposited the various pulsed plasma conditions where the samples were treated with *S. epidermidis* and stained as before.

High levels of safranin stained biofilm are visible on the control sample and samples P1 and P2. Sample P3 shows a high level of antifouling ability comparable to the original 12 sccm sample, as shown by the decrease in safranin (red) stained biofilm on the surface. This result indicates that the antifouling ability of the ppOx is not solely dependent on thickness. Due to the long plasma “on” time of sample P3, film chemistry would be highly comparable to the standard 12 sccm minute sample as seen in C:N ratio. The precursor deposited onto samples P1 and P2 would undergo less fractionation due to the “off” time of the cycle not suppling energy to fractionate the precursor vapour within the chamber. This means that there would be vast differences between P1 and P2, and the 12 sccm sample made under regular CW plasma. From the literature it is known that pulsed plasmas tend to have lower levels of crosslinking within the deposited organic layer and increased chemical functionality. This change in surface properties will strongly influence the adhesion of the bacteria. This reveals that extreme oxazoline functionality retention is not ideal for antifouling applications and a certain degree of cross linking is required.

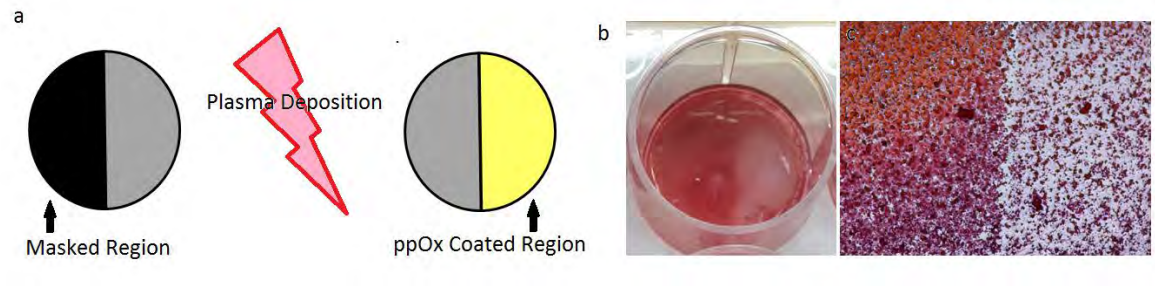


Figure 6.6 a) Schematic of masked region and plasma depostion showing the half of the well coated with ppOx b) Biofilm growth on ppOx film in half treated 12-well tissue culture plate c) the interface of the coated\uncoated areas on the well as observed via microscopy.

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To demonstrate the controllability of the plasma deposition, half of a well of a standard 12-well tissue culture plate was coated with the ppOx. Figure 6.6a shows a schematic of the protocol used to deposit ppOx to the selected area of the plate. Half of the individual wells were masked during the plasma deposition. Upon removing the mask, half of the individual wells were functionalised with ppOX while the other half remained untreated. Using the same biofilm formation and staining protocols as above, the strongly adhered biofilm was visualised on the interface of the coated and uncoated areas. Figure 6.6b shows a photo of the stained biofilm and a microscope image a coated\uncoated interface in the well. This shows a clear reduction in the total biofilm adhering to the ppOx coated half of the well.

**Protein binding and implications**

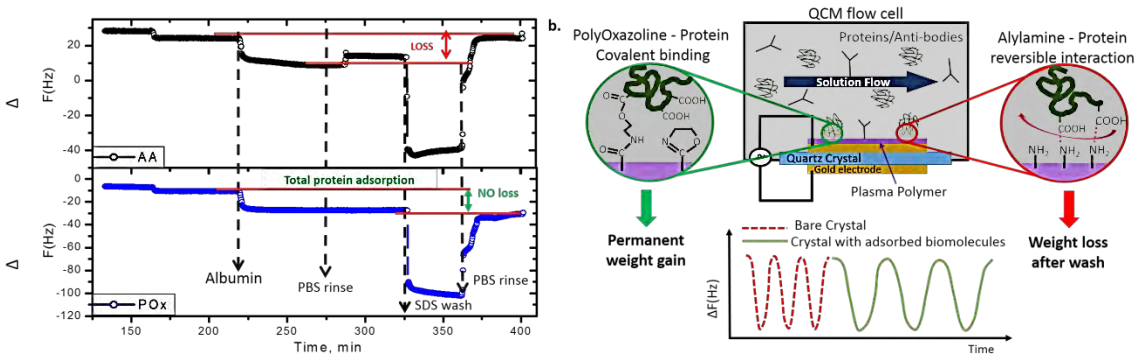


Figure 6.7 a) Real time QCM frequency trace demonstrating that the mass gained almost instantly following exposure to albumin solution (negative deflexion) is retained after PBS rinse and SDS wash (10 v% in PBS) on the ppOx (50W 5 min) coated QCM crystal (bottom trace), while it is lost for the allylamine equivalent (top). b) Schematic of the different protein adsorption mechanism occurring on the ppOx (50W 5 min) and ppAA coated QCM crystals.

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The capacity of the plasma deposited ppOx films unique chemistry to covalently bind biomolecules was examined in real time using QCM-D measurements. The experiments were conducted under flow conditions in physiological buffers and amine group-rich ppAA coatings were used as controls. Exposure to a 0.1 mg/mL albumin (BSA) solution resulted, for both control ppAA and ppOx coated sensors (Figure 6.7a and 6.7b, respectively), in a sharp decrease in frequency, corresponding to a mass increase due to protein attachment to both plasma polymer films. After equilibration, the sensors were subjected to thorough *in situ* rinsing and washing steps, first with PBS, then with concentrated SDS (10%) and finally, with PBS again. The purpose of the SDS was to remove from the surface proteins that were physically adsorbed only. Analysis of the frequency trace throughout these washing steps revealed differences in the protein binding behaviour dependent on the nature of the plasma polymer film.

The frequencies of ppAA coated sensors increased after SDS wash and PBS rinse recovering completely their initial values, which reveals that all adsorbed protein had

been washed off the sensor surface. On the other hand, no frequency change was observed for ppOx coated samples following PBS rinse relative to the change in frequency after initial adsorption. The permanent negative deflection remained after washing with SDS and rinse with PBS, thus providing evidence of irreversible attachment and indicative for covalent protein binding on ppOx plasma films. The average amount of BSA covalently bound to ppOx plasma film was estimated using the Sauerbrey equation and found to be 70 ± 7 ng.cm$^{-2}$. This corresponds to approximately 25% surface coverage which is appropriate for biosensor platforms as it allows sufficient spacing between protein molecules for conducting binding reactions.

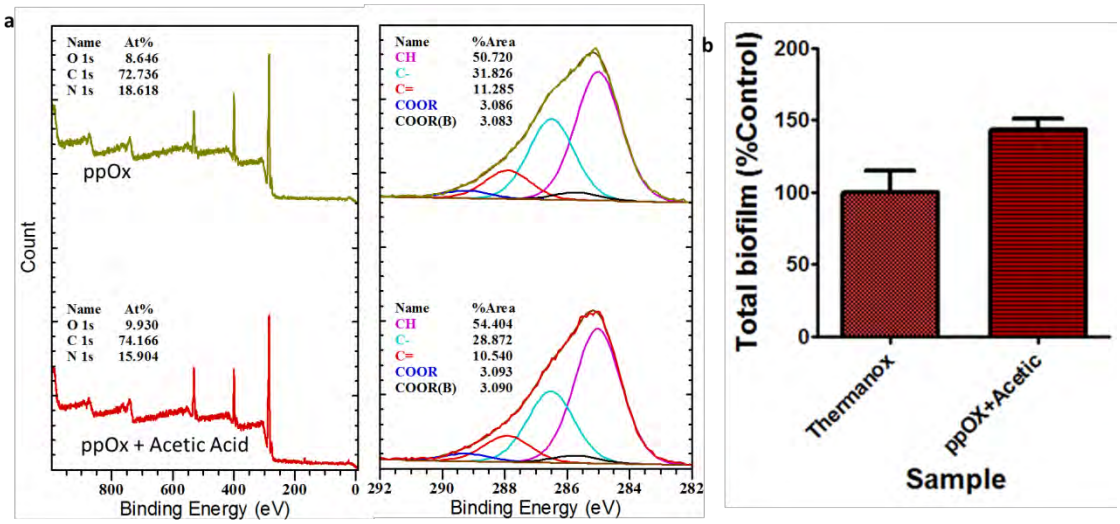


Figure 6.8 a) XPS high resolution C1s showing original chemistry of ppOx (top) the difference in surface chemistry caused by the addition of acetic acid to ppOx (bottom) b) Total biofilm on the surface of 12sccm 50W ppOx treated with acetic acid showing the difference in total biofilm on the surface. Error bars show ±SEM.

As demonstrated above carboxillic acids (-COOH) can be irrervesibly bound to oxazoline rings retained on ppOx surfaces.[31] It is hypothesized that the retention of the oxazoline ring is important to the ppOx ability to prevent strong bacterial adhesion. Thus, it was expected that covalent binding of the carboxylic acids to active sites in the polymer would mask these sites and increase biological adhesion. Acetic acid was bound to ppOx and changes in the antifouling ability of the polymer were investigated. Intuitively this will utilise any functionality retained from the oxazoline rings to bind the acid to the surface. This will leave a less functional surface to which bacteria can effectively adhere. The original ppOx contains an assortment of surface chemistries and active groups including some retianed oxazoline rings. Upon treatment with the acetic acid, the acid binds and breaks the oxazoline ring changing the chemistry of the surface by adding more carbon and oxygen.

XPS was used to obtain a survey and high resolution C1s spectrum of the acetic acid treated ppOx. Figure 6.8a shows the chemistry of ppOx (top) and ppOx+acetic acid (bottom). The total percentage of carbon and oxygen present on the surface increases, from 72.7 to 74.2 At% and 8.6 to 9.9 At% respectively, and total nitrogen decreases from 18.6 to 15.9 At% with the addition of the acetic acid. The XPS survey confirms that the surface coating is still present after the binding of the acid as there is no underlying silicon detected. Figure 6.8a also shows changes in the shape of the C1s peak. After deconvolution it is revealed that there is an increase the C-H (285.0 eV) from 50.72% to 54.40% and an increase of the C- at 286.5 eV indicating a greater proportion of single aliphatic bonds.

Figure 6.8b shows the effect on the antifouling ability of the ppOx after post modification with the acetic acid. The addition of acetic acid to the surface disrupted the antifouling ability of the ppOx. This shows the delicate balance of chemistry and surface properties required to retain the antifouling ability of the ppOx.

**Plasma deposited 2-Ethyl-2-Oxazoline**

The antifouling ability of plasma deposited 2-ethyl-2-oxazoline (Et-ppOx) was also investigated. Et-ppOx was deposited at 10, 20, 30, 40 and 50 W for 2 minutes at 8 sccm (Et-10W Et-20W, Et-30W, Et-40W and Et-50W) on Thermanox coverslips. Figure 6.8a shows the total biofilm grown on the surface after 24h as quantified using safranin staining (as above). Et-20W, Et-30W, Et-40W and Et-50W all showed a significant decrease in total biofilm on the surface after washing $p<0.001$. This indicates a loose binding of the bacteria and extracellular matrix. Although there was a slight increase in total biofilm on Et-10W this was not statistically significant ($p>0.05$). Figure 6.9b shows safranin (red) stained biofilms grown on ppAA (top-right), ppOx (bottom-left) and Et-ppOx (bottom-right) and an uninoculated glass coverslip as a control (top-left). The dramatic decrease in up taken safranin clearly shows the reduction of biofilm on the deposited oxazoline samples. Figure 6.9ci shows a typical safranin biofilm grown on glass and autofluorescence image of the biofilm (inset). The addition of the Et-ppOx decreases biofilm on the surface (Figure 6.9c). Figure 6.9cii also shows the autofluorescent nature of the Et-ppOx (inset). More work into the optimisation of the Et-ppOX is currently being conducted by Vasilev and co-workers.

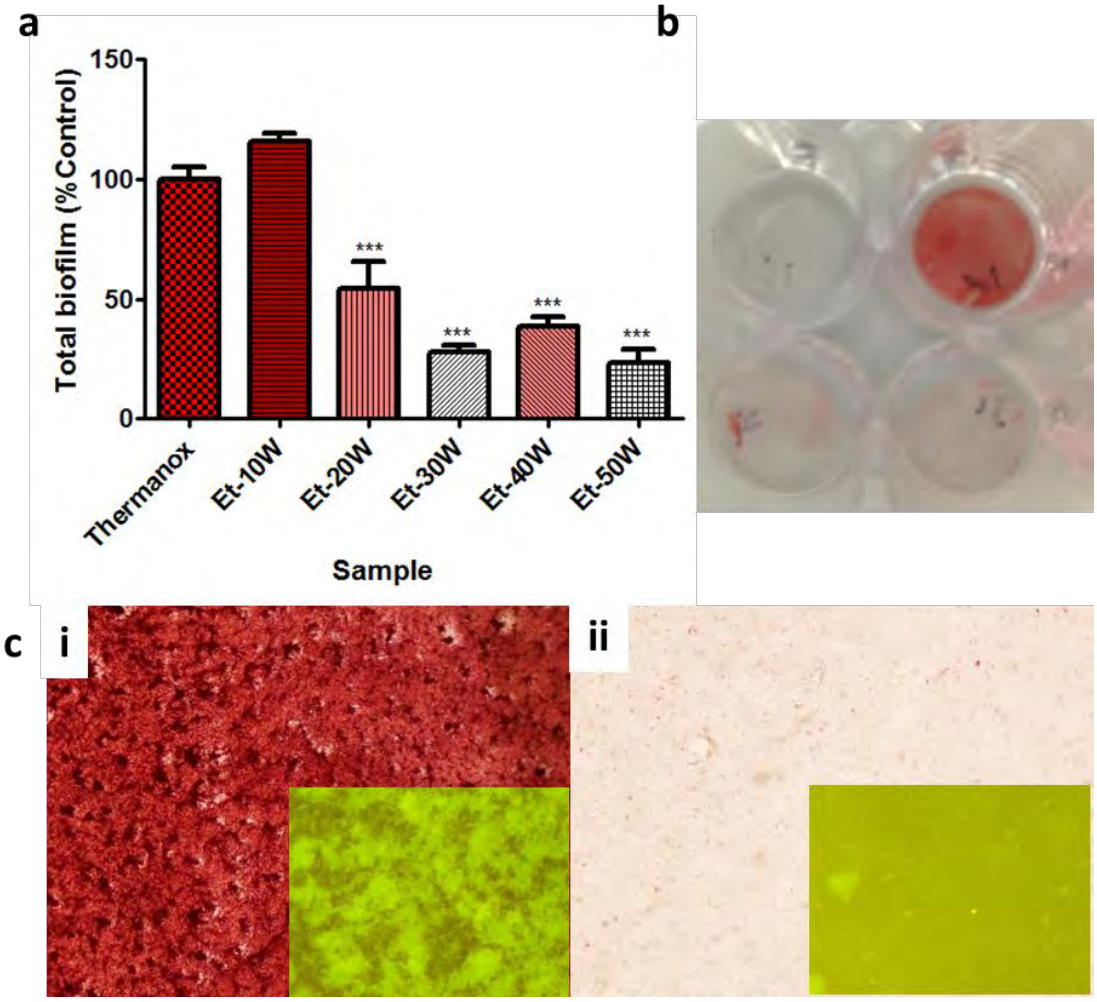


Figure 6.9 a) Total biofilm on the surface of 8sccm 50W Et-ppOx deposited for 2 minutes showing the difference in total biofilm on the surface. Error bars show ±SEM b) Stained biofilms on uninoculated glass (top-left), ppAA (top-right), ppOx (bottom-left) and Et-ppOx (bottom-right) c) (i) Microscope image of stained biofilm on untreated thermanox and autoflourence of typical biofilm on ppAA (inset) and (ii) microscope image of stained biofilm on Et-ppOx and autofluorescence typical remaining biofilm on low-fouling Et-ppOx (inset)

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POx surfaces made via wet chemical processes have been proposed for use as antifouling surfaces for biomedical applications.[21, 50] Here it was demonstrated that antifouling properties are achievable through plasma deposition of 2-methyl-2-oxazoline and 2-ethyl-oxazoline. By utilising a deposition method that does not require

the use of solvents or catalysts, oxazoline-based polymers can be made sustainably. Varying and optimising the plasma deposition conditions of the oxazoline precursor can create ppOx films with different chemistries and controllable thickness. Furthermore, as plasma deposition processes are substrate independent and can be up scaled, the prospect for ppOx in industrial use is foreseeable. However, it must be noted that polymer stability should be investigated before use in the biomedical field. Indeed, the potential cytotoxic or genotoxic effects, for example those produced by some oxazoline derivatives,[51] of any dissolved polymer or released polymer fragments from unstable films needs further investigation before any biomedical use of ppOx.

## 6.4 Conclusions

Plasma polymerisation of oxazolines provides a reproducible and substrate-independent method to produce oxazoline based organic films (ppOx) with controllable functionalities. It was shown that the adhesion of *S. epidermidis* biofilms ppOx and Et-ppOx coated surfaces, produced under specific parameters, was significantly reduced and 63% of the biofilm on the surface detached during routine washing steps. Furthermore, the ability to use a "pulsed" plasma polymerisation method was shown. Interestingly, the antifouling properties were only retained when the plasma conditions closely matched those of CW plasma polymerisation. Masking the outer surface chemistry by the covalent acetic acid to the surface reduced any bio-passive properties of the surface. The variety of plasma polymerisation conditions not only allows for the generation surfaces with bio-passive attributes but can also retain some chemical functionality that can be used for further functionalisation. Although,

initial investigation into the plasma deposition of 2-ethyl-2-oxazoline has shown promising antiboifouling properties, further optimisation of these films is required.

## 6.5 References


1. Banerjee, I.; Pangule, R. C.; Kane, R. S., Antifouling Coatings: Recent Developments in the Design of Surfaces That Prevent Fouling by Proteins, Bacteria, and Marine Organisms. *Advanced Materials* 2011, 23, 690-718.

2. Callow, J. A.; Callow, M. E., Trends in the development of environmentally friendly fouling-resistant marine coatings. *Nature Communications* 2011, 2, 244.

3. Vasilev, K.; Cook, J.; Griesser, H. J., Antibacterial surfaces for biomedical devices. *Expert Review of Medical Devices* 2009, 6, 553-567.

4. Darouiche, R. O., Current concepts - Treatment of infections associated with surgical implants. *New England Journal of Medicine* 2004, 350, 1422-1429.

5. Scott, R. D. *The Direct Medical Costs of Healthcare-associated Infections in U.S. Hospitals and the Benefits of Prevention*; 2009.

6. Guajardo, R. A., Marine Biofouling as a Business Opportunity. *https://smallbusinessonlinecommunity.bankofamerica.com/thread/9694* 2008.

7. Ke, X.; Hongqiang, R.; Lili, D.; Jinju, G.; Tingting, Z., A review of membrane fouling in municipal secondary effluent reclamation. *Environmental science and pollution research international* 2013, 20, 771-7.

8. Bernbom, N.; Ng, Y. Y.; Jorgensen, R. L.; Arpanaei, A.; Meyer, R. L.; Kingshott, P.; Vejborg, R. M.; Klemm, P.; Gram, L., Adhesion of food-borne bacteria to stainless steel is reduced by food conditioning films. *Journal of Applied Microbiology* 2009, 106, 1268-1279.

9. Knight, G. C.; Craven, H. M., A model system for evaluating surface disinfection in dairy factory environments. *International Journal of Food Microbiology* 2010, 137, 161-167.

10. Flemming, H. C., Biofouling in water systems - cases, causes and countermeasures. *Applied Microbiology and Biotechnology* 2002, 59, 629-640.

11. Rao, T. S.; Kora, A. J.; Chandramohan, P.; Panigrahi, B. S.; Narasimhan, S. V., Biofouling and microbial corrosion problem in the thermo-fluid heat exchanger and cooling water system of a nuclear test reactor. *Biofouling* 2009, 25, 581-591.

12. Cavallaro, A.; Majewski, P.; Barton, M.; Vasilev, K., Substrate independent approach for immobilisation of quaternary ammonium compounds to surfaces to reduce bio-burden. *Materials Science Forum* 2014, 783-786, 1389-1395.

13. Tiller, J. C.; Liao, C.-J.; Lewis, K.; Klibanov, A. M., Designing surfaces that kill bacteria on contact. *Proceedings of the National Academy of Sciences* 2001, 98, 5981-5985.

14. Simovic, S.; Losic, D.; Vasilev, K., Controlled drug release from porous materials by plasma polymer deposition. *Chemical Communications* 2010, 46, 1317-1319.

15. Taheri, S.; Cavallaro, A.; Christo, S. N.; Smith, L. E.; Majewski, P.; Barton, M.; Hayball, J. D.; Vasilev, K., Substrate independent silver nanoparticle based antibacterial coatings. *Biomaterials* 2014, 35, 4601-4609.

16. Cavallaro, A.; Taheri, S.; Vasilev, K., Responsive and "smart" antibacterial surfaces: Common approaches and new developments. *Biointerphases* 2014, 9, 029005.

17. Danese, P. N., Antibiofilm Approaches: Prevention of Catheter Colonization. *Chemistry & Biology* 2002, 9, 873-880.

18. Zanini, S.; Müller, M.; Riccardi, C.; Orlandi, M., Polyethylene Glycol Grafting on Polypropylene Membranes for Anti-fouling Properties. *Plasma Chem Plasma Process* 2007, 27, 446-457.

19. Dong, B.; Manolache, S.; Somers, E. B.; Lee Wong, A. C.; Denes, F. S., Generation of antifouling layers on stainless steel surfaces by plasma-enhanced crosslinking of polyethylene glycol. *Journal of Applied Polymer Science* 2005, 97, 485-497.

20. Branch, D. W.; Wheeler, B. C.; Brewer, G. J.; Leckband, D. E., Long-term stability of grafted polyethylene glycol surfaces for use with microstamped substrates in neuronal cell culture. *Biomaterials* 2001, 22, 1035-1047.

21. Konradi, R.; Acikgoz, C.; Textor, M., Polyoxazolines for Nonfouling Surface Coatings — A Direct Comparison to the Gold Standard PEG. *Macromolecular Rapid Communications* 2012, 33, 1663-1676.

22. Sharma, S.; Johnson, R. W.; Desai, T. A., Ultrathin poly(ethylene glycol) films for silicon-based microdevices. *Applied Surface Science* 2003, 206, 218-229.

23. Hoogenboom, R., Poly(2-oxazoline)s: A Polymer Class with Numerous Potential Applications. *Angewandte Chemie International Edition* 2009, 48, 7978-7994.

24. Wiesbrock, F.; Hoogenboom, R.; Leenen, M. A. M.; Meier, M. A. R.; Schubert, U. S., Investigation of the Living Cationic Ring-Opening Polymerization of 2-Methyl-, 2-Ethyl-, 2-Nonyl-, and 2-Phenyl-2-oxazoline in a Single-Mode Microwave Reactor†. *Macromolecules* 2005, 38, 5025-5034.

25. Tauhardt, L.; Frant, M.; Pretzel, D.; Hartlieb, M.; Bucher, C.; Hildebrand, G.; Schroter, B.; Weber, C.; Kempe, K.; Gottschaldt, M.; Liefeith, K.; Schubert, U. S., Amine end-functionalized poly(2-ethyl-2-oxazoline) as promising coating material for antifouling applications. *Journal of Materials Chemistry B* 2014, 2, 4883-4893.

26. Jordan, R.; Ulman, A., Surface initiated living cationic polymerization of 2-oxazolines. *Journal of the American Chemical Society* 1998, 120, 243-247.

27. Bouten, P. J. M.; Hertsen, D.; Vergaelen, M.; Monnery, B. D.; Boerman, M. A.; Goossens, H.; Catak, S.; van Hest, J. C. M.; Van Speybroeck, V.; Hoogenboom, R., Accelerated living cationic ring-opening polymerization of a methyl ester functionalized 2-oxazoline monomer. *Polymer Chemistry* 2015, 6, 514-518.

28. Zhang, N.; Steenackers, M.; Luxenhofer, R.; Jordan, R., Bottle-brush brushes: cylindrical molecular brushes of Poly(2-oxazoline) on glassy carbon. *Macromolecules* 2009, 42, 5345-5351.

29. Wang, H.; Li, L.; Tong, Q.; Yan, M., Evaluation of photochemically immobilized Poly(2-ethyl-2-oxazoline) thin films as protein-resistant surfaces. *ACS Applied Materials & Interfaces* 2011, 3, 3463-3471.

30. Pidhatika, B.; Rodenstein, M.; Chen, Y.; Rakhmatullina, E.; Mühlebach, A.; Acikgöz, C.; Textor, M.; Konradi, R., Comparative stability studies of poly(2-methyl-2-oxazoline) and poly(ethylene glycol) brush coatings. *Biointerphases* 2012, 7, -.

31. Ramiasa, M.; Cavallaro, A.; Mierczynska-Vasilev, A.; Christo, S.; Gleadle, J.; Hayball, J. D.; Vasilev, K., Plasma Polymerised PolyOxazoline Thin Films for Biomedical Applications. *Chemical Communications* 2015, 51, 4297-4282.

32. Vasilev, K., Nanoengineered Plasma Polymer Films for Biomaterial Applications. *Plasma Chem Plasma Process* 2013, 1-14.

33. Coad, B. R.; Jasieniak, M.; Griesser, S. S.; Griesser, H. J., Controlled covalent surface immobilisation of proteins and peptides using plasma methods. *Surface and Coatings Technology* 2013, 233, 169-177.

34. Swaraj, S.; Oran, U.; Lippitz, A.; Friedrich, J.; Unger, W., Study of influence of external plasma parameters on plasma polymerised films prepared from organic molecules (acrylic acid, allyl alcohol, allyl amine) using XPS and NEXAFS. *Surface and Coatings Technology* 2005, 200, 494-497.

35. Yasuda, H., *Plasma polymerization*. Academic press: 1985.

36. Gleason, K. K., A Chemical Engineering Perspective on “Views on Macroscopic Kinetics of Plasma Polymerisation”. *Plasma Processes and Polymers* 2010, 7, 380-381.

37. Vasilev, K.; Britcher, L.; Casanal, A.; Griesser, H. J., Solvent-induced porosity in ultrathin amine plasma polymer coatings. *The Journal of Physical Chemistry B* 2008, 112, 10915-10921.

38. Abdull Rasad, M. S. B.; Halim, A. S.; Hashim, K.; Rashid, A. H. A.; Yusof, N.; Shamsuddin, S., In vitro evaluation of novel chitosan derivatives sheet and paste cytocompatibility on human dermal fibroblasts. *Carbohydrate Polymers* 2010, 79, 1094-1100.

39. Werner, S.; Krieg, T.; Smola, H., Keratinocyte-Fibroblast Interactions in Wound Healing. *Journal of Investigative Dermatology* 2007, 127, 998-1008.

40. Bose, R. K.; Lau, K. K. S., Initiated CVD of Poly(2-Hydroxyethyl Methacrylate) Hydrogels: Synthesis, Characterization and In-vitro Biocompatibility. *Chemical Vapor Deposition* 2009, 15, 150-155.

41. Wang, A.; Pu, K.; Dong, B.; Liu, Y.; Zhang, L.; Zhang, Z.; Duan, W.; Zhu, Y., Role of surface charge and oxidative stress in cytotoxicity and genotoxicity of graphene oxide towards human lung fibroblast cells. *Journal of applied toxicology : JAT* 2013, 33, 1156-64.

42. Yang, A.; Cardona, D. L.; Barile, F. A., In vitro cytotoxicity testing with fluorescence-based assays in cultured human lung and dermal cells. *Cell Biology and Toxicology* 2002, 18, 97-108.

43. Hamerli, P.; Weigel, T.; Groth, T.; Paul, D., Enhanced tissue-compatibility of polyethylenterephtalat membranes by plasma aminofunctionalisation. *Surface and Coatings Technology* 2003, 174–175, 574-578.

44. Lerouge, S.; Major, A.; Girault-Lauriault, P.-L.; Raymond, M.-A.; Laplante, P.; Soulez, G.; Mwale, F.; Wertheimer, M. R.; Hébert, M.-J., Nitrogen-rich coatings for promoting healing around stent-grafts after endovascular aneurysm repair. *Biomaterials* 2007, 28, 1209-1217.

45. von Keudell, A.; Benedikt, J., A Physicist's Perspective on “Views on Macroscopic Kinetics of Plasma Polymerisation”. *Plasma Processes and Polymers* 2010, 7, 376-379.

46. McCann, M. T.; Gilmore, B. F.; Gorman, S. P., Staphylococcus epidermidis device-related infections: pathogenesis and clinical management. *Journal of Pharmacy and Pharmacology* 2008, 60, 1551-1571.

47. O'GARA, J. P.; HUMPHREYS, H., Staphylococcus epidermidis biofilms: importance and implications. *Journal of Medical Microbiology* 2001, 50, 582-587.

48. Katti, K. S., Biomaterials in total joint replacement. *Colloids and Surfaces B: Biointerfaces* 2004, 39, 133-142.

49. Rinsch, C. L.; Chen, X.; Panchalingam, V.; Eberhart, R. C.; Wang, J.-H.; Timmons, R. B., Pulsed radio frequency plasma polymerization of allyl alcohol: controlled deposition of surface hydroxyl groups. *Langmuir* 1996, 12, 2995-3002.

50. Konradi, R.; Pidhatika, B.; Mühlebach, A.; Textor, M., Poly-2-methyl-2-oxazoline: A Peptide-like Polymer for Protein-Repellent Surfaces. *Langmuir* 2008, 24, 613-616.

51 Suter, W.; Jaeger, I.; Racine, R. R.; Donatsch, P.; Neumann, P.; Matter, B. E., Mutagenicity evaluation of amino-oxazoline derivatives using in vitro and in vivo short-term tests. *Environmental Mutagenesis* 1983, 5, 527-540.

# 7 Controlled and sustained release of pharmaceuticals via a single step solvent-free encapsulation*

## 7.1 Preamble

Herein, a novel and solvent-free technique for the encapsulation of pharmaceuticals that allows controllable release rates of antibiotics. The method utilises the deposition of a plasma polymer coating of controlled chemistry and thickness on the outer surface of drug particles placed under continuous agitation.

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## 7.2 Introduction

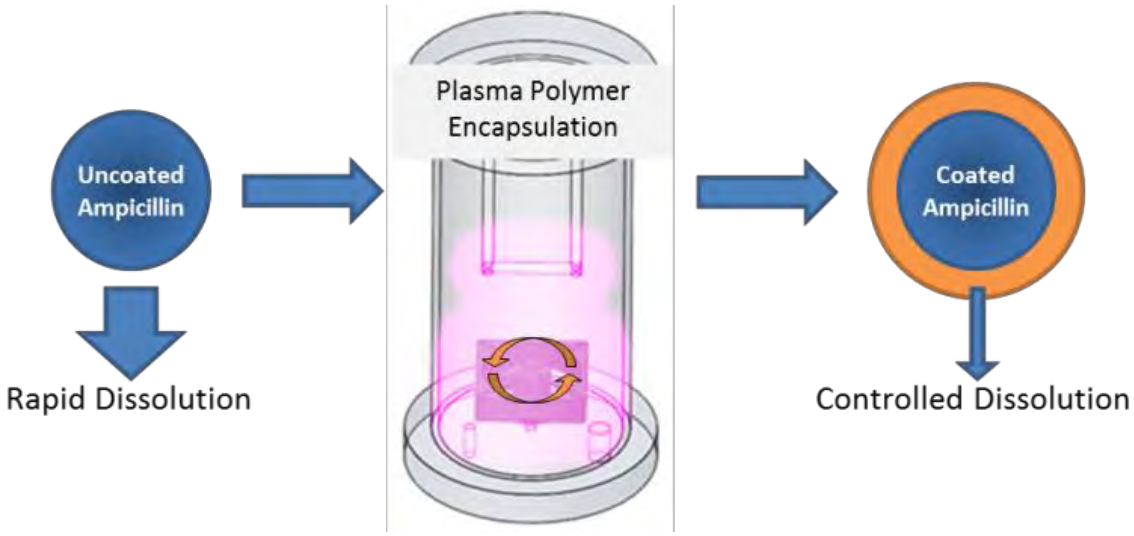
Today, the benefits of using controlled drug release systems in medical therapies are well recognised.[1-3] In conventional drug administration, the substance is administered in high doses only to be repeated hours or a day later. The drawbacks of this approach for the patient are high costs and possible systemic toxicity. In addition, many patients have issues with compliance to the administration regime. [4, 5] These are some of the reasons that brought into light the concept of controlled release. Over the last decades, a vast number of technologies have been proposed to provide controlled, targeted and sustained release of pharmaceuticals delivered by oral or intravenous administration, via specifically designed carriers or implantable devices. [2, 6-8] Most of the technologies involve polymeric systems which entrap the drug into capsules or into surface coatings. The preparation of such formulations require wet processes which demand multistep processing and solvents (such as LBL and miniemulsion techniques) that have a high cost and often limited encapsulation efficacy. [9, 10] The use of nanoparticles and liposomes as drug carriers has also been extensively investigated. [11-14]

In this chapter, a facile, one step and dry process for encapsulation and control of the release rate of pharmaceuticals is reported. Importantly, as demonstrated in this report, the technology can be applied to “off the shelf” commercially formulated compounds without the need for any pre-treatment steps. The process is based on plasma polymer encapsulation of drug powders placed on a moving platform that provides random motion of the particles. A schematic of the experimental approach is shown in the Scheme. 7.1 and Appendix A.2.1 (Fig. S.7.1). Ampicillin was selected as model drug to demonstrate the technology for two reasons. Firstly, ampicillin is a well-

established, often prescribed broad-spectrum antibiotic commonly used in pharmacokinetic and antibiotic resistance studies, which makes the compound an excellent model drug. Secondly, providing advances into the delivery of antibiotics which may make a contribution towards easing the mortality and morbidity caused by infectious diseases: the second leading cause of death after heart diseases. [15]

Antibiotics have previously been used with plasma polymers with the purpose of generating antibacterial coatings. Examples are those loaded into the plasma polymer films[16] or into porous materials. [17] Covalent attachment to plasma polymer coatings has also been reported. [18]

However, this is the first report that demonstrates encapsulation of individual antibiotic particles via plasma polymers. The goal is to achieve a sustained and controlled release of concentrations above the minimum inhibitory concentration (MIC) for a period of at least 5 days. Since ampicillin is highly hydrophilic, as many common antibiotics, a plasma polymer deposited from vapour of 1,7-octadiene (ppOD) as the encapsulating material was chosen. The resulting coatings are relatively hydrophobic (Fig. 7.1b) and hence it can be hypothesised that this type of encapsulation will reduce the interactions between the drug and aqueous medium. In addition, 1,7-octadiene is easy to deposit from plasma and there are published evidence that the coatings are not cytotoxic.[19]




Scheme 7.1 Plasma polymer encapsulation of ampicillin for sustained release using a plasma reactor with an additional platform to induce continuous agitation of powdered substances within the plasma field.

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## 7.3 Results and Discussion

To control the rate of release of ampicillin the thickness of the capsule wall was varied. Figure 7.1a shows the polymer film thickness, determined by ellipsometry, as a function of time when deposited on planar surfaces. The film thickness increases linearly in the studied time of 20 minutes with a rate of 28 nm per minute.

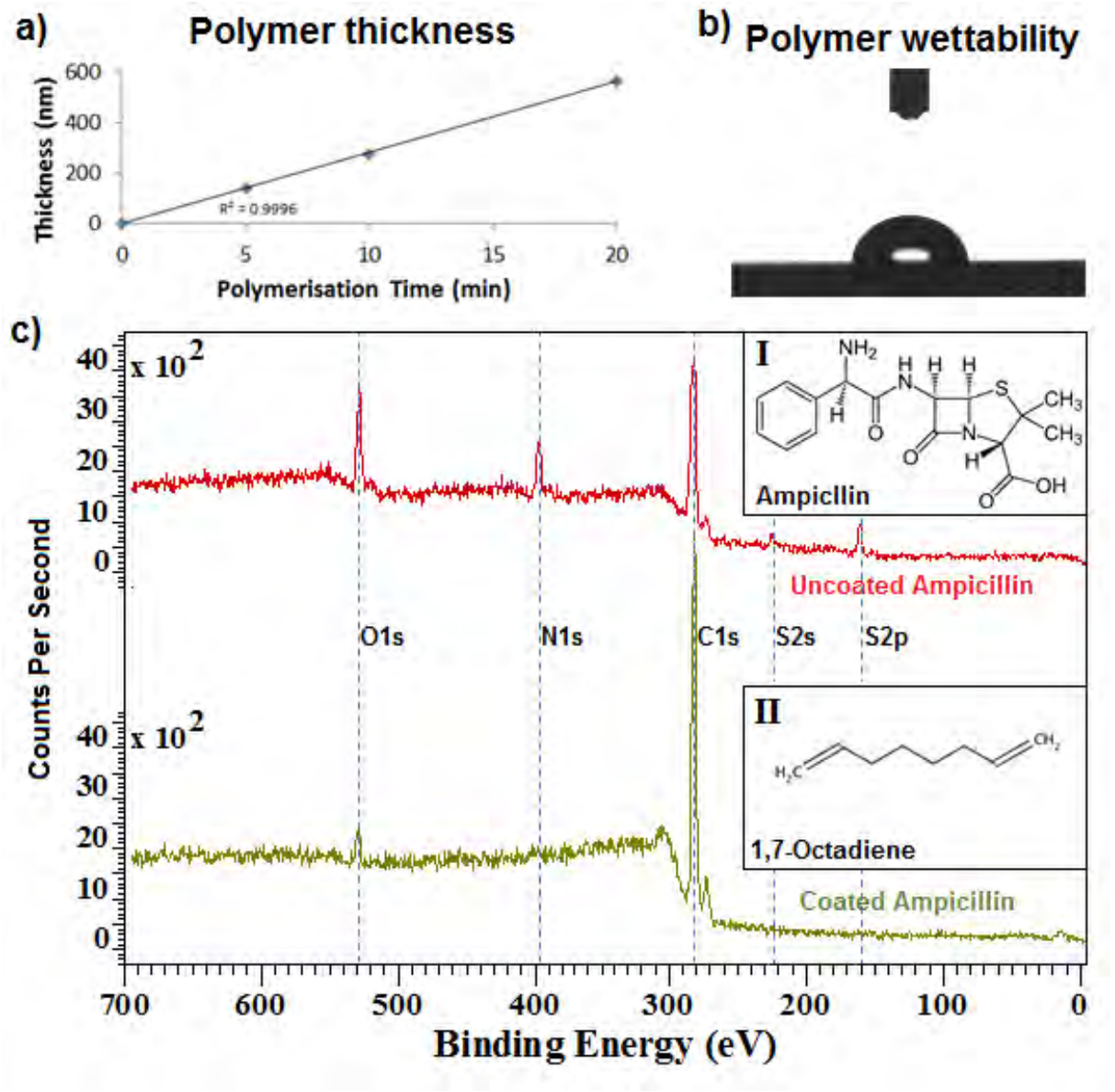


Figure 7.1(a) Thickness of plasma deposited 1,7-octadiene layer on planar samples for various time periods. (b) Water contact angle of plasma polymer coating deposited from 1,7-octadiene. (c) XPS spectrum of uncoated ampicillin (top) and after encapsulated with ppOD for 5 minutes (bottom), and the corresponding to chemical structures of ampicillin (inset I) and 1,7-octadiene precursor (inset II).

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When deposited on particles, the thickness of the coating would be lower compared to that deposited on a planar surface for the same time. This is intuitive to understand taking into account the high surface area of the particles compared to planar surface.

However, the principle of increased coating thickness with longer deposition time would still hold. This allows for the control of the thickness of the capsule outer wall. The plasma polymer coatings deposited from 1,7-octadiene had advancing water contact angles of 81° ±2°, image shown in Figure 7.1b which is consistent with published studies. [20, 21]

Ampicillin powder was added to the plasma chamber as received and coated whilst being continuously agitated (details of the samples holder are given in Appendix. A.2.1 Fig. S.7.1). The chemical composition of the powders was determined via XPS before and after coating. Figure 7.1c shows a clear difference in the intensity of the N1s and S2p peaks, at 399.5 eV and 163.0 eV, respectively, between the uncoated ampicillin (top spectrum) and the 1,7-octaidene encapsulated ampicillin (bottom spectrum). As seen in the chemical structure of ampicillin, the molecule contains nitrogen and sulphur which give clear peaks in the XPS survey spectrum. After coating for 5 minutes nitrogen and sulphur can no longer be seen. This suggests that the coating (1,7-octaidene does not contain nitrogen or sulphur) of the outer surface of the powder is uniform and thicker than the sampling depth of XPS (ca 10 nm). Three different coating times were used to establish different thicknesses of the outer shell: 5, 10 and 20 minutes (A05, A10 and A20 respectively).

Figure 7.2 shows the release profile of the encapsulated ampicillin. The concentration of dissolved drug in aqueous medium over time was determined by UV-Vis spectroscopy using the intensity of the absorbance band at 268 nm.

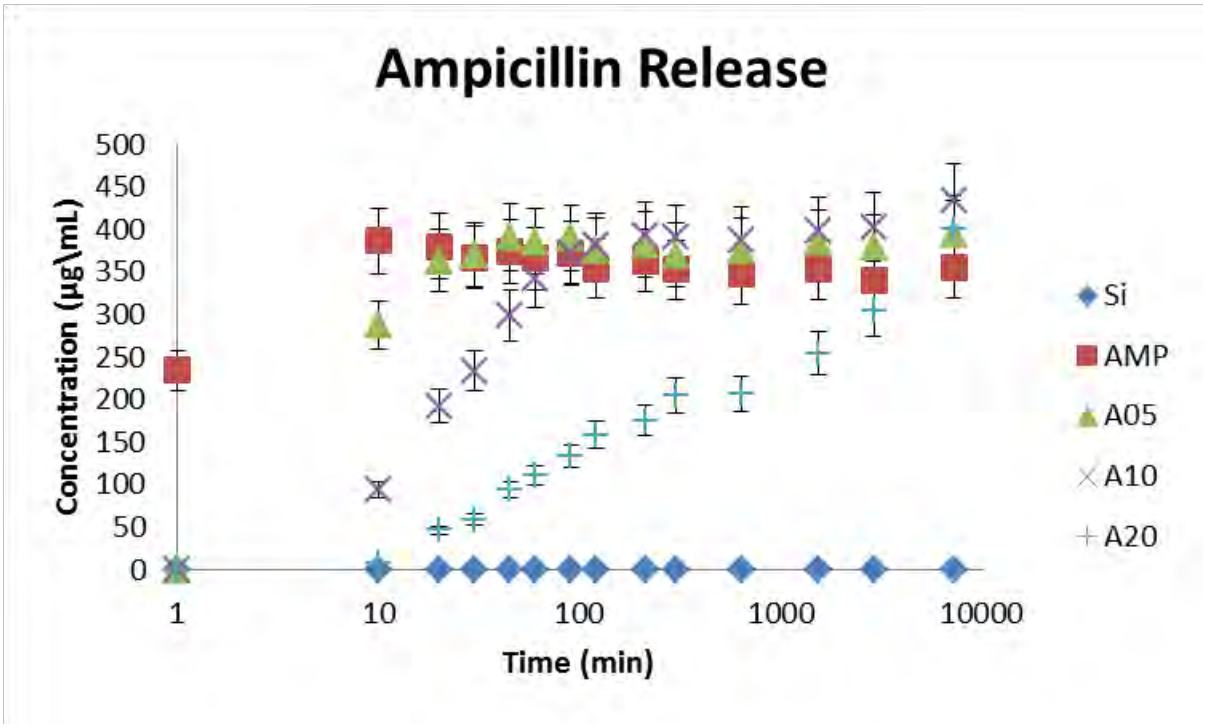


Figure 7.2 Ampicillin release rates over 5 day period. Where release of ampicillin into MIlli-Q water from coated and uncoated ampicillin powder based on UV-Vis absorbance band (268nm) where AMP (■ ), A05 (▲ ), A10 (X), and A20 (+) (uncoated ampicillin and ampicillin with 5, 10 and 20 minute ppOD coatings respectively) showed different release rates depending on polymerisation time. Si powder coated with ppOD (♦) was used as a control. All error assumes a ±10% deviation.

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Uncoated ampicillin was fully dissolved within 10 minutes. Powders coated for 5 and 10 minutes (A05 and A10) released all their contents within 20 and 90 minutes, respectively (Appendix. A.2.1 Fig. S.7.2). The sample coated for 20 minutes (A20) showed sustained release of the ampicillin for 5 days. Si powder coated with ppOD was used as an ampicillin-free control. There is a clear correlation between the time taken to fully dissolve the coated ampicillin and the plasma polymer deposition time used for encapsulation. This indicates that the capsule thickness plays a crucial role in regulating ampicillin release through restricting the ability of water to penetrate the

ppOD coating and dissolve the ampicillin, and also the capacity of the dissolved ampicillin to pass through the coating.

It is interesting to note that capsules built for 5 and 10 minutes were not capable of significantly reducing the release rate of the drug. This suggests that the thickness of the coating may not have been sufficient to completely encapsulate the drug particles. Although the particles were continuously agitated to provide uniform coating, the thickness of the capsule wall (few tens of nanometers) is very small relative to the size of the particles (few tens to few hundreds of microns as seem from microscopy images in Figure 7.3).

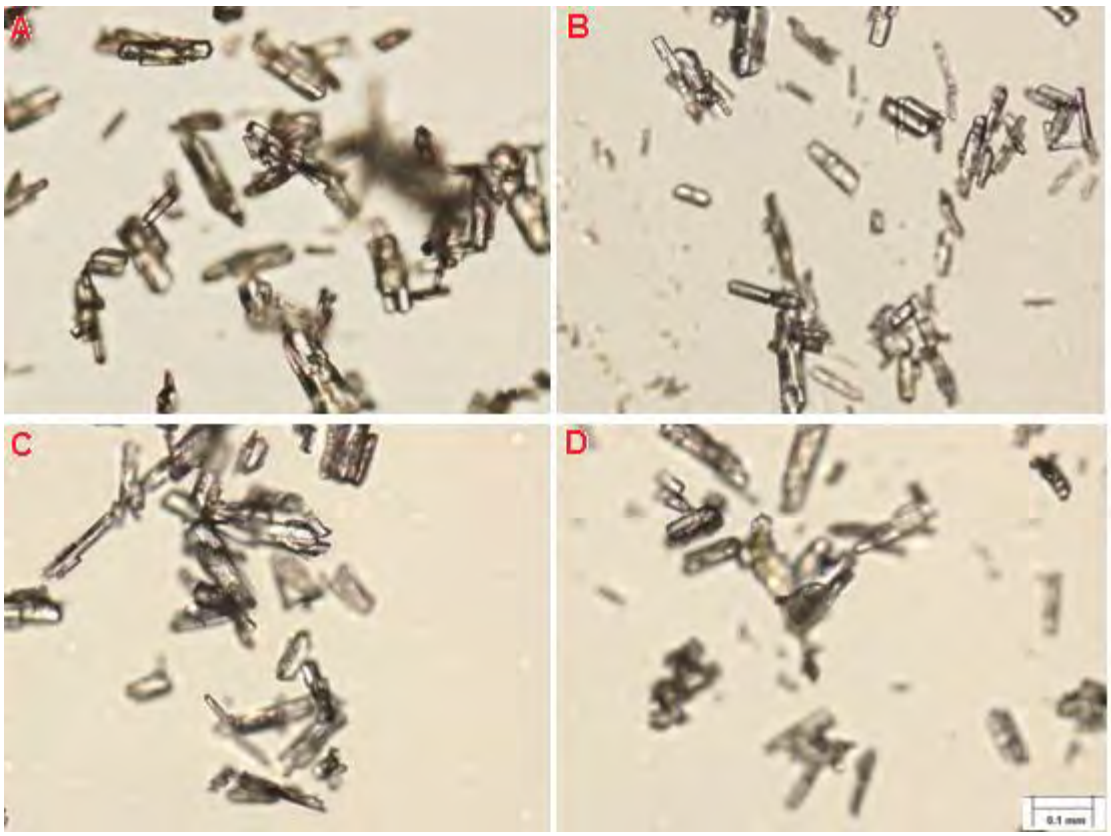


Figure 7.3 Optical microscope images of ampicillin powder. A) uncoated ampicillin B) ampicillin with 5 minute coating (A05) C) ampicillin with 10 minute coating (A10) D) ampicillin with 20 minute coating (A20). Scale: 0.1mm

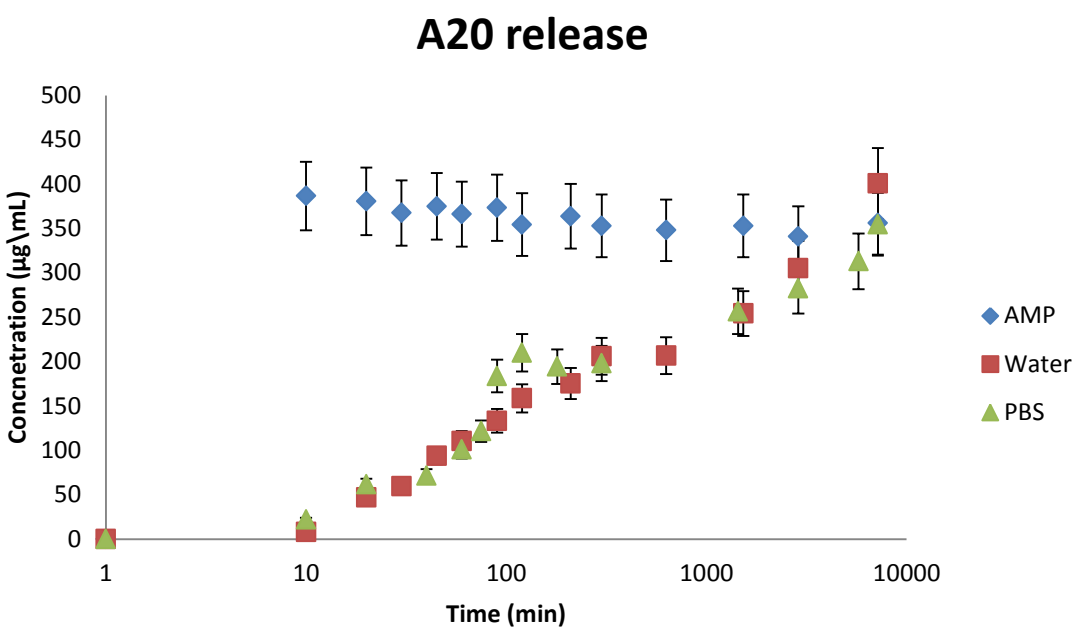


Figure 7.4. Ampicillin release from A20 samples over 5 days from water and PBSWhere release of ampicillin into filtered water (■) and PBS (▲) from coated ampicillin powder based on UV-vis absorbance (268nm) where AMP (♦), was used as a reference. All error assumes a ±10% deviation.

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In addition, particle shape is not spherical. Thus, irregularities in particle shape, such as edges and groves, may not have been fully coated allowing direct exposure to the solvent when deposition times were 5 and 10 minutes. These issues were fully overcome when the deposition time was 20 minutes. This shows the capacity of the technology to provide encapsulation of particles of complex shapes and diversity of sizes. However, it also shows that the coating thickness needs to be optimised for a particular pharmaceutical based of the size and shape of the respective particles.

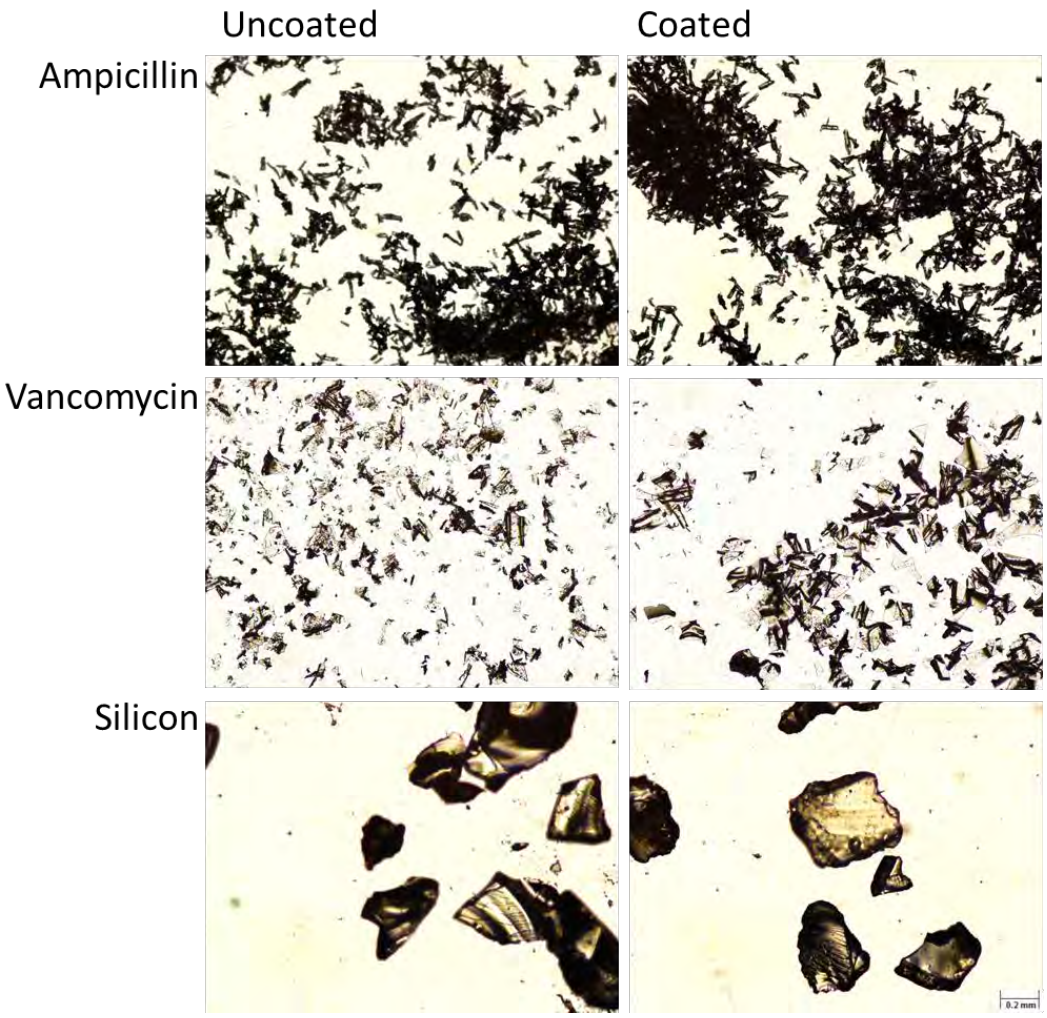


Figure 7.5. Optical microscope images of ampicillin, vancomycin and Si with 20 minute with and without ppOD coating showing no apparent change in size of the coated particles. Scale: 0.2mm

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Importantly for biological applications, comparative release studies of the A20 sample in PBS were conducted. The results are shown in Figure 7.4 demonstrates almost identical release kinetics. To demonstrate that the technology is applicable to other drugs, vancomycin particles (as supplied) were coated with ppOD for 20 minutes. However, since the vancomycin particles size distribution is different to that of ampicillin, as shown in Figure 7.5, the resultant release profile follows a different kinetic (Figure 7.6). This demonstrates that the condition of encapsulation need be

optimised for every individual drug. Future studies in our lab will aim at establishing (if possible) a universal relationship between drug particle size distribution and capsule thickness and material.

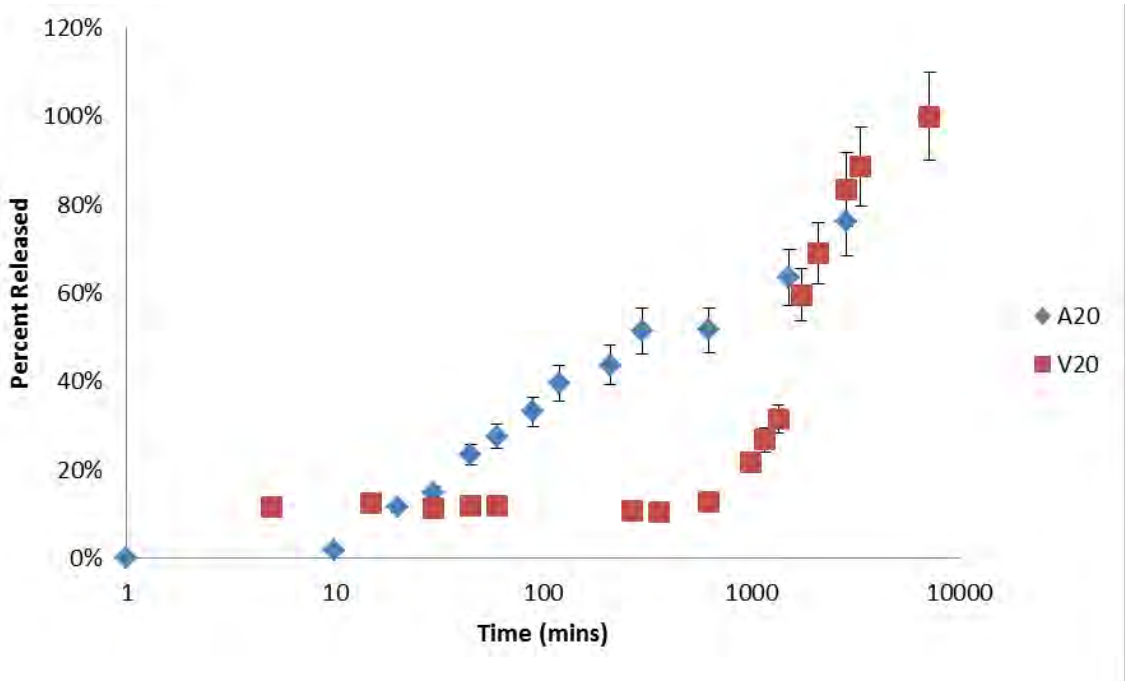


Figure 7.6 Comparative release of ampicillin and vacomycin from ppOD capsules (A20 (♦) and V20 (■) respectively) showing the difference in accumulative release relative to the final concentration released. All values based on UV-vis absorbance bands for individual antibiotics (268nm for ampicillin and 280nm for vancomycin) All error assumes a ±10% deviation.

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Ultimately, it is important to demonstrate that in addition to a sustained and controlled release the drug is in active form after release. Table 7.1 shows the MIC values to *Staphylococcus epidermidis* and *Escherichia coli* obtained using the dissolved ampicillin with and without encapsulation. The values for *S. epidermidis* and *E. coli* were 50 and 3.125 µg\mL respectively. There are no differences in the MIC values between the coated and the uncoated ampicillin. This result suggests that the plasma deposition process did not affect the activity of the drug. The MIC values and the

release kinetics shown in Figure 7.2 also demonstrate that at any point of time the drug concentration is above the MIC values. The effect of the capsule material on the MIC value was also investigated. The data shown in Appendix. A.2.1 Table S.7.1 shows negligible effect on *E. coli* and no *S. epidermidis* when tested against coated silica powder.

Table 7.1 MIC values obtained from leached ampicillin from neat ampicillin and ppOD encapsulated ampicillin (A05, A10 and A20) for *S. epidermidis* and *E. Coli* showing no change in efficacy.

| **Sample** | ***S. epidermidis* MIC (µg\mL)** | ***E. coli* MIC (µg\mL)** |
|---|---|---|
| **AMP** | 50 | 3.125 |
| **A05** | 50 | 3.125 |
| **A10** | 50 | 3.125 |
| **A20** | 50 | 3.125 |

## 7.4 Summary

In summary, a novel solvent-free encapsulation technology which can be used to control the rate of drug release was developed. The feasibility of the technology with ampicillin as a model drug was demonstrated. Sustained release over 5 days was achieved and evidence that the encapsulation process does not alter the activity of the compound was provided. The technology presented in this communication has an enormous potential for application with variety of drugs and medical treatments. Considering the vast variety of plasma polymerisable precursors, the physical and chemical properties of the outer capsule, as well as the wall thickness, are easily customizable and could be fine-tuned to suit different applications. Herein, the controlled release of hydrophilic drugs was demonstrated, however, it is hypothesised that utilising the same technology but involving hydrophilic coatings can be used to enhance the dissolution and bioavailability for poorly-soluble drugs. The capsules can

also be prepared using functional plasma polymers and further decorated with specific ligands that would open enormous opportunities into the field of targeted drug delivery for treating diseases such as cancer.

## 7.5 References


1. Zhang, Y.; Chan, H. F.; Leong, K. W., Advanced materials and processing for drug delivery: The past and the future. *Advanced Drug Delivery Reviews* 2013, 65, 104-120.

2. Uhrich, K. E.; Cannizzaro, S. M.; Langer, R. S.; Shakesheff, K. M., Polymeric Systems for Controlled Drug Release. *Chemical Reviews* 1999, 99, 3181-3198.

3. Allen, T. M.; Cullis, P. R., Drug delivery systems: entering the mainstream. *Science* 2004, 303, 1818-1822.

4. Pechère, J.-C.; Hughes, D.; Kardas, P.; Cornaglia, G., Non-compliance with antibiotic therapy for acute community infections: a global survey. *International Journal of Antimicrobial Agents* 2007, 29, 245-253.

5. Blaschke, T. F.; Osterberg, L.; Vrijens, B.; Urquhart, J., Adherence to Medications: Insights Arising from Studies on the Unreliable Link Between Prescribed and Actual Drug Dosing Histories. *Annu. Rev. Pharmacol. Toxicol.* 2012, 52, 275-+.

6. Langowska, K.; Kowal, J.; Palivan, C. G.; Meier, W., A general strategy for creating self-defending surfaces for controlled drug production for long periods of time. *Journal of Materials Chemistry B* 2014, 2, 4684-4693.

7. Jeong, B.; Bae, Y. H.; Lee, D. S.; Kim, S. W., Biodegradable block copolymers as injectable drug-delivery systems. *Nature* 1997, 388, 860-862.

8. Yang, P.; Gai, S.; Lin, J., Functionalized mesoporous silica materials for controlled drug delivery. *Chemical Society Reviews* 2012, 41, 3679-3698.

9. Naik, J.; Lokhande, A.; Mishra, S.; Kulkarni, R., Development of sustained release micro/nanoparticles using different solvent emulsification technique: A review. *Int J Pharm Bio Sci* 2012, 3, 573-590.

10. de Villiers, M. M.; Lvov, Y. M., Layer-by-layer self-assembled nanoshells for drug delivery. *Advanced Drug Delivery Reviews* 2011, 63, 699-700.

11. Baier, G.; Cavallaro, A.; Vasilev, K.; Mailänder, V.; Musyanovych, A.; Landfester, K., Enzyme Responsive Hyaluronic Acid Nanocapsules Containing Polyhexanide and Their Exposure to Bacteria To Prevent Infection. *Biomacromolecules* 2013, 14, 1103-1112.

12. Yandrapu, S. K.; Upadhyay, A. K.; Petrash, J. M.; Kompella, U. B., Nanoparticles in Porous Microparticles Prepared by Supercritical Infusion and Pressure Quench Technology for Sustained Delivery of Bevacizumab. *Molecular Pharmaceutics* 2013, 10, 4676-4686.

13. Labuschagne, P. W.; Adami, R.; Liparoti, S.; Naidoo, S.; Swai, H.; Reverchon, E., Preparation of rifampicin/poly(d,l-lactice) nanoparticles for sustained release by

supercritical assisted atomization technique. *The Journal of Supercritical Fluids* 2014, 95, 106-117.

14. Celia, C.; Ferrati, S.; Bansal, S.; van de Ven, A. L.; Ruozi, B.; Zabre, E.; Hosali, S.; Paolino, D.; Sarpietro, M. G.; Fine, D.; Fresta, M.; Ferrari, M.; Grattoni, A., Drug Delivery: Sustained Zero-Order Release of Intact Ultra-Stable Drug-Loaded Liposomes from an Implantable Nanochannel Delivery System (Adv. Healthcare Mater. 2/2014). *Advanced Healthcare Materials* 2014, 3, 153-153.

15. World Health Organization, Annex Table 2: Deaths by cause, sex and mortality stratum in WHO regions, estimates for 2002. *The world health report* 2004.

16. Vasilev, K.; Poulter, N.; Martinek, P.; Griesser, H. J., Controlled Release of Levofloxacin Sandwiched between Two Plasma Polymerized Layers on a Solid Carrier. *ACS Applied Materials & Interfaces* 2011, 3, 4831-4836.

17. Simovic, S.; Losic, D.; Vasilev, K., Controlled drug release from porous materials by plasma polymer deposition. *Chemical Communications* 2010, 46, 1317-1319.

18. Braceras, I.; Oyarbide, J.; Azpiroz, P.; Briz, N.; Ipiñazar, E.; Álvarez, N.; Atorrasagasti, G.; Fratila, R. M.; Aizpurua, J. M., "Plasma-Click" Based Strategy for Obtaining Antibacterial Surfaces on Implants. *Plasma Processes and Polymers* 2013, 10, 328-335.

19. Liu, X.; Feng, Q.; Bachhuka, A.; Vasilev, K., Surface chemical functionalities affect the behavior of human adipose-derived stem cells in vitro. *Applied Surface Science* 2013, 270, 473-479.

20. Vasilev, K.; Mierczynska, A.; Hook, A. L.; Chan, J.; Voelcker, N. H.; Short, R. D., Creating gradients of two proteins by differential passive adsorption onto a PEG-density gradient. *Biomaterials* 2010, 31, 392-7.

21. Michelmore, A.; Mierczynska, A.; Poh, Z.; Goreham, R. V.; Losic, D.; Short, R. D.; Vasilev, K., Versatile gradients of chemistry, bound ligands and nanoparticles on alumina nanopore arrays. *Nanotechnology* 2011, 22, 415601.

# 8 Conclusions and Outlook

## 8.1 Conclusions

The aims of this thesis were to generate novel antimicrobial surfaces to contribute to new fundamental knowledge of bacteria / surface interactions and develop novel applications. This was achieved by developing antimicrobial surface coatings based on plasma polymerisation. In this work three coatings of differing chemistries were proposed for use antimicrobial surfaces and interrogated *in vitro*. The ability to utilise plasma polymers for controlled dissolution of pharmaceuticals was also demonstrated.

Firstly, a quaternary ammonium was immobilised to the surface. This was achieved through the deposition of an amine rich film onto the surface and the covalent addition of the quaternary ammonium using epoxy-amine chemistry. XPS was used to carefully characterise the surfaces and quantify the total quaternary ammonium groups. Coatings were optimised for maximum $NR_4^+$ addition by varying pH with KOH. It was demonstrated that the immobilised quaternary ammonium could effectively decrease the viability of *E. coli* on the surface as determined via LIVE\DEAD staining. Interestingly, these surfaces did not seem to exhibit any antimicrobial effect on *S. epidermidis*.

The immobilised quaternary ammonium coatings were further developed into gradients of controlled density of quaternary ammonium as a useful tool to determine the minimum concentration and charge threshold required to induce antimicrobial action. Using a precise dip coating method, the density of immobilised quaternary ammonium was precisely controlled in a linear manner over 13 mm coverslips. XPS

was used to determine the total $NR_4^+$ along the gradient and AFM was used to characterise the surface potential along the gradient. It was shown that a specific threshold of total of 4.18% NH4+ of total nitrogen and 120.4 mV was required to induce death against E. coli.

Furthermore, the lack of observable cytotoxicity of the coating *in vitro* was demonstrated using primary human fibroblasts. This was attributed to the constituents of the culture medium attaching to the quaternary ammonium coatings. The adsorption of environmental components onto the coating was demonstrated using QCM and its implications on the antimicrobial ability of quaternary ammonium based surfaces, and other antimicrobial surfaces, were elucidated.

Secondly, the ability to use the peptide-like antibiotic polymyxin as a contact killing antimicrobial was investigated. These surfaces were generated via a simple two-step process. An aldehyde rich surface was generated through the plasma deposition of propionaldehyde and polymyxin was immobilised. XPS was used to confirm the presence of polymyxin on the surface. Using autofluorescent *E. coli* it was shown that the aldehyde rich coating decreases bacterial adhesion under high nutrient conditions. Although the addition of the polymyxin had no biocidal effect on the bacteria, QCM was used to demonstrate that the immobilised polymyxin retained its ability to bind up to 52 ng of LPS per $cm^2$.

Next, capabilities of plasma deposition for the generation of polyoxazoline-based antifouling coatings were also investigated. 2-methyl-2-oxazoline and 2-ethyl-2-oxazoline were plasma deposited under a range of conditions. The stability of these plasma polymers was examined and there was a clear relationship between stability

and deposition power. *S. epidermidis* was grown directly on the polyoxazoline coatings. When deposited at high flow rates, the plasma polymerised form of 2-methyl-2-oxazoline can reduce biofilm formation by 63%. However, it was also noted that modification of the coating chemistry by acetic acid significantly reduces its antifouling ability. A greater decrease in biofilm attachment was seen on plasma deposited 2-ethyl-2-oxazoline with a decrease of total biofilm by 77%.

Finally, translating simple plasma deposited films directly to the surface of antibiotic powders was explored. By adding model powdered antibiotics, ampicillin and vancomycin, directly to the plasma chamber and providing continuous agitation to the powders, a homogenous polymer can be formed on the antibiotic particles which encapsulates the particles. For this purpose plasma deposited octadiene was chosen as a test polymer due to its hydrophobicity. The ability to deposit directly on the drugs was confirmed via XPS. The release rates of the coated antibiotics were determined via UV-vis spectroscopy. It was established that a 20 minute deposition of octadiene gave sustained release over a 5 day period. This opens the possibility for adding functional polymers, to which molecules or peptides can be bound, to particles to develop site-targeting drugs.

Ultimately, the work of this thesis was to develop antimicrobial films and characterise the interaction between the films and bacteria. Plasma polymerisation is a useful tool to achieve this as it can be used to produce films of controlled chemistry in a substrate independent manner. In this work, the substrate independence of plasma polymerisation was exploited to develop a novel method of sustained release of various drugs without the need for a carrier.

## 8.2 Future directions

Overall, this work illustrates the complex bacteria\surface interactions and a range of possibilities of plasma deposited polymeric films for antimicrobial applications. In this work, plasma deposition was used to facilitate the addition of antimicrobial agents directly to a surface, the generation of antifouling surfaces and the encapsulation of pharmaceuticals. With that said, more research in this field is needed, especially towards understanding the bacteria\surface.

In this work, two different surface coatings composed of a base polymer and an immobilised antimicrobial compound were developed; ppAA+GTAC and ppA+PMX. As demonstrated, the different surfaces affected bacteria differently. That being said, the use of gradients of controlled density of immobilised antimicrobials can be used to readily assess the effects of concentrations of these antimicrobials on the bacteria. This opens up a model for rapid screening of minimum bactericidal concentrations for immobilised antimicrobials. The same gradient technology can be used to determine the amount of antimicrobial that has negative implications on mammalian cells. This will allow researchers to find medically relevant "therapeutic" concentrations of grafted antimicrobials that are bactericidal and non-cytotoxic.[1]

As outlined in Chapter 4, fouling of antimicrobial surfaces may be beneficial in a biological setting as it would mask any potential toxic effects of the surface *in vivo*. However, biological fouling, in particular protein adhesion due to changing chemistries, needs to be assessed. The effect of the surface itself and the nature of any bound proteins would lead to different in vivo responses.[2] An assessment on the immunological and

other secondary responses to the surfaces is required before the use of any antimicrobial surface, including ppAA+GTAC, on implantable medical devices.

The ability to use ppA+PMX for extracorporeal endotoxin removal or sepsis treatment can be elaborated. However, the clinical use for this coating is not limited to direct treatments. The prospect for the use of ppA+PMX as an endotoxin sensor is currently being investigated. As the current ppA+PMX is limited to the absorption of LPS, work is being conducted to use other immobilised agents to the range of toxin removal. This will open up the use of the same coating and immobilisation technique for other purification technologies, including environmental and water purification.

Plasma deposited oxazolines exhibit the ability to modulate bacteria binding and\or protein binding. As the precise mechanism of this is still unknown, further investigation of this is required. In Chapter 6, it was shown that plasma deposited 2-ethyl-2-oxazoline exhibits greater antibiofouling properties than the plasma deposited 2-methyl-2-oxazoline. It is intended that this work be built upon to determine the optimal deposition parameters to generate highly antifouling surfaces for biomedical applications.[3]

However, due to these unique properties of the plasma deposited oxazolines,[4] a range of applications can be envisioned. For example, work currently being conducted by Vasilev *and co-workers* is exploiting the ability for ppOx to covalently bind proteins for cancer detection.

Surfaces with multiple mechanisms of action are highly desirable as antimicrobial surfaces.[5, 6] Combinations of the surfaces developed in this work could be developed

and investigated. For example, a surface which has the ability to bind endotoxins (ppA+PMX) and an antifouling surface (ppOX) may be combined to utilise the properties of both. This opens up the possibility of surfaces that exhibit multiple mechanisms of antimicrobial action or combination with stimuli-responsive surfaces.[7]

As the drug release system developed in this work is completely independent of the chemistry of the pharmaceutical used, this can be easily translated to any powdered drug. However, a greater insight into the effects of the plasma deposition on the activity of the drug needs to be investigated. As the substrate is completely removed, any toxicological effect of the deposited polymer also needs to be investigated as it will remain at the site of treatment after the complete dissolution of the drug. Ultimately, this method will allow encapsulation of pharmaceuticals with a specific chemical functionality. From a practical perspective, the attachment of an antibody or targeting molecule or peptide to the plasma polymer may allow for direct site-specific drug delivery.

## 8.3 References

1. Vasilev, K.; Cook, J.; Griesser, H. J., Antibacterial surfaces for biomedical devices. Expert Rev Med Devices 2009, 6, 553-67.

2. Thevenot, P.; Hu, W.; Tang, L., SURFACE CHEMISTRY INFLUENCE IMPLANT BIOCOMPATIBILITY. Current topics in medicinal chemistry 2008, 8, 270-280.

3. Schubert, U. S.; Tauhardt, L.; Pretzel, D.; Gottschaldt, M.; Kempe, K.; Pohlers, D., Zwitterionic poly (2-oxazoline) s as promising candidates for blood contacting materials. Polymer Chemistry 2014, 5, 5751-5764

4. Ramiasa, M.; Cavallaro, A.; Mierczynska-Vasilev, A.; Christo, S.; Gleadle, J.; Hayball, J. D.; Vasilev, K., Plasma Polymerised PolyOxazoline Thin Films for Biomedical Applications. Chemical Communications 2015, 51, 4297-4282.

5. Stobie, N.; Duffy, B.; Colreavy, J.; McHale, P.; Hinder, S. J.; McCormack, D. E., Dual-action hygienic coatings: Benefits of hydrophobicity and silver ion release for protection of environmental and clinical surfaces. Journal of Colloid and Interface Science 2010, 345, 286-292.

6. Sambhy, V.; MacBride, M. M.; Peterson, B. R.; Sen, A., Silver bromide nanoparticle/polymer composites: dual action tunable antimicrobial materials. Journal of the American Chemical Society 2006, 128, 9798-9808.

7. Cavallaro, A.; Taheri, S.; Vasilev, K., Responsive and “smart” antibacterial surfaces: Common approaches and new developments. Biointerphases 2014, 9, 029005.

# APPENDIX

## A1.1 Supporting information for Chapter 5

*Immobilised antibiotics on plasma polymers for dual mechanism antimicrobial coatings*

**Generation of plasma coated Carbonyl Iron for a retrievable endotoxin removal system**

Powders were coated by using a shaking platform comprised of a centre motor with DC voltage speed dependence and an offset weight to induce a shaking motion. The shaking platform was loosely attached to the plasma reactor's anode using a wire bracket. DC power was provided to the shaking platform using an external power supply. Powder substrates were loaded into disposable petri dishes directly adhered to the shaking platform. Powder substrates were chosen based on their relative magnetic properties and availability. Carbonyl iron was purchased from Sigma-Aldrich were used as substrate controls. A 10 minute polymer deposition was performed with a base pressure of $2x10^{-2}$ mbar and precursor vapour added until a steady $2.1x10^{-1}$ mbar was achieved. Plasma was ignited at 40W. Samples were left to under continuous agitation during the deposition.

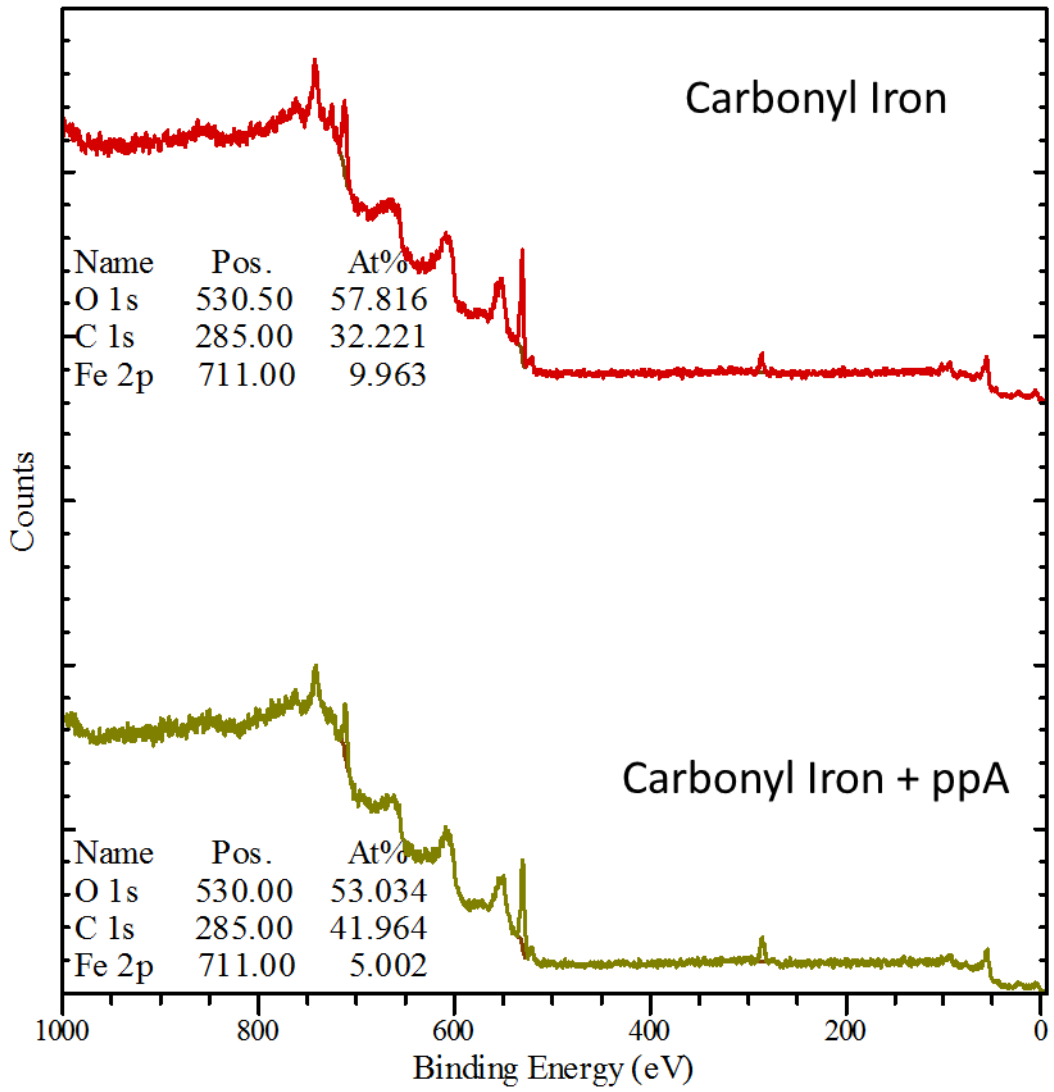


FIG. S.5.1. XPS spectrum of Carbonyl Iron (top) and Carbonyl Iron with plasma deposited propionaldehyde (bottom) showing a decrease in total surface iron from 9.96% - 5.00% after the addition of the aldehyde coating.

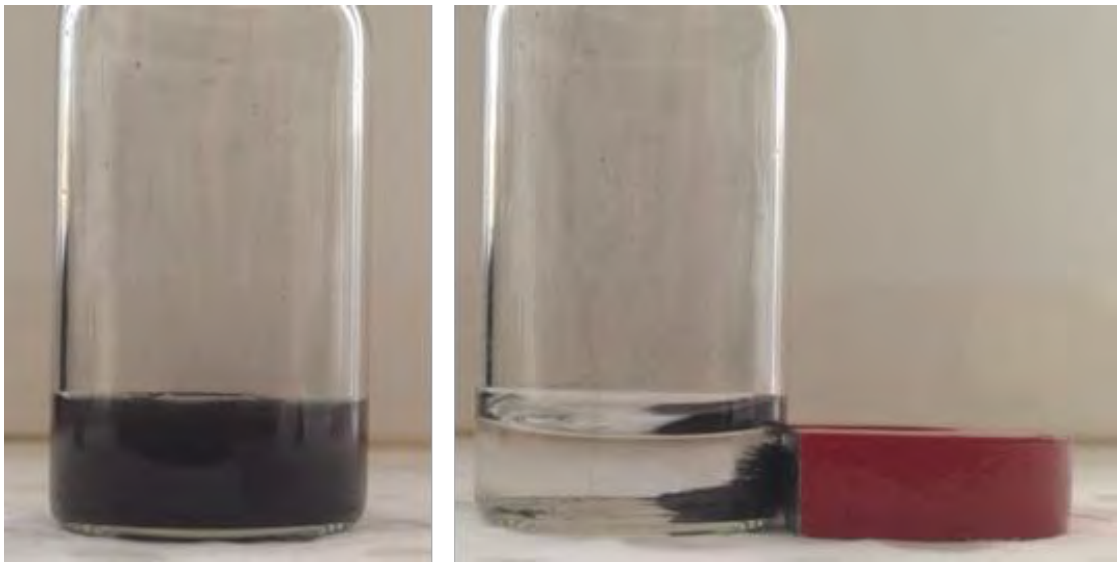

FIG. S.5.2. Photographs showing the retrievable nature of the magnetic carbonyl iron particles.

## A.1.2 Supporting information for Chapter 7

*Controlled and sustained release of pharmaceuticals via single step solvent-free encapsulation*

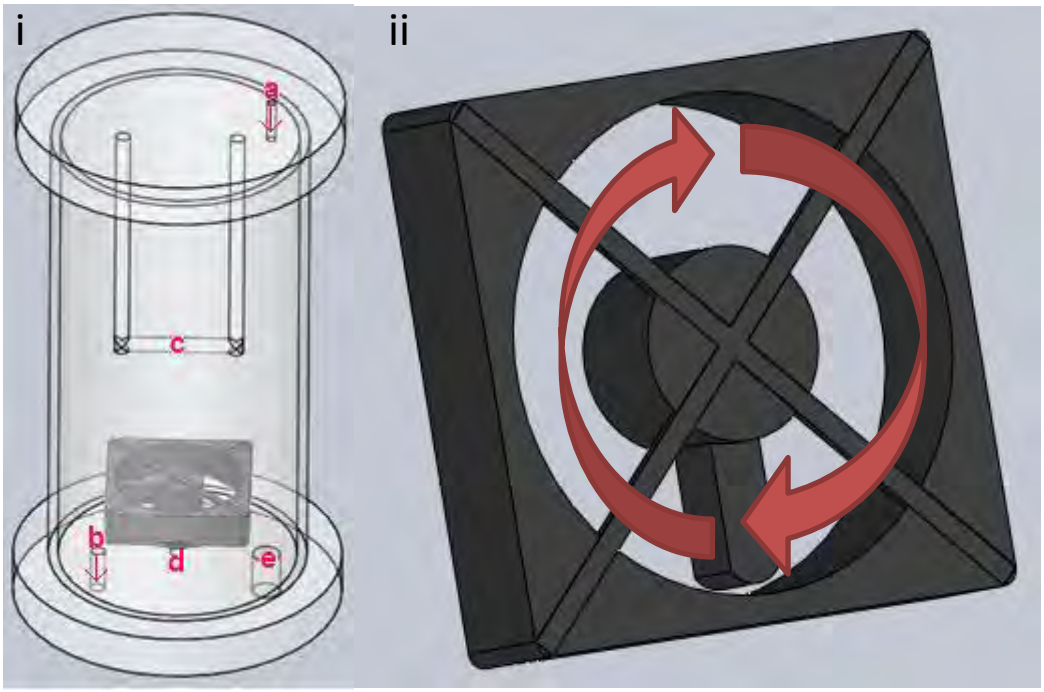


FIG. S.7.1. i) Schematic of plasma reactor setup with additional DC power input and off–centre weighted motor for inducing shaking motion a) monomer inlet b) monomer outlet c) RF cathode d) RF anode with attached motor e) DC power in port. ii) Schematic of shaking motor.

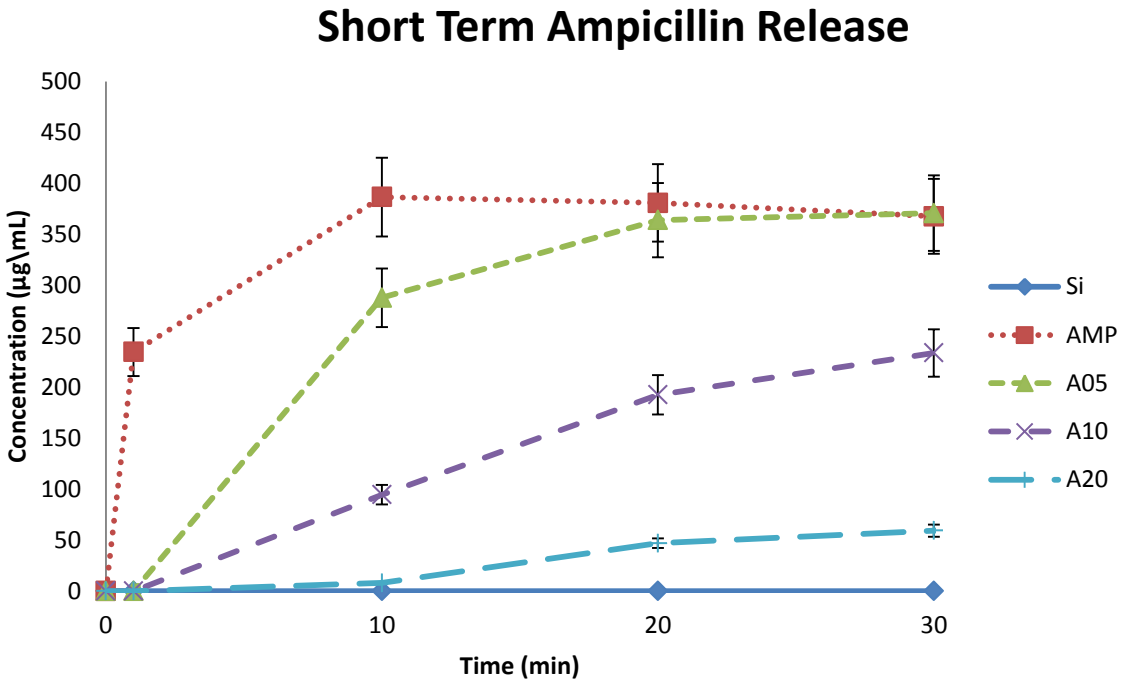


FIG. S.7.2. Initial ampicillin release over 30 minutes. Total ampicillin release rates over 5 day period. Where release of ampicillin into filtered water from coated and uncoated ampicillin powder based on UV-vis absorbance where AMP (■ ), A05 (▲ ), A10 (**X**), and A20 (**+**) (uncoated ampicillin and ampicillin with 5, 10 and 20 minute plasma polymerized octadiene coatings respectively) showed different release rates depending on polymerisation time. Si powder coated with octadiene (♦) was used as a control. All error assumes a ±10% deviation.

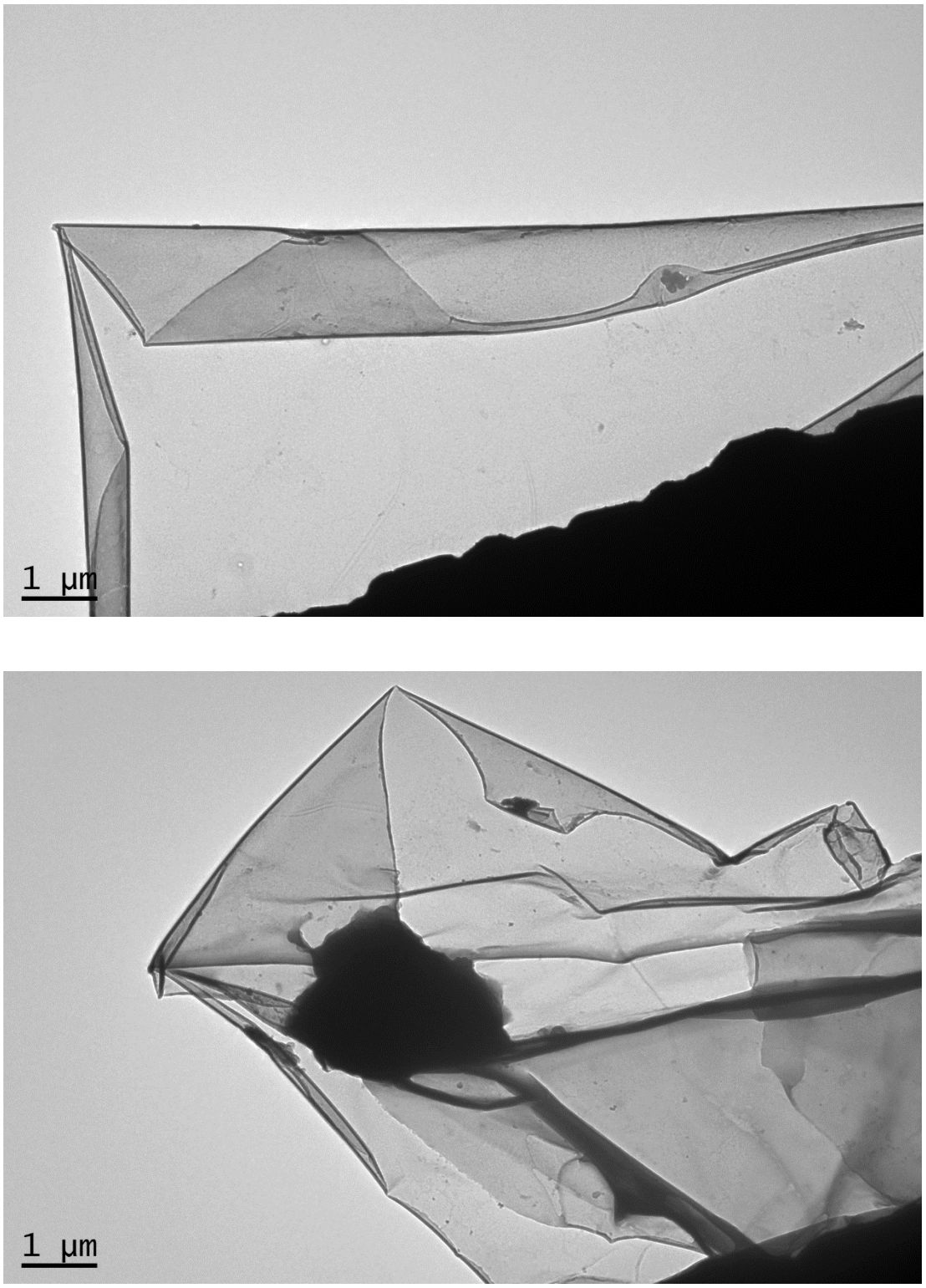


FIG. S.7.3. TEM images of remaining polymer flakes from solution post release of ampicillin.

| Sample | Concentraion (µg\mL) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 50 | 25 | 12.5 | 6.25 | 3.125 | 1.5625 | 0.78125 | 0.390625 | 0.1953125 | 0.09765625 | 0 | BLANK |
| Solution control | 0.859 | 0.912 | 0.904 | 0.888 | 0.889 | 0.897 | 0.893 | 0.895 | 0.917 | 0.892 | 0.900 | -0.001 |
| Coated Si | 0.178 | 0.659 | 0.862 | 0.879 | 0.878 | 0.894 | 0.890 | 0.890 | 0.901 | 0.875 | 0.886 | -0.001 |
| AMP | 0.000 | 0.000 | 0.000 | 0.000 | 0.051 | 0.563 | 0.818 | 0.866 | 0.865 | 0.879 | 0.875 | 0.000 |
| A05 | 0.000 | 0.001 | 0.000 | 0.000 | 0.023 | 0.432 | 0.806 | 0.852 | 0.864 | 0.891 | 0.895 | 0.000 |
| A10 | 0.003 | 0.000 | 0.000 | 0.004 | 0.052 | 0.629 | 0.840 | 0.879 | 0.863 | 0.849 | 0.857 | 0.000 |
| A20 | 0.003 | 0.002 | 0.002 | 0.000 | 0.122 | 0.666 | 0.871 | 0.896 | 0.868 | 0.886 | 0.880 | 0.001 |

Low turbidity

High turbidity

TABLE S.7.1. Turbidity ($OD_{600nm}$) caused by E. coli post treatment with serial diluted coated and uncoated ampicillin showing MIC of ampicillin and, coated Si showing some inhibition of *E. coli* due to the interaction between the octadiene and bacteria.

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# A.4 Chapter 3 Author Declaration for the Referred Manuscript

**Chapter 3 Author Declaration for the Referred Manuscript**

Cavallaro A, Majewski P, Barton M, Vasilev K. Substrate independent approach for immobilisation of quaternary ammonium compounds to surfaces to reduce bio-burden. **Materials Science Forum**. 2014;783-786:1389-95

**Declaration by the Candidate:**

Extent of contribution of the first author (Alex Cavallaro)

- *Prepared all substrates used for generation of immobilised QAC*
- *Optimised all plasma deposition conditions and surface immobilisation*
- *Performed surface characterisation*
- *Developed assay protocols for bacterial interaction with surfaces*
- *Analysed and interpreted results and wrote the initial draft of the manuscript*

Extent of contribution of the second author (Peter Majewski)

- *Co-supervised work done*
- *Assisted in interpretation of chemical analysis*
- *Edited the manuscript prior to submission*

Extent of contribution of the third author (Mary Barton)

- *Co-supervised work done*
- *Assisted in interpretation of microbiological results*
- *Edited the manuscript prior to submission*

Extent of contribution of the final author (Krasimir Vasilev)

- *Supervised all work done*
- *Assisted in interpretation of all results*
- *Edited the manuscript prior to submission*

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# A.5 Chapter 6 Author Declaration for the Referred Manuscript

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Ramiasa M*, Cavallaro A*, Mierczynska-Vasilev A, Christo S, Gleadle J, Hayball JD, et al. Plasma Polymerised PolyOxazoline Thin Films for Biomedical Applications. **Chemical Communications.** 2015;51:4297-82.

*equal contribution

**Declaration by the Candidate:**

Extent of contribution of the first author (Melanie Ramiasa)

- *Prepared substrates used for generation of coatings*
- *Plasma deposition and surface immobilisation*
- *Performed surface characterisation and stability study*
- *Consultation of assay methodology\experimental design*
- *Analysed and interpreted results and wrote the initial draft of the manuscript*

Extent of contribution of the second author (Alex Cavallaro)

- *Prepared substrates used for generation of coatings*
- *First plasma polymerisation of oxazolines*
- *Preliminary surface chemistry characterisation*
- *Conducted antimicrobial studies, analysed and interpreted results*
- *Conducted fibroblast biocompatibility study*
- *Consultation of assay methodology\experimental design*
- *Edited the manuscript prior to submission*

Extent of contribution of the third author (Agnieszka Mierczynska-Vasilev)

- *Performed protein interaction assays*
- *Assisted in interpretation of microbiological results*

Extent of contribution of the forth author (Susan Christo)

- *Performed immunological assays*
- *Edited the manuscript prior to submission*

Extent of contribution of the fifth author (Jonathan Gleadle)

- *Co-supervised work conducted*
- *Consultation of biological interactions*

Extent of contribution of the sixth author (John Hayball)

- *Co-supervised work conducted*
- *Edited and reviewed manuscript prior to submission*

Extent of contribution of the final author (Krasimir Vasilev)

- *Supervised all work done*
- *Assisted in interpretation of all results*
- *Edited the manuscript prior to submission*

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Macgregor-Ramiasa M, Cavallaro A, Vasilev K. Properties and Reactivity of Polyoxazoline Plasma Polymer Films. **Journal of Materials Chemistry B.** Published Online (DOI:10.1039/C5TB00901D)

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- *Prepared substrates used for generation of coatings*
- *Plasma deposition and surface immobilisation of AuNP*
- *Performed surface characterisation and stability study*
- *Consultation of assay methodology\experimental design*
- *Analysed and interpreted results and wrote the initial draft of the manuscript*

Extent of contribution of the second author (Alex Cavallaro)

- *Prepared substrates used for generation of coatings*
- *First plasma polymerisation of oxazolines*
- *Analysed samples and interpreted results (ellipsometry)*
- *Consultation of assay methodology\experimental design*
- *Edited the manuscript prior to submission*

Extent of contribution of the final author (Krasimir Vasilev)

- *Supervised all work done*
- *Assisted in interpretation of all results*
- *Edited the manuscript prior to submission*

*This article was peer reviewed and corrections made according to the referees' comments*

The Candidate's signature ..... DATE: 20-07-15

*The above Candidate's declaration correctly reflects the contribution of each co-author. The author permits the publication to be included in the candidate's thesis.*

First author's signature..... DATE:.....

*The above Candidate's declaration correctly reflects the contribution of each co-author. The author permits the publication to be included in the candidate's thesis.*

Final author's signature..... DATE: 20-07-15

## A.6 Chapter 7 Author Declaration for the Referred Manuscript

Cavallaro A, Vasilev K. Controlled and sustained release of pharmaceuticals via single step solvent-free encapsulation. **Chemical Communications**. 2015;51:1838-41

**Declaration by the Candidate:**

Extent of contribution of the first author (Alex Cavallaro)

- *Prepared full experimental design*
- *Prepared all substrates used for generation of coated particles and planar substrates*
- *Performed surface characterisation*
- *Developed novel platform for plasma polymerisation on powders*
- *Optimised all plasma deposition conditions for complete drug encapsulation*
- *Analysed drug release kinetics*
- *Tested and confirmed the retention of antimicrobial activity*
- *Analysed and interpreted results and wrote the initial draft of the manuscript*

Extent of contribution of the final author (Krasimir Vasilev)

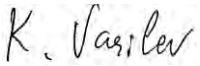
- *Supervised all work done*
- *Directed experimentation pathway*
- *Assisted in interpretation of all results*
- *Edited the manuscript prior to submission*

*This article was peer reviewed and corrections made according to the referees' comments*

The Candidate's signature  DATE: 15-07-15

*The above Candidate's declaration correctly reflects the contribution of each co-author.*

*The author permits the publication to be included in the candidate's thesis.*

Final author's signature  DATE: 15-07-15

## A.7 List of Publications

**List of Publications**

**Cavallaro A**, Majewski P, Barton M, Vasilev K. Substrate independent approach for immobilisation of quaternary ammonium compounds to surfaces to reduce bio-burden. Materials Science Forum. 2014;783-786:1389-95.

**Cavallaro A**, Taheri S, Vasilev K. Responsive and "smart" antibacterial surfaces: Common approaches and new developments. Biointerphases. 2014;9:029005.

**Cavallaro A**, Vasilev K. Controlled and sustained release of pharmaceuticals via single step solvent-free encapsulation. Chemical Communications. 2015;51:1838-41.

Bachhuka A, Christo SN, **Cavallaro A**, Diener KR, Mierczynska A, Smith LE, et al. Hybrid core/shell microparticles and their use for understanding biological processes. Journal of Colloid and Interface Science. 2015;457:9-17.

Baier G, Cavallaro A, Friedemann K, Müller B, Glasser G, Vasilev K, et al. Enzymatic degradation of poly (l-lactide) nanoparticles followed by the release of octenidine and their bactericidal effects. Nanomedicine: Nanotechnology, Biology and Medicine. 2014;10:131-9.

Baier G, **Cavallaro A**, Vasilev K, Mailänder V, Musyanovych A, Landfester K. Enzyme Responsive Hyaluronic Acid Nanocapsules Containing Polyhexanide and Their Exposure to Bacteria To Prevent Infection. Biomacromolecules. 2013;14:1103-12.

Delalat B, Mierczynska A, Ghaemi SR, **Cavallaro A**, Harding FJ, Vasilev K, et al. Materials

Displaying Neural Growth Factor Gradients and Applications in Neural Differentiation of Embryoid Body Cells. Advanced Functional Materials. 2015;25:2737-44.

Goreham RV, Mierczynska A, Pierce M, Short RD, Taheri S, Bachhuka A, et al. A substrate independent approach for generation of surface gradients. Thin Solid Films. 2013;528:106-10.

Kafshgari MH, **Cavallaro A**, Delalat B, Harding FJ, McInnes SJ, Mäkilä E, et al. Nitric oxide-releasing porous silicon nanoparticles. Nanoscale research letters. 2014;9:1-9.

Macgregor-Ramiasa M, **Cavallaro A**, Vasilev K. Properties and Reactivity of Polyoxazoline Plasma Polymer Films. Journal of Materials Chemistry B. Published Online (DOI:10.1039/C5TB00901D)

Ramiasa M, **Cavallaro A**, Mierczynska-Vasilev A, Christo S, Gleadle J, Hayball JD, et al. Plasma Polymerised PolyOxazoline Thin Films for Biomedical Applications. Chemical Communications. 2015;51:4297-82.

Taheri S, **Cavallaro A**, Barton M, Whittle J, Majewski P, Smith L, et al. Antibacterial Efficacy and Cytotoxicity of Silver Nanoparticle Based Coatings Facilitated by a Plasma Polymer Interlayer. Plasma Medicine. 2014;4:101-15.

Taheri S, **Cavallaro A**, Christo SN, Smith LE, Majewski P, Barton M, et al. Substrate independent silver nanoparticle based antibacterial coatings. Biomaterials. 2014;35:4601-9.

Vasani RB, Losic D, **Cavallaro A**, Voelcker NH. Fabrication of stimulus-responsive diatom biosilica microcapsules for antibiotic drug delivery. Journal of Materials

Chemistry B. 2015;3:4325-9.

**Cavallaro A**, Majewski P, Barton M, Vasilev K. Influence of Immobilized Quaternary Group Density, Surface Charge and Environmental Factors on the Coating Antimicrobial Efficacy and Cytotoxicity (Submitted to RSC Biomaterial Sciences)

Krismastuti F, **Cavallaro A**, Prieto-Simon B, Voelcker N. Multiplexed Wound Biomarker Detection on Porous Silicon Resonant Microcavities (Submitted to Angewandte Chemie)

**Patents**

PLASMA POLYMERISED OXAZOLINE COATINGS AND USES THEREOF "Australian provisional patent application No. 2014903447".

**Manuscripts in Prep.**

**Cavallaro A**, Macgregor-Ramiasa M, Vasilev K. Modulating biological interactions with surfaces using plasma deposited oxazoline thin films (Draft in progress)