Acknowledgements

I would like to thank my supervisor, Toby, for providing me with the opportunities, ideas and support during this work. Also thanks for the frequent trips abroad and occasional beer! To this end I would also like to thank members of the Jenkins group; Charlotte, Jin, Loftus, Xavi, Matt D, Dave, Tom and Jon who were a great help and/or hindrance, the Cameron group; Ellie and Becky, and the EMBEK1 consortium; in particular Renate, Luis, Thet and Alex.

Thanks also go to Nick Waterfield and group (Maria, Matt and Jay) for patience and plenty of microbiological consumables. With their assistance I was able to design and execute assays and crucially, was able to develop skills and understanding in the area of microbiology. Also thanks to Andy J for help with monomers and XRD, the Marken group (Stuart, Mike, Rob and Charlie) for QCM and games of pool, along with Andrea and Richard for eukaryotic cell fun in Falmouth and the Chemistry football team.

A great deal of thanks must also go to my parents, for limitless support over my student career (!) and most importantly of all I would like to thank my wife, Marina for everything she has done for me and contended with over these past few years. I truly would not be where I am today without your support.
Abstract

Bacterial infection is a growing concern in hospital and community settings, where the issue of biofilms is a major problem. Most current methods of preventing microbial attachment and biofilm formation are limited due to application, process or inherent flaws. It was proposed that thin films containing an organometallic element could be deposited using plasma, a quick, clean surface modification technique; to create antimicrobial films which could then be applied to a range of substrates.

Several novel antimicrobial monomer systems were synthesised and characterised based on silver, copper and zinc as the active constituent with phosphines, phosphites, maleimide and a novel Schiff base among the ligand systems. All monomers were found to greatly inhibit the growth of *P. aeruginosa* and *S. aureus* in solution and on solid media. Successful monomers were deposited onto suitable substrates (glass, gold, plastics, non-woven polypropylene) using continuous wave and pulse plasma, with the films characterised and low levels of active metal found in analysis using XPS and SIMS. Films were tested against solutions of pathogenic bacteria using a number of traditional and modern microbiological techniques and found to inhibit growth under a range of conditions, potentially due to the synergistic action of metal and ligand on bacterial cells. Effective control of bacteria was exhibited at times varying from 1h to 24h+. Highly volatile compounds were produced which allowed quick deposition of plasma films, which showed excellent activity against bacteria (99.9%+ growth reduction), indicating viability for potential application. All films tested showed no inhibition or toxicity to eukaryotic cells.
# Table of contents

**Chapter 1: Introduction** ........................................................................................................ 1-1  
1.1 Bacterial infections ........................................................................................................... 1-1  
  1.1.1 Hospital-acquired infections ....................................................................................... 1-1  
  1.1.2 Causes of infection ....................................................................................................... 1-2  
  1.1.3 Infection sites ............................................................................................................... 1-3  
    1.1.3.1 Implant-based infection ....................................................................................... 1-3  
    1.1.3.2 Burns and wounds .............................................................................................. 1-4  
    1.1.3.3 Dermatological infection ................................................................................... 1-5  
1.2 Biofilms ................................................................................................................................ 1-6  
  1.2.1 Biofilm formation ......................................................................................................... 1-7  
  1.2.2 Biofilm structure and growth .................................................................................... 1-8  
  1.2.3 Inherent resistance of biofilms .................................................................................. 1-10  
1.3 Current antimicrobial approaches .................................................................................... 1-12  
  1.3.1 Non-fouling approaches ............................................................................................ 1-12  
  1.3.2 Antibiotic and biocidic approaches .......................................................................... 1-13  
  1.3.3 Metal-based approaches ........................................................................................... 1-14  
    1.3.3.1 Silver ................................................................................................................... 1-14  
    1.3.3.2 Other metals ......................................................................................................... 1-17  
  1.3.4 Antimicrobial challenges ......................................................................................... 1-18  
1.4 Plasma processing ............................................................................................................ 1-19  
  1.4.1 Application of plasma ............................................................................................... 1-20  
1.5 References ....................................................................................................................... 1-21  

**Chapter 2: Instrumentation and theory** ............................................................................. 2-29  
2.1 Plasma reactor ................................................................................................................ 2-29  
  2.1.1 Plasma ...................................................................................................................... 2-29  
  2.1.2 Plasma physics ............................................................................................................ 2-31  
  2.1.3 Plasma processes ....................................................................................................... 2-34  
  2.1.4 Plasma polymerisation ............................................................................................... 2-37  
    2.1.4.1 Plasma polymerisation mechanism ................................................................... 2-39  
    2.1.4.2 Effect of deposition parameters ........................................................................ 2-41  
  2.1.5 Plasma reactor ............................................................................................................ 2-43
2.2 Characterisation methods ................................................................. 2-45
  2.2.1 Fourier-transform infra-red spectroscopy ................................. 2-45
    2.2.1.1 Self-assembled monolayers ........................................... 2-46
  2.2.2 Nuclear magnetic resonance .................................................. 2-48
  2.2.3 X-ray crystallography .............................................................. 2-50
  2.2.4 Melting point analysis ............................................................. 2-51
  2.2.5 Atomic absorption spectroscopy ............................................. 2-51
  2.2.6 X-ray photoelectron spectroscopy ......................................... 2-52
  2.2.7 Mass spectrometry ................................................................. 2-54
  2.2.8 Secondary ion mass spectrometry .......................................... 2-54
  2.2.9 Electron microscopy .............................................................. 2-55
    2.2.9.1 Energy dispersive x-ray spectroscopy .............................. 2-57
  2.2.10 Quartz crystal microbalance ............................................... 2-58
  2.2.11 Alpha-step profiling ............................................................. 2-58
  2.2.12 SPR ...................................................................................... 2-59

2.3 Microbiological assays .................................................................. 2-61
  2.3.1 Microbiology .......................................................................... 2-61
  2.3.2 The cell .................................................................................. 2-62
    2.3.2.1 Prokaryotic cell ................................................................. 2-62
    2.3.2.2 Eukaryotic cell ................................................................. 2-63
  2.3.3 Genotype and phenotype ......................................................... 2-64
  2.3.4 Bacterial cell growth ............................................................... 2-64
  2.3.5 Model organisms ..................................................................... 2-66
    2.3.5.1 Pseudomonas aeruginosa ................................................. 2-66
    2.3.5.2 Staphylococcus aureus ........................................................ 2-66
    2.3.5.3 Micrococcus luteus ............................................................. 2-67
  2.3.6 Bacterial detection ................................................................. 2-67
    2.3.6.1 Colony counting ............................................................... 2-67
    2.3.6.2 Solution turbidity .............................................................. 2-68
    2.3.6.3 Bright-field microscopy .................................................... 2-69
    2.3.6.4 Fluorescent tagging .......................................................... 2-70
  2.3.7 Microbial safety and sterility .................................................... 2-73
  2.3.8 Antibacterial evaluation of materials ....................................... 2-73
    2.3.8.1 Disc diffusion assays ......................................................... 2-74
Chapter 3: Experimental methods

3.1 Plasma reactor
   3.1.1 Monomer deposition
   3.1.2 Pulse plasma
   3.1.3 Oxygen/fluorocarbon treatment

3.2 Plasma film characterisation
   3.2.1 Substrate preparation
      3.2.1.1 Glass preparation
      3.2.1.2 Gold evaporation
      3.2.1.3 SAM preparation
   3.2.2 FTIR
      3.2.2.1 Transmission spectra
      3.2.2.2 Absorption spectra
   3.2.3 AAS
   3.2.4 XPS
   3.2.5 Electron microscopy
   3.2.6 SPR
   3.2.7 QCM
   3.2.8 α-step profiling

3.3 Monomer characterisation
   3.3.1 FTIR
      3.3.1.1 Transmission spectra
      3.3.1.2 Absorption spectra
   3.3.2 NMR
   3.3.3 XRD
   3.3.4 Melting point

3.4 Microbiological techniques
   3.4.1 Aseptic techniques
   3.4.2 Monomer microbiological assays
3.4.2.1 Disc diffusion assay ............................................................... 3-87
3.4.2.2 MIC assay ........................................................................... 3-88
3.4.3 Plasma film microbiological assays ........................................... 3-89
3.4.3.1 Petri dish assay .................................................................... 3-90
3.4.3.2 JIS assay ............................................................................ 3-90
3.4.4 Antibacterial analysis ............................................................... 3-90
3.4.4.1 Colony count ....................................................................... 3-90
3.4.4.2 Live/Dead analysis ............................................................... 3-90
3.4.4.3 Microscopy ......................................................................... 3-91
3.5 Cytotoxicity tests ....................................................................... 3-92
3.6 Appendix .................................................................................... 3-93
3.6.1 Pulse operation ....................................................................... 3-93
3.6.2 Oxygen/fluorocarbon treatment .............................................. 3-94

Chapter 4: A novel silver compound for the plasma deposition of antimicrobial films .................................................................................................................. 4-95
4.1 Antimicrobial silver ..................................................................... 4-95
4.2 Silver chemistry ........................................................................... 4-97
4.3 Novel silver complexes for plasma deposition ............................. 4-97
4.3.1 Ligand selection ....................................................................... 4-98
4.3.2 Lewis adduct selection ............................................................. 4-99
4.4 Preliminary plasma study – maleimide ......................................... 4-102
4.5 bis-triphenylphosphino silver maleimide ..................................... 4-103
4.5.1 PSSM preparation method ....................................................... 4-104
4.5.2 PSSM characterisation ............................................................. 4-104
4.6 Plasma deposition of PSSM ......................................................... 4-108
4.7 Microbiological assays .................................................................. 4-116
4.7.1 Assays versus Micrococcus luteus .......................................... 4-116
4.7.1.1 Monomer zone of inhibition .............................................. 4-117
4.7.1.2 Initial plasma film tests: visualisation .................................. 4-118
4.7.1.3 Traditional counting methods .......................................... 4-119
4.7.1.4 Live/Dead cell staining ....................................................... 4-121
4.7.2 Assays versus Staphylococcus aureus and Pseudomonas aeruginosa 4-123
4.7.2.1 Monomer zone of inhibition .............................................. 4-123
4.7.2.2 Minimum inhibitory concentration ..................................... 4-125
Chapter 1: Introduction

1.1 Bacterial infections

1.1.1 Hospital-acquired infections

Nosocomial, or hospital-acquired infections (HAIs) and community-acquired infections are a growing health hazard, particularly in intensive care units, the chronically ill and imuno-compromised people. Infections are becoming increasingly prevalent with the advent of micro-organisms which can resist antibiotic action, known as hospital ‘superbugs’ e.g. methicillin-resistant Staphylococcus aureus (MRSA) and C. difficile. Indeed, infectious diseases are the second highest cause of death worldwide, of which bacteria account for around two-thirds. Approximately 5-10% of hospitalised patients experience an HAI and the average time-period for bacterial blood-stream infection in hospitals is thirteen days from admission, with a mortality rate of 12%, increasing to 80% in intensive care units. Not only are infections a major personal danger, they also create financial and logistical problems for hospitals and health organisations as hospitalisation time can be prolonged by up to thirty-two days by infection, costing the NHS over one billion pounds a year and resulting in the deaths of thousands of people in the process. Despite this, several criteria must be assessed by a health service or hospital when considering the feasibility of implementing new infection controls; the most important of which are effectiveness and cost relative to non-prevention.

Propensity for infection is directly correlated to bacterial colonisation on surfaces. In open systems such as solid/air and solid/liquid interface, bacteria may or may not be able to propagate but can pose a bio transfer threat in either case e.g. MRSA on hands in hospitals, Salmonella in food industry. Surfaces with potential for bio transfer include gloves, ward curtains and hospital scrubs; although the outright transfer source of nosocomial infections in general is still unclear. A way of avoiding contamination is to create antimicrobial materials or impart antimicrobial activity onto existing surfaces. The importance of engineering antimicrobial surfaces
is clear in this context alone, yet there are a multitude of applications for such solutions. The aim of this work was to create novel antimicrobial molecules and incorporate them into thin films using plasma deposition for this purpose.

1.1.2 Causes of infection

Many pathogenic bacteria exist, yet some are more deadly than others. Variables such as toxin production, biofilm-forming tendency and prolific nature of growth can influence pathogenicity of different species of bacteria towards humans. Action of bacteria also slows or prevents healing of wounds. Common human pathogens and their prevalence in intensive care units (ICUs) is summarised in fig. 1.1.

*Figure 1.1: The prevalence of pathogens in intensive care units in Western Europe*  

*Staphylococcus aureus*, the most prevalent pathogen in ICUs is commonly found on human skin and hair yet plays an important role in dermatitis and chronic infections. Around 20% of the population carry *S. aureus* at all times and are known as persistent carriers; 60% are intermittent carriers and 20% are non-carriers. MRSA, the resistant strain of *S. aureus* accounting for up to 50% of clinical isolates in some cases is typically resistant to all antibiotics bar vancomycin and teicoplanin, causing major problems for treatment.

*Pseudomonas aeruginosa*, a prolific biofilm-forming pathogen is known as a factor in the mortality of immunocompromised patients and those with cystic fibrosis and
cancer.\textsuperscript{17} Most cystic fibrosis patients have their lower respiratory tract colonised by \emph{P. aeruginosa} by adolescence or adulthood.\textsuperscript{18} Proteases released by \emph{P. aeruginosa} are thought to damage host tissues and upset antibacterial defence mechanisms.\textsuperscript{19} This second most commonly found strain found in ICUs was shown to be present in sink drains\textsuperscript{11} in hospitals, which is a potential source of infection.

\emph{P. aeruginosa} can only survive for a relatively short period of time as individual cells so transfer must occur rapidly from infection source to the body. \emph{S. aureus} have been shown as much harder organisms, surviving for up to two days on abiotic surfaces in comparison to around seven hours for \emph{P. aeruginosa}. Studies have shown differences between bacteria found in endogenous flora of patient’s skin and those found on abiotic surfaces of hospitals, suggesting many nosocomial infections can be self-administered. Concurrently, other studies have shown incidence of moist materials hosting a variety of bacterial strains in hospitals including MRSA and \emph{P. aeruginosa}.

### 1.1.3 Infection sites

#### 1.1.3.1 Implant-based infection

Infection is easily contracted in hospital via surgical procedures or implanted medical devices such as joint replacements,\textsuperscript{20} heart valve prosthesis and pacemakers\textsuperscript{21} (category I); or IV lines and catheters\textsuperscript{22-23} (category II). These devices can be colonised by bacteria prior to application if not thoroughly cleaned,\textsuperscript{22} by inoculation from patient’s skin or the hands of surgical staff.\textsuperscript{21} Category I devices only carry an infection threat at implantation, whereas category II devices carry a cumulative risk.\textsuperscript{24} Despite best sterilisation efforts, infection is a major cause of implant failure.\textsuperscript{25} For instance in the case of urinary catheters, bacteria may migrate along the surface of a patient’s skin where glycoprotein has built up, onto the device and build up subcutaneously as an infection.\textsuperscript{22,24}
Three million catheters are used on a yearly basis in the US alone and lead to up to one million infections,\textsuperscript{23} 20% of which are serious, with a mortality rate of 28%.\textsuperscript{24} Studies have shown that catheter-associated urinary tract infection (CAUTI) contributes $>40\%$ of all institutionally acquired infections,\textsuperscript{27-28} between 250,000 and 500,000 in the United States annually\textsuperscript{29} and a quarter of all catheterised patients become infected within a week, with a cumulative daily risk of 5%.\textsuperscript{30} Removal of a severely infected device is difficult, unpleasant to the patient and expensive,\textsuperscript{20} with a large quantity of CAUTIs (e.g. fig. 1.2) caused by antibiotic-resistant pathogens such as Enterobacter, \textit{Pseudomonas aeruginosa}, \textit{Proteus mirabilis} and various staphylococci.\textsuperscript{31} The prevalence of CAUTIs has been proven to increase death rates in hospitals,\textsuperscript{23,32} with up to a quarter of these infections classed as fatal.\textsuperscript{21,29}

\subsection{1.1.3.2 Burns and wounds}

Burns and wounds are prime sites of infection for opportunistic pathogens, where \textit{P. aeruginosa} and \textit{S. aureus} are of the greatest importance\textsuperscript{33-34} with \textit{Staphylococcus} the most prevalent coloniser of burn sites.\textsuperscript{35} The surface of a burn is protein-rich which provides an excellent environment for bacteria to propagate.\textsuperscript{34} MRSA cells are frequently found in burns units, with incidence rising to 64\% from 18\% in regular hospital wards.\textsuperscript{11} An exotoxin released by \textit{S. aureus}, toxic shock syndrome toxin-1 (TSST-1) can cause a disease in burns patients, particularly young children who possess lower levels of TSST-1 antibodies than the general populous, called toxic shock syndrome (TSS).\textsuperscript{36} TSS is a severe systemic illness characterised by shock,
pyrexia, rash, gastrointestinal disturbance and lethargy or irritability.\textsuperscript{37} \textit{P. aeruginosa} has also been associated with TSS\textsuperscript{35} and the condition is associated with high mortality rate of up to 50\%,\textsuperscript{38} so eradication of bacteria from burn sites is of paramount importance.

1.1.3.3 Dermatological infection

Outside of hospitals nappy rash, or diaper dermatitis is a source of infection in the community, accounting for 20\% of dermatological cases in childhood. Commonly thought of as ammonia-mediated, recent work\textsuperscript{17,39-40} has shown the influence microorganisms such as \textit{S. aureus} and \textit{Candida albicans} can have in such environments.

Intact human skin naturally preserves body fluid homeostasis and contains a natural defence mechanism of bactericidal lipids which kill bacteria such as streptococci.\textsuperscript{34,41} However, skin moistened by damp materials is damaged by rubbing motion and subsequent increased permeability allows skin irritants access to the lower layers of the skin. It has been postulated\textsuperscript{42} that increased pH caused by urine allows the activity of lipases and proteases of micro-organisms to act on the skin. Indeed, people with dermatitis show increased susceptibility to secondary-type infections of \textit{S. aureus}.\textsuperscript{41,43}

The top layer of modern disposable nappies typically consists of non-woven polypropylene that allows movement of liquid away from the skin surface in order to prevent skin dampening. Non-wovens are also used in wound dressings and other hospital applications; with greater than half of all surgical gowns used in the United States composed of non-woven material. However, non-wovens can also serve to pool microorganisms which cause infection,\textsuperscript{44} creating problems for both hospital patients and in the community at large.
1.2 Biofilms

A major factor in the pathogenicity of bacterial infection is that more than 99% of all bacteria are contained in biofilms, which prolong inflammation in chronic wounds by secretion of enzymes, toxins and extracellular substances. Of all the diseases treated in the 1990s, 60% were reported as the result of biofilm infection and it is believed that beneficial or pathogenic biofilms are present in all chronic wounds.

A biofilm is defined as a structured; viscoelastic community of bacterial cells enclosed in a self-produced polymeric matrix adhered to an inert or living surface at a solid-liquid interface (fig. 1.3). These sessile communities are very hardy and have been proven to withstand extreme antibiotic treatment, much more so than planktonic cells (up to 1000 times), possibly due to the difference in diffusion of antibiotics or complex changes in physiology of the film. It is perhaps because of cellular cooperation that bacteria constitute the most successful form of terrestrial life.

CAUTIs and prosthetic infections for instance are generally caused by biofilm formation, as implanted surfaces can be easily colonised due to the high availability of nutrients and appropriate fluid for growth at the liquid/solid interface in the body. In addition to localised infection, cells may detach from a biofilm to seed ‘downstream’ and cause acute infection.

![Figure 1.3: Biofilm structure](image-url)
1.2.1 Biofilm formation

Biofilms were only recently discovered.\textsuperscript{52} Previously, it was assumed that all bacteria are planktonic (free swimming\textsuperscript{56}), but in the 1970s microbial communities were reported\textsuperscript{46} and in recent years much work has been done to establish the nature and structure of biofilms. Current understanding of biofilm formation is as follows:\textsuperscript{57-59}

(i) Initial, reversible attachment of bacteria to substrate. (Net product of electrostatic forces, Brownian motion and flagella propulsion.\textsuperscript{56})

(ii) Attachment becomes irreversible upon movement of bacteria using twitching motility and production of extracellular polysaccharides (EPS).

(iii) Microcolony formation by reproduction and twitching motility.

(iv) Biofilm matures via increased synthesis of EPS matrix and formation of complex structure.

A schematic of biofilm formation is shown in fig. 1.4.

\textbf{Figure 1.4: Biofilm formation}\textsuperscript{46}

Twitching motility via type IV pili allow initial attachment\textsuperscript{57} and movement\textsuperscript{60-61} of bacteria on a surface. Pili are helical polymeric fibres 6nm in diameter and several micrometers long attached to the outside of a bacterial cell,\textsuperscript{60} which act by extension, tethering and retraction in a similar way to a grappling hook. Twitching motility also plays a role in host colonisation and pathogenesis, including activation of host cell responses.\textsuperscript{62}
Physiological co-operativity between cells contributes greatly to shaping biofilms. Cell-to-cell signalling has been shown to be a factor in differentiation of biofilm structure, with two systems identified in *Pseudomonas aeruginosa*, *lasR-lasI* and *rhlR-rhlI*. Both gene products direct synthesis of homoserine lactones, which at sufficient population density reaches the concentration required for gene activation. Gene regulation of this kind is known as ‘quorum sensing’, which also allows bacteria to react as a group in response to stimuli. Communication in this manner, it has been reported allows the release of degradative extracellular enzymes and cytotoxins. Unlike planktonic cells where signals may be diluted and taken away in medial flow, the diffusion-limiting nature of a biofilm environment allows lactones to reach critical concentration level.

1.2.2 Biofilm structure and growth

Biofilm structures can be heterogeneous, in that several species of bacterium may form a cohesive unit. Within these communities, different species of bacteria may interact and exchange such information as genes. The morphology of mature biofilms is such that bacteria are embedded in differentiated mushroom- and pillar-like structures consisting of EPS matrix or glycocalyx, separated by water channels. Within the matrix, each bacterium occupies a specific microenvironment determined by surrounding cells, proximity to water channel and the EPS matrix itself. Channels provide the flow of nutrients dissolved in bulk solution necessary to allow bacterial growth and removal of waste products. Such is the complexity of channel systems in biofilms, they have been compared to circulatory systems and biofilms may even be considered as primitive multicellular organisms, as evidenced by their complex nature summarised in fig. 1.5.
Despite the growing work on biofilms, knowledge is limited about interactions between EPS secreted by two different bacteria and how they form a cohesive matrix. Recent work has shown that electrostatic interactions between like charged polymer stands bridged by multivalent counter ions such as $\text{Fe}^{3+}$ and $\text{Al}^{3+}$ may play a part, but other models include electrostatics between oppositely charged polymer chains, hydrogen bonding, covalent bonding, chain entanglement and hydrophobic interactions.

Hydrodynamics regulate the growth of a mature biofilm, with accumulation rate determined by the net result of bacterial attachment, detachment and growth rates. For instance, attachment is controlled by the flow of microbes, detachment rate by erosion (shear stress) and growth by nutrient transport. Biofilm structure in turn determines the rate of nutrient transport to lower layers, which therefore controls bacterial growth and the rate of biofilm colonisation. Hydrodynamics and biofilm structure are therefore co-dependent.
Although nutrient flow rate is a major factor in biofilm growth, the chemical activity of reactants is equally important. If a concentration gradient is established by continual uptake of chemicals at the surface, increased flow of nutrients to the biofilm occurs. This contrasts with planktonic bacteria, for which availability of nutrients is diffusion controlled. Interaction of the biofilm with chemicals is therefore further complicated by surface properties such as charge, hydrophobicity etc.

Therefore, for a successful biofilm community a balance must be reached by which the medium is not overly nutrient heavy (increased viscosity) or nutrient poor (decreased growth rate). Other factors influencing the growth of biofilms include environment, bacterial components and modes of attachment.

1.2.3 Inherent resistance of biofilms

Naturally occurring and infection based biofilms have been proven to resist various bacteriophage, amoebae, chemical biocides and human immune system responses. For example, a 600-fold increase in the biocide chlorine is required to kill biofilm cells of *Staphylococcus aureus* versus the planktonic variety and *P. aeruginosa* resistance to antibiotics is up to 100-times greater as a biofilm. It has been proposed that bacterial communities avoid antibiotic action via a concerted effort of several defence mechanisms.

Biofilm structure (EPS matrix) slows the diffusion of antibiotics, which allows deactivation via a penetration barrier, although this may be overcome during long-term treatment. The inherent negative charge carried by the EPS matrix also serves to chelate positively charged antibiotic ions.

Core cells in biofilm structure are nutrient deficient, existing in a slow/non-growing or ‘starved state’, which is not very susceptible to antimicrobial agents (fig. 1.6) Cell viabilities reduce from around 100% in the planktonic phase to around 10% in a biofilm. Curiously however, slow-growing *P. aeruginosa* biofilm cells have shown decreased tolerance to chlorine under anaerobic conditions when response to conventional antimicrobials remains constant. Experimental work has proven that
planktonic *P. aeruginosa* is significantly less affected by antibiotics in the stationary phase,\textsuperscript{74} correlating to that of biofilm core cells. However, planktonic bacteria and sheared-off biofilm once again become susceptible to antibiotics upon dilution in an appropriate medium as nutrients become available and cell-to-cell signalling is reduced.\textsuperscript{75}

![Figure 1.6: Core, ‘starved state’ cells which have low susceptibility to antibiotics survive and re-grow after antimicrobial treatment\textsuperscript{49}](image)

Structural and bacterial heterogeneity of biofilm provides dual defence method:\textsuperscript{46}  
a) Differentiated structure ensures cells are in a variety of metabolic states, therefore denying blanket metabolically directed action.  
b) Possibility that at least one species of bacteria may be resistant to antibiotic attack.

In addition to biofilm-specific modes of antibiotic action, existing knowledge of planktonic bacterial protection mechanisms include the expulsion of foreign bodies such as drugs, toxic metal ions and organic solvents (efflux pumps)\textsuperscript{49} and gene mutation, with consequential evolution of antibiotic resistant microbial strains\textsuperscript{54}. Indeed, it has been suggested that there is a link between antibiotic action and programmed cell death (PCD)\textsuperscript{76}, whereby an antimicrobial agent damages a cell to a degree, causing it to self-destruct. If some bacteria contain defective genes coding for PCD, then they will persist and are able to replicate again once antibiotic treatment has ceased. Evidence of such a mechanism may justify current concern in the medical world regarding over use of antibiotics.\textsuperscript{54}
1.3 Current antimicrobial approaches

The prevention of bacterial infection and potential biofilm formation in medical and industrial environments is required as once an infection begins and a biofilm takes hold, it is very difficult to remove without serious harm. Much work has therefore been carried out to engineer antimicrobial methods and materials in recent years which protect against biofilms and their associated problems. Such work has focused primarily on areas of preventing cell adhesion and proliferation of microorganisms both in solution and on surfaces. Surface-active polymeric coatings often prove advantageous over monomeric antimicrobials due to the inherent properties of non-volatility and chemical stability.

1.3.1 Non-fouling approaches

Antimicrobial properties can be greatly influenced by the topography of surfaces e.g. factors such as hydrophobicity and roughness. The physical modification of medical devices by tailoring of surface properties for microbe repulsion or development of ultra-smooth surfaces have been attempted, which rely on preventing the initial stages of biofilm formation namely initial attachment of cells.

A variety of functional groups on surfaces can encourage and discourage cell adhesion e.g. ion-ion, ion-dipole interactions with proteins of bacteria and growth factors. Positive interaction can lead to the initial stages of biofilm formation or conditioning of the surface for this process. Most studies have shown a clear correlation between the hydrophobicity of surfaces and detachment or reduced attachment of cells. If positive surface free energy of adhesion is imparted on the interface, cell attachment is reduced. However, electrostatic considerations are usually small under physiological conditions, meaning such approaches are limited for use in applications such as implants.

Microbe repulsion techniques generally involve the formation of non-charged hydrogel-forming coatings e.g. polyethylene glycol (PEG), polyethylene oxide (PEO), ultra hydrophobic e.g. Lotus effect surfaces which exhibit very high water contact angle or the development of Teflon-type technology using
It is important to note that these methods do not provide an totally or indefinitely repulsive surface to all bacteria.8,79

1.3.2 Antibiotic and biocidic approaches

Antibiotics are commonly and have historically been used in the aqueous phase and can be administered orally to combat general infections87 e.g. flucloxacillin. More recently, work has been undertaken to produce surfaces impregnated with antibiotics, which can allow the initial attachment but subsequent killing of cells whilst simultaneously releasing antibiotic into solution to prevent proliferation of planktonic phase bacteria. Proteins or synthetic binding agents can ionically trap antibiotic e.g. ciprofloxacin77 and release on contact with an appropriate medium e.g. blood.25 However, studies have shown that activity of antibiotics such as biguanides is reduced 1000-fold by biofilm formation.55 Only two new classes of antibiotic have been developed since 1968 though and due to emerging resistant bacterial strains, antibiotics are set to be superseded in coming years. In their place is likely to be a promising class of antimicrobial macromolecules which mimic the natural activity of host-defence peptides.88

Chemical modifications such as the incorporation of biocidic agents into polymers20,29,79 have been employed. Alternatively, methods of loading traditional antiseptics such as chlorhexidine gluconate29 into existing polymeric coatings by dipping, padding or spraying44 have shown good efficacy against antibiotic-resistant Staphylococci, yet safety concerns and potential for resistance have blighted this approach. Other approaches include tackling enzymes such as GlmU, responsible for cell wall production.27 Proteins such as these can be deactivated by thiol-specific units, although such broad spectrum activity will inevitably lead to problems with eukaryotic cells.

Quaternary ammonium compounds (QACs) are cations of the general formula NR₄⁺, e.g. tetraethylammonium bromide. They are widely used as disinfectants in the aqueous phase, acting on microorganisms by cation targeting the negatively charged exterior of the cell and fatally releasing potassium ions and other vital cell
components. Recent work has shown that long-chain QA-polymers can be grafted from surfaces or polymerised onto surfaces to good antimicrobial effect. However, QACs are associated with causing anaphylactic reactions to neuromuscular blocking drugs during general anaesthesia in surgery and skin and respiratory irritations, so their incorporation into medical equipment or materials seems unlikely.

Natural remedies have also been reconsidered in recent years due to bacterial resistance to modern antibiotics. Tea tree oil for instance, consisting of terpene hydrocarbons and associated alcohols is proposed to act in a manner similar to QACs, by disrupting cell membrane structure and function. Unfortunately there is very little clinical evidence of activity of such remedies against micro-organisms.

1.3.3 Metal-based approaches

Metals ions, particularly silver, copper, bismuth and zinc have been shown to reduce microbial growth. Metals are ranked thusly in order of general antimicrobial activity: Ag > Hg > Cu > Cd > Cr > Ni > Pb > Co > Zn > Fe. Consequently most attention has been focused on silver as an antimicrobial metal.

1.3.3.1 Silver

Silver is non-essential to cell function, but the use of silver as an antibacterial agent is well known since ancient times and in addition to biocidal activity, appears to have anti-inflammatory properties. At the same concentration levels as chloride, silver is 500-times more effective as an antimicrobial and on a par with antibiotics such as tobramycin but has shown to be effective against MRSA in the nanocrystalline dressing Acticoat. Silver is one of the most popular biocidic materials, as unlike antibiotics and halogens, it does not display toxicity or carcinogenicity in relatively low concentrations; although acute poisoning at higher concentrations (>0.35 mg/day) can result in allergic reactions such as toxic epidermal necrolysis, hepatic dysfunction, nephropathy and leukopaenia. Prolonged exposure to colloidal silver has proven to be physically harmless but can lead to argyria, a condition evidenced by the primary symptom of blue/grey skin. Silver also displays high thermal stability and long-term activity. Simple application use is in water purification, dental fillings and in treatment of burns.
and wound bandaging, in the form of silver sulfadiazine.\textsuperscript{22} Low concentration solutions of silver nitrate are used in baby’s eyes under statutory law in the US and many other countries to prevent infection,\textsuperscript{104-105} yet in common with silver sulfadiazine is limited in activity due to the rapid deactivation of free ions by complexation under physiological concentrations of chloride and protein.\textsuperscript{93,100} However, the silver ion remains the number one topical antimicrobial in burn care.\textsuperscript{106}

Many studies have shown the antimicrobial properties of silver, but very few surface-active silver-containing materials are convincing in clinical studies.\textsuperscript{107} For example, in the mid-nineties work was done on impregnation of collagen catheters with silver ions, in order to inhibit bacterial growth and prevent migration. While effects were seen, the biodegradable nature of collagen meant depletion of all Ag\textsuperscript{+} ions in as little as five days.\textsuperscript{108} More recently, galvanic deposition of elemental silver onto prosthetic surfaces has also been investigated\textsuperscript{28,109} and while \textit{in-vivo} testing showed reduced infection rates, elevated silver concentrations were seen in blood and organs.

Another potential method of incorporating silver onto medical surfaces is via magnetron sputtering at high pressure\textsuperscript{95} and under RF plasma,\textsuperscript{96} although these require too high a temperature for practical applications. However, coatings containing silver have been produced using a cold plasma sputtering technique,\textsuperscript{82} and magnetron sputtering combined with a neutral atom beam\textsuperscript{96} or ion beam implantation\textsuperscript{110} which allow the deposition of silver nanoparticles into a polymeric matrix, in comparison to wet polymerisation methods. Such a coating provides silver ions at the surface without waste in the bulk, so maximising the efficiency of the material. These techniques however, do not allow precise control over thickness and silver content\textsuperscript{94} and if a material releases silver too readily, cytotoxicity will result by which the material becomes harmful to human cells.\textsuperscript{96} Consequently, implanted medical devices containing purely silver are limited by leaching and toxicity.\textsuperscript{28}

A recent development has been encapsulation of silver nanoparticles into polymeric matrix via dip coating.\textsuperscript{111} This approach utilises anionic carboxylate functionality of polymethacrylate copolymer and ion-exchange with Na\textsuperscript{+} ions replaced by Ag\textsuperscript{+} ions, before UV irradiation reduces the silver to nanoparticulate form. It has also been
suggested that the high surface area inherent in colloidal particles may allow a high level of antibacterial drug delivery. Use of such particles which can be controlled by external forces such as magnetic fields, would be useful in bulk solutions and piping, but less effective for practical applications on biomedical surfaces. Dendrimer-silver complexes as nanocomposite solutions have also shown effect, but with similar difficulties. A silver iontophoretic catheter has been proposed as another alternative treatment, consisting of silver wires wound around the catheter and connected to an electrical source creating a field of silver ions. This method has shown some antimicrobial activity and is shown to be non-toxic during long term animal model in-vivo studies, although practical human studies have yet to be evaluated.

Recent work has taken the logical progression and combined the obvious benefits of non-fouling coatings with silver materials. Copolymers loaded with silver nanoparticles resulted in a material containing hydrophobic chains which both inhibits microbial attachment and prevents attachment (fig. 1.7). Another approach is that of surface grown polyelectrolyte brushes, ion-exchanged to trap silver ions within the matrix. Unfortunately, this material does not provide lasting antimicrobial action and as is common with many current antimicrobial methods is not long-lasting due to facile release of silver.

Figure 1.7: Structure of a potentially non-fouling and microbial growth inhibiting material
1.3.3.2 Other metals

Copper and zinc, despite being essential to cell function, can be toxic to both humans and microorganisms at high concentrations, (or low concentrations as free ion)\textsuperscript{114-115} inhibiting many enzymatic activities\textsuperscript{116} and cell reactions.\textsuperscript{117} A safe level of copper intake for humans has been recommended at between 1.5-3.0 mg per day, although the actual requirement may be less than 1.5 mg daily.\textsuperscript{118} The recommended daily allowance of zinc is much higher, around 15 mg.\textsuperscript{119}

Copper is required by human enzymes such as cytochrome oxidase, copper-zinc SOD, and metallothionein; with the main function being catalysis of oxidation-reduction (or electron transfer) reactions involving oxygen.\textsuperscript{115} Copper toxicity is not as apparent as silver, at around 10 mg per day but is manifested in liver cirrhosis, hemolysis and damage to renal tubules, brain, and other organs. Typical applications of copper as an antimicrobial are as fungicide in ponds and copper door knobs in hospitals.

Zinc acts as an essential antioxidant and anti-inflammatory agent,\textsuperscript{120} without which humans suffer slow healing, immune system dysfunction and growth retardation.\textsuperscript{114} It is used in over three hundred enzymes, which is far more than other trace metals, including iron.\textsuperscript{121} Consequently, it is far more bio available than other metals and therefore far less toxic to humans in high concentrations, unlike all other pre- post- and transition metals. However, zinc is still harmful to humans at levels of over 100 mg/day, causing nausea, vomiting, epigastric pain, cramps and diarrhoea.\textsuperscript{119} Zinc toxicity to microorganisms is utilised in toothpaste\textsuperscript{117} and zinc oxide is typically used in cream form for protection against skin irritation, a historical solution to nappy rash in babies.\textsuperscript{122}

Surface-based copper- and zinc-containing antimicrobials have been developed in a similar vein to those with silver, but to a much lesser extent. For example, ion implantation reminiscent of the method discussed for silver can be used to introduce a large amount of copper into medical grade polyethylene, thereby imparting long-lasting activity against microorganisms.\textsuperscript{78} Implantation of metal in this manner does not alter bulk properties of the polymer.\textsuperscript{110} Additionally, nanocrystalline copper films
can be prepared using magnetron sputtering techniques\textsuperscript{123} and loading into cellulose fibres,\textsuperscript{124} although these methods suffer from ion leaching. A promising new development is wet incorporation of copper nanoparticles into a polymer matrix, analogous to the silver approach, which shows fair antibacterial surface activity and significant retention of metal in the polymer.\textsuperscript{125}

Less work still has centred on zinc exclusively, with most research focused on monomeric compounds and suspensions like zinc oxide. Both copper and zinc oxide nanoparticles have proven to be useful antimicrobials.\textsuperscript{126} Studies have shown that the efficacy of metal oxides is reduced when immobilised on surfaces,\textsuperscript{127} but activity can be improved by up to 50\% by coating with carbon and introducing into a polymeric network.\textsuperscript{128} Zinc cations have been incorporated into poly(esteramide-urethane) materials with good microbial efficacy but require high levels of zinc for activity.\textsuperscript{129}

### 1.3.4 Antimicrobial challenges

Current surface-active antimicrobial materials appear to have a multitude of different advantages associated with inherent flaws. Traditional antibiotics and antimicrobial agents are becoming less useful as bacteria build resistance through overuse.\textsuperscript{6,88} Some multidrug-resistant bacteria currently have no known treatment methods.\textsuperscript{2} In-built resistance mechanisms of planktonic bacteria and biofilms, in conjunction with the need for agents which are non-toxic to humans, brings about the requirement for more subtle techniques as treatment than the simple use of chemicals such as chlorine as an antibiotic.

Modification or coating of abiotic surfaces in order to prevent the initial attachment, accumulation and growth of microorganisms has become quite popular, although no satisfactory solution has been found as yet.\textsuperscript{20} Treatment of surfaces with metal ions or nanoparticles has received a great deal of attention, but problems experienced include lengthy and labourious processing,\textsuperscript{93} consumption of hazardous materials and \textit{in-situ} use of solvents\textsuperscript{111} (e.g. dipping); alongside activity-based problems such as solvent encapsulation,\textsuperscript{130} overloading and the facile release of active
ingredients,\textsuperscript{108} manifested in apparently effective tests which in fact result in cytotoxicity and quick depletion.

Despite the obvious need for new antimicrobial agents, development on this front is waning.\textsuperscript{2} Focus has switched to engineering material solutions using existing antimicrobials, yet in light of increasing resistance and problems of current agents it is suggested that a synergistic approach of creating novel antimicrobials designed with cell targets in mind alongside material development would best suit future developments.

1.4 Plasma processing

One of the most promising areas for biomedical coatings and surface modifications in recent years is that of plasma processing. Properties such as inertness, hydrophobicity, nano-patterning, sterilisation, and most importantly in the context of this work, non-fouling chemistry and selection of chemical group functionality may be imparted on surfaces.\textsuperscript{82} The major advantages of plasma use include ability to alter surface chemistry and physical properties without altering the bulk components, inherent sterility and potential of up-scaling for industrial applications.\textsuperscript{131}

\textbf{Figure 1.8:} Schematic of plasma polymerisation: monomer fragments depositing onto a substrate alongside a soup of ions radicals, photons and electrons, a plasma
Plasma polymerisation or deposition involves grafting of molecules onto a substrate in a glow discharge chamber to form a thin, pin-hole free, conformal organic or inorganic film.\textsuperscript{132} The plasma glow is a combination of electrons, photons, ions and radicals\textsuperscript{131} (fig.1.8). Plasma is usually ignited using a 13.56 MHz radio frequency (RF) modulator (although atmospheric plasma also exists)\textsuperscript{103} and monomer units are introduced via vapour flow from an external chamber and can be deposited onto a variety of substrates.\textsuperscript{133} For this reason, all potential monomers (usually small organic compounds e.g. alcohols, carboxylic acids, anhydrides etc)\textsuperscript{134} must have a relatively high vapour pressure in addition to capability of ionic or radical polymerisation.\textsuperscript{131} Pseudo-conventional polymers can be formed in a plasma glow, although repeat units are usually oligomeric at only 3 groups in length and tend to be highly crosslinked e.g. oligo ethylene oxide.\textsuperscript{135} Film structure is typically dependent on reaction conditions such as monomer flow rate, RF input power, plasma on/off time (pulsing), reaction time and pressure (discussed in chapter 2).\textsuperscript{136}

### 1.4.1 Application of plasma

Plasma reaction conditions can be tailored to produce polymers of precise surface functionality under low temperature and pressure,\textsuperscript{135} so allowing for simple biomedical application. For example, the polymerisation of PEO under mild reaction conditions shows high retention of ethylene oxide groups, which provides very good non-fouling properties.\textsuperscript{137} In one study, this reaction has been coupled with silver atom sputtering to produce an antibacterial material, although this suffers from metal ion leaching.\textsuperscript{82} Deposition in this manner is a simple one-step, one-pot, clean, dry process, allowing the simple modification of three-dimensional surfaces\textsuperscript{131} which compares favourably to multi-step conventional polymerisation reactions as it does not require the use of environmentally damaging reagents.\textsuperscript{103} However, comparison of resulting films from different reactors is difficult due to the non-conformity of reaction chamber characteristics such as reactor dimension, electrode configuration and positioning of substrates.\textsuperscript{70}

The use of organic molecules in plasma polymerisation is important for bio-applications, for instance groups such as amines may be incorporated into films,
which can influence protein adsorption\textsuperscript{71} and provide immobilisation of biomolecules.\textsuperscript{138} Alternatively, monomers like acrylic acid can be used to produce films providing a similar function and improve the adhesion of eukaryotes on surfaces.\textsuperscript{80} Additionally, metal-containing species have previously been deposited in this manner\textsuperscript{139-141} albeit resulting in thin metallic films evocative of traditional chemical vapour deposition.

1.5 References


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Chapter 2: Instrumentation and theory

The general experimental process of this work was multi-step, as shown in fig. 2.1. The approach was taken to investigate each novel monomer system through plasma deposition and antimicrobial testing.

![Diagram of experimental approach](image)

*Figure 2.1:* The general experimental approach to antimicrobial surface generation

Most concepts, instruments and equipment utilised in this work were therefore common to each section of results. Consequently, the universal theoretical subject matter and instrument application is presented in this section along with technical information on the equipment used.
2.1 Plasma reactor

2.1.1 Plasma

States of matter are generally split into three groupings: solid, liquid and gas in which a substance may exist according to environmental factors such as temperature and pressure. However, more than 99% of all universal matter is in the form of plasma, a state above that of gas in energy.¹ Despite this it is not readily seen on Earth, although humans regularly observe it in stars (the Sun) and the aurora borealis (fig. 2.2). Man-made plasmas exist in the form of neon signs, fluorescent bulbs, arc welders and sodium vapour lamps.

![Figure 2.2: The aurora borealis (Northern lights), a plasma discharge²](image)

Altering the state of matter generally involves heating or cooling until the desired state is achieved. Heating a solid until the enthalpy of fusion \((\Delta_{\text{ fus}} H^\circ)\) is added at the melting temperature causes the change of state to liquid. Until this point, the thermal motion of molecules contained within the solid is restricted, but may allow a small vibration about a mean position. If the liquid form is heated further, it evaporates upon addition of the enthalpy of vapourisation \((\Delta_{\text{ vap}} H^\circ)\), where the kinetic energy of molecules becomes larger than their potential energy.³ Addition of more energy (typically thousands of degrees centigrade) until the enthalpy of ionisation

¹

²

³
\((\Delta_{\text{ion}} F^\circ, X(g) \rightarrow X^+(g) + e^-(g))\) is reached, causes the gas to first dissociate and then ionise creating a mixture of ions, electrons, neutral gaseous atoms and light quanta; a plasma.\(^1\,^4\)

Plasma was first named by Irving Langmuir in 1928, during his studies of electrified gases in vacuum tubes,\(^4\) although it was previously described by Crooks in 1879 as ‘a world where matter may exist in a fourth state’.\(^5\) Plasma can be formed by physical treatment other than temperature change, for instance plasma created using an electric discharge is known as low-temperature, or cold plasma.\(^4\) Due to the instantaneous loss of plasma energy to the environment, laboratory plasma study requires use of electric discharge in order to supply energy as quickly as it is lost. In addition, reduced pressure (\(\approx 10^{-2} \text{Torr}\)) can be used to perpetuate the plasma state at a lower input energy than atmospheric plasma.\(^6\)

Electric energy suitable for the generation of plasma includes direct current (DC) at a frequency of 50 and 60 Hz or alternating current (AC) of greater than 60 Hz. AC methods include 10 or 20 kHz audio frequency, 13.56 MHz radio frequency or 2.45 GHz microwave frequency. Power can be supplied to a reaction chamber containing atoms and molecules directly via a pair of electrodes.\(^6\) In the case of a DC glow discharge, power is transferred from an electric field to electrons. AC glow discharges are dependent on the frequency of alternation. Low frequency is comparable with DC glows, but with alternating polarity. At increased frequency positive ions become immobilised due to the extremely fast field polarity changes, until at around 500 kHz the half-cycle becomes so short that all electrons stay within the inter-electrode volume. Therefore, the system sustains charged particles much better than DC discharges and becomes dependent on internal collisions to perpetuate the plasma, allowing an external electrode source to be used in place of contact electrodes, at a much lower voltage e.g. copper coil.\(^5\)

2.1.2 Plasma physics

Plasmas are extremely complex in terms of physical considerations, due to the various states present, movement and interactions of these species, and the effect of
external sources. Energy is transferred by particle collision and photon absorption to achieve ionisation. Collisions occur in atom-atom, atom-ion, atom-electron, ion-ion, ion-electron and electron-electron systems.

The fact that plasma contains both positively, negatively and non-charged particles; means plasmas are described as ‘quasi-neutral’. Neutrality is maintained by what is known as Debye shielding, where it is necessary for many charged species to exist within a sphere with a radius of Debye length, \( \lambda_D \) a parameter of plasma. The concentration of charged species in the sphere, \( N_D \) is shown below:

\[
N_D = n_e \frac{4}{3} \pi \lambda_D^3 > 1
\]

*Equation 2.1*

Concentration of charged species within the sphere of Debye length, where \( n_e \) is the concentration of electrons (i.e. \( n_e = n_i \), ionic concentration)\(^6\)

Therefore, if ion concentration is equivalent to electron concentration; negatively charged ion quantity is negligible. In addition plasma length, \( L \) must be expanded to longer than Debye length. These conditions are satisfied in experimental use of plasma\(^6\) where a Debye sheath region exists when plasma is in contact with a substrate that contains an excess of positive ions which are accelerated toward the negative charge of the substrate.\(^8\) This situation occurs due to the high energy of electrons relative to their mass, which escape the sheath region and charge the substrate resulting in positive ion movement toward the substrate.

Since electrons and positively charged ions are the major components of plasma, their interactions must be considered. Electron behaviour in an electric field is governed by Newton’s law (equation 2.2).

\[
a = \frac{q}{m_e} E
\]

*Equation 2.2*

Newton’s law; where \( a \) is electron acceleration, \( q \) its electric charge, \( m_e \) its mass and \( E \) an electric field.\(^4\)

An electron subject to a continuous electric field will continue to accelerate (i.e. gain energy) until a collision occurs. Collisions involve transfer of energy \( K \) from an
electron to an electron in an atom. The effect of electron-atom collision can therefore be quantified using the magnitude of $K$:

- $K > qV_i$, where $V_i$ is the ionisation potential of the atom. The atom becomes ionised by an inelastic collision and becomes positive with a $+q$ charge.

- $qV_i > K > 0$, an inelastic collision where an electron in the atom is excited to a higher energy level, but below that of the ionised state. After a short period of time ($\sim 10^8$ s), the electron returns to the ground state, possibly via lower energy levels as it cannot persist in the excited state. An excess energy gained is released by the electron in the form of a photon, the frequency of which is described below.

$$E = h \nu = E_m - E_n$$

Equation 2.3

*The energy released by an electron upon return from an excited state ($E_m$) to the ground state ($E_n$), where $h$ is Plank’s constant and $\nu$ is photon frequency.*

- $K = 0$, an elastic collision where electrons in the atom remain in the ground state and no structural effect is seen to the atom.

The number of collisions an electron experiences for time $\Delta t$ and energy lost during these collisions in a plasma is seen in equation 2.4.

$$a) \quad N = \sqrt{\frac{3kT_e}{m_e}} \left( \frac{\Delta t}{\lambda_e} \right) \quad b) \quad Q = 4kT_e \frac{m_e}{m_m}$$

Equation 2.4

*a) The number of electron collisions, where $k$ is the Boltzmann constant, $T_e$ is electron temperature and $\lambda_e$ is the mean free path of electrons; b) Energy lost by an electron in a collision where $m_m$ is the mass of neutral atom or molecule and $T_e > T_m$ ($T_m$ is the atom or molecule temperature), true in plasma conditions.*

Therefore electrons encounter a great deal of collisions and lose energy, but due to the very low $m_e/m_m$ ratio, do not lose a large amount of their kinetic energy upon collision in plasma. Electrons can then participate in multiple inelastic collisions, thus perpetuating the ionisation of the plasma.
The reasoning behind the very high electron temperature in plasma is shown in equation 2.5. Since the ratio of \( m_m/m_e \) is very high in plasma, the electron temperature is extremely high (ca. \( 10^4 \) K).

**Equation 2.5**

\[
T_e = \left( \frac{e}{k} \right) \left( \frac{E \lambda_e}{2 \sqrt{2}} \right) \left( \frac{m_m}{m_e} \right)^{1/2} \left( \frac{\pi}{6} \right)^{1/4}
\]

*Electron temperature, \( T_e \)*

Ion temperature, on the other hand is much lower (ca. 500 K) because ions have a near equivalent mass to neutral atoms and molecules, therefore lose a great deal of energy during collision. In fact, average ion temperature is only slightly higher than the atom and molecule temperature in plasma (ca. 300 K).

In addition to collision ionisation, photons created either externally (sunlight) or internally (excited states) may participate in plasma formation. Photons of wavelength \( \lambda \) possess energy of \( hc/\lambda \), where \( c \) is speed of light. Photo-ionisation occurs when atoms absorb photon energy which is greater than the ionisation potential (equation 2.6). Photo-excitation is possible in a similar manner to the inelastic collision \( qV_i > K > 0 \), if absorbed energy is greater than excitation energy but less than ionisation energy.\(^6\)

**Equation 2.6**

\[
\lambda \leq \frac{1239.8}{\phi}
\]

*Photo-ionisation of an atom, where \( \phi \) is the ionisation potential in eV\(^6\)*

### 2.1.3 Plasma processes

The principle process in initiation and maintenance of the plasma state is clearly ionisation. However, several other types of collision occur which are also discussed here.\(^4\)

**Ionisation**, as discussed above results primarily from collisions where the energy imparted by an incoming electron meets or exceeds the ionisation potential of an atom:
Equation 2.7

\[ \text{He} + e^- \rightarrow \text{He}^+ + 2e^- \]

*Ionisation of a helium atom*

In addition to simple electron collision, at extremely high temperature molecule-molecule collision may result in ionisation. Thermal ionisation occurs where the kinetic energy \((kT)\) of a collision becomes larger than the ionisation potential, similarly to the electron-atom process.

Formation of **excited states** during an inelastic non-ionising collision with a lower energy electron is an alternative to the ionisation pathway:

Equation 2.8

\[ \text{He} + e^- \rightarrow \text{He}^* + e^- \]

*Excitation of a helium atom*

In the majority of cases, the excited electron returns to the ground state after a very short time via radiative decay, as described earlier:

Equation 2.9

\[ \text{He}^* \rightarrow \text{He}^- + h\nu \]

*De-excitation of an excited helium atom*

Some excited states however, can persist for 1 ms or longer. These states are known as **metastable states**. Although such a state is not supplied with enough energy to ionise, it may reach this level via impact with another electron. If an electron of the energy \(e_1\) collides with a metastable atom with energy \(qV_m\) and provides ionisation energy of the atom \(e_1 \geq q(V_i - V_m)\), the metastable atom becomes ionised (equation 2.10).

Equation 2.10

\[ \text{He}^* + e^- \rightarrow \text{He}^+ + 2e^- \]

*Ionisation of a metastable helium atom*

Metastable states may also play a part in plasma **catalysis**. Penning (1937) discovered that metastable neon atoms are of a higher energy than the ionisation energy for argon. Metastable neon can therefore catalyse ionisation of argon atoms, themselves returning to the ground state upon collision (equation 2.11). Indeed, addition of only 0.1% argon to a neon gas lowers voltage required to initiate plasma
of the system significantly. Plasma containing any mixture of gas a and gas b can be
be catalysed in this manner provided $V_m^a > V_i^b$.

\textbf{Equation 2.11} \quad \text{Ne}^* + \text{Ar} \rightarrow \text{Ne} + \text{Ar}^+ + e^-

\textit{Catalysis of argon ionisation by metastable neon}

Unlike electron-atom interactions where ionisation, excitement and non-change are
the three pathways available, \textbf{electron-molecule collision} involving a low energy
electron can result in an inelastic collision where an elastic collision would be seen
in an atom. This is due to the presence of quantised vibrational and rotational levels
that are available to molecules consisting of atoms, at energy generally much lower
than electron energy levels. The result of this is that more inelastic collisions take
place in molecular gas than atomic gas and subsequently electrons lose more energy
in molecular gas.

\textbf{Negative ion} formation occurs in plasma where highly electronegative atoms exist,
such as F, Cl, Br, I and O. Electrons can be captured upon impact with these
elements due to the high electron affinity they possess (equation 2.12). If energy in
the amount $qV_a$ is added to the negative ion, liberation of an electron and a neutral
atom occurs.

\textbf{Equation 2.12} \quad (i) \text{Cl}_2 + e^- \rightarrow \text{Cl}^- + \text{Cl} \\
(ii) \text{Cl}^- \rightarrow \text{Cl} + e^-

\textit{(i) Neutral ion formation and (ii) dissociation}

\textbf{Charge transfer} can occur between an ion and neutral atom, where an electron
passes from an atom to the ion species during collision (equation 2.13). These
collisions are important in the context of ion flow in a discharge system. For
instance, an ion with high velocity may collide with a molecule with low velocity,
therefore transferring charge to a slower moving species.

\textbf{Equation 2.13} \quad \text{Ar}^+ + \text{H}^+ \rightarrow \text{Ar}^+ + \text{H}

\textit{Charge transfer between a hydrogen ion and argon atom}
Charged species collisions have less significance in a plasma glow discharge than those between neutral atoms and molecules as neutral species predominate in a partially ionised gas. However, they do occur in the form of **electron-ion and ion-ion recombination** (equation 2.14), with ion-ion the more frequent reaction of the two. Gas phase recombination results in release of a photon of energy $qV_i + K$.

\[
\begin{align*}
(i)\ Ar^+ + e^- & \rightarrow Ar + hv \\
(ii)\ O^- + Ar^+ & \rightarrow O + Ar
\end{align*}
\]

*(i) Electron-ion (gas phase) and (ii) ion-ion recombination*

Due to the favouring of ion-ion interactions, recombination in electronegative gases (e.g. $O_2$) tends to follow negative ion formation. This is also the case for **surface recombination**, where the surface (S) is first activated by formation of a negative ion, then neutralised by recombination with a gas phase ion with positive charge (equation 2.15). Such a process raises the temperature of the surface by absorption of excess energy.

\[
\begin{align*}
(i)\ S + e^- & \rightarrow S^- \\
(ii)\ S^- + Ar^+ & \rightarrow S + Ar
\end{align*}
\]

*(i) Electron-surface followed by (ii) ion-ion recombination*

**Fragmentation** of molecules is an important plasma process caused by electron bombardment of a chemical bond. This can lead to homolytic or heterolytic fission of the bond, thereby allowing formation of free radicals in plasma. Indeed, dependent on the chemical content of the plasma, free radical concentration may be up to six orders of magnitude higher than ionic concentration. This process has a major role in polymerisation of organic molecules in plasma.\(^4\)

### 2.1.4 Plasma polymerisation

Plasma polymerisation (PP) is essentially a low temperature derivative of plasma-enhanced chemical vapour deposition (PECVD)\(^9\) but is quite removed from traditional ‘wet’ polymerisation and chemical vapour deposition methods despite many similarities. The closest forms of polymerisation are radiation polymerisation
(RP) involving ionisation of monomers as an initiation step and Parylene polymerisation (PAP), by which polymer deposits in a vacuum. However, RP does not allow deposition in a vacuum and PAP is not initiated by ionisation. Therefore, plasma polymerisation can be viewed as a hybrid of the two, combined with chemical vapour deposition.

Polymerisation in plasma can only take place where organic gases or vapours are present, inorganic gases such as O₂ perform non-polymeric etching and atom implantation reactions. Once introduced to the plasma, an organic gas will deposit polymer-like products onto the surfaces of all substrates in the reactor.

Unlike conventional polymerisation where a polymer formed has the same elemental composition as the monomer, plasma polymers generally show distinguished differences such as the possession of less hydrogen and more oxygen content regardless of monomer composition. This is due to the presence of residual radicals in deposited films reacting with atmospheric oxygen upon removal of the substrate from a reactor. Polymers created in a plasma glow also do not show regular repeat units like conventional polymers. Consequently, the irregularity of film structure leads to broadening of spectral peaks in FTIR and XPS analysis. Plasma polymers can also be formed from monomers containing no discernable starting point for polymerisation such as saturated organic compounds, due to the fragmentation and recombination processes in plasma.

Plasma polymerisation in this work was undertaken using a home-made reactor (fig. 2.3) which is explained in detail in section 2.1.5. The chamber (centre) was evacuated using a high level vacuum, with organic vapour allowed to flow from a Youngs flask. Plasma was ignited using RF power via copper wire wrapped around the chamber (blue), and polymers were deposited onto the substrate located in the chamber for a set time period.
2.1.4.1 Plasma polymerisation mechanism

The mechanism of plasma polymerisation is highly complex and not very well elucidated, although it is understood to be radical mediated,\(^7\) with recent work implicating ion fragmentation contribution.\(^{13}\) It has been proposed by Yasuda\(^4\) that rapid step-growth, rather than chain-growth polymerisation is responsible for polymerisation in plasma:

\[
\text{Equation 2.16} \quad [M_m^* + M_n^* \rightarrow M_{m+n}]^N
\]

*Step-growth plasma polymerisation, where M* is a reactive species and N the number of similar reactions\(^4\)*

This process differs from conventional step-growth polymerisation, where the reaction occurs between molecules. A reaction is initiated by plasma processes, with monomer groups forming primarily radical species. Free radicals detected via electron spin resonance (ESR) in plasma polymers seem to suggest radical growth methods. The reactive species may be free radical groups, leading to the termination of growth; radical-molecule, creating a larger radical; diradical-radical, again creating a larger radical; diradical-molecule, or diradical-diradical, leading to a larger diradical. This process is summarised in the schematic shown in fig. 2.4.
Figure 2.4: The bicyclic step-growth mechanism of proposed plasma polymerisation, where \( M_i \), \( M_j \) and \( M_k \) are different sized reactant groups\(^4\)

As can be seen from this visualisation, there are two cyclical step-growth reaction pathways; (i) where the reaction is repeatedly re-initiated via plasma after termination, (ii) by perpetuation of the diradical species. This mechanism of plasma film growth is widely accepted but likely to be too simple as it does not account for ion contribution, e.g. in the sheath region, which could be quite significant under certain plasma conditions\(^{13}\).

It is assumed that monomeric gas travels through an ‘active’ zone of bulk plasma where radical formation takes place, then enters the sheath region resulting in recombination\(^{14}\). Deposition of the polymer onto a substrate occurs upon the loss of kinetic energy, or from formation of a chemical bond with the target atom or molecule\(^4\). The deposited polymer is usually highly cross-linked\(^{15}\) due to the reaction mechanisms and conformal\(^{16}\).
2.1.4.2 Effect of deposition parameters

Due to the complex nature of plasma glow discharge, it is difficult to exert homogeneity and retain functional groups present in the monomer in polymeric composition. In recent years, much work has been done in this area to provide ways of controlling chemistry of films deposited in this manner.

Good functional group retention and a decrease in cross-linking in polymers is seen at low input power. A boundary exists between two deposition conditions, the power-deficient (very low power) and monomer-deficient regimes (high power). This boundary provides the optimum deposition power in continuous plasma, where maximum polymer deposition rate is achieved. However, at very low input power the degree of cross-linking becomes so low that problems arise with adhesion to substrate, with more fragmented films shown to produce a more stable film.

Pulsing of the input power allows a high degree of film chemistry control and functional group retention, far more than that of PECVD for instance. Pulse is generated by applying a determined frequency of plasma on and off times via the use of an oscilloscope which allows for initiation of reaction pathways unavailable in continuous wave (CW) mode. Equivalent input power can be determined by the product of peak power and duty cycle (equation 2.17).

\[
P_{eq} = P_{peak} \left( \frac{t_{on}}{t_{on} + t_{off}} \right)
\]

*Equation 2.17*

There are two distinct reaction regimes at work under pulse plasma polymerisation, monomer activation/surface activation (UV irradiation, ion or electron bombardment) followed by conventional radical-mediated polymerisation i.e. initiation-propagation. Pulsing allows polymerisation under conditions where less ion bombardment is seen on the substrate, therefore providing lower surface temperatures and reduced UV effects. The lack of constant exposure to ions means the resulting films are generally stronger and more flexible.
It has been seen that low RF duty cycle polymerisation produces less branched and more highly ordered polymers with greater functional group retention than conventional high power CW plasma. Indeed, in the case of maleic anhydride pulsed plasma polymerisation (PPP), anhydride functionality incorporation is favoured at short on-times and long off-times. The proposed reaction mechanism for PPP using maleic anhydride as a model is summarised in fig. 2.5. Essentially, the reaction is initiated during the on-time by radical step-growth as described above, the polymer is deposited then a chain-growth mechanism takes over during the off-time.

![Figure 2.5: The proposed chain-growth of a polymer chain during the pulse-off period of PPP](image)

Due to the similarity between the deposited material and monomer at long off-times, it has been shown that such material has a very low degree of polymerisation, (similarly to very low-input power CW films) with a great deal of soluble and non-covalently bonded material on the surface. It is thought that the bulk material undergoes additional reactions due to availability of free radicals in the system, so providing greater stability at depth. Pulse plasma suffers from potentially large amounts of unreacted monomer and adhesion problems during long $t_{off}$ times and low input wattage, or surface activation (ablation) by ions during $t_{on}$ phase of high duty cycle reactions. Adhesion can be improved by oxygen-plasma activation of polymer surfaces prior to deposition, or the use of self-assembled monolayers on metallic substrates. Surface activation in this manner is also subject to changes ranging from cleaning through to etching depending on the input energy.

**Monomer flow rate** affects the polymerisation process by influencing the structure of deposited film. A pinhole-free solid film is generally formed at high flow rate and low pressure, whereas lower flow rate and higher pressure results in powder
Flow rate can be calculated from change in pressure when the vacuum tap is closed for time $\Delta t$ (equation 2.18).

\[
\chi = \frac{dn}{dt} = \left( \frac{P_f - P_s}{\Delta t} \right) \left( \frac{V}{RT} \right)
\]

**Equation 2.18**

Monomer flow rate ($\chi$) in mol min$^{-1}$ when $P_s$ is starting pressure (Pa), $P_f$ is final pressure (Pa), $T$ (K), $V$ (m$^3$) and $\Delta t$ (minutes)$^{10}$.

As one mole of an ideal gas at STP occupies 2.4 x 10$^4$ cm$^3$, the value $\chi$ in moles per minute can be converted to standard cubic centimetres per minute (sccm) by multiplying this figure.

**Co-polymerisation** in which a mixture of monomer gases are deposited concurrently can provide control over concentration of surface functionality and increase adhesion properties of polymers.$^{16}$ For example, in the plasma glow of 1,7 octadiene and acrylic acid the former compound acts as a diluent of the latter, allowing control of acrylic acid functional group concentration and improving stability of the coating.

Additional factors affecting the products of plasma polymerisation include **substrate temperature**, which is largely dependent on input power, flow rate and pressure; **reactor geometry**, which determines the plasma glow size and varies from reactor to reactor; and **substrate position**, where it has been demonstrated that positioning ‘downstream’ from the main plasma zone allows further control of film structure,$^{19}$ for example decreased cross-linking due to reduced ion bombardment.$^{25}$

### 2.1.5 Plasma reactor

Two plasma reactors were used during this work. Both were of a home-made design with one built with help from Dr. Stuart Fraser of the University of Sheffield based on the design described by Bullett et al.$^{16}$ (fig. 2.6).
The second, newer reactor was built with assistance from Dr. Renate Förch of Max-Planck Institute for Polymer research, Mainz, Germany (fig. 2.7).

**Figure 2.6:** The plasma reactor in use in the University of Bath Chemistry department

**Figure 2.7:** Second plasma reactor
Each reactor comprised a Pyrex cylindrical tube, 30cm long and 10cm in diameter. Reactor one had a round stainless steel flange at either end, reactor two had the vacuum line exit moulded as part of the cylinder design with a heat jacket at the other end. Substrates were positioned at an optimised point for deposition according to the conditions and monomer used. The system was kept under vacuum by an Edwards RV5 pump (ca. \(10^{-2}\) mbar), gases for reactor cleaning or film tailoring (e.g. oxygen) fed in via a gas control valve and monomer vapour allowed to flow from the Youngs tap (far right in fig. 2.6), rate of which was controlled by adjustment of the tap and/or external heat applied to the monomer chamber. Power was supplied via a 13.56 MHz RF coaxial system and connected to the reactor via a coil of solid copper wire of 2mm diameter and was earthed through the electrodes or vacuum line. A manual matching network was used to adjust input impedance so that maximum power was transferred from the generator to the load. The RF power supply was introduced in CW or pulse mode by control use of a pulse generator. An oscilloscope was used to alter the width and amplitude of the pulse, allowing high levels of control over reaction conditions.

### 2.2 Characterisation methods

Following the synthesis of compounds and plasma polymerisation in this work, identification of molecule and polymer composition was required. In order to appreciate the value of the analytical techniques, some brief background is provided here.

#### 2.2.1 Fourier-transform infra-red spectroscopy

Fourier-transform infra-red (FTIR) spectroscopy is primarily used to determine energy differences between vibrational states of molecules. FTIR spectroscopy requires the change in dipole moment of a molecule upon vibration and is realised when an antisymmetric stretch occurs, or the bending of a bond results in departure of the dipole moment from zero. This is true for such bonds as N-H, C=O, C-N etc so can be applied for use in functional group characterisation.
As discussed in the plasma physics section, photo-excitation can occur in molecules where a photon of the correct energy is absorbed. This is the basis if IR spectroscopy. If a photon of infra-red radiation of frequency $\nu$ is absorbed which matches the separation in vibrational levels, the molecule is excited to a higher vibrational level. Bonds vibrate at certain energy according to their components, so an IR spectrum may be obtained by measuring percentage transmission or absorption of an IR beam against wavenumber after data processing (Fourier-transform). The chemical composition of a molecule may then be determined from this spectrum.\(^{27-28}\)

Two FTIR spectrometers were primarily used during this work, a Nicolet-Nexus spectrometer for transmission work using potassium bromide pellets and a Perkin-Elmer FT-IR spectrum 100 spectrometer with an ATR attachment for absorption spectra, which offers multiple reflections from thin films.

### 2.2.2 Self-assembled monolayers

Plasma polymers can be deposited onto a variety of substrates including glass, plastics and metals, depending on the ability of the surface to attract and interact with deposition material. As described in 2.1.4.2, oxygen plasma is a useful way of activating a substrate for attachment.\(^ {19}\) Alternatively, the surface may be activated prior to a substrate being placed into the reactor. An advantage of using a metal such as gold as a substrate is that molecules may self-assemble themselves on the surface for this purpose. Another useful property of gold is that it may be used in conjunction with the phenomenon of surface plasmon resonance (SPR) for measuring film thickness.\(^ {29}\)

LaSFN9 glass (Berliner glass) with high refractive index (1.8502) and borosilicate glass (BK7) was used as a base substrate for gold evaporation using an Emitech thermal gold evaporator.

Surface-assembled monolayers (SAMs) are ‘ordered molecular assemblies formed by the adsorption of an active surfactant on a solid surface’ (fig. 2.8).\(^ {30}\) Order in these systems results from a spontaneous chemical reaction at the interface, as equilibrium is reached. A comparable system in nature is the formation of lipid bilayers, which constitute the basis of cell membranes.
Molecules such as thiols, which can possess long chain hydrocarbons with sulphur containing head groups, have the ideal structure for SAM formation. Sulphur has a strong affinity for heavy transition metal surfaces such as gold, and many gold-organosulphur SAMs have been reported. Reaction between gold and the sulphur head group is thought to be driven by exothermic release of hydrogen, as seen below:

\[
\frac{1}{2} R - S - H + Au \rightarrow R - S - Au + \frac{1}{2} H_2
\]

The most widely studied thiol is propan-2-ene thiol, or allyl mercaptan. This is a very short chain molecule, so forms a monolayer very readily. Tails of SAMS do not generally appear perpendicular to the substrate, but are angled slightly. The presence of an unsaturated tail is the basis for interest in plasma polymerisation, as radical reaction with free monomer units may provide a useful form of adhesion. This process is shown in fig. 2.10, where plasma polymerisation of \(N\)-substituted maleimide is proposed.
2.2.3 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is a technique for studying molecular structure and shape where magnetic nuclei are present (e.g. spin of greater than zero). Since a nucleus is a charged particle in motion, it will develop a magnetic field. When a magnetic field is applied, nuclei line up parallel to the applied field either spin aligned or spin opposed. The more highly populated state is the lower energy spin aligned state. In NMR, electromagnetic radiation ‘flips’ the alignment of spin to the higher energy spin opposed state and the energy of transition is measured by observing the frequency of the resonant electromagnetic field. Fourier transform is employed to process the data and an absorption spectrum presented, with the peaks at frequencies corresponding to transitions between nuclear energy levels.

NMR transition frequency depends on the local magnetic field a nucleus experiences. The position of an NMR peak is given in terms of chemical shift, $\delta$, which is dependent on the difference between the resonance frequency of the nuclei studied and a reference compound (e.g. tetramethylsilane (TMS) is used as a reference for $^1$H and $^{13}$C):
Equation 2.19

\[ \delta = \left( \frac{V - V_{ref}}{V_{ref}} \right) \times 10^6 \text{ ppm} \]

Chemical shift in an NMR spectrum\(^{27}\)

Where chemical shift is negative, the nucleus involved is shielded relative to the reference standard and positive chemical shift indicates deshielded nuclei. The extent of shielding is dependent on such factors as the proximity of neighbouring atom electron density and the environment of the nucleus.

In addition to chemical shift, spin-spin coupling of nuclei provides information on the position of atoms in a molecule. The magnitude of spin-spin coupling is often greatest when two atoms are directly bonded to each other and is stated as the spin-spin coupling constant, \(J\). Coupling in this manner causes splitting of peaks \((J)\) on the spectrum, according to the spin-active nuclei ‘seen’ by the nuclei in question using the equation \(2nI + 1\), where \(I\) is the spin of nuclei ‘seen’ and \(n\) the number of nuclei. For example, coupling of \(^{13}\text{C}\) with two \(^1\text{H}\) nuclei in a -CH\(_2\)- moiety provides a triplet in the NMR spectra, as two \(I = \frac{1}{2}\) nuclei are seen, therefore \((2 \times 2 \times \frac{1}{2}) + 1 = 3\). The relative intensities of each split peak (multiplet) are given by Pascal’s triangle (e.g. 1:2:1 for coupling to two spin \(\frac{1}{2}\) nuclei), as there are four ways of orienting two spin \(\frac{1}{2}\) nuclei; +1, 0, 0 and -1, therefore intensity of the 0 state is twice that of the other states.

The relative integrated intensity of a peak in an NMR spectrum correlates to the number of nuclei in the set, so can be used as a quantitative measure. The nuclei \(^1\text{H}\) (natural abundance 99.99%), \(^{13}\text{C}\) (1.11%), \(^{15}\text{N}\) (0.37%), \(^{31}\text{P}\) (100%) and \(^{107/9}\text{Ag}\) (100%) for instance are all spin \(I = \frac{1}{2}\), so making NMR a useful tool for their analysis.\(^3\)\(^{,27}\) Copper (II) on the other hand, is paramagnetic so cannot be observed using NMR.

Certain \(^1\text{H}\) NMR were run on a Bruker AV250 MHz spectrometer and the remaining \(^1\text{H}\), \(^{13}\text{C}\) and \(^{31}\text{P}\) NMR run on a Bruker AV300 MHz spectrometer.
2.2.4 X-ray crystallography

X-ray diffraction (XRD) is the least ambiguous method for precisely determining molecular structure. X-ray structure determination is more important for inorganic compounds than organic as inorganic molecules and solids are more structurally diverse and frequently contain more varied bonding.

A crystal sample is required for XRD, which is usually made using recrystallisation of a solid product. Recrystallisation allows the purification and structural analysis of a compound. The standard method for recrystallisation is to dissolve a solid into the minimum amount of solvent in an attempt to grow crystals from the resulting solution. The pure compound desired drops out to form crystals first, leaving impurities in the solution and the more slowly a solution is cooled, the larger the individual crystal growth. Trial and error using various solvents should eventually allow crystals of suitable size for XRD, although like-dissolves-like so generally polar solvents should be used with polar compounds, non-polar solvents with non-polar compounds.

XRD involves the irradiation of a single crystal mounted in a diffractometer by an x-ray source of a fixed wavelength. The crystal position and that of an x-ray detector are controlled by a computer. At certain orientations of the crystal relative to the x-ray beam the crystal diffracts the beam at a fixed angle and intensity is measured by the detector when placed in the direction of the diffracted beam. The computer allows the detector to scan through each reflection (over 1000) whilst storing intensities and obtaining more than ten reflections for each structural parameter to be determined (positions of atoms and a number of locations associated with their thermal motion). A ‘trial’ structure is established by the computer and refined by systematic shifts in atom position until agreement between the observed and calculated XRD intensities is achieved.27,31

XRD is useful for confirmation of the monomer structure in this work and provides other data such as bond lengths and angles. Data collection was implemented on a Nonius Kappa CCD diffractometer, using Mo-Kα radiation (\(\lambda = 0.71073 \text{ Å}\)). Data were corrected for Lorentz and polarization effects, and structure refinement was by
full-matrix least squares on $F^2$. Structure solution and refinement was performed using SHELX-86$^{32}$ and SHELXL-97$^{33}$ software, respectively. Data was uniformly corrected for Lorentz and polarisation. Hydrogen atoms were included at calculated positions throughout, and refined using a riding model.

2.2.5 Melting point analysis

The melting point range of a monomer gives an indication of its purity. A narrow melting point range means the product is purer than a wide ranging melting point. Melting point is also useful as a diagnostic tool to ensure monomer reproducibility. Melting point readings of monomers were taken using a Stuart SMP10 melting point apparatus.

2.2.6 Atomic absorption spectroscopy

Atomic absorption spectroscopy (AAS) is a method of quantitatively assessing a sample for elemental content and is widely used to analyse samples for metal content.$^{34}$ The principle relies on the excitation of electrons in a sample to higher energy levels. Elements absorb light of a particular wavelength which is specific to electron excitation in that element. Atomic absorption spectroscopy exploits this property, meaning the technique is highly element specific and makes the identification of elemental composition unequivocal.$^{35}$

The energy required for each transition of a particular metal is known, so providing the energy put in is known and the energy transmitted is detected, the amount of energy absorbed can be calculated and the corresponding number of transitions correlated to elemental concentration using the Beer’s law (equation 2.20).

\[
{kC} = \log \left( \frac{I}{I_0} \right)
\]

*Equation 2.20* 

*Beer’s law; absorption in an atomic gas where $C$ is concentration, $I_0$ and $I$ light intensity before and after absorption, and $k$ is a constant*
A typical AAS set-up (fig. 2.11) uses a flame to atomise a sample solution into a beam of light at the selected wavelength specific to the element which is to be analysed for. Incident light is then absorbed by atoms and transmitted light carries onto the monochromator and onward to the detector. The intensity is recorded and outputted to the appropriate data system.\(^{34}\)

![Figure 2.11: Schematic of a flame atomic absorption spectrometer\(^{35}\)](image)

In order to quantify concentrations of metal in samples, the AAS is calibrated using standard solutions of known concentration of the specified metal. If absorption is plotted versus concentration, a calibration curve is obtained from which metal content in unknown samples can be derived principally in parts per million (ppm). This analytical method was used to determine the leached metal content in solution and the metal content of dissolved plasma films.

### 2.2.7 X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) is a surface analysis tool which is used to quantitatively examine elemental composition within the top layer of a material.\(^{36}\) The basic principle is that a sample is exposed to an x-ray beam and the kinetic energy and numbers of electrons which are ejected are measured. Detection limits are typically in the region of parts per thousand and XPS is primarily used to monitor surfaces to a depth of around 10nm.

XPS is element specific, as each element ejects electrons of binding energy characteristic of electron configuration e.g. \(1s\), \(2s\), \(2p\) etc. If the input energy i.e. x-
ray wavelength is known and the kinetic energy of electrons is detected, then the binding energy may be determined using equation 2.21.

\[ E_b = E_p - (E_k + \phi) \]

Equation 2.21

Binding energy \( E_b \), where the x-ray energy \( (E_p) \), kinetic energy of electrons \( (E_k) \) and work function of spectrometer \( (\phi) \) is known

The number of ejected electrons that are detected at each binding energy is directly correlated to elemental composition in the area analysed. This information is processed to produce an XPS spectral plot of number of electrons detected versus binding energy in electron volts. Peaks in an XPS plot may be subject to deviation which provides further information such as the oxidation state of an element in a film. If an element is in a higher oxidation state then the binding energy increases from the neutral atom due to greater interaction between the ionic core and the electron being ejected. An additional consideration is splitting of \( p \), \( d \) and \( f \) orbitals due to spin-orbit coupling, which is evident at high resolution of spectral peaks.

An XPS system schematic is shown in fig. 2.12. In order to prevent collision of electrons with gas particles, an ultra high vacuum (UHV) is required. A sample is loaded into the vacuum chamber and is irradiated by an x-ray source (commonly Al-K\( \alpha \) or Mg-K\( \alpha \) radiation) with the resulting ejected electrons dispersed and detected according to their kinetic energy.

![Figure 2.12: Schematic of an x-ray photoelectron spectrometer](image)

XPS was used to analyse the surface of thin films generated by plasma deposition. A Kratos Axis Ultra spectrometer operating at a base pressure of 3x10\(^{-9}\) Torr was used in this work. Samples were irradiated with Al-K\( \alpha \) X-rays (1486.6 eV) using an X-ray
analysis spot size of 700 µm x 300 µm and ~225 W power. Additionally, an Axis Ultra DLD spectrometer producing 450 W power was used.

### 2.2.8 Mass spectrometry

Mass spectrometry (MS) is an extremely accurate way of effectively weighing a molecule in order to determine its molar mass. Molecules are ionised and the mass-to-charge number ratio \((m/z)\) of all ions is measured, with the resulting mass spectrum presented as a plot of relative abundance of produced ions against \(m/z\).

Macromolecules are generally difficult to ionise without fragmentation, and although this isn’t such a problem for plasma treated surfaces where fragmentation has likely taken place, it is for identification of pure compounds. Matrix-assisted laser desorption/ionisation (MALDI) and electrospray ionisation (ESI) are techniques which avoid this problem. Using ESI uncharged compounds are ionised by adducts such as protons and sodium ions available in solution (e.g. from glassware). Positive or negative ions may be formed, depending on the choice of ionisation for a sample. Positive ESI is the ionisation method used in this work, in combination with a time-of-flight (TOF) ion detector. A sample undergoes ESI, followed by the acceleration of particles using an electric field of known strength, then received by the TOF detector. The TOF detects ions in real-time according to their \(m/z\) and velocity at high vacuum. For example, larger ions carry less velocity as they require more energy to accelerate them so are received later by the TOF detector than smaller ions. From the time recorded at the detector, very precise \(m/z\) of an ion can be determined and subsequently the molecular structure may be deduced from fragment size.\(^3,\textsuperscript{37}\)

### 2.2.9 Secondary ion mass spectrometry

Secondary ion mass spectrometry (SIMS) is a highly sensitive surface-specific form of mass spectrometry, able to detect elements in the parts per billion (ppb) range. By bombarding the surface with highly energetic ions, atoms are thrown from their original positions as secondary ions. These ions can then be detected, identified and
in some cases, related back to their original positions on the surface. Static secondary ion mass spectrometry, SSIMS is used to study monolayer coverage of surfaces as the ion beam penetrates around 10nm into the surface, ejecting neutral and a small amount of ionised molecules from the top surface. SIMS is conducted within a UHV, with pulsed primary ions (source is usually Cs$^+$ or more recently cluster ions e.g. C$_{60}^+$) fired at the sample and secondary ions are sputtered from the surface. Secondary ions are then electrostatically accelerated (given the same kinetic energy) towards a time-of-flight detector, where ions arrive according to their mass to charge ratio and outputted to a spectra of intensity versus mass to charge ratio. Mapping of ion distribution can be achieved by recording the image position on an immersion lens whilst simultaneously detecting secondary ions.

SSIMS was used in conjunction with ion mapping to analyse plasma deposited films using an ION-TOF spectrometer, with a bismuth ion beam for impacting the surface. The SIMS software also allowed imaging of the surface, looking at the variation in surface density of various ion fragments.

### 2.2.10 Electron microscopy

Electron microscopes are used to magnify surfaces far beyond the capabilities of an optical microscope, up to a thousand times greater. This is achieved through use of an electron beam in place of a photon of light, which has a much smaller wavelength and consequently results in improved resolution using equation 2.22.

\[
R = 0.5 \frac{\lambda}{(\mu \sin \alpha)}
\]

*Equation 2.22*

The resolution of a microscope, where $\lambda$ is the wavelength of the incident beam, $\mu$ is the refractive index of the medium between specimen and lens and $\alpha$ is the semi-angle of the lens

There are two principle types of electron microscopes, scanning and transmission. A scanning electron microscope (SEM) relies on reflectance of electrons that are fired at a sample surface as a beam is scanned across a designated area by rapidly changing magnetic fields. Upon impact with the sample, incident electrons are
backscattered or secondary electrons are generated which are collected by the detector. An image is then generated as a function of the number of electrons collected for a particular area, yielding topographical information. Transmission electron microscopy (TEM) on the other hand, is a direct observation of electrons through an ultra-thin sample. Electrons are fired at the sample, resulting in an image of electron concentration per unit area, with the final image projected onto a fluorescent screen which is viewed through leaded glass. Parts of the sample which hinder or prevent electrons passing through will appear darker and provide topographical information. Diagrams of typical electron microscopes are detailed in fig. 2.13.

As with XPS, a vacuum source is generally used to reduce the mean free path of electrons and the source of electrons in both SEM and TEM is commonly a tungsten filament or lanthanum hexaboride. Sample preparation for both microscopes requires coating with an electrically conductive material e.g. gold and must be electrically grounded to prevent a charge build-up.
Greater resolution is observed in TEM as the smaller sample area allows for a more powerful objective lens. However, very thin samples are required and only a small area of the sample may be covered.

2.2.10.1 Energy dispersive x-ray spectroscopy

Energy dispersive x-ray spectroscopy (EDS) is an additional analytical tool offered by electron microscopy. It works in a similar vein to XPS, with a similar sensitivity of ca. 0.1%. Unlike XPS which monitors electrons ejected by a sample, EDS works by detecting the x-rays produced by the impact of electrons on the surface. X-rays are evolved when incident electron bombardment excites an electron and ejects it from its shell creating an electron hole, which a higher energy electron from an outer shell fills resulting in excess energy being released as an x-ray in the process. In common with XPS the technique is element-specific; the x-rays evolved as a result of excess energy in the atom are characteristic of this atom, although x-ray energies evolved from different elements may overlap.40 X-rays are collected concurrently with electrons by different detectors and presented as spectra in a plot of x-ray count versus energy in eV or KeV.

Figure 2.14: The Scanning (l) and transmission (r) electron microscopes used at the University of Bath Centre for electron optical studies40

A JEOL JSM6310 scanning electron microscope (fig. 2.14) with EDS capabilities was used during this work to generate images of plasma deposited films. Additionally, a JEOL JEM1200 transmission electron microscope with an Oxford
Instruments INCA x-sight x-ray detector was used for attempts at further resolution of samples.

2.2.11 Quartz crystal microbalance

A quartz crystal microbalance is a highly sensitive miniature balance used to monitor very slight changes in the weight of thin films down to nanogram scale. It utilises the piezoelectric (pressure-electric) effect inherent in certain crystalline materials, for example quartz. Essentially, the frequency of resonance can be measured in a crystal upon application of an electric field. If the mass of a crystal is increased by deposition of a film then the resonance frequency decreases and the difference in measured frequencies yields change in mass. The crystal frequency is measured using a quartz crystal oscillator circuit connected to a frequency counter.

This technique was employed to obtain mass readings for plasma deposited films. An Oxford electrodes quartz crystal oscillator circuit was used in conjunction with Fluke PM6680B frequency counter. The Sauerbrey equation (2.23) was used to derive film masses from frequency readings.

\[ \frac{\Delta m}{\Delta f} = -\frac{A\sqrt{\mu_q \rho_q}}{2f_0^2} \]

*Equation 2.23*

*The Sauerbrey equation; where \( \Delta m \) = mass change, \( \Delta f \) = frequency change, \( A \) = area of the crystal, \( \mu_q \) = shear modulus of quartz, \( \rho_q \) = density of quartz and \( f_0 \) = frequency of resonance*  

For the quartz crystals used in this work, area was 0.2 cm\(^2\), shear modulus was 2.947 x 10\(^{11}\) gcm\(^{-1}\)s\(^2\) and density 2.648 gcm\(^{-3}\); resulting in a measured frequency change corresponding to a mass increase with \( \Delta m/\Delta f = -1.05 \) ng Hz\(^{-1}\).

2.2.12 Alpha-step profiling

Alpha-step profiling (\( \alpha \)-step) is a simple method of measuring film thickness and provides topographical information on surfaces. A stylus tip is applied to the surface
of a film and the vertical movement as the tip is rastered across the surface is directly proportional to surface characteristics, reminiscent of atomic force microscopy.

\( \alpha \)-step was used in this work to provide plasma film thickness information. Films were deposited onto silicon wafer and the surface was scratched with a steel pin, providing a zero depth for thickness to be measured against. A stylus tip of a width of ca. 5\( \mu \)m was passed across the surface using a TLC Tencor P-10 \( \alpha \)-step profiler, with a 50\( \mu \)m/s scan rate.

### 2.2.13 SPR

Surface plasmon resonance (SPR) is a growing tool for measuring surface properties such as biomolecule attachment and film thickness. SPR is an energy wave caused when visible light is coupled to the electron field (plasmon wave) in a thin metal film. This wave extends out to the dielectric material on the metal film and decays exponentially\(^{43-44}\). Gold and silver are the most commonly used metals due to their noble character and inherent stability.

The propagation constant of a SP wave is always higher than the optical wave propagation in the dielectric therefore SPR cannot employ incident optical wave only. The momentum of the incident optical wave must be enhanced to match that of the SP wave, which is achieved via attenuated total reflection (ATR)\(^ {45-46}\).

![Figure 2.15: The basic SPR setup showing a prism using ATR to couple laser light to the metal film. The dielectric is on the underside of the metal film\(^ {46}\) ](image)

\( \text{Optical wave} \)
\( \text{Prism} \)
\( \text{Gold film} \)
\( \text{Dielectric} \)
A glass prism may be used to couple laser light into the metal film using ATR (fig. 2.15) and at a certain angle of incidence, maximum coupling of the light with the surface plasmon occurs. At this angle, light is not reflected but interacts with free electrons in gold to form a resonant energy wave (surface plasmon). This is known as the angle of resonance, and is detected by a sharp minimum in the reflected light monitored (fig. 2.16) as all the light is fed into the surface mode.

![Figure 2.16: An SPR curve showing minimum as angle of resonance](image)

As the angle of resonance is dependent on refractive index, the thickness of a film may be estimated from this angle. In addition, the change in resonance angle can be followed in real-time allowing the attachment rate of biomolecules in a label-free manner or swelling properties of a film to be monitored. In this work, SPR was used to determine the thickness of deposited films on gold discs.

Experiments on polymer coated gold discs were made using a home-made SPR instrument (fig. 2.17).
2.3 Microbiological assays

2.3.1 Microbiology

Microbiology is essentially the study of organisms that may only be seen through a microscope\(^4\). This encompasses viruses, bacteria, archaea, fungi and protista. Microbes can be split into three distinct groupings (or kingdoms) according to DNA structure; Bacteria, Archaea and Eukarya. The former two kingdoms have what is known as prokaryotic cell structure, the latter eukaryotic cell structure. Eukaryotes are a more advanced form of cell, not just restricted to microbes but include higher plants and animals, of which humans are one example.
2.3.2 The cell

2.3.2.1 Prokaryotic cell

Bacteria are around 0.1-750µm in diameter and contain a large amount of variation in appearance, although two particular shapes predominate, in the form of cocci (spherical) and bacillus (rod). A generic view of a prokaryotic cell is shown below:

![Diagram of a prokaryotic cell](image)

**Figure 2.18:** Diagram of a prokaryote cell

The exterior of most bacterial cells consists of a rigid cell wall, made of sugars and amino acids (peptidoglycan), with a plasma membrane internal to this. The cell wall acts as a protective sheath, preventing lysis resulting from osmotic pressure and providing shape.

The plasma membrane separating the interior of the cell from the environment is a semi-permeable lipid bilayer around 5-10nm thick. Phospholipids consisting of a polar head group attached via glycerol to two long-chain, non-polar fatty acids constitute this bilayer. Self-assembly occurs so that the hydrophilic head groups interact with water molecules in solution, with the hydrophobic fatty acid groups orientated away from aqueous media, due to the thermodynamic drive to achieve the lowest energy conformation. Due to the hydrophobicity of the membrane interior, charged species such as ions are repelled. This is part of the simple defence mechanism bacteria have against uncomplexed metal ions.
Flagella are situated on the outside of a cell and provide a form of propulsion for bacteria. The position and number of flagella a cell possesses is commonly used for classification. Flagella are rigid protein structures bound to the membrane of a cell, rotation of which is driven by the basal body. Counter-clockwise rotation drives the cell forward and clockwise rotation allows the cell to tumble. The direction a cell moves is determined by chemotaxis, or movement to or from a chemical source according to signals received by a cell. For instance, in biofilm formation, the surface acts as an attractant so flagella movement is a response to this.

Prokaryotic cytoplasm is aqueous and contains a cocktail of molecules, proteins (e.g. ribosomes) necessary for cell function and RNA. Prokaryote DNA is also found in the cytoplasm, which consists of a single chromosome, varying in size between different species of bacteria. The DNA is tightly wound and associated with proteins to form structure. Additionally, metal cations associate with DNA to allow replication to take place, a process which, as mentioned previously can be disrupted by presence of silver ions. As the DNA is not bound by a membrane, this allows simple access for such ions. The area in the cell where DNA resides is known as the nucleoid, which is accompanied by additional small DNA molecules called plasmids.

2.3.2.1.1 Gram-staining

Bacteria are often split into two groups according to the structure of the cell wall and its response to staining. Gram-positive bacteria have a thick layer of peptidoglycan (~20-80 nm), whereas Gram-negative cell walls comprise a thin layer of peptidoglycan and an additional protective outer membrane. The Gram stain (crystal violet) once applied to Gram-positive cells cannot be washed out using alcohol, thus staining them dark purple. Gram-negative cells stain a much lighter purple or pink due to their thin cell wall.

2.3.2.2 Eukaryotic cell

By comparison to prokaryotes, the eukaryotic cell is far more complex. There are many differences between the two types of cell, but fundamental differences are that
the eukaryotic cell contains a distinct nuclear membrane and complex organelles such as mitochondria or chloroplasts, alongside mitotic cell division. Such organelles closely resemble prokaryotic cells and it is thought that some form of endosymbiotic event occurred early in their evolution.\(^48\)

### 2.3.3 Genotype and phenotype

The genotype of an organism is the genetic information carried forward from the parent organism. These genes provide the organism with its resulting phenotype. The phenotype of an organism is an observable trait due to the combination of gene expression with environment, such as cell function, behaviour and appearance. Therefore, organisms may contain the same (or very similar) genotype but exhibit very different phenotype or vice-versa.\(^49\)

**Figure 2.19:** Phenotype variation in *Pseudomonas aeruginosa*

This is evidenced in *Pseudomonas aeruginosa* bacteria where phenotypical expression is altered in cells with identical genotype due to their position in a biofilm structure e.g. starved state cells at the centre of a biofilm slow metabolism compared to cells at the periphery of a biofilm. Equally, some *P. aeruginosa* cells have different genotype resulting in differing phenotype such as those with and without pili (fig. 2.19) which show different attachment characteristics to surfaces.

### 2.3.4 Bacterial cell growth

Bacteria can be grown (or cultured) in aqueous or solid media by the inoculation of the media with a small amount of cells. Most bacteria have individually defined
minimum, maximum and optimum growth temperatures, ranging from -10°C to over 80°C, although the majority of pathogenic bacteria tend to have an optimum of 37°C, or human body temperature. Equally, bacteria can grow in a range of oxygen concentrations, pH and water content; although culture shaking can facilitate dynamic aerobic growth whereas static cultures predominantly grow anaerobically. The most important cells for study in this work are those which grow best in conditions experienced in the body. Thus, incubators with and without shaking capabilities were used during this work at a set temperature of 37°C.

The method of growth in bacterial cells is called binary fission, that is, each cell divides in two and cultures grow exponentially. Cells first accumulate nutrients; grow to a critical size where DNA replication takes place, then divide by the formation of a new inter cell wall. Upon inoculation in growth media bacterial cultures do not initially grow, as cells must adapt to their new environment and increase in size. This is known as the lag phase. Following this stage is the exponential growth phase, where very fast cell division takes place and is dependent on the division time of the bacteria involved. Due to the limited availability of nutrients, growth after a certain period slows and eventually levels off as cell division is balanced by cell death. The final stage of culture growth is where cell death becomes greater than division, cells lyse and the culture becomes less turbid (fig. 2.20).48,50

![Figure 2.20: Bacterial culture phases](image-url)
2.3.5 Model organisms

The bacteria used in antimicrobial tests in this work were chosen due to their importance in the context of nosocomial infection, owing to the potential application fate of these materials. As shown in chapter 1, two of the most important bacterial types in these environments are *P. aeruginosa* and *S. aureus*, hence were used here.

2.3.5.1 *Pseudomonas aeruginosa*

A Gram-negative bacterium, *P. aeruginosa* is an opportunistic pathogen which naturally resides in damp habitats such as marshes and has the ability to colonise mammalian hosts. *P. aeruginosa* is characterised by its fast growth rate and propensity to form large green clusters. It is motile, propelled by flagella and at the microscopic scale appears as a unicellular rod, typically 0.5-1.0 μm in width and 1.5-4.0 μm in length.\(^{51}\) *P. aeruginosa* is a major source of infections in humans, the major reason for which is its prolific biofilm-forming nature aided by speed of growth, which also allows for antibiotic resistance.\(^{52}\) The strain used in this work was *Pseudomonas aeruginosa* PAO1.

2.3.5.2 *Staphylococcus aureus*

*S. aureus* is a Gram-positive organism first isolated in pus from human wounds. Cells are spherical (hence ‘coccus’) non-motile, ranging from 0.8-1.0 μm in diameter and typically cluster together to form golden-coloured chains. The name derives from this characteristic in the Greek for ‘bunch of grapes’ and ‘berry’.

Predominantly an aerobic bacterium (although can respire anaerobically), *S. aureus* requires a medium containing a sugar source and amino acids in order to propagate and is mesophilic, growing optimally between 35-40ºC.\(^{53}\) As discussed previously it is responsible for hospital- and community-acquired infections. The strain ‘methicillin-resistant’ *Staphylococcus aureus* (MRSA), as its name suggests has developed resistance to methicillin antibiotics. Along with another evolved strain, ‘vancomycin-resistant’ *Staphylococcus aureus* (VRSA), these organisms create a huge problem in hospital settings where antibiotics are the major source of infection
prevention.\textsuperscript{54} Due to safety concerns, the strain used in this work was ‘methicillin-susceptible’ \textit{Staphylococcus aureus}, MSSA 476.

\textbf{2.3.5.3 \textit{Micrococcus luteus}}

\textit{M. luteus} is a close relation to \textit{Staphylococcus}, albeit non-pathogenic and forms yellow clusters rather than golden ones. The other notable difference from \textit{S. aureus} is the inability to anaerobically ferment glucose.\textsuperscript{53} Found in soil, air and the natural flora of human skin, \textit{M. luteus} is ubiquitous and even colonises human respiratory tracts.\textsuperscript{55} \textit{M. luteus} was used as a model organism during preliminary work owing to safety concerns regarding more pathogenic strains. It has also been proposed as a potential nosocomial pathogen to immunocompromised patients.

\textbf{2.3.6 Bacterial detection}

Due to the inherently small size of micro-organisms, it is difficult to observe them without using special equipment. In the context of this work, it was important to determine whether bacteria which interact with plasma films and free compounds are viable so microbiological methods were employed to enable this.

There are several methods for bacterial observation and qualitative or quantitative assessment of cell viability, of varying age and accuracy in determining both the numbers and homogeneity of bacteria present. Quantitative methods can only estimate numbers present in cultures, which can exceed $10^{10}$ cells per millilitre so making direct counting difficult. Methods used in this work included culturing cells as larger visible colonies, using cell light scattering properties, fluorescent tagging of cells and direct viewing of cells through microscopes.

\textbf{2.3.6.1 Colony counting}

The simplest method of detecting bacteria is through cell culture in appropriate media followed by streaking onto solid nutrient agar and incubation at optimal
growth temperature. After a strain-dependent period of time (usually ca. 18h) visible cell colonies appear on the agar surface, (fig. 2.21) each of which corresponds to an initial cell which has reproduced sufficiently. Therefore by counting cell colonies it is possible to estimate a quantity of bacteria which were present in the original solution, typically expressed as colony forming units per millilitre (CFU ml⁻¹).⁴⁸

![Figure 2.21: M. luteus cell colonies on an LB-agar plate grown from individual cells](image)

**Figure 2.21:** *M. luteus* cell colonies on an LB-agar plate grown from individual cells

This is the ‘gold standard’ of bacterial detection as it provides both an estimation of cell quantities and shows any contamination within the culture e.g. other micro-organisms. Downsides to this methodology are that it is a lengthy process requiring an entire day to estimate cells present in a stock solution and does not necessarily present all viable cells as those which cannot replicate do not show.

### 2.3.6.2 Solution turbidity

A more facile yet slightly more inaccurate and indirect approach to cell quantification is utilising solution turbidity or the light scattering properties of cells. Due to the fact that cell propagation results in a less transparent solution than pure media alone, the difference in light absorption can be measured and related back to the initial bacterial concentration.⁴⁸ This is typically achieved using a UV-vis spectrophotometer, with the transmission of light through a known path length reduced by cells resulting in an optical density reading (OD) for the sample (fig. 2.22).
Optical density is plotted versus CFU ml\(^{-1}\) generated by colony counting the same sample at various known dilution factors to yield a calibration plot. This plot is used to easily determine the bacterial concentration of solutions, but is highly strain-specific so a calibration plot must be generated for every bacterial strain under particular experimental conditions. Also, due to the small nature of bacterial cells a great deal of them is required to scatter sufficient light to monitor by UV-vis, but too many and light cannot pass through. Therefore, the limit of detection of this method is in the region of 10\(^5\)-10\(^8\) CFU ml\(^{-1}\) and cell viability cannot be deduced. The UV-vis spectrophotometer used in this work was a Thermos Helios Gamma single beam model.

### 2.3.6.3 Bright-field microscopy

A method of directly viewing cells, bright-field microscopy has been used for a number of years. Using visible light (as opposed to electron sources for electron microscopes) to illuminate a specimen mounted on a stage, the image is magnified up to 1000-times by the objective and ocular lenses (fig. 2.23) to reveal the structure and dispersion of micro-organisms in two dimensions.
This methodology is useful for detecting the presence and position of cells on surfaces but is limited as quantification is difficult on heterogeneous or three-dimensional substrates and no cell viability information can be obtained. The bright-field microscope used in this work was a combination microscope which was also used for fluorescence microscopy.

2.3.6.4 Fluorescent tagging

Bacterial cells may be tagged with a fluorescent dye in order to identify their presence and/or viability. Such dyes utilise the principle of fluorescence, which is evidenced by decay of short-lived excited states in an atom or molecule through the emission of a photon. Such states are created by the excitation of fluorophores with light of a certain wavelength (fig. 2.24).
\[
S_0 + h\nu_{\text{ex}} \rightarrow S_1 \\
S_1 \rightarrow S_0 + h\nu_{\text{em}} + \text{heat}
\]

**Figure 2.24:** The basic principle of fluorescence, where \( S_0 \) is the ground electronic state, \( S_1 \) is the first excited state, \( h\nu_{\text{ex}} \) is the energy of excitation and \( h\nu_{\text{em}} \) is the energy of the emitted photon

Conventional fluorescent dyes for investigating cell viability use a single dye which only binds bacteria with intact membranes or functioning metabolism. Newer dyes have dual-function combination dyes which bind both viable and non-viable cells.\(^{56}\)

The dye used in this work was the Invitrogen Molecular probes LIVE/DEAD Baclight cell viability kit (Live/Dead stain), which consists of two dyes; SYTO-9 and propidium iodide which both stain the nucleus of bacteria. The SYTO-9 element tags all cells with green fluorescence while propidium iodide enters and tags those cells with damaged membranes with red fluorescence, displacing SYTO-9 in the process. Fluorescently labelled cells can be detected via two methods, fluorescence microscopy for surfaces or use of a fluorimeter for solutions.

### 2.3.6.4.1 Fluorescence spectroscopy

A spectrofluorimeter is an instrument which both excites fluorophores and detects emission of fluorescence (fig. 2.25). Light of a defined wavelength emitted by a laser or lamp is monochromated and irradiates the sample solution. Fluorophores within the sample are excited and the resulting emitted photons of light are detected either singly or simultaneously.

![Schematic of a basic spectrofluorimeter](image)

**Figure 2.25:** Schematic of a basic spectrofluorimeter

The spectrofluorimeter used in this work was an Ocean optics USB4000-FL with a multichannel detector. The emission/excitation maxima for the Live/Dead stain are 480/500nm for SYTO-9 and 490/635nm for propidium iodide. This technique was
principally employed for measuring bacterial viability in solutions of cells removed from plasma films and control substrates by the ratio of green/red fluorescence counts.

### 2.3.6.4.2 Fluorescence and confocal microscopy

Light microscopy can be adapted to utilise fluorescence in visual representation of cell viability. In place of visible light used in bright-field microscopy, ultraviolet light is the light source in a fluorescence microscope. Fluorophores are excited similarly to fluorescence spectroscopy and emit photons which can be viewed optically. This method was used to take images of plasma- and non-treated surfaces post bacterial attachment.

Along the same lines as a conventional fluorescence microscope is the confocal scanning laser microscope (fig. 2.26), which allows the construction of a three-dimensional sample image. Typically, incident light is passed through an objective lens and onto the sample. Emitted light then passes onward to the detector via a pinhole which allows detection of light from the focal plane only. The sample is scanned in the x, y and z directions, with the detected light processed by a computer to create the three-dimensional image.57

![Figure 2.26: Confocal scanning laser microscope set-up](image-url)
Of particular advantage is the ability to assess bacterial viability when attached to surfaces, although each confocal scan can take around 15 minutes. The confocal microscope used during this work was a Dual laser Nikon Eclipse 90i microscope. Confocal imaging was of use in examining three-dimensional materials exposed to bacterial cultures e.g. non-wovens.

2.3.7 Microbial safety and sterility

In order to work safely with bacterial samples, maintenance of sterile conditions was required. All work was carried out with class II pathogens (MSSA 476, PAO1) in a class II safety cabinet. Such a cabinet comprises a glass screen to protect the user with an air circulation system containing filters. A small opening at the front of the cabinet allows access to work with samples safely and without disrupting sterility. Disinfection of surfaces outside of the cabinet was through use of bleach or 70% alcohol.

Sterilisation of media and equipment is generally achieved by use of heat, commonly in the form of an autoclave. Autoclaving is essentially decontamination of materials with pressurised steam and in order to sterilise, an autoclave must run at 121ºC and 15 psi for 15 minutes.48

Although samples which were to be exposed to bacteria could have been sterilised using an autoclave, it was decided to use a quick and dry method of UV/ozone treatment. An ultraviolet light source attacks bacteria on contaminated surface through high energy bombardment of chemical bonds within the cell.

2.3.8 Antibacterial evaluation of materials

There are a multitude of methods for measuring the antimicrobial properties of solutions and surfaces. Although there are American and Japanese standards for example, to date there is no definitive method or procedure for assessing antimicrobials. The methods used in this work were chosen according to their applicability and are detailed here. Common amongst all assays of course was the need to test all potential antimicrobials against a non-treated control.
2.3.8.1 Disc diffusion assays

A simple way of qualitatively assessing the effectiveness of free compound or the leaching properties of plasma films is to place the antimicrobial onto a solid growth medium coated with bacteria and gauge visible colony growth or otherwise after a defined period of time. If a zone of clearing surrounding the antimicrobial is evidenced, then it can be assumed that diffusion of the agent has inhibited microbial growth. Although this method is a useful indicator of antimicrobial efficacy and helpful for comparing material leaching, it is not necessarily reliable for comparing different free compounds which can exhibit vastly different solubilities in aqueous media.

2.3.8.2 Minimum inhibition assays

Another method for determining the efficacy of free compounds is to quantitatively assess the minimum concentration of an antimicrobial to inhibit microbial growth in solution. Differing quantities of antimicrobial agent are dissolved in an appropriate media for bacterial growth, with a set concentration of bacteria added to the mix and incubated at optimum growth. After a defined time period, OD of each sample is taken and a plot of OD or CFU ml\(^{-1}\) versus antimicrobial concentration is plotted. Commonly assessed is the ability of the antimicrobial to inhibit either 50% or 90% of bacterial cell growth compared to the control.

2.3.8.3 Solution assays

If an antimicrobial agent is leached from a surface, it is useful to know how it affects bacterial growth in solution. In order to do this, a solution of bacteria which is in the vicinity of an antimicrobial surface can be tested for bacterial cell viability through use of colony counting, optical density or Live/Dead stain. Due to the high concentration of bacterial cells in some solutions, multiple dilutions and platings are required when utilising the colony count method.
2.3.8.4 Surface assays

A simple method of assessing surface-active antimicrobials is by allowing growth for a set period of time and comparing visual biofilm formation. Other visualisation methods include the use of a light or fluorescent microscope. In order to quantify the surface-activity of antimicrobials it is necessary to remove those cells which have attached to the surface. Typically, the surface is lightly rinsed to remove free cells, replenished with fresh media and vortexed to remove surface-attached cells. These cells can then be assessed by colony counting, OD or fluorescence in the same manner as solutions.

This general assay type was employed for a range of substrates from plastics to glass to gold, with some modifications along the way. A Japanese industry standard (JIS L 1902) for quantification of bacterial growth on textiles which follows a similar method was also used.

2.4 References

(5) van Os, M. T., University of Twente, 2005.
(6) Inagaki, N. *Plasma surface modification and plasma polymerisation*; Technomic, 1996.
(9) Forch, R.; Chifen, A. N.; Bousquet, A.; Khor, H. L.; Jungblut, M.; Chu, L.


Chapter 3: Experimental methods

In keeping with chapter 2, all common experimental methods used are detailed here.

3.1 Plasma reactor

3.1.1 Monomer deposition

Use of the reactor for plasma polymerisation required loading of substrate to the main chamber, then adding monomer to the Young’s flask and proceeded as detailed in appendix. In brief, the chamber was evacuated, allowed to reach a suitable base pressure, the monomer tap was opened and either continuous wave or pulse plasma was ignited using the radio frequency source.

A continuous wave plasma reaction can be seen below:

![Figure 3.1: Plasma polymerisation in reactor 1](image)

3.1.2 Pulse plasma

Pulse plasma deposition was operated using the standard monomer deposition procedure. Duty cycles of 1/40 (T=41ms) 10/40 (T=50ms) and 40/40 (T=80ms) were
commonly employed by altering on/off times on the oscilloscope to give equivalent
input powers of 1.2W, 10W and 25W respectively at a peak power of 50W.

3.1.3 Oxygen/fluorocarbon treatment

Oxygen and fluorocarbon gas sources were introduced to the plasma chamber via
inlet taps on the flange of reactor 1 and the glass attachments of reactor 2. Treatment
using these gases was used before or after the deposition of complex, never
concurrently.

3.2 Plasma film characterisation

3.2.1 Substrate preparation

Plasma films were deposited onto a wide range of substrates including NaCl and
KBr discs, glass slides, SAM coated gold slides, polystyrene petri dishes and non-
woven materials. Preparation of polymeric surfaces for the acceptance of plasma
films or for use as controls in microbiological assays was achieved using either
oxygen plasma or UV/O₃ treatment. The preparation of gold films however, required
a series of procedures to clean glass, deposit gold and coat with SAMs.

3.2.1.1 Glass preparation

Slides of glass were cleaned by sonication with KI/I₃ solution for 15 minutes in an
ultrasonic bath to remove old gold and rinsed 20 times in MilliQ water. They were
then sonicated for 15 minutes with 2% Hellmanex (an alkaline solution), rinsed 20
times in MilliQ water and sonicated twice in absolute ethanol for 15 minutes. Slides
were then dried with nitrogen and stored under nitrogen at 4°C.

3.2.1.2 Gold evaporation

Slides for gold evaporation were subjected to UV radiation, before being placed in a
gold evaporator. Gold of mass 118mg was cut from a reel of gold wire (diameter
0.5mm), sonicated in ethanol for 10 minutes and dried in a nitrogen stream. Clean
gold was then coiled and placed in the appropriate basket of the evaporator. The system was put under high vacuum until a pressure of $3 \times 10^{-6}$ was reached, at which point evaporation of gold took place at 3-4 amps. This allowed deposition of a gold film around 50nm thick. After evaporation, gold coated slides were placed in an oven at 550°C for 1½ minutes to allow the gold to anneal. Once cool, the slides were then exposed to UV/O$_3$ for 15 minutes and stored under nitrogen at 4°C until required.

3.2.1.3 SAM preparation

Gold slides were placed in a solution of 5mM allyl mercaptan in ethanol for 30 minutes, then dried in N$_2$ immediately prior to plasma deposition.

3.2.2 FTIR

3.2.2.1 Transmission spectra

300 mg anhydrous potassium bromide was ground to a fine powder using an agate mortar and pestle. The minimum amount of powder was then placed in between two anvils of a stainless steel dye under vacuum and a pressure of 7 tonnes was applied to the dye for 15 seconds, yielding a homogenous KBr disc of 13mm diameter. Plasma films were deposited onto the discs and FTIR measurements of the discs taken after a background reading. For each sample, 128 scans were recorded at a resolution of 4 cm$^{-1}$.

3.2.2.2 Absorption spectra

Plasma films were deposited onto SAM-coated gold chips and FTIR measurements taken after a background reading. For each sample, 32 scans were recorded at a resolution of 4 cm$^{-1}$.
3.2.3 AAS

Plasma films were deposited onto glass slides and either placed in 2 ml of phosphate buffered saline for 24 h or dissolved using 2 ml of ultra pure nitric acid. The resulting solutions were subsequently fed into an atomic absorption spectrometer after calibration with the appropriate metal-containing standards (Ag = 1, 2.5 and 5 ppm; Zn = 0.4, 1 and 2 ppm) and elemental concentration results were obtained. All operations were carried out by Alan Carver at the University of Bath.

3.2.4 XPS

Plasma films were deposited onto Si-wafer after UV/O3 treatment to activate and sent for analysis at either CERAM (Stoke) or NPL (Teddington).

3.2.5 Electron microscopy

Samples for electron microscopy were prepared in different manners according to the method i.e. scanning or transmission used. For SEM, plasma films were deposited onto Si-wafer after UV/O3 treatment to activate or SAM-coated gold chips and then coated with a thin film of carbon or gold to increase the conductivity. For TEM, plasma films were deposited onto carbon coated copper grids. Electron microscopes were operated by Hugh Perrot at the Centre for Electron Optic Studies (CEOS) at the University of Bath.

3.2.6 SPR

SPR cells were set up with a LaSFN9 glass prism placed on top of the non-coated side of slides, with immersion oil separating them. The coated side was placed onto a Teflon base containing a rubber O-ring and then secured into the SPR holder. Once in place on the SPR instrument, the holder platform axes were varied until total internal reflection of incident light was achieved. The angle was then scanned through from 19° until after the resonance angle was reached (minimum reflectance)
and the SPR curve plotted on a computer programme. In order to obtain a plasma film thickness measurement, bare gold was scanned and a curve recorded before plasma deposition to allow comparison. A fitting programme (WINSPALL, Max Plank Institute for Polymer Research, Mainz, Germany) was then used to determine an estimated thickness using the parameters for the bare gold slide:
- Prism: thickness = 0, dielectric constant (real) = 3.404, (imaginary) = 0
- Gold layer: thickness = 50nm, dielectric constant (real) = -12.5, (imaginary) = 1.5
- Air: thickness = 0, dielectric constant (real) = 1, (imaginary) = 0

The resulting curve simulation was fitted to the experimentally observed curve (critical angle and resonance angle) to estimate thickness of the gold layer:
Thickness of the plasma film was estimated by entering the following parameters:
- Plasma film: thickness = 30nm, dielectric constant (real) = 2.25, (imaginary) = 0
- (Optional) Water: thickness = 0, dielectric constant (real) = 1.778, (imaginary) = 0

The simulated curve was fitted to the experimental curve by only allowing for change to plasma film thickness parameter, so yielding this value post-fitting.

### 3.2.7 QCM

Plasma films were deposited onto SAM-coated 10 MHz gold ITO crystals. The crystals were weighed prior to- and post- deposition by attachment to a potentiostat, placement in a Faraday cage and recording the frequency after five minutes.
3.2.8 α-step profiling

Films were plasma deposited onto Si-wafer after UV/O₃ treatment to activate. Profiling was performed by Alex Lotz at Max Plank Institute for Polymer Research, Mainz, Germany.

3.3 Monomer characterisation

3.3.1 FTIR

3.3.1.1 Transmission spectra

A small amount of sample (ca. 3mg) was added to 300 mg anhydrous potassium bromide and ground to a fine powder using an agate mortar and pestle. The minimum amount of powder was then placed in between two anvils of a stainless steel dye under vacuum and a pressure of 7 tonnes was applied to the dye for 15 seconds, yielding a homogenous KBr disc of 13mm diameter. An FTIR measurement of this disc was taken after a background reading. For each sample, 128 scans were recorded at a resolution of 4 cm⁻¹.

3.3.1.2 Absorption spectra

A small amount of sample was placed on the crystal of the FTIR spectrophotometer and crushed. An FTIR measurement was taken after a background reading. For each sample, 32 scans were recorded at a resolution of 4 cm⁻¹.

3.3.2 NMR

Samples for NMR analysis were prepared by adding a small amount (<5mg) to the minimum amount of solvent in a clean NMR tube. All NMR experiments were carried out in either deuterated chloroform or dimethyl sulfoxide solvent, depending on solubility of the monomer. Samples were immediately loaded to the appropriate spectrometer queue and run automatically.
3.3.3 XRD

Crystals suitable for single crystal X-ray diffraction studies were formed by the recrystallisation of crude monomer from an appropriate solvent followed by slow evaporation of solvent. Samples were submitted to Dr. Andrew Johnson at the University of Bath for analysis.

3.3.4 Melting point

Glass capillary tubes were melted at one end and allowed to set, blocking one end. A small amount of sample was loaded to the open end and moved to the blocked end. The blocked end of the tube was then loaded to a melting point apparatus and the melting point observed by slow heating.

3.4 Microbiological techniques

Biological work was undertaken in both Biology and Biochemistry, and Chemistry at University of Bath under the supervision and guidance of Dr. Nick Waterfield and Pete Jewell. Safety precautions taken when working with microbes included lab coat, gloves and use of a flow hood. Work with class II pathogens was undertaken in a class II safety cabinet. Any contaminated waste was disposed of via autoclave and/or use of bleach.

3.4.1 Aseptic techniques

Of utmost importance during microbiological work is sterility, both for the safety of the user and to prevent cross-contamination of samples. As such, all equipment used for contact with microbes (e.g. pipette tips, tubes) was either pre-sterilised, filter syringed or autoclaved at 121°C. Prior to microbiological work the safety cabinet interior, pipette fillers, gloves and exterior of bottles etc were sterilised with 70%
ethanol. During microbiological work, great care was taken to prevent contamination by minimising lid-removal time, avoiding contact of pipette fillers with the inside of tubes and jars, and use of new pipette tips and tubes for each sample. Post assay, the area was decontaminated with 70% ethanol.

### 3.4.1.1 Bacterial culture

Cultures of bacteria were prepared in two ways:

1. Inoculation of a solution with a streak of bacteria. A sterile loop was used to pick several colonies of bacteria from a stock plate, added to 10ml LB broth and incubated at 37°C for 24 hours to allow growth.

2. Inoculation of a solution with a single colony of bacteria. A sterile pipette tip was used to pick a single colony of bacteria from a stock plate, added to 10ml of LB broth and incubated at 37°C for 24 hours with shaking at 200rpm to agitate growth. Each culture was prepared in triplicate against a negative control of pure LB to identify contaminated media.

The second method was preferred for latter work as it provided greater reproducibility of culture. After incubation, 1ml of each culture was centrifuged for 3 minutes at 1200 r.p.m. and excess media poured off to be replaced by fresh media. 100µl of culture was added to 900µl of fresh media and OD taken at 600nm versus a zeroed control of pure LB in order to estimate CFU ml⁻¹.

Stock solutions of bacteria were made adding 800µL of a grown culture to 800µL glycerol and storing at -80°C. These stocks were then used to create fresh cultures by spreading of 50µL onto an agar plate and growing up again.
3.4.2 Monomer microbiological assays

3.4.2.1 Disc diffusion assay

The disc diffusion or zone of inhibition (ZOI) assay was used as an indicator of the antibacterial efficacy of free compound. Pellets of compound were prepared using a 13 mm die and 10 tonne press. The required molar quantity of compound (and KBr) was weighed, added to the die and pressed at 8 tonnes for 30 s. Overnight bacterial cultures in LB media were adjusted to $10^5$ CFU ml$^{-1}$. 100μl of solution was spread onto a 2.5% LB agar plate, and a pellet placed onto the dish immediately. Plates were inverted and incubated at 37°C for 18-24h. After this time, the zone of inhibition was observed between the edge of the pellet and the edge of the bacterial growth ring (fig. 3.2)

![Figure 3.2: Zone of inhibition assay](image)

3.4.2.2 MIC assay

The MIC or Minimum Inhibitory Concentration of free compound was determined as follows, shown in fig. 3.3. An initial 6 solution dilution series of compound was made up in molecular biology grade DMSO (>99.99% purity) over a suitable
concentration range by serial dilution. DMSO was used due to the insoluble nature of monomers and of high purity to avoid presence of contaminants such as iron, which is seen in >98% pure DMSO. Once the initial dilution series was made up, 100µl of each solution was added to 800µl LB and 100µl $10^6$ CFU ml$^{-1}$ of resuspended bacterial culture in LB. Samples were incubated at 37°C for 18 hours, then removed from incubation and the optical density was determined by UV-vis spectrophotometry at 600nm versus a zeroed blank of 9:1 LB:DMSO. Optical density was correlated to bacterial growth by cell counting calibration and plotted against the monomer concentration range. An MIC$_{50}$ for each compound was estimated at 50% bacterial growth reduction.

![Figure 3.3: Minimum inhibition concentration assay](image)
3.4.3 Plasma film microbiological assays

All assays followed the general procedure shown in fig. 3.4 with varying modifications.

![Diagram](image)

**Figure 3.4**: General method of antimicrobial surface-activity

3.4.3.1 Petri dish assay

Monomer was plasma deposited onto three 50mm diameter Petri dishes in three separate depositions, post 10min O₂ 50W CW plasma. Control Petri dishes were subjected to 10min UV/O₃ treatment in order to sterilise.

Overnight cultures of bacteria were adjusted to ca.10⁵ CFU ml⁻¹ in either minimal LB (10x dilution of regular LB) or regular LB media. 40μl of culture was added to each Petri dish with sufficient minimal/full LB to make working solutions of 4ml at 10³ CFU ml⁻¹. Dishes were incubated at 37°C for desired time period. After this time, effluent media was poured off and the surface indirectly washed with 2x 2ml physiological saline (0.9% w/v NaCl). 1ml of physiological saline was applied to the dish surface before each dish was wrapped in parafilm and vortexed for 30s.

3.4.3.2 JIS assay

Monomer was plasma deposited onto three 9x9cm strips of non-woven (PP), which were subsequently cut into three sets of two 3x3cm squares (end pieces only used).
Control non-woven were cut into 3x3cm squares and subjected to 10min UV/O₃ treatment in order to sterilise.

Overnight cultures of bacteria were adjusted to ca.10⁵ CFU ml⁻¹ in LB media, 200μl of which was added to each square of non-woven in a sterile 50mm petri dish. Infected non-woven was incubated at 37ºC for a specified time. After this time, non-woven squares were removed from the Petri dish, lightly washed in 2ml physiological saline (0.9% w/v NaCl) dips and placed into a 50ml tube containing 20ml of physiological saline. Each tube was then vortexed for 5 x 5s.

3.4.4 Antibacterial analysis

3.4.4.1 Colony count

100μl of the vortexed solution in 3.4.3.1 or 3.4.3.2 was applied to the surface of a 2.5% LB agar plate and spread. Further vortexed solutions at 20-, 400-, 8000-, 1.6x10⁵-, and 3.2x10⁶-times dilution in physiological saline were also made and spread in the same manner. Plates were incubated for 18-24h until bacterial colonies were observed.

After incubation, agar plates containing colonies of bacteria were assessed for viable cell count. A dilution factor was chosen where individual colonies were evident, but at countable and statistically viable levels (ca.30-300). Colonies were then counted and scaled up (multiplied by 10x dilution factor) to give an estimation of viable cell count, colony-forming units per millilitre (CFU ml⁻¹).

3.4.4.2 Live/Dead analysis

Live/Dead stain stock solution was prepared by adding pre-measured quantities of SYTO-9 and propidium iodide in the BacLight kit to a common 5ml of sterile water. This solution was stored under foil at -18°C.
900µl of the vortexed solution in 3.4.3.1 or 3.4.3.2 or of the effluent solution was added to 100ml of Live/Dead stain, shaken and allowed to diffuse for ten minutes. After this time, samples were loaded to 1.6ml disposable cuvettes and fluorescence spectra obtained at an excitation wavelength of 490nm. Emission intensities observed at the appropriate wavelengths for the two dyes were used to estimate bacterial viability.

In order to create a calibration plot, bacterial cultures of estimated live/dead ratios were created using live cultures and identical dead cultures; cells were killed by heat shock at 60°C for 30 minutes.

### 3.4.4.3 Microscopy

Samples were prepared for fluorescent microscopy by staining washed but non-vortexed surfaces on appropriate substrates with 50µl of Live/Dead stain. After 10 minutes, samples were observed on either a fluorescence or confocal microscope.

### 3.5 Cytotoxicity tests

The cytotoxicity of plasma films was studied by culturing cells from embryonic Swiss mouse fibroblasts (NIH 3T3) and human neonatal epidermal keratinocytes (HEK102-05n) and monitoring growth on treated and non-treated glass coverslips.

NIH 3T3 cells were seeded onto coverslips at high density and allowed to grow for 24h. HEK 102-05n cells were much slower growing and were only available for use at a lower density, so were allowed to grow on the treated coverslips for 72h prior to cytotoxicity testing. An XTT/MPMS assay\(^1\) was used to assess cell viability after this time, where XTT tetrazolium is converted to orange formazan crystals by mitochondrial enzymes in live cells, thereby giving a colourimetric response measured by light absorption. Therefore, greater cell viability is indicated by increased absorption.

PMS stock was diluted to 1:100 and 5µl XTT was added per ml. The resulting
solution was added to media containing the cells to be tested (pre-prepared) in a 1:4 ratio, shaken and incubated at 37°C for 2h under 5% CO₂. Media was removed from cultures after this time and added to a 96 well plate, and then absorbance was measured at 450nm.


### 3.6 Appendix

Detailed operation of plasma reactor:

- Reactor cleaned manually: the reactor chamber and both flanges (reactor 1) were washed using acetone, water and ethanol, allowed to dry
- Reactor cleaned using oxygen plasma
- Monomer loaded to Young’s flask. Freeze thawed several times (3+) by cooling flask with liquid nitrogen followed by gentle heating
- Substrate loaded to the main chamber at one end: Vacuum was removed, one electrode was detached using wing-nuts (reactor 1) or retaining strap (reactor 2), the substrate loaded, lines reattached and vacuum applied. System was allowed to reach base pressure (ca. 2 × 10⁻² mbar)
- Monomer chamber was opened to vacuum, pressure was allowed to stabilise
- Monomer flow rate was measured by closing the vacuum tap for 30s, pressure readings were taken at start ($P_\text{s}$) and end ($P_\text{f}$) of this time period then applied to equation 2.18 of chapter 2
- The RF generator was switched on, mode set to ‘CW’, plasma ignited (RF on) and desired input power set. (If pulse required, see 3.6.1)
- Input impedance was manually matched using ‘Tune’ and ‘Load’ to minimise the reflected power. The reflected power was reduced to <0.5% of the input power at all times during deposition
- Deposition pressure was recorded along with the input and reflected power
- Deposition was stopped after desired time, then the monomer chamber was closed
• The substrate was removed after allowing the reactor to cool, then generally used immediately for analysis or biological assay

NB: The initial step of degassing or removal of impurities was applied only to non-melting monomers as those compounds which melt when heated or are liquid at room temperature instantly degas under vacuum.

### 3.6.1 Pulse operation

- Mode was set to ‘Pulse’ using toggle switch at the rear of the RF generator
- The pulse generator and the oscilloscope was switched on
- Wavelength and period was altered using dials on pulse generator. Pulse ‘on’ and ‘off’ appeared visually on oscilloscope. With ‘time divisions’ set to 10ms, each square equated to 10ms
- Commonly used duty cycles were 10/40, 1/40 and 40/40. 1/40 pulse was set by dialling ‘on’ time to 1ms and ‘off’ time to 40ms

Some compounds struggled to maintain a plasma glow when pulse operation was used. If a monomer with a particularly high flow-rate was used, the flow rate was reduced using monomer tap. The tap was opened slowly until a pulse plasma glow was maintained.

### 3.6.2 Oxygen/fluorocarbon treatment

- Cylinder tap was opened \((\text{O}_2/\text{C}_2\text{F}_6)\) (to 1 bar for \(\text{O}_2\)), the gas was allowed to flow to the inlet tap
- Monomer taps were closed, the desired inlet tap opened until the reactor pressure reached \(1-2 \times 10^{-1}\) mbar
- The RF generator was switched on, mode set to ‘CW’, plasma ignited (RF on) and desired input power set (usually 50W)
- The reaction was stopped after desired time
- Cylinder tap was closed, the gas was allowed to dissipate. Inlet tap was closed
Chapter 4: A novel silver compound for the plasma deposition of antimicrobial films

A novel organosilver complex was synthesised, characterised and found to inhibit the growth of *M. luteus*, *P. aeruginosa* and *S. aureus*. The complex was used as a monomer unit in the plasma deposition of thin films and these films were found to inhibit microbial growth on a range of substrates by a number of traditional and modern microbiological techniques. Films were also found to be completely harmless to eukaryotic cells after exposure of between one and three days. Aspects of this chapter were summarised in the Royal Society of Chemistry journal *Chemical Communications* in 2009.¹

4.1 Antimicrobial silver

The use of silver as an antimicrobial agent is discussed in chapter 1, but the precise mode of toxic action against micro-organisms is a contentious issue. It has been proposed that silver cations (Ag⁺) act by binding strongly to electron donor groups containing sulphur, oxygen or nitrogen, which are plentiful in bacterial cells and replacing essential metal ions such as calcium and zinc.²⁻³ For instance, Ag⁺ ions can deactivate the enzymes required for DNA replication⁴ by irreversibly blocking the helix from unwinding.⁵ Silver ions may also perform activity at cell membranes, where they react with thiol (-SH) groups,⁶⁻⁷ causing damage to the microbe and possibly activating self destruct genes⁸ (fig. 4.1). Both of these mechanisms have been observed in electron microscope studies of cells treated with silver ions.⁹
Due to the multiple biocidal mechanisms employed by silver, it is believed that there is little chance bacteria may become resistant to it,⁶ although some evidence for resistance has been shown. Ag⁺ exposure has proven non-toxic to mammalian cells in low concentrations,³,⁷ but silver effects in the body can be inhibited by complexation with organic compounds present in biofluids,¹⁰ e.g. albumin in blood³,¹¹ therefore long term release issues remain a problem with silver applications. Indeed, frequent treatment can allow bacterial resistance to be built up¹² and some silver coatings can be denatured by blood plasma over time.¹³

A recent study has shown that silver efficacies are greatest when the free ionic content is lowest,⁵ possibly due to instant chelation or complexation in a media environment as silver has a propensity to form insoluble silver chloride, which is a sink source for silver. This study also indicates that DNA replication prevention, rather than membrane disruption is the key aspect of silver ion effect as an antibacterial agent, due to the fact that silver requires complexation in order to diffuse through the cell membrane and cytoplasm. Complexed silver prevents activation of bacterial ion efflux mechanisms,¹⁴ which actively pump out free silver ions in the cell and enzymatic detoxification (redox chemistry).¹⁵ Therefore, pure silver application is less effective where required at low concentrations, i.e. in the body, so the most efficient biocidal material for medical use is one which carries
silver in complexed form, but bound such that ion-exchange is possible to allow silver release at DNA binding sites.

4.2 Silver chemistry

Silver is a ‘noble metal’, found toward the right-hand side (group 11, row 2) of the transition metal series, the $d$-block. Due to the poor nuclear shielding of the $4f$ orbitals, second and third row transition metals are larger and softer than those of the first row. Silver is resistant to oxidation, or ‘noble’ with only one stable oxidation state ($+1$). With the exception of silver nitrate, silver (I) salts are nearly always anhydrous and sparingly soluble in water due to formation of a three-dimensional polymer network. The poor reactivity and high conductivity of silver lies in the ground state electron configuration of $[\text{Kr}]5s^14d^{10}$. Silver preferentially forms $\text{Ag}^+$ over other oxidation states as removal of the $5s^1$ electron leaves a highly stable filled $4d^{10}$ set.

4.3 Novel silver complexes for plasma deposition

The ideal way to incorporate silver into plasma films is through deposition with an organic or reactive component. Previous efforts discussed in chapter 1 have shown organic frameworks impregnated with silver nanoparticles can be an effective surface-active antimicrobial agent. However, such an approach is limited by unsolicited release of silver which can render the surface itself inactive. The approach taken here was reaction of silver salt with an organic molecule which can act as a polymeric linker e.g. containing double bond functionality, thus theoretically maintaining silver at the surface in complexed form in the polymer structure (fig. 4.2).
A number of organo-silver complexes have been reported over the years, although the inherent light and thermal instability of silver means simple silver compounds (Ag-R) readily decompose upon subjection to vacuum and UV light in a plasma monomer chamber. In addition, organo-silver complexes are inherently involatile and poor antimicrobials on account of their oligomeric supramolecular structure owing to intermolecular forces.\textsuperscript{19-20} To avoid these undesirable characteristics it is possible to both stabilise and reduce the intermolecular forces in Ag-R compounds by use of bulky ligand systems which provide steric hinderence.\textsuperscript{20}

4.3.1 Ligand selection

Prior work in the area of plasma polymerisation has established the use of small organic molecules containing unsaturation as good precursors for thin films. If a silver containing unit could be incorporated into such a molecule, then polymerised, a potential antibacterial agent with desired properties of complexation and homogeneity would result. For this purpose, recent studies on plasma deposited maleic anhydride\textsuperscript{21-22} and acrylic acid\textsuperscript{23} were of interest, which demonstrated good compatibility with biomolecules. Unfortunately in the case of maleic anhydride, a bond with silver cannot form without disrupting ring structure or polymerisation characteristics. Alternatively, the analogue maleimide (fig. 4.3) contains an amide group which can undergo reaction with silver compounds to provide a silver-nitrogen bond.
The result of this reaction is silver maleimide (fig. 4.4). Bonding between the metal centre and nitrogen is relatively simple due to sp$^3$ hybridisation of tetrahedral first row elements. Silver salts or precursors (nitrate, oxide) provide a source of oxidised silver, therefore an empty 5s orbital. If the nitrogen atom of maleimide is deprotonated, a situation can arise with complexation to silver which satisfies the electronic requirements of each atom.

4.3.2 Lewis adduct selection

Several potential Lewis adducts are available for silver stabilisation including bipyridine (bipy) and diphenylphosphinoethane (dppe), but triphenylphosphine ligands were chosen due their inexpensive, highly available nature and relatively low toxicity. Silver binds to phosphorus very well due to interaction between the similarly diffuse 4d and 3d orbitals.$^{20}$
As can be seen from fig. 4.5 a), ligands such as these provide a large amount of steric bulk about the central phosphorus atom (orange), which is the binding point for silver. When two PPh$_3$ adducts coordinate to silver, reductive elimination is blocked by steric hindrance. The Tollman cone angle for PPh$_3$ is 145°, which although not the largest steric bulk in comparison with other analogous Lewis adducts$^{26}$ still allows bonding of the silver atom with the organic component. Higher angle phosphines such as PtBu$_3$ (182°) can only be used singularly and consequently provide less overall bulk.

Phosphines coordinate to the metal centre through the lone pair as sigma (σ)-donors i.e. form a dative bond and are favoured due their ‘soft’ nature.$^{26}$ Phosphines also act as good π-acceptors, ‘back-bonding’ with the metal through P-C σ* orbitals for added stability (fig. 4.6).
Thus, triphenylphosphine when used in 2:1 molar ratio with an Ag-R complex provides light and air stable silver compounds. Using this rationale, the novel compound bis-triphenylphosphino silver maleimide was designed (fig. 4.7).

![Figure 4.6: Stabilisation of a metal centre using phosphine ligands](image)

**Figure 4.6:** Stabilisation of a metal centre using phosphine ligands

![Figure 4.7: The reaction scheme for formation of novel phosphine-stabilised silver maleimide complex](image)

**Figure 4.7:** The reaction scheme for formation of novel phosphine-stabilised silver maleimide complex
4.4 Preliminary plasma study – maleimide

In order to ascertain the feasibility of using maleimide as a polymeric base-unit, an initial plasma deposition study was undertaken. Here, information on functional group retention and thickness at different input power and pulsing was observed. All plasma polymers were deposited at flow rates of around 0.27 sccm onto NaCl discs (FTIR) or gold films (SPR).

A variety of parameter combinations were experimented with in order to establish the optimum parameters for maleimide polymerisation. At 50W peak input power the greatest FG retention was seen under deposition parameters which allowed a long pulse ‘off-time’ i.e. 1/40 duty cycle. Sharp peaks were detected for this polymer, presences of which were also detected as broader absorptions in CW FTIR spectra. For other selected duty cycles however, much broader peaks were evidenced, indicating less FG retention and a much more random polymeric coating (fig. 4.8).

Figure 4.8: FTIR spectra of maleimide plasma films deposited onto NaCl discs
As can be seen from the FTIR spectra, each film showed different functionality according to duty cycle and each was reproducible according to the pulse parameters used. The main areas of functionality retained during such polymerisation appeared to be the N-H stretch, carbonyl region and associated conjugation. In addition, weak shoulder peaks due to alkane vibration were established. This was consistent with radical polymerisation to produce an alkane based polymer i.e. removal of alkene functionality.

Further investigation of each system revealed a steady increase in transmission IR absorbance for each band over time, indicating increased deposition at longer polymerisation times. This was confirmed by film thickness measurements using SPR.

**4.5 bis-triphenylphosphino silver maleimide**

Initial methods for preparing the novel complex (PSSM) began using an adapted synthesis of Schwartz\(^{27}\) with a simple reaction of silver nitrate and maleimide in absolute ethanol and presence of base. These reactions yielded a very high level of decomposed silver (Ag\(_2\)O and intermediates e.g. Ag-OH)\(^{28}\) from the reaction of base with silver ions. To avoid decomposition, silver nitrate was stabilised with triphenylphosphine before reaction with sodium maleimide (fig. 4.9). Although this methodology afforded a reasonably high yield, residual base present in products still allowed silver decomposition.

![Figure 4.9](image)

**Figure 4.9:** The stabilisation of silver with phosphine prior to reaction with base

An alternative methodology was reaction of silver oxide with carboxylic acids detailed by Edwards\(^{29}\) with maleimide substituted as the ligand in water at 1:1 molar ratio. This required filtration of large amounts of excess Ag\(_2\)O and a low yielding product (ca. 30%), which was unsatisfactory. A mixture of EtOH with water provided a solvent in which the majority of silver nitrate was converted to Ag-R, while a modified version of silver carboxylate synthesis employed by Jakob et al\(^ {30}\)
where EtOH with minimum MeCN to dissolve silver nitrate provided an even more satisfactory and high yielding solvent system for this reaction, so became method of choice for silver salt synthesis. Reaction of the resulting compound with two equivalents of PPh₃ generated the target molecule (fig. 4.10).

\[
\text{AgNO}_3 + \text{Maleimide} \xrightarrow{\text{Base}} \text{Ag-maleimide}
\]

\[
\text{Ag-maleimide} + 2\text{PPh}_3 \xrightarrow{} (\text{Ph}_3\text{P})_2\text{-Ag-maleimide}
\]

**Figure 4.10:** A two-step synthetic route to phosphine stabilised silver maleimide

### 4.5.1 PSSM preparation method

To a stirred solution of maleimide (10mmol, 0.98g) in ethanol (20ml), a 10ml solution of silver nitrate (10mmol, 1.70g) in an ethanol/acetonitrile mixture (minimum MeCN) was added and the solution stirred for 5 minutes. After this time a solution of triethylamine (10mmol, 1.01g) in ethanol (5ml) was added dropwise over 30 minutes, resulting in the formation of an insoluble white precipitate. The solid residue was isolated by filtration, washed three times with ethanol and dried *in-vacuo*, yielding silver maleimide (1.92g, yield 94%).

Addition of silver maleimide (5mmol, 1.02g) to a stirred solution of triphenylphosphine (10mmol, 2.62g) in toluene resulted in the formation of a light yellow precipitate after 1 hour. The solid residue was isolated by filtration, washed three times with toluene and dried *in-vacuo*, yielding *bis*-triphenylphosphino silver maleimide (PSSM) (3.35g, yield 92%). Anal. Calc. for C₄₀H₃₆O₂AgP₂N: C, 65.48; H, 5.12; N, 1.78. Found: C, 64.7; H, 5.40; N, 1.38. Mp: 158-160°C.

### 4.5.2 PSSM characterisation

The novel molecule, PSSM was recrystallised from minimal hot *isopropyl* alcohol and a crystal structure obtained by single crystal analysis (fig. 4.11).
The crystal structure reveals a three-coordinate silver atom central to the two triphenylphosphine groups and maleimide in a trigonal arrangement. Maleimide aligns itself perpendicularly to the electron-rich phosphine groups for reduced repulsion and is coordinated to silver in a similar manner to that of phthalimide in an analogous complex containing an Ag-N bond. Discrepancies between the two silver-phosphorus bond lengths are likely to result from electrostatic repulsion of the phenyl groups creating increased steric hindrance.

Re-crystallised complex was also dissolved in the minimum deuterated chloroform and proton, carbon and phosphorus NMR spectra generated.
Fig. 4.12 shows a visual representation and direct interpretation of NMR spectra. Proton NMR shifts were consistent with literature values for phenyl and olefin groups, with some solvent present from recrystallisation product. For each subsequent NMR observed however, the solvent contribution was ignored and only relevant functionality presented in formal assignment as displayed below:

<table>
<thead>
<tr>
<th>Chemical shift (ppm), multiplicity</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.3, multi.</td>
<td>Phenyl ring</td>
</tr>
<tr>
<td>4, sep.</td>
<td>¹PrOH (CH)</td>
</tr>
<tr>
<td>3.5, s</td>
<td>Olefin protons (maleimide)</td>
</tr>
<tr>
<td>1.85, s</td>
<td>H₂O</td>
</tr>
<tr>
<td>1.2, d</td>
<td>¹PrOH (CH₃)</td>
</tr>
</tbody>
</table>

Fig. 4.12: ¹H NMR spectra of PSSM crystals and assignment

¹H NMR (300MHz) (ppm): δ = 7.3 (m, 30H, P(C₆H₅)₃), 3.5 (s, 2H, -CH-CH-).

13C{¹H} NMR (75 MHz) (ppm) δ = 38.98, 129.28, 130.49, 133.01, 134.41.

31P{¹H} NMR (121 MHz) (ppm) δ = 10.00.
As was the case with proton, the carbon NMR correlated to literature values for such functionality and presence of a single phosphorus environment confirms the bonding mode identified in fig. 4.11.

An FTIR spectrum was also obtained from PSSM crystals.

*Figure 4.13: FTIR spectrum of PSSM*

FTIR peaks (cm$^{-1}$): 3132, 2967 (C-H, Ph alkene); 1616 (C=O); 1478, 1433 (ring deformation); 1327, 1281, 1249 (conjugation); 1155 (P-C) and 997, 694 (C-H).

Amide stretching seen in pure maleimide FTIR is not present in the novel product, therefore indicating the removal of the amide proton. The carbonyl peak in PSSM is of significantly lower wavenumber than free maleimide, dropping from around 1700 cm$^{-1}$ to 1616 cm$^{-1}$, owing to a reduction in bond order of the carbonyl functional group caused by delocalisation of electron density within the maleimide ring. Intermolecular hydrogen bonding between the amide proton and carbonyl predominates in solid maleimide over resonance. Once bonded with silver, hydrogen bonding ability is removed and therefore conjugation dominates (fig. 4.14) which reduces the double-bond character of the C-O bond, creating a lower absorption frequency for the carbonyl peak of PSSM.
Presence of extra bands around 3000 cm\(^{-1}\) in the silver compound is characteristic of phenyl groups in addition to alkene stretching modes also present in maleimide and a shoulder peak attributed to the alkene stretch is also seen at around 1600 cm\(^{-1}\). In addition, carbon-hydrogen bending modes associated with \textit{cis}-alkenes appear at around 940 cm\(^{-1}\) for both compounds, while additional peaks diagnostic of triphenylphosphine in the fingerprint region are shown, allowing for extra confirmation of the new molecule. These absorptions are also not seen for pure silver maleimide.\(^{27}\)

**4.6 Plasma deposition of PSSM**

Due to the results of the preliminary plasma deposition experiments with pure maleimide it was decided to use the duty cycles which showed best functional group retention for PSSM studies, namely 1/40 and CW. Initial efforts were at 50W and 100W input power for 30 mins onto sodium chloride discs. Unfortunately these early experiments did not yield particularly strong FTIR spectra, implying slow deposition of pp-PSSM (although this could also have been due to poor adhesion to the substrate). It appeared the stabilisation with triphenylphosphine had increased the stability of silver maleimide but not sufficiently increased volatility. The cause of this was likely \(\pi\)-stacking (p-p interactions), or hydrogen bonding where interaction of protons of phenyl groups with delocalised electron density on adjacent phenyl groups created a large supramolecular structure, reducing the propensity for monomeric units to escape to the gas phase.

Nevertheless further study of the PSSM system was sought, with reaction time increased to three hours. Resulting FTIR spectra of depositions at 100W input power...
for 3 hours are shown in fig. 4.15, which were readily reproducible. The 1/40 duty cycle film spectrum was of far lower intensity than that of the CW film indicating slower film growth, although some functional group retention was seen. The continuous wave plasma film showed broadening of FTIR peaks indicative of fragmentation caused by ionisation effects present under such conditions, in agreement with discussion in chapter 2. Due to the faster deposition rate evidenced in CW plasma and reasonable levels of functional group retention, early antimicrobial work was carried through using CW plasma deposition.

![FTIR Spectra](image.png)

**Figure 4.15:** Comparative FTIR spectra for PSSM 1/40 and CW duty cycle 100W 3h plasma depositions on NaCl discs

Film thickness under these parameters was monitored using SPR, with a resonance angle shift of 21° observed after deposition of pp-PSSM (fig. 4.16) converted to film thickness of 25nm by use of fitting software. Such observed thickness was reproducible and proved stable upon washing.
Plasma films were also observed visually by the use of bright-field microscopy. Non-woven polypropylene material was viewed pre- and post-plasma deposition with PSSM and noticeable dark deposits were evidenced (fig. 4.17), suggesting localised rather than homogenous spread of silver through the film. This was also observed in films deposited onto SAM-coated gold under SEM (fig. 4.18).
Figure 4.17: Light microscope images of untreated (top) and pp-PSSM treated (bottom) non-woven (fibre strands ca.10 μm in diameter)

Figure 4.18: Scanning electron micrograph of pp-PSSM deposited on gold

It is likely that silver exposed to intense UV radiation and high energy ions such as continuous wave plasma was subject to decomposition as silver nanoparticles or
nanocrystals, regardless of stabilisation using phosphines cf. ‘wet’ nanoparticle formation (fig. 4.19).

![Diagram](image)

**Figure 4.19:** Conventional ‘wet’ nanoparticle formation on non-woven strands

Subsequent work was carried out under the slightly milder conditions of 50W continuous wave in order to preserve functional group structure, whilst heating the monomer chamber to around 50°C. Silver decomposition within the monomer chamber was observed at temperatures above 60°C and did not greatly increase the monomer flow rate. Standard reaction conditions and parameters are shown in fig. 4.20, below:

<table>
<thead>
<tr>
<th>Duty cycle (peak power)</th>
<th>Flow rate (sscmm$^3$ min$^{-1}$)</th>
<th>Base pressure (mbar)</th>
<th>Film deposition rate</th>
<th>Deposition time</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW (50 W)</td>
<td>0.19 +/- 0.05</td>
<td>0.012</td>
<td>0.2 nm min$^{-1}$</td>
<td>1h – 3h</td>
</tr>
</tbody>
</table>

**Figure 4.20:** Standard reaction conditions and parameters for pp-PSSM deposition

Under these conditions the film deposition rate was increased by around 3 nm per hour from that seen at 100W, due to a reduction in surface etching by high energy particles and a slightly elevated monomer flow rate. Enhancement in characterisation was possible through use of ATR-FTIR in conjunction with a highly reflective substrate in gold. An example ATR-FTIR spectrum for a 50W deposition of PSSM is shown in fig. 4.21.
As can be seen in fig. 4.21, despite the shorter deposition time, much greater sensitivity was observed for plasma films using gold and ATR-FTIR than was achieved for NaCl discs and transmission IR in fig. 4.15. Functional group retention is seen from the pure monomer spectrum, albeit with much broadened peaks. A broad absorption is seen at around 3400 cm\(^{-1}\), indicating presence of an O-H or N-H group. Carbonyl functionality is retained from the monomer structure and there is evidence of triphenylphosphine ligand retention, although possibly oxidised by residual oxygen present either during reaction or post deposition on exposure to air.

Mass spectrometry was used to evaluate film composition by washing the surface of a long film deposition with hot solvent and analysing the resulting solution (fig. 4.22). Of particular interest was a strong peak between 631-633 \(m/z\) which was assigned to \(\text{Ag(PPh}_3\text{)}_3^+\). Presence of silver is detected by isotopic ratio, i.e. two major peaks correspond to ions containing Ag \{107\} and \{109\}. The peak at around 774 \(m/z\) also contained silver with presumably artefacts of ionisation within the plasma glow.
In addition to evidence of silver in films from microscopic means and mass spectroscopy, attempts were made to quantify content within films using XPS but it appeared low sensitivity of this approach (ca. 0.1% at) precluded detection of silver (fig. 4.23). A great deal of oxygen content was presumably due to incorporation from air, but importantly carbon, nitrogen and phosphorus; the key elemental constituents of PSSM, were all detected. Significant fragmentation had occurred in the film, as concentrations of each were proportionally different to what may be expected and low levels of contaminant were also observed; likely to arise from glass substrate. It could be inferred that inhomogeniety of the film structure, accompanied by insensitivity of XPS and low volatility of the silver monomer contributed to making detection of silver difficult.
Further attempts were made to quantify silver content through alternative means, namely atomic absorption spectroscopy. Films were deposited onto polystyrene Petri dish substrates, a solution of phosphate-buffered saline added and AAS reading taken after 24h to assess the leaching of silver into solution. This is summarised in fig. 4.24, where it can be seen that silver transport into solution from the surface results in a solution containing around 70 parts per billion of silver, whereas a non-treated surface contains/leaches no silver.
Figure 4.24: Atomic absorption of PBS solutions exposed to pp-PSSM treated and non-treated Petri dish for 24h

Such a result confirms the presence of silver in very low levels and also provides information on the potential antimicrobial activity of the film. It would appear that leached silver is at suitably non-toxic levels to humans after this time, but at concentrations which are potentially lethal to bacteria.

4.7 Microbiological assays

A range of microbiological assays were employed to assess the antimicrobial properties, as detailed in chapters 2 and 3. Initial testing of pp-PSSM films was against Micrococcus luteus.

4.7.1 Assays versus M. luteus

Conditions were established with M. luteus (ML) which allowed the accurate and reproducible measurement of bacterial growth, with procedural modifications made along the way.
4.7.1.1 Monomer zone of inhibition

A disc diffusion assay was used to confirm antibacterial properties of PSSM, as seen in fig. 4.25.

![Image of disc diffusion assay](image)

**Figure 4.25:** Disc diffusion of PSSM monomer (top) and silver nitrate (bottom) versus Micrococcus luteus on LB agar

A halo of inhibition was observed around the PSSM monomer pellet where bacteria could not grow. The edge of this halo showed a dark crust, indicating an area where silver is possibly decomposed through catalysis by bacteria. This halo exists even with inherently limited solubility of PSSM, contrasting sharply to the high solubility of silver nitrate. Interestingly, there was a visually smaller inhibition halo for silver nitrate than PSSM, but a much larger solubility ring.
4.7.1.2 Initial plasma film tests: visualisation

A simple method used to gauge the effect of pp-PSSM films was to inspect the surface for bacterial fouling after exposure to a solution containing ML. The image in fig. 4.26 shows two Petri dishes, one which was treated with pp-PSSM, the other a non-treated control. A solution of minimal media and ML was applied to the surface, poured off after 24h and the surface lightly rinsed. It was quite clear that under these conditions pp-PSSM had considerable antibacterial potential as the surface was almost clean whereas the control showed biofilm formation on the surface.

**Figure 4.26:** Visible *M. luteus* biofilm reduction on pp-PSSM treated Petri dish (l) versus non-treated control (r)

Similarly, gold chips coated with pp-PSSM were placed in a solution of ML and incubated for 24h before the surface was vortexed to remove bacteria and plated. A visual representation in terms of colonies is seen in fig. 4.27. It was instantly apparent before colony counting, that bacteria from treated films were much less viable than those of non-treated surfaces.
Figure 4.27: *M. luteus* colonies on agar plates from pp-PSSM treated (l) and control (r) gold substrates after 24h

The left-hand agar plate shows cells grown from the treated surface, the right-hand plate cells grown from the non-treated surface; with significantly less growth from the treated surface.

4.7.1.3 Traditional counting methods

The cell viability observed above was quantified using colony counts. Several dilution factors of the initial vortexed solution were plated and the most statistically viable (between 30-300 cells) counted and used for cell growth quantification. This is shown in fig. 4.28, where *M. luteus* growth was assessed against treated and non-treated surfaces in triplicate at the appropriate dilution factor.
As can be seen above, growth on pp-PSSM treated surfaces was reduced to around a third of the growth evidenced on non-treated samples, on average. This compares markedly to the growth-promotion effect that pp-maleimide only films produce, displayed graphically in fig. 4.29 which demonstrated the necessity of a silver-containing moiety in the film.
4.7.1.4 Live/Dead cell staining

As discussed previously, a new method of observing bacterial viability is through fluorescent labelling. Before the analysis of antibacterial surfaces, a calibration plot of Live/Dead ratio versus cell viability was obtained by combining proportional quantities of fresh bacteria and heat shocked bacteria vortexed from Petri dishes over a range from 0% live to 100% live. Cells in each solution were tagged with Live/Dead stain and fluorescence measurements taken, resulting in fig. 4.30 by calculating the ratio of live (green) to dead (red) cells.

![Graph showing fluorescence intensity against emission wavelength for different live/dead ratios.](image)

**Figure 4.30:** Live/Dead stain calibration for ML fluorescence readings (l) and the resulting calibration plot (r)

Although the emission curves for 90% and 50% live cells appear similar with near-identical green readings, the absolute intensity given off by the 50% live sample is lower therefore live/dead ratios fit nicely into the calibration plot. This plot was difficult to obtain as making bacterial solutions of precise viability is tricky, although calibration was ultimately reasonably reproducible. The calibration plot was used to calculate the percentage of ML cells which were viable when removed from Petri dish surfaces (fig.4.31).
The plasma input power was reduced from 100W to 50W for this assay in an attempt to increase functional group retention and improve still further antimicrobial properties. It is clear from this graph that very few ML cells were bound to the pp-PSSM surface and around 1% of these were live, compared to virtually all for pp-maleimide and non-treated control. This trend was reproduced in fluorescence microscopy of the Petri dish surface in fig. 4.32, where the early stage of biofilm growth was seen in the control while dead cells were observed on the pp-PSSM films. Dead cells were seen to clump in an attempt at self-preservation, much like the function of biofilm formation.

Figure 4.31: ML cell viability using Live/Dead stain after 24h on surface
4.7.2 Assays versus S. aureus and P. aeruginosa

After general antimicrobial procedures were established with *Micrococcus luteus*, the more virulent chosen model pathogens *S. aureus* (MSSA 476) and *P. aeruginosa* (PAO1) were introduced, starting with MSSA.

4.7.2.1 Monomer zone of inhibition

As with ML, disc diffusion assays were used as a tool for visual observation of free PSSM efficacy against bacterial lawns.
As observed in fig. 4.33, PSSM was active against MSSA in a similar manner to the action against ML, likewise silver nitrate. The activity of maleimide was extremely high due to very high solubility in aqueous media, resulting in complete dissolving of the pellet and the opposite effect was seen in the triphenylphosphine pellet.

Zone of inhibition study for PAO1 was conducted using varying concentrations of potassium bromide in the pellets as a rudimentary attempt at visual quantification of activity (fig. 4.34). Unfortunately the inhibition halos merged at points but importantly a great deal of inhibition was seen overall, especially in the 100% PSSM pellet with correspondingly less activity as the KBr concentration was increased.
4.7.2.2 Minimum inhibitory concentration

In addition to disc diffusion, minimum inhibition concentration (MIC) assays were used to quantify antimicrobial activity of PSSM versus MSSA and PAO1. Solutions containing a range of known PSSM content were incubated with bacteria and OD measured after 18h (fig. 4.35).
The MIC$_{50}$ of a compound is the concentration required to reduce bacterial population by 50%, which was calculated for both MSSA and PAO1 as and $38 \times 10^{-6}$ and $0.8 \times 10^{-6}$ mol dm$^{-3}$ respectively for the PSSM system. The MIC$_{50}$ for PAO1 compares favourably with that reported for silver sulfadiazine$^{33}$ at $125 \times 10^{-6}$ mol dm$^{-3}$, although it is difficult to directly compare MIC results due to differences in experimental procedure. As controls the starting materials silver nitrate, maleimide and triphenylphosphine were also subjected to MIC$_{50}$ tests against PAO1, again requiring much higher concentrations to inhibit cell growth of 48µM, 52µM and
>16.5mM respectively. This result suggested a synergistic action of ligand and metal on cells.

### 4.7.2.3 Surface assays

pp-PSSM plasma films were tested against MSSA and PAO1 to gauge their efficacy. Initial colony count data when deposited on gold chips seemed to suggest similar activity as seen with ML (fig. 4.36), with viable cell reduction of over 50% on pp-PSSM films from control.

![Figure 4.36: Quantification of MSSA growth from pp-PSSM treated and non-treated gold surfaces after 24h](image)

Additionally, fluorescence microscopy was used to monitor the Petri dish surface after exposure to bacteria (fig. 4.37).
Figure 4.37: MSSA growth and viability on non-treated (left) and pp-PSSM treated (right) Petri dishes after 24h

Note much fewer cells overall were seen on the treated surface and the viability of cells was very low compared to near-100% live cells on the control. Up until this point all work was carried out using minimal media for bacterial growth, which generally doesn’t allow for rapid cell reproduction and growth. It was decided to use LB media in place of the minimal variety, which would exceed the favourable conditions found in the human body. Optimised growth conditions allowed facile bacterial propagation and overwhelming of surfaces by cells from solution to provide an extreme test of antimicrobial efficacy. Also reduced was deposition time, to a standardised 1h.

As seen in fig. 4.38, bacterial viability measured in effluent solution was not greatly reduced in Petri dishes coated with pp-PSSM than the control. Unfortunately, it proved too difficult under these conditions to provide calibration of Live/Dead stain in solution due to large amounts of bacteria creating problems with the excitation/emission of light. When attempts were made to heat-shock cells, a large amount of clumping occurred, creating uncertainty in results. Therefore it was only possible to compare cell viability to the control, not outright quantify.
The surface cell viability was not too dissimilar for MSSA than solution tests, but the reduction in viability of attached PAO1 was marked (fig. 4.39). For the MSSA assay cell viability was reduced for the Petri dish relative to the final control, but also evidenced was a significant amount of live cells relative to the initial control taken after 10 minutes. This situation was reversed in the PAO1 samples, where cell viability was reduced from both the initial and final controls. The discrepancy observed between *S. aureus* and *P. aeruginosa* susceptibility to both free PSSM compound and pp-PSSM films is likely due to the inability of *P. aeruginosa* to form a protective biofilm quickly enough in response to the toxin, whereas *S. aureus* cells do not use biofilms as a primary form of protection so showed better survival rates.
Initial control populations showed lower levels of live cells than the final control due to the change stage of growth the population was experiencing. When culture was added to the Petri dishes, the growth was likely stationary so there was considerable levels of dead cells in solution hence the low live/dead ratio. Once adapted to the environment in the Petri dish, cells which attached to the control surface experienced an environment ideal for their development and were consequently more viable when removed than the initial culture.

The combination of data in figures 4.38 and 4.39 in conjunction with AAS data indicates that pp-PSSM films are surface active rather than demonstrating leaching meaning that they will not present a toxic hazard to humans, and provided the surface is not swamped with enormous quantities of cells will remain active for a prolonged period of time. It is possible that fragmentation and cross-linking of organic components of the monomer effectively act to trap the active ions within the film, which may only act upon interaction with bacteria.

Another interesting study was the investigation of PAO1 cell viability relative to film deposition time. Fig. 4.40 shows the increasing IR absorbance as a function of plasma reaction length, indicating increased film deposition over time. Film content was compared with cell viability at each time-point and it was observed that the kill-rate of pp-PSSM increased as greater film content went down, although not linearly.
Greatest reduction in absolute cell viability was seen after 2h deposition, with a 50% further reduction after 4h and a four-fold decrease from this point to 16h film deposition. It would suggest that the initial film deposition killed a reasonable amount of cells but subsequent deposition only served to homogenise the surface activity or provide additional active material which was able to find its way to the surface.

Figure 4.40: FTIR absorbance intensity of pp-PSSM as a function of time at 50W CW (top) and associated decrease in PAO1 cell viability after 24h as the film deposition time was increased (bottom)
4.7.2.4 Non-woven assays

Up to this point all bacterial work had been carried out on largely 2-dimensional surfaces in Petri dishes and gold. It was decided to move onto depositing pp-PSSM films on 3-dimensional polymeric networks in order to assess viability of this procedure for treating such materials as wound dressings. Polypropylene taken from inlay of disposable nappies was chosen as a cheap model system.

An initial modified disc diffusion assay was performed on a lab culture of Gram-negative *Escherichia coli* DH5α. Treated and non-treated non-woven was placed on a lawn of bacteria and incubated in the same manner as a disc diffusion assay. The image in fig. 4.41 dramatically displays activity of pp-PSSM on the bacterial cells, where growth was almost completely inhibited below the squares of treated non-woven, whilst the areas underneath the control non-woven showed normal growth.

![Image](image_url)

**Figure 4.41**: Modified diffusion assay demonstrating effect of pp-PSSM deposited squares of non-woven on *E. coli*

Also observed was the zone around non-woven materials, where growth was not inhibited beyond the limit of material in pp-PSSM or control but silver nitrate treated non-woven showed inhibition as a result of leaching. This further confirmed the pp-PSSM mode of action against bacteria to be contact-killing rather than solution-based.
Due to the multi-faceted nature of non-woven materials, confocal microscopy was employed to assess cell viability visually. This method also presented an opportunity to simply determine the best position to place samples in the plasma reactor for optimised activity against bacteria (fig. 4.42).

These confocal images show the greatest reduction in cell viability was further from the monomer source, most noticeably in the plasma afterglow region. This is in agreement with previous work discussed in chapter 2 where greater functionality is retained in the region after the main plasma glow. Consequently, this region was used for all subsequent pp-PSSM depositions and confocal images from non-wovens deposited on in this region are shown in figures 4.43 and 4.44.
Confocal images on non-wovens displayed the basic activity characteristics of previous assays where *P. aeruginosa* was more susceptible to pp-PSSM films than *S. aureus*. The reduction in MSSA cell viability was less than seen for PAO1, although was still significant versus the control. Plasma film efficacy was dramatic against PAO1, with the vast majority of cells bound to the material killed. An additional note of interest was bright spots on the treated non-woven which could be indicative of localised silver.
A quantitative method of assessing microbial growth on non-woven materials was use of the Japanese industry standard assay (JIS). This technique is a derivation of the general assay procedure used previously, but with a much smaller amount of solution and a carefully controlled initial concentration of bacteria which allows easy comparison between assays. Squares of non-woven were inoculated with bacteria, incubated, then the cells shaken off and plated. Results of the first attempt at this assay are shown in fig. 4.45.

![Figure 4.45: JIS assay of PAO1 on pp-PSSM treated squares of non-woven after 18h](image)

Despite the antibacterial nature of pp-PSSM films displayed via alternative means, the JIS assay showed a slight increase in microbial growth on these films over the control. It was seen during application of culture to the treated non-woven that a great deal of hydrophilicity was imparted due to plasma deposition, which was possibly due to oxygen incorporation into the film as evidenced in XPS. Subsequently, the solution found it far easier to reach all of the non-woven extremely quickly carrying with it bacterial cells. This therefore not only aided transport of cells to the non-woven surface, but provided an ample supply of cells in solution suspended between fibres which were not affected due to the contact-killing mechanism these films employ. Additionally, it was found by confocal microscopy
that all cells were not removed from the surface by vortexing the non-woven, which provided another source of underestimation of film activity (fig. 4.46). To counter-act these effects, an additional hydrophobic layer was plasma deposited onto the pp-PSSM film.

**Figure 4.46:** Confocal microscope images of PAO1 cell viability on control (top) and pp-PSSM treated non-woven (bottom) post-JIS assay vortex

### 4.8 Fluroinated adlayer

As discussed in chapter 1, hydrophobic polymers and plasma films are commonly used to impart non-fouling characteristics onto materials. With this in mind, it was decided to modify plasma films containing active metals with a very thin top layer which would prevent or reduce initial bacterial attachment through either blocking or hydrophobic effects. Attempts were made within the group to use a PEG-like monomer (ethylene glycol diacrylate) to produce non-fouling films, with mixed results. Use of the liquid monomer was expensive and long effective deposition time required meant other avenues were investigated. Subsequently, hexafluoroethane was used as a non-fouling layer. This compound offered a simple,
Gaseous solution to non-fouling surfaces through plasma deposition. FTIR of a pp-C₂F₆ reaction on polypropylene non-woven is shown in fig. 4.47.

**Figure 4.47:** FTIR spectrum of fluorinated non-woven versus non-treated non-woven. A large peak assigned to fluorocarbon deposition was observed at ca. 1660 cm⁻¹.

Once proof of deposition onto non-woven was observed, the plasma parameters were optimised to 50W CW for 30s. A short deposition such as this proved sufficient for significant hydrophobic behaviour (fig. 4.48).
As can be seen, the plasma-treated non-woven with fluorocarbon showed vastly increased hydrophobicity relative to the control, which was less hydrophilic (absorbed water less facilely) than previous pp-PSSM treated non-woven. This ability was also passed over to pp-PSSM treated non-woven when subject to pp-C_2F_6 adlayer, thereby reducing total liquid in contact with surface of non-woven.

### 4.8.1 pp-PSSM + pp-C_2F_6 assays

A fluorinated adlayer was applied after pp-PSSM film deposition *in-situ* and the JIS assay was repeated at 18h, with non-bound cells washed from the solution before the attached cells were vortexed into solution and plated. This gave a much better antimicrobial result for PAO1 than without the adlayer, as shown in fig. 4.49.

**Figure 4.49:** JIS assay of PAO1 on pp-PSSM + pp-C_2F_6 treated squares of non-woven after 18h
Less absolute cell growth (i.e. control) was observed on non-woven than without a wash step (~3 versus ~6 x 10^7 CFU ml^{-1}) and the growth on treated material was reduced to about 40% relative to the control.

All assays to this point had been conducted with a bacterial incubation time of between 18-24h, but with the intended use of such films in applications which may require shorter exposure to large amounts of bacteria such as wound dressings, investigation of shorter incubation time was required. As such, an assay was performed with 3h incubation, the results of which are shown in fig. 4.50.

**Figure 4.50:** JIS assay of MSSA (l) and PAO1 (r) on pp-PSSM + pp-C_2F_6 treated squares of non-woven after 3h

PAO1 growth relative to the control was around 10% after 3h exposure to treated non-woven. This was around a four-fold increase in the antimicrobial efficacy when compared to activity after 18h, which suggests further evidence for a contact-killing mechanism as a foundation of dead cells after this time will allow a platform of live cells to attach on top thereby reducing efficacy as time wears on. A common theme observed was greater activity of the treated film against PAO1 rather than MSSA, for reasons discussed previously. MSSA growth however, was still around a 6^{th} of that seen for the control.
4.9 Cytotoxicity study

In addition to antibacterial activity, an important aspect of this study in the context of potential application was testing for surface toxicity against eukaryotic cells. An independent test on pp-PSSM treated Petri dishes with a covered centre during deposition creating an internal control showed preference of amoeba to settle only on the control area through visualisation.

In order to carry out quantitative cytotoxicity tests, plasma films were deposited onto glass coverslips and alongside oxygen plasma treated and non-treated coverslips were exposed to cultures of Swiss mouse fibroblasts and human neonatal epithermal keratinocytes in suitable media. Due to the sensitive nature of studying eukaryotic cells and ease of contamination, work was carried out in conditions with the minimum chance of compromising sterilisation. Cells were allowed to attach to the films and their viability observed by optical absorbance after 1-3 days. Results of this experiment are shown in fig. 4.51.
No cytotoxic effect was evidenced in the tests of pp-PSSM films against either type of eukaryotic cell. There were negligible differences in optical density measurements recorded for all surface tests which related to viability of cell population.
4.10 References


(34) Vice, B., University of Bath, 2008.

**Chapter 5:**
Chapter 5: Novel zinc- and copper-Schiff base complexes for the plasma deposition of antimicrobial films

Novel zinc and copper Schiff base complexes were synthesised, characterised and found to inhibit the growth of *P. aeruginosa* and *S. aureus*. Both complexes were used as precursors in the plasma deposition of thin films, of which pp-ZSB 1/40 pulse duty cycle parameters were found to retain much structural integrity of the monomer thorough FTIR, XPS and SIMS mapping. pp-ZSB films were found to inhibit microbial growth according to duty cycle, with a correlation drawn between monomer retention and efficacy. 1/40 duty cycle films were found to be bacteriostatic and significantly slowed bacterial growth over a period of 24h when deposited on polystyrene and non-woven materials. The films were also found to be completely harmless to eukaryotic cells after exposure of between one and three days.

5.1 Antimicrobial zinc and copper

The mechanism by which zinc and copper act against micro-organisms is, like silver, somewhat of a mystery and appears to be complex. Zinc and copper exhibit strong binding characteristics to intra- and extra-cellular proteins and active sites such as amino groups e.g. cysteine, histidine; causing similar problems to silver. Unlike silver, both metals are required in cell function e.g. zinc fingers and as such mechanisms of import and export are facilitated by proteins contained in the cell membrane. In the case of excess intra-cellular zinc, Gram-negative bacteria pump ions from the cell via a three-polypeptide complex and *Pseudomonas* contains a four-polypeptide complex which expels Cu (II) to maintain cationic homeostasis. Due to the ubiquitous nature of zinc, the general trend of toxicity to higher organisms follows for bacteria with copper more harmful than zinc. As such, resistance can be built by cells exposed to very high levels of these metals.
5.2 Metal chemistry

5.2.1 Zinc

Zinc is a metallic element found at the far right-hand side (group 12, row 1) of the d-block, ground-state configuration [Ar] $4s^2\, 3d^{10}$. It is a ‘harder’ element than silver due to less shielding effects and has one principle oxidation state of +2 resulting from removal of the 4s electrons. The cation $\text{Zn}^{2+}$ is known as a ‘borderline hard’ Lewis acid, partly as a consequence of ionic radius contraction so tends to bind ‘borderline hard’ Lewis bases.\(^{6,7}\)

5.2.2 Copper

Copper is a metal found directly above silver and to the left of zinc in the periodic table (group 11, row 1). The ground-state configuration of copper, [Ar] $4s^1\, 3d^{10}$ would suggest a tendency to form predominantly ‘soft’ Cu\(^+\) analogous to silver, but due to the stabilising Jahn-Teller distortions caused by the orbital degeneracy of $d^9$ configuration can also form the ‘borderline hard’ oxidation state (II) analogous to zinc.\(^{5-7}\)

5.3 Novel compound selection

Previous work in the area of zinc polymer synthesis showed a bipyridyl-zinc (II)-bicarboxylate system was stable enough for radical-initiated polymerisation into a coherent material.\(^8\) On account of this, work was conducted within the Jenkins group focused on synthesising these and analogous compounds for potential plasma deposition (fig. 5.1).
Unfortunately, very high melting points resulting from the high intermolecular forces such as π-stacking and hydrogen bonding meant these compounds had limited potential for plasma application and were subsequently utilised in conventional polymers instead.

Due to the time spent investigating monomers such as those depicted above only to find them unsuitable through attempted plasma deposition, a set of criteria was drawn up for preparing new metal-containing antimicrobial plasma monomers. Compounds should be antimicrobial, cheap to make, of low melting point or high volatility, of reasonable stability, unsaturated and the ligand of limited toxicity. Consequently, a step-wise approach was taken to quickly establish monomer potential for use as an antimicrobial plasma precursor (fig. 5.2).
Potential ligand systems were identified, synthesised and complexed with the metal, then characterised with a primary concern being melting point in the absence of thermogravimetric analysis. Monomers of suitably low melting point were subjected to antibacterial quantification, with a positive result giving the green light for further exploration using plasma.

The first compound to be successfully produced via this systematic approach was a Schiff base coordination complex. Schiff bases are a class of compound formed by the condensation of a primary amine with a carbonyl, named after Hugo Schiff. Such a broad description of compound allows scope for much functional group substitution and complexes incorporating nitrogen and oxygen have proven excellent coordinators of transition metals. In particular, Schiff bases resulting from the reaction of salicylaldehyde and primary amines complexed with metals have shown good antibacterial efficacy due to metal coordination reducing the polarity of the metal ion, so reducing recognition by the cell and increasing lipophilicity of the molecule. Additionally, inhibition of microbial growth by free Schiff base has been
evidenced, albeit to a much lesser degree than when complexed by metal (ca. 10-fold).\textsuperscript{11}

Salicylaldehyde is liquid at room temperature, as are simple primary amines meaning the resulting product (dependent on substitution) is likely to be liquid or of low melting temperature. Therefore allylamine, a short-chain alkene terminated primary amine was chosen to react with salicylaldehyde for the synthesis of a novel ligand system with the subsequent coordination of zinc and copper.

### 5.4 Novel Schiff base complexes

Synthesis of Schiff Base complexes was carried out in a two step process. The initial step was synthesis of the Schiff Base ligand using the general procedure of You et al\textsuperscript{11} followed by complexation with zinc or copper.

#### 5.4.1 Schiff base ligand synthesis

Salicylaldehyde (150mmol, 15.99ml) was dissolved in 30 ml of methanol. Allylamine (150mmol, 10.92ml) was added and the reaction refluxed for 2 hours (figure 5.3). The solution was allowed to cool and the solvent was removed under vacuum resulting in a yellow oil. The product was re-solvated in dichloromethane and dried using anhydrous magnesium sulphate, which was removed via gravity filtration. The solvent was removed under vacuum producing (E)-2-((allylimino)methyl)phenol, a yellow oil. (18.11g, yield 75%).

![Figure 5.3: The reaction of salicylaldehyde with allylamine to produce Schiff base ligand](image)

5-148
5.4.1.1 Schiff base ligand characterisation

The ligand product was dissolved in deuterated chloroform and characterised by NMR to obtain necessary detail on successful synthesis.

\[
\begin{align*}
\text{1H NMR (250 Hz) (ppm): } & \delta = 4.2 (d, 2H, -CH_2 (H_A)), 5.2 (m, 2H, =CH_2 (H_B)), 6.1 (m, 1H, -CH= (H_C)), 7.4 (m, 4H, C_6H_4OH (H_D)), 9.9 (s, 1H, -CH= (H_E)), 11.1 (s, 1H, OH). \\
\text{13C NMR (75 MHz) (ppm): } & \delta = 61.7, 116.9, 117.4, 119.0, 119.3, 131.8, 132.7, 135.2, 161.6, 166.1.
\end{align*}
\]

5.4.2 Zinc Schiff base synthesis

Schiff base ligand \((E)-2-((allylimino)methyl)phenol\) (100mmol, 16.12g) was dissolved in 30ml of methanol. Triethylamine (100mmol, 13.94ml) and zinc chloride (50mmol, 6.80g) were added and the reaction refluxed for two hours (fig. 5.4). The solution was allowed to cool and the solvent was removed under vacuum. The resulting orange/yellow oil was re-solvated in dichloromethane and water washed. The organic layer was removed and dried using anhydrous magnesium sulphate. The solvent was removed under vacuum and the yellow oil cooled to allow crystallisation. The yellow solid was isolated by filtration, washed with ice-cold hexane and dried \textit{in-vacuo} to yield the zinc-Schiff base complex, ZSB. (15.03g, yield 78%). Mp: 112-114°C.
**Figure 5.4:** Complexation reaction of zinc or copper with Schiff base ligand

5.4.2.1 ZSB characterisation

The novel complex ZSB was recrystallised from minimal hot toluene and a crystal structure obtained (fig. 5.5).

![Crystal Structure](image)

**Figure 5.5:** The crystal structure of zinc-Schiff base monomer ZSB

Selected bond lengths (Å): Zn(1)–O(1) 1.988(2); Zn(1)–O(2) 2.048(2); Zn(1)–O(2)_#1 2.085(2); Zn(1)–N(1) 2.051(3); Zn(1)–N(2) 2.070(3). Selected bond angles (º): O(1)–Zn(1)–O(2) 99.58(9); O(1)–Zn(1)–N(1) 91.27(10); O(2)–Zn(1)–N(1) 110.49(10); O(1)–Zn(1)–N(2) 90.43(10); O(2)–Zn(1)–N(2) 125.62(10); N(1)–Zn(1)–N(2) 122.65(11).

The ligand was reacted with zinc in the ratio 2:1, but the monomeric structure observed was a dimer containing four ligand units to two metals. No formal bonding was assigned between the metal centres. It would appear that due to the ionic size of Zn$^{2+}$ and the small nature of coordinating elements, greater coordination than tetrahedral arrangement was achieved. In addition to the expected coordination of...
nitrogen and oxygen, two oxygen atoms also acted as bridges between the metal centres resulting in the pentahedral coordination of each zinc ion. Bond lengths showed a slightly increased distance between the zinc ion and bridging oxygen over the non-bridging oxygen.

As with the pure ligand, ZSB was soluble in deuterated chloroform for NMR study.

\[ \text{1H NMR (250 Hz) (ppm): } \delta = 4.1 \text{ (d, 2H, -CH}_2\text{- (H}_A\text{)), 5.0 \text{ (m, 2H, =CH}_2\text{ (H}_B\text{)), 5.8 \text{ (m, 1H, -CH}=\text{ (H}_C\text{)), 6.8 \text{ (m, 4H, C}_6\text{H}_4\text{OH (H}_D\text{)), 8.3 \text{ (s, 1H, -CH}=\text{ (H}_E\text{)).} \]

\[ \text{13C NMR (75 MHz) (ppm): } \delta = 61.9, 113.5, 117.0, 118.6, 122.1, 132.2, 134.0, 134.7, 169.6, 170.4. \]

Reproduction of resonance shifts from the ligand NMR coupled with the appearance slightly further upfield due to the electropositive influence indicated the ligand was coordinated to the metal centre.

An FTIR spectrum was also obtained from ZSB crystals (fig. 5.6).
FTIR peaks (cm\(^{-1}\)):
- 3053, 2909 (C-H, Ph aliphatic);
- 1613 (C=C);
- 1594 (C=N);
- 1465, 1432 (C-H, Ph aliphatic);
- 1287 (C-O);
- 760 (C-H, Ph ortho disubstituted).

Of interest was the identification of key functional groups from both salicylaldehyde and allylamine precursors, notably the presence of phenyl and C=N groups. Peaks characteristic of functional groups present in the Schiff base were observed in alkene, phenyl, carbon-oxygen and carbon-nitrogen groups; and a lack of characteristic carbonyl peak confirmed the complete condensation reaction of the ligand. Additionally, absence of an O-H peak in the 3500cm\(^{-1}\) region in lieu of an alcoholic solvent confirmed deprotonation of the alcohol group in the salicylaldehyde portion, therefore complexation with zinc.

### 5.4.3 Copper Schiff base synthesis

The analogous complex containing copper, CSB was prepared in an identical manner to ZSB with copper acetate (50mmol, 9.10g) used in place of zinc chloride. A dark crystalline green solid was obtained. (14.17g, yield 74%). Mp: 120°C.
5.4.3.1 CSB characterisation

The copper-Schiff base complex was recrystallised from hot toluene and an x-ray crystal structure obtained (fig. 5.7).

![Figure 5.7: The crystal structure of CSB monomer]

Selected bond lengths (Å): Cu(1)–O(1) 1.8980(13); Cu(1)–O(2) 1.8899(13); Cu(1)–N(1) 2.0126(14); Cu(1)–N(2) 2.0007(14). Selected bond angles (º): O(2)–Cu(1)–O(1) 173.43(6); O(1)–Cu(1)–N(1) 91.19(6); O(2)–Cu(1)–N(1) 88.05(5); O(1)–Cu(1)–N(2) 88.25(6); O(2)–Cu(1)–N(2) 92.57(6); N(2)–Cu(1)–N(1) 178.78(6).

As evidenced from the crystal structure, CSB is unlike ZSB in that it forms a monomeric rather than dimeric structure. CSB also forms a square planar geometry, confirmed by O-Cu-O and N-Cu-N angles approaching 180º rather than tetrahedral geometry in a four coordinate system. This was due to copper predominantly forming octahedral structures in the oxidation state (II), owing to Jahn-Teller distortions providing increased stability through elongation of bonds along the z-axis. Although the monomeric crystal structure appears square planar, this geometry was effectively maintained through interactions with adjacent groups which provided a pseudo-octahedral geometry (fig. 5.8).
An extended lattice structure was observed where copper was weakly coordinated by aliphatic groups of the Schiff ligand through the z-axis and lead to \( \pi \)-stacking through phenyl rings and aliphatic protons. A result of this semi-ordered lattice was an increase in the melting temperature over ZSB despite the dimerisation experienced in ZSB.

Due to the paramagnetic \( d^0 \) configuration of copper (II), NMR analysis was unavailable. However, the FTIR spectrum of CSB (fig. 5.9) was almost identical to ZSB indicating Schiff base ligand presence and the green colour observed is characteristic of complexed copper (II).
FTIR peaks (cm⁻¹): 3019, 2922 (C-H, Ph aliphatic); 1619 (C=C); 1594 (C=N); 1465, 1428 (C-H, Ph aliphatic); 1318 (C-O); 760 (C-H, Ph ortho disubstituted).

Slight differences in peak shift between CSB and ZSB were ascribed to coordination and supramolecular structure.

Preparation of both ZSB and CSB were also attempted at up to 150mmol with no change in yield, proving scaleability of these syntheses which is important for potential application.

### 5.5 Plasma deposition of ZSB and CSB

Both zinc and copper Schiff base complexes were found to have suitably low melting points and acceptable minimum inhibition concentrations (see 5.6) so were investigated for plasma deposition. As with PSSM, plasma parameters were altered to obtain optimal functional group retention in the resulting films.

First attempts at pp-ZSB and pp-CSB deposition were conducted at room temperature i.e. no monomer heating, in a 50W CW plasma for 1h at monomer flow rates of 0.11 and 0.09 s cc⁻¹ min⁻¹ respectively (fig. 5.10). Under these conditions
only a thin film was formed with little functional group retention from the monomer structure.

![Figure 5.10: FTIR spectra of pp-ZSB and pp-CSB at 50W CW without monomer heating for 1 hour]

Therefore, monomers were heated to melting point in order to increase volatility. Subsequent flow rates were increased to 0.37 and 0.26 sscm min\(^{-1}\) for pp-ZSB and pp-CSB depositions reflecting the melting point order, with a marked improvement in absorption reflecting increased film deposition despite halving the reaction time to 30 minutes.

A problem arising from heating the monomer chamber was considerable condensation of gaseous complex on cold areas of glass, notably the glass neck of the monomer chamber leading to the plasma chamber. This had an influence on the monomer flow rate; therefore new glass chambers with short necks were created in
order to reduce the distance between the monomer source and the substrate (fig. 5.11).

![Figure 5.11: Comparison of old (l) and new (r) monomer chambers](image)

A result of this was much improved flow rate for pp-ZSB in particular (0.53 sscm$^3$ min$^{-1}$ versus 0.26 sscm$^3$ min$^{-1}$ for pp-CSB) which was reflected in increased film deposition and functional group retention. Subsequent reactions were carried out under a range of duty cycles (CW, 1/40, 10/40 and 40/40) in order to ascertain which conditions provided a film with greatest functionality (fig. 5.12).
Continuous wave films showed a marked improvement again from those deposited under the same conditions with longer-necked monomer chambers with notable peaks relating to the original monomer structure present. However, due to ionising effects the CW films showed far broader peaks in the FTIR spectra than pulse duty cycle reactions. Both 10/40 and 40/40 reactions showed improved film definition over CW conditions but as expected from theory, 1/40 duty cycle with least ionisation and longest off time relative to pulse on time provided the films with greatest functionality. This was important as the trend of functionality retention creating more antimicrobial films was seen in the antibacterial effects of these plasma films (section 5.6). Therefore, the standard deposition duty cycle used was 1/40 pulse.

Absorbance intensity of pp-ZSB and pp-CSB increased in the heated monomer film spectra from the cold monomer spectra according to the relative flow rate increase but there was substantial functional group discrepancy evidenced between the two metal centred complexes, with pp-ZSB showing more resemblance to the monomer. FTIR spectra of pp-ZSB films also showed much greater IR activity than pp-CSB.

**Figure 5.12:** FTIR spectra of pp-ZSB (l) and pp-CSB (r) films at 50W under a range of duty cycles with heating for 30 minutes.
films. Additionally, films deposited from ZSB were strongly yellow in colour whereas those of CSB showed no noticeable colour change in the substrate. These factors were possibly due to break-up of the inherent crystal structure of CSB which may have been necessary for complex stability and consequently did not carry over to the plasma film. Subsequently, the greatest film characterisation was focused on the pp-ZSB 1/40 system (fig. 5.13).

Figure 5.13: FTIR spectrum of pp-ZSB at 50W under 1/40 pulse duty cycle for 30 mins

pp-ZSB film structure was considered to be very close in composition to the precursor, indicating the mild conditions experienced during pulse plasma reactions. Oxidation occurred as evidenced by the insertion of a strong O-H peak in the FTIR spectrum, along with broadening of the band in the 1600cm⁻¹ region. Other peaks present in the monomer were retained such as C-H stretches, with C=N obscured by peak broadening.

Standard pp-ZSB and pp-CSB deposition parameters established and used during this study are displayed in fig. 5.14 below:
<table>
<thead>
<tr>
<th>Monomer</th>
<th>Monomer temperature (°C)</th>
<th>Duty cycle (peak power)</th>
<th>Flow rate (sscm(^3) min(^{-1}))</th>
<th>Base pressure (mbar)</th>
<th>Film deposition rate (nm min(^{-1}))</th>
<th>Deposition time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZSB</td>
<td>140</td>
<td>CW, 40/40,10/40, 1/40 (50 W)</td>
<td>0.53 +/-0.07</td>
<td>0.020</td>
<td>1.1 nm min(^{-1})</td>
<td>30 min</td>
</tr>
<tr>
<td>CSB</td>
<td>140</td>
<td>CW, 40/40,10/40, 1/40 (50 W)</td>
<td>0.26 +/-0.05</td>
<td>0.022</td>
<td>1 nm min(^{-1})</td>
<td>30 min</td>
</tr>
</tbody>
</table>

**Figure 5.14:** Standard reaction conditions and parameters for pp-ZSB and pp-CSB deposition

In place of SPR, the use of alpha-step, in conjunction with a quartz crystal microbalance was used to define physical film characteristics. A combination of these techniques was used to generate film thickness and mass measurements, which were processed to give estimated film properties (fig. 5.15).

<table>
<thead>
<tr>
<th></th>
<th>pp-ZSB</th>
<th>pp-CSB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass (µg cm(^{-2}))</td>
<td>14.57</td>
<td>6.42</td>
</tr>
<tr>
<td>Moles of monomer (nmol cm(^{-2}))</td>
<td>37.80</td>
<td>16.67</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>32 +/-2</td>
<td>30 +/-4</td>
</tr>
<tr>
<td>Density (g/cm(^3))</td>
<td>4.55</td>
<td>2.14</td>
</tr>
</tbody>
</table>

**Figure 5.15:** Estimated plasma film physical properties for 50W 1/40 pulse duty cycle deposition for 30 minutes

Absolute mass of films deposited varied quite considerably between ZSB and CSB, with the zinc-containing films containing more material despite similar thicknesses, which was in agreement with flow rate measurements. Density of the films was therefore significant as greater metal content would increase density, suggesting that metal content of pp-ZSB was around twice that of pp-CSB. This was important for
antimicrobial implications of the film, although the relative toxicities to of the two metals to bacteria (i.e. copper>zinc) meant the films may still show similar activity.

SEM of pp-ZSB/CSB films did not provide sufficient information so the more sensitive approach of TEM was utilised (fig. 5.16). Zinc-containing films appeared to show a microstructure repeat pattern indicative of polymeric structure whereas the copper films were considerably smoother in microstructure but with greater topographical deviation.

![Figure 5.16: TEM images of pp-ZSB (l) and pp-CSB (r) films at 50W 1/40 pulse duty cycle for 30 minutes](image)

EDS did not pick up presence of zinc or copper in the films, although the inherent use of copper grids as base for the carbon substrate precludes the area-specific identification of copper. The limit of detection of around 0.1%at may also have caused problems in observing the metals. XPS however, which offers a similar level of detection picked up zinc and fluorine content in pp-ZSB/pp-C\textsubscript{2}F\textsubscript{6} films at around 0.1 and 1.1% respectively while showing evidence of Schiff base ligand retention (fig. 5.17). The survey spectrum of this film gave the following atomic compositions (with Si from underlying substrate):
Si 2p 47.51%; C 1s 7.37%; N 1s 0.92%; O 1s 42.89%; F 1s 1.11%; Zn 2p 3/2 0.09%

**Figure 5.17:** XPS spectra of C 1s binding energies in pp-ZSB film at 50W 1/40 pulse duty cycle for 30 minutes

The carbon 1s peaks appeared in an approximate ratio of 3:2:1 (low-high energy), where the ‘low’ energy peak was assigned to hydrocarbon constituents of the Schiff base ligand; the ‘middle’ energy (1.0-1.7 eV higher) to amine and carboxy functionality; and the high energy peak to oxidation of the film (as seen in FTIR spectra) with C=O and O-C=O expected at around 3.0-4.0 eV above the hydrocarbon. Oxidation and potential fragmentation disallowed the possibility of accurate ligand quantification within the film. This data coupled with FTIR spectra show that despite potential fragmenting conditions experienced in plasma, structural retention can still be achieved to a high degree under mild conditions.

In place of dissolving films for regular mass spectrometry, the surface-specific technique secondary ion mass spectrometry was used to identify the location and distribution of zinc in pp-ZSB films (fig. 5.18).
Although at appreciably low levels confirmed by XPS, zinc (red) appeared to be evenly distributed throughout the film alongside Schiff base ligand. SIMS and XPS analysis of pp-CSB films did not detect presence of copper.

AAS detected leached zinc and copper in solution at only 20ppb and 10ppb respectively, therefore confirming the reason for low density observed for pp-CSB films relative to pp-ZSB films. Measurement of zinc in the dissolved pp-ZSB films deposited under standard conditions for 30 minutes showed low concentrations of metal, confirming the data from other analytical techniques (fig. 5.19).
5.6 Microbiological assays

Assays developed for assessing the antibacterial activity of PSSM and plasma films were used to analyse the efficacy of zinc and copper monomers and polymers.

5.6.1 Monomers

5.6.1.1 Disc diffusion

Due to the highly hydrophobic nature of the Schiff base ligand, both ZSB and CSB could not be pelleted for the disc diffusion assay. Instead, the compound was placed loose on the agar surface to judge for antibacterial properties (fig. 5.20).
Even accounting for the lack of pellet, the zone of inhibition surrounding ZSB was quite considerable and the yellow colour was spread throughout the agar surface, indicating the volatility of the compound. By contrast, CSB did not seem to disperse to the same extent perhaps reflecting the crystalline nature of the complex and a more modest inhibition ring was observed despite the stronger antimicrobial nature of Cu over Zn.

5.6.1.2 MIC

As with PSSM, minimum inhibition concentrations were observed for ZSB and CSB in solution. These are summarised in fig. 5.21.

<table>
<thead>
<tr>
<th>Complex</th>
<th><em>P. aeruginosa</em> PAO1 MIC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th><em>S. aureus</em> MSSA MIC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZSB</td>
<td>1.8</td>
<td>210.6</td>
</tr>
<tr>
<td>CSB</td>
<td>13.5</td>
<td>97.6</td>
</tr>
<tr>
<td>Schiff base ligand</td>
<td>900.1</td>
<td>2710.0</td>
</tr>
</tbody>
</table>

Figure 5.21: MICs determined for ZSB, CSB and Schiff base versus MSSA and PAO1
It was clear from these experiments that the Schiff base ligand alone has a negligible impact on bacterial growth compared to both metal-containing moieties. Interestingly a counter-result to the disc diffusion assay was observed in CSB and ZSB solution activity against MSSA, where CSB was around twice as effective as ZSB, possibly as a result of liberated Cu\(^{2+}\) ions upon crystal structure collapse. It could be the case that solvent molecules acted as coordinating ligands to displace intermolecular forces and maintain \(d^0\) configuration, thereby altering interaction between complex and cells. Also of interest was a much lower MIC for ZSB versus PAO1 than CSB which was the opposite case than for MSSA. ZSB MIC was also over twenty times less than observed for zinc acetate versus PAO1, indicating the synergistic impact of ligand composition and perhaps coordination energy. It would appear that zinc acetate allows free Zn\(^{2+}\) ions in solution which are recognised by the cell and subsequently pumped out, whereas complexed zinc in ZSB is not as readily identified and ligand exchange may occur within the cell, causing damage to proteins and DNA.

5.6.2 Plasma film assays

5.6.2.1 Efficacy confirmation

Initial antibacterial tests were carried out as cursory examinations on non-woven polypropylene as a pseudo-disc diffusion assay (cf. pp-PSSM – fig. 5.22).

**Figure 5.22:** Untreated (l) and pp-ZSB treated non-woven (r) diffusion assay on MSSA coated agar
It was observed that analogous to pp-PSSM there was very little diffusion of active product while the area immediately beneath the treated fabric was almost completely clear of visible bacterial growth. This confirmed the viability of pp-ZSB as a contact-killing antimicrobial surface. Subsequent assays were therefore carried out on Petri dishes and non-wovens to quantify the degree of activity.

As expected from low diffusion and limited metal content in solution, bacteria suspended in media exposed to pp-ZSB and pp-CSB surfaces were largely unaffected in growth. Despite the ionising conditions in continuous wave plasma and subsequent fragmentation of the molecule, no noticeable difference was observed in Live/Dead solution tests between CW and 1/40 pulse plasma duty cycle films. Bacteria attached to surfaces however were much more limited in viability (fig. 5.23). Bacteria were exposed to plasma films deposited at 50W under 1/40 duty cycle for 30 minutes in minimal media.

Figure 5.23: Live/Dead cell ratios of MSSA (l) and PAO1 (r) from pp-ZSB treated and non-treated Petri dish surfaces after 24h

It was clear from these results that plasma reaction duty cycle and resulting monomer structural retention in films was directly related to antimicrobial efficacy of the surface. Continuous wave plasma films showed least contact-killing of both PAO1 and MSSA of all pp-ZSB films but were relatively more effective against PAO1. In terms of functional group retention, both 10/40 and 40/40 films produced similar FTIR spectra and consequently similar activity versus both bacterial strains; while 1/40 films showed the greatest monomer retention and efficacy against
bacterial cells. This provided further evidence for the requirement of the metal centre to be coordinated to the Schiff base for optimised activity.

As observed in FTIR and other characterisation methods, pp-CSB films did not contain as much monomer functionality or indeed metal content as pp-ZSB, so it was no surprise that reproduction of surface assays on these films resulted in less relative reduction in bacterial growth, although interestingly there was a large discrepancy between CW and 1/40 pulse duty cycle film efficacy for PAO1 again indicating influence of duty cycle. Therefore, the following assays were therefore all carried out on pp-ZSB 1/40 pulse duty cycle films.

5.6.2.2 Time dependence

Up to this point, all assays on plasma films had been carried out after a standardised incubation time of between 18-24h, with the exception of a 3h assay on pp-PSSM. In order to assess how film activity altered over time, an assay was conducted over a period of 24 hours where 72 Petri dishes were plasma treated, inoculated with bacteria and three control and three pp-ZSB treated Petri dishes removed from incubation every hour. Bacteria were removed from the surface in the standard procedure and between three and five dilution factors were spread on agar for counting the following day. It was therefore an extremely time- and material-expensive process but yielded very useful information on the antibacterial efficacy of the zinc-Schiff base plasma film over time (fig. 5.24).
The results of this study were quite revealing as the bacteriostatic effect pp-ZSB has on *Pseudomonas* cells over time was clearly seen. Initial concentrations of bacteria were low on both the control and treated surfaces but grew extremely rapidly on control dishes during early hours of the assay when cell viability declined on treated dishes. This is seen more clearly in fig. 5.25. After this time, bacterial concentrations were seen to increase on pp-ZSB surfaces with a growth lag to control evident which stayed relatively constant for the length of the assay, resulting in large differences in viable cell count at the end of the assay. This effect was probably seen, because bacteria which attached and subsequently died during the early stages of the assay then provided a platform for bacterial cells growing in exponential phase in solution to attach and proliferate i.e. early stage biofilm growth.
5.6.2.3 Non-wovens

To supplement quantitative data derived from assays on Petri dishes, treated non-woven materials were also tested. Initial JIS assays suffered from the same problem of hydrophilicity imparted on the fabric as pp-PSSM non-woven assays (fig. 5.26).

**Figure 5.25**: First 4h of time-dependence assay in fig. 5.24

**Figure 5.26**: JIS assay of PAO1 on pp-ZSB treated squares of non-woven after 18h
Growth evidenced on the treated non-woven appeared to slightly exceed that of the control but essentially the same level of growth was seen. This observation was again contrasted by confocal microscope images of treated and non-treated non-woven which showed much reduced viability of attached cells (fig. 5.27).

**Figure 5.27:** Confocal microscope images of MSSA (l) PAO1 (r) on pp-ZSB treated non-woven after 24h

Activity of pp-ZSB films observed via this method was in agreement with both MIC data and Live/Dead stained cells vortexed from the surface shown above, where MSSA was not quite as susceptible as PAO1. Nevertheless, red cells of both bacteria appeared on non-woven strands although interestingly live planktonic phase MSSA cells were also visualised, highlighting the contact-kill mechanism of these films.

pp-ZSB films were coated with a pp-C$_2$F$_6$ adlayer to reduce hydrophilic effects in the fabric and a shorter assay run with results of the time dependence assay on Petri dishes in mind (figures 5.28 and 5.29). pp-CSB films were also tested and treated as an additional control.
A massive decrease in viable *Pseudomonas* cell count was observed for the pp-ZSB treated non-woven after four hours compared to both the initial and final controls, and pp-CSB films. This was important as it validated the results of the Petri dish assay and proved the effectiveness of deposition on a different substrate. As can be seen from the image of colonies present on agar plates, there were similar numbers of viable cells from both control and pp-CSB treated non-woven which are both of 400x dilution. By comparison, the dish from pp-ZSB film was of 20x dilution and showed virtually no growth at all.
The evidence of growth on pp-CSB in the same magnitude as the control showed the ineffectiveness of the fluorinated adlayer without a truly active base film. When media was initially applied to non-wovens, the pp-CSB film caused the solution to ‘bead’ the most therefore was the most hydrophobic substrate and consequently would have shown most bacterial inhibition if this was a major factor alone. The presence of Schiff base in pp-CSB films also confirmed the need for a metal centre to coordinate activity against bacterial cells.

![Graphical data](image)

**Figure 5.29:** JIS assay of MSSA on pp-ZSB + pp-C₂F₆ and pp-CSB + pp-C₂F₆ treated squares of non-woven after 4h. Graphical data (top) and visual representation of bacterial colonies (bottom) from control (l), pp-CSB (c) and pp-ZSB (r) non-woven

Viable cell count (CFU ml⁻¹)

Surface

- Control Initial
- Control Final
- pp-ZSB
- pp-CSB
An almost identical scenario was presented when pp-ZSB films were tested against MSSA (fig. 5.29) with negligible growth versus the control, albeit with slightly less viable growth on pp-CSB films. Due to the impressive activity of pp-ZSB films deposited on non-woven materials after this short time-period, the time dependence assay used for Petri dishes was repeated for non-wovens against both MSSA and PAO1 (figures 5.30 and 5.31). This assay was conducted starting with 96 treated squares of treated and 96 of non-treated non-woven, taking three pieces of each from incubation every two hours and plating the solution derived of 3-5 dilution factors for each sample.

![Graph showing viable cell count (CFU ml⁻¹) over time (h) for control and pp-ZSB treated samples.](image)

**Figure 5.30:** Time-dependence assay of PAO1 on pp-ZSB treated non-woven

The non-woven time course assay showed an even greater lag period than for the Petri dish version versus PAO1, with around 10% growth on treated fabrics after 16h relative to the control. Importantly, all treated fabrics showed much less growth than the control fabric taken at the same time. The early stage of the assay was where the activity of pp-ZSB film was most apparent, with virtually no growth seen up to six hours. Growth seen after this point is likely due to multilayer formation of cell as discussed previously. The control meanwhile showed a drop in viable cells after two hours, most likely due to planktonic bacteria adapting to their environment during this time and suffering as a consequence; but proliferated extremely rapidly after this point.
Treated fabrics tested against MSSA showed a very similar trend to that seen for PAO1, although even less viable cells were observed at the final time point relative to control. Growth was also seen after six hours, albeit control growth was far in excess of this. Control growth followed the same pattern as for PAO1 where initial cell count was higher than that at two hours, which preceded rapid growth.

Time-dependence tests of pp-ZSB against both PAO1 and MSSA were very instructive in helping determine the mechanism by which the surface acts on bacteria. It appeared initial contact-killing was seen during the hours following inoculation with cells, followed by a period of slow growth which was potentially caused by transfer of ZSB throughout the bacterial population. The effects seen in this assay were seen despite using a higher initial inoculated cell count of bacteria in stationary phase (slow growing i.e. stronger defence) than would be experienced in an application setting, therefore it is likely these tests still underestimate real-life bacterial viability on films.

5.7 Cytotoxicity study

Despite excellent antimicrobial properties, it was necessary to expose eukaryotic cells to pp-ZSB surfaces to assess cytotoxicity. The same assay as for pp-PSSM was
followed, using cultures of Swiss mouse fibroblasts and human neonatal epidermal keratinocytes (fig. 5.32).

Figure 5.32: Optical absorbance readings from cytotoxicity tests of pp-ZSB films (red), pp-O2 treated (blue) and non-treated (green) glass coverslips against human epidermal keratinocytes (l) and Swiss mouse fibroblasts (r)

In keeping with pp-PSSM films, no cytotoxicity was observed as a result of eukaryotic cell interaction with pp-ZSB films.
5.8 References

Chapter 6: A novel highly volatile silver compound for the plasma deposition of antimicrobial films

A novel liquid organosilver complex was synthesised, characterised and found to inhibit the growth of *P. aeruginosa*. The complex was used as a monomer unit in the rapid plasma deposition of thin films, which demonstrated functional group retention from the precursor and considerable silver presence. These films were found to significantly inhibit microbial growth on non-woven polypropylene using a standard industrial assay.

6.1 Altering metal compound characteristics

As discussed in preceding chapters, antimicrobial metal-containing complexes can be produced quickly and cheaply through selection of the appropriate starting materials. Perhaps the most important single factor when choosing unsaturated ligands for potential plasma deposition of antimicrobial organometallics was inherent high volatility in the resulting product, which was not necessarily the case in some earlier molecules. Melting points of all compounds made previously were above 100°C and consequently required heating to encourage molecules into the gas phase. This would be undesirable in an application setting where monomers would be required to have a high flow-rate for quick deposition without heating. Therefore, significant efforts were made to reduce melting points of compounds to avoid intermolecular forces holding monomeric complex units together in a supramolecular structure. This approach was taken in the two broad manners: (i) systematic ligand substitution and (ii) Lewis-adduct substitution.
6.1.1 Ligand alteration

Previous efforts had shown maleimide with triphenylphosphine as suitable constituents in an organo-silver complex for plasma deposition, albeit with reasonably low volatility. The melting point of pure maleimide was observed at around 91°C, producing a silver salt silver maleimide and Lewis-base stabilised PSSM of melting points 228°C and 158°C respectively. Potential for strong intermolecular forces exists between monomers in PSSM, in the form of π-stacking and hydrogen bonding between carboxyls and protons of other maleimide olefin groups or triphenylphosphine groups. A route to avoid such interaction is to increase freedom of movement within the ligand system i.e. use ‘floppier’ ligands which are not as easily constrained within a crystal structure.

Phosphine-stabilised silver (I) carboxylates have previously been reported of the general formula R-Ag-(PPh3)2 where R = acrylic acid and 4-pentenoic acid,\(^1\) so proved a useful starting-point for adaptation with unsaturated moieties such as 3-butenolic acid (liquid) and maleic acid (fig. 6.1).

![Figure 6.1: 3-butenolic acid (l) and maleic acid (r)](image)

As seen in fig. 6.1, maleic acid has dicarboxylic acid functionality so could be used to introduce two units of silver per mole. Using the general synthetic route for PSSM preparation, silver carboxylates were made using a range of unsaturated ligands and subsequently stabilised with triphenylphosphine (fig. 6.2). Silver was also unsuccessfully reacted with the Schiff base used in chapter 5.
Melting points of these compounds however, did not prove a great improvement upon the PSSM system (fig. 6.3). It was seen that lengthening the alkyl chain on linear carboxylates from 4 carbons to 5 carbons reduced intermolecular forces within the compound, possibly as a regular alignment and packing was less easily achieved. When the double bond in the pentenoic acid precursor was replaced with a triple bond, the melting point was seen to decrease again due to the reduction in availability and correct alignment of protons for intermolecular bonding. The maleic acid derivative showed an increased melting point over the precursor as the di-metal centre and triphenylphosphine structure offered interaction between phenyl and olefin groups on adjacent molecules.

Cursory plasma deposition of these monomers was attempted but not continued due to low flow rates. Although altering ligand composition had some effect on increasing volatility in triphenylphosphine-stabilised silver complexes, it was clear that the major factor influencing intermolecular forces was the Lewis adduct.
6.1.2 Lewis adduct alteration

Alongside triphenylphosphine, the options for Lewis-base stabilisation of silver salts include alkyl phosphines as displayed previously,\(^2\) which provide the ‘floppy’ ligands required to reduce propensity of the compound to crystallise i.e. reduce symmetry and intermolecular forces. Therefore, trimethylphosphine (PMe\(_3\)), triethylphosphine (PEt\(_3\)) and tri-\(n\)-butylphosphine (P\(^n\)Bu\(_3\)) (fig. 6.4) were considered as potential alternative Lewis bases which remove the potential for \(\pi\)-stacking. Tri-\(tert\)-butylphosphine was also considered due to large steric bulk which would further stabilise the metal centre but was not of viable cost for potential upscale.

\[ \text{Figure 6.4: Potential alkyl phosphines for silver stabilisation: trimethylphosphine (l), triethylphosphine (c), tri-\(n\)-butylphosphine (r)} \]

Initial efforts in stabilising silver maleimide with two equivalents of PMe\(_3\) were conducted using the same synthesis as PPh\(_3\) under inert atmosphere on a Schlenk line. A white solid was yielded each time which decomposed during drying \textit{in-vacuo}. The difficulty of working with such air-sensitive compounds as alkyl phosphites (e.g. P\(^n\)Bu\(_3\) is pyrophoric, Schlenk techniques must be used) alongside unstable products meant an alternative Lewis base was sought.

Phosphites, a class of compound analogous to phosphines with an ester rather than alkyl linkage presented themselves as a viable option as they can be used under atmospheric conditions and are very cheap. The presence of a pre-oxidised alkyl chain was also favourable for ligand retention in the plasma glow. Triethylphosphite (P(OEt)\(_3\)) was investigated for use with silver maleimide, maleate and allylacetate using the synthesis in 6.2.1 producing yellow, purple and colourless liquids respectively (fig. 6.5).
Figure 6.5: Triethylphosphite (top) and liquid silver compounds synthesised (bottom)

These complexes dramatically demonstrated the ability of phosphites to sufficiently reduce intermolecular forces in order to produce a highly volatile monomer for plasma deposition. Subsequent plasma deposition attempts with both silver maleate and allylacate resulted in decomposition of silver in the monomer flask, which was proposed to occur via decarboxylation\(^2\)-\(^3\) (fig. 6.6). Decomposition was not evident for the Ag-N bound silver maleimide analogue and this system was therefore taken forward for more detailed analysis.

Figure 6.6: The possible decomposition pathway of silver vinyl carboxylates\(^2\)-\(^3\)
The results of this investigation into increasing silver compound volatility were transferred to zinc and copper compound synthesis. Attempts were made at making Zn$^{2+}$ and Cu$^{2+}$ analogues were unsuccessful due to the hard-soft acid-base principle meaning phosphorus was not of suitable orbital size to interact with either ‘hard’ ion. It is possible that the ‘softer’ Cu$^+$ ion would be more conducive to phosphorus reception to create a directly analogous compound of silver (I) complexes.

6.2 *tris*-triethylphosphito silver maleimide

The novel compound (PTSM) was prepared in a two step method similar to the one used for PSSM (fig. 6.7).

![Figure 6.7: Synthesis of tris-triethylphosphito silver maleimide](image)

**6.2.1 Synthesis**

To a stirred solution of maleimide (20mmol, 1.96g) in ethanol (20ml), a 10ml solution of silver nitrate (20mmol, 3.40g) in an ethanol/acetonitrile mixture (minimum MeCN) was added and the solution stirred for 5 minutes. After this time a solution of triethylamine (20mmol, 2.02g) in ethanol (5ml) was added dropwise over 30 minutes, resulting in the formation of an insoluble white precipitate. The solid
residue was isolated by filtration, washed three times with ethanol and dried in-vacuo, yielding silver maleimide (2.84g, yield 94%).

To a stirred solution of silver maleimide (10mmol, 2.04g) in THF (30ml), a stirred solution of triethylphosphite (30mmol, 4.98g) in THF was added and the solution stirred for 30 minutes. After this time the solvent was removed under vacuum, yielding a yellow liquid, tris-triethylphosphito silver maleimide (PTSM) (6.16g, yield 88%).

6.2.2 Characterisation

Unlike previous monomers it was not possible to crystallise PTSM, therefore a crystal structure was not obtained. PTSM was soluble in deuterated chloroform for NMR study with a very clean spectrum observed.

$^1$H NMR (300MHz) (ppm): $\delta = 6.4$ (s, 2H, -CH-CH-), 3.9 (p, 18H, P(O-CH$_2$-CH$_3$)$_3$), 1.2 (t, 27H, P(O-CH$_2$-CH$_3$)$_3$). $^{13}$C NMR (75 MHz) $\delta = 16.2$, 59.7, 136.0, 186.2.

An FTIR spectrum was also obtained (fig. 6.8).

![FTIR spectrum of PTSM](image)

**Figure 6.8:** FTIR spectrum of PTSM
FTIR peaks (cm⁻¹): 2980, 2900 (C-H, aliphatic); 1633 (C=O); 1608 (C=C); 1476, 1444, 1390 (C-H alkane); 1310, 1181 (C-O), 1013 (C-H vinyl) and 924 (C-H alkane).

In addition to peaks ascribed to silver-maleimide formation in chapter 4, absorptions diagnostic of triethylphosphite were seen in the PTSM FTIR spectrum. Carbonyl and alkene presence from the maleimide ligand was supplemented by the appearance of peaks relating to alkane and carboxy functionality. This gave additional confirmation of product formation in addition to NMR data.

### 6.3 Plasma deposition of PTSM

Due to the high volatility of PTSM and evidence of previous investigations pointing towards low duty cycle parameters contributing towards high levels of functional group retention and antimicrobial activity in the resulting films, initial plasma deposition of PTSM was run at 50W under 1/40 pulse duty cycle. An FTIR spectrum of such a deposition is seen in fig. 6.9 below:

![Figure 6.9: FTIR spectrum of pp-PTSM 50W at 50W 1/40 pulse duty cycle for 30 minutes](image)

Figure 6.9: FTIR spectrum of pp-PTSM 50W at 50W 1/40 pulse duty cycle for 30 minutes
As with pp-ZSB films, a strong correlation between monomer and plasma film structure was seen in using IR. Key absorbances assigned to alkanes were observed as sharp peaks identical to the monomer, while C=O and C-O bands were broadened by oxidation of the film. Removal of the strong vinyl C-H bend and C=C stretch indicate the elimination of alkene functionality consistent with radical polymerisation through the maleimide unit. The strength of film absorbances outweighed contribution of O-H band appearance due to oxidation, indicating a relatively thick and less ionised film was deposited which was related to very high flow rate of monomer (fig. 6.10). Identical FTIR spectra were observed regardless of substrate position within the reactor which was presumably due to the high flow of monomer ensuring similar reaction conditions throughout the reactor.

<table>
<thead>
<tr>
<th>Duty cycle (peak power)</th>
<th>Flow rate (sccm’ min⁻¹)</th>
<th>Base pressure (mbar)</th>
<th>Film deposition rate</th>
<th>Deposition time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/40 (50 W)</td>
<td>0.58 +/- 0.10</td>
<td>0.034</td>
<td>0.46 µg cm⁻² min⁻¹</td>
<td>30 minutes</td>
</tr>
</tbody>
</table>

**Figure 6.10:** Standard reaction conditions and parameters for pp-PTSM deposition

Reaction conditions experienced using pp-PTSM (fig. 6.10) were similar to that of pp-ZSB, with slightly higher flow rate seen for PTSM than ZSB despite no heat being applied to the liquid silver system. The deposition rate was also similar to pp-ZSB (fig. 6.11), with a slightly higher molar deposition per reaction indicating a likely more antimicrobially active film due to the relatively high toxicity of silver to zinc. Such observations also suggest this system is more viable for potential application as the heat source is not required.

<table>
<thead>
<tr>
<th>pp-PTSM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass (µg cm⁻²)</td>
<td>13.87</td>
</tr>
<tr>
<td>Moles of monomer (nmol cm⁻²)</td>
<td>19.76</td>
</tr>
</tbody>
</table>

**Figure 6.11:** Estimated plasma film physical properties for 50W 1/40 pulse duty cycle deposition for 30 minutes
Due to the low detection limit, x-ray photoelectron spectroscopy could not detect silver in pp-PTSM films, but potential evidence of ligand retention was observed. The survey spectrum of this film gave the following atomic compositions (with Si from underlying substrate):

Si 2p 45.24 %; P 2s 3.14 %; C 1s 12.76 %; N 1s 0.88 %; O 1s 37.87 %

As with pp-ZSB films, the relative intensity of carbon 1s peaks gave clues to functionality present within pp-PTSM films (fig. 6.12). Additionally, oxidation of the pp-PTSM film seen in FTIR spectrum was confirmed by the high oxygen content observed in XPS.

![XPS spectra of C 1s binding energies in pp-PTSM film at 50W 1/40 pulse duty cycle for 30 minutes](image)

**Figure 6.12:** XPS spectra of C 1s binding energies in pp-PTSM film at 50W 1/40 pulse duty cycle for 30 minutes

The low energy peak constituted around 50% of the total C 1s intensity, the middle energy around 41% of total and the high energy peak approximately 9%. This corresponded to the ‘low’ energy peak assigned to hydrocarbon i.e. the terminus of the triethylphosphite group and the base of maleimide, the middle energy (ca. 1.6 eV higher energy) to the C-O of triethylphosphite and the high energy (ca. 3.2 eV higher energy than hydrocarbon) to amide functionality. Relative intensity ratios and
assignment were therefore consistent with monomer retention of 1 maleimide to 9 triethylphosphite groups.

Like pp-ZSB films, SIMS was used to map silver content on the surface of pp-PTSM on silicon wafers (fig. 6.13). Unlike pp-PSSM films where phosphine-stabilised silver was observed in dissolved films, peaks assigned to pure silver ions and phosphate-silver moieties were seen and interestingly no phosphite bound silver was detected in major quantities. It could be the case that ion impact on the surface caused cleavage of these bonds, or they were broken by ionisation in the plasma reactor. In either scenario, silver was observed inhomogenously in large quantities across the surface and generally accompanied by organic constituents, indicating the potential for a surface which is highly antimicrobial.

![Figure 6.13: SIMS map and spectrum of metal and ligand content within pp-PTSM films at 50W 1/40 pulse duty cycle for 30 minutes](image)

If ionisation of bonds is caused within the plasma glow it is possible that the equivalent power of reaction may be sufficiently reduced to prevent this, through
lowering of 50W peak power or changing on or off time of duty cycle. However, if ionisation or fragmentation of bonds is required for plasma glow sustenance, then it is suggested unlikely for silver to be retained in monomeric fashion during plasma deposition.

AAS showed agreement to XPS and SIMS data, with low silver concentrations observed in dissolved and leached films.

6.4 Microbiological assays

As this system was developed later on in the project, extensive microbiological examination was not possible. However, work was conducted on monomer and plasma film which allowed for comparison with previous work on other surfaces. Additionally the system was utilised in direct comparison with commercial dressings (chapter 7).

6.4.1 Minimum inhibitory concentration

As for all other monomer systems, a minimum inhibition concentration was determined (fig. 6.14).
Interestingly, the MIC$_{50}$ of the phosphite-stabilised analogue of silver maleimide against PAO1 was measured as less antimicrobial than when phosphine-stabilised, at around $51 \times 10^{-6}$ mol dm$^{-3}$. Triethylphosphite ligands however, showed little to no activity against bacteria much like triphenylphosphine with an MIC$_{50}$ of over $16.5 \times 10^{-3}$ mol dm$^{-3}$, which leads to the conclusion that perhaps coordination number about the central silver atom plays a part in antimicrobial action. As ligand number was increased from two to three, a 50+ fold increase in MIC$_{50}$ was observed from PSSM to PTSM. This effect may also be due to composition of coordinating ligands in that phosphine ligands may assist interaction with or transport through the cell membrane where phosphites do not. Previous work$^5$ has shown silver compounds coordinated by phosphorus groups show no antimicrobial activity, which was clearly not the case with either PSSM or PTSM.

### 6.4.2 Plasma film assay

A single assay was conducted on this film with a fluorinated adlayer on non-woven against *Pseudomonas aeruginosa*, using the same conditions which gave the best result for pp-PSSM films (fig. 6.15). This would allow simple comparison between the films in terms of activity (see chapter 8).
Figure 6.15: JIS assay of PAO1 pp-PTSM + pp-C$_2$F$_6$ treated squares of non-woven after 3h

The result of this assay was dramatic, with a huge difference in cell viability between those taken from the treated and non-treated squares of material of around 99.9% reduction. The impact of this film decreased the number of cells from those initially inoculated so showing outright killing of cells on the surface, not just growth inhibition. This becomes significant when comparing antimicrobial surfaces for application. It was a disappointment that time limitations prevented further exploration of this highly promising system.

6.5 References


Chapter 7: Application of antimicrobial plasma films

The use of plasma deposition in an industrial setting for antimicrobial surfaces is a viable option, especially when introduced to existing manufacturing steps utilising plasma. pp-ZSB and pp-PTSM films were shown to be effective antimicrobials on a range of substrates for a range of applications and hold their own when subjected to scrutiny versus commercially produced antimicrobial materials. It is quite feasible that such films could supplement or even supersede current methods for producing antimicrobial surfaces for many applications due to the ease of production.

7.1 Application of plasma

It was demonstrated in chapter 1 that there is a need for antimicrobial surfaces in hospital settings to prevent both the accumulation and transfer of bacteria which cause nosocomial infection. Plasma deposition of antimicrobials may provide a satisfactory solution.

7.1.1 Application viability

Plasma deposition has potential as a technique to easily impart an antimicrobial coating onto a range of substrates without altering the bulk properties of the material and can be easily incorporated into the manufacture of such materials as non-wovens. Indeed, plasma has been widely used in applications such as thin film vacuum coating, microelectronic deposition (PECVD of metallic silver and copper) and sterilisation of biotechnology equipment, with the plasma industry worth over $1 billion per annum and growing rapidly. However, some issues created by plasma processing for industry include the inherent use of vacuum systems and the high initial cost for equipment (pumps, chamber, RF generator etc.). These factors are overcome when potential for ultra-green, clean products is realised which can pay for the original cost very quickly; and when plasma deposition is
utilised *in-situ* as supplementary to standard plasma processes e.g. sterilisation-coating.

Alternatively, an atmospheric plasma system has been developed\(^2\) which allows generation of a one atmosphere uniform glow discharge plasma (OAUGDP), which is a ‘cold’ RF glow analogous to low-pressure plasma operating on displacement currents (fig. 7.1).

![Atmospheric plasma system MOD VI OAUGDP\(^{TM}\) operating in air\(^2\)](image)

**Figure 7.1:** Atmospheric plasma system MOD VI OAUGDP\(^{TM}\) operating in air\(^2\)

Use of a plasma technique such as this would remove the need for expensive vacuum systems and batch processing, so making plasma a more attractive proposition to industry.

Currently, large-scale batch plasma processors are used in industry (fig. 7.2) which allow quick treatment of materials for a range of applications. Reels of flexible material e.g. non-wovens, are loaded to the reactor and pressure is rapidly reduced within the system in a similar manner to laboratory-scale reactors. Material is loaded from one reel to another at speeds between 1-30 m min\(^{-1}\) and is treated during transport.\(^7\)
The width of material treated can be anywhere up to 60 inches, meaning a substantial amount of processing can occur in a very short space of time, depending on the volatile nature of precursor; adhesion characteristics and the required quantity of deposited material.

7.1.2 Alternative methods

Recently new chemical vapour deposition techniques have been developed which could provide an alternative to plasma, in iCVD and oCVD (initiated and oxidative). These methods also allow for the potential to apply polymers to low melting point substrates, much like plasma but minus the low pressure requirement, although thermal energy in the system (ca.300°C) plus use of an initiator would prove inhibitive for stability of metal constituents, particularly silver.8

Most other methods (e.g. spin-coating, dipping) are solvent based and therefore not environmentally friendly and expensive. For example, in the industrial manufacture of thin films, most operational cost is tied up in wastewater treatment which is many times more than for a plasma set-up or vacuum cost.6

Figure 7.2: The wide web plasma industrial plasma system7
Plasma deposition for industry therefore has the following major advantages:

- Cheap long term operation
- Highly efficient
- Deposition onto a range of substrates (inc. 3D)
- Films unique to plasma
- Little/no by-products or waste

However, as discussed previously, difficulty has been reported in transferring plasma deposition characteristics from one reactor to another which appears to be the biggest problem facing plasma deposition for application.

### 7.2 Potential plasma substrates

For industrial application of plasma deposition with minimal cost, it makes sense to incorporate film formation into a manufacturing process which inherently requires plasma. This is the case with materials such as nappy linings, wound dressings and medical disposables e.g. gloves, scrubs etc; so plasma depositing antimicrobials on the surface would require little change in the production method but with a great deal of added value to the finished product. With this in mind, plasma films were deposited onto a range of substrates supplementary to those already previously coated (SAM-gold, polystyrene Petri dish, silicon wafer etc) and assays conducted against both *P. aeruginosa* and *S. aureus*. Deposition of plasma films onto non-woven polypropylene of nappy lining was observed for all three main systems used in this work with good efficacy against these bacteria already observed (chapters 4-6). This indicates a thin coating of antimicrobial via plasma deposition could help make nappy rash a thing of the past.

Other non-wovens tested included polypropylene material used in hospital scrubs, supplied by an industrial partner. The material was already highly hydrophobic due to plasma treatment and a pp-ZSB film (best efficacy at this stage) was deposited to assess whether the antimicrobial effects were enhanced (fig. 7.3).
Despite the hydrophobic effects exhibited by the non-woven and massively reduced cell count observed on the control squares over that seen for other control non-woven, a huge reduction was still seen from control to treated samples in both PAO1 and MSSA cell viability. This showed plasma deposition of antimicrobials was a viable second-step in manufacture of these materials in terms of bacterial efficacy and could provide a route to preventing bio transfer within hospitals over short periods of time.

Subsequent tests were carried out on glass and polystyrene over short periods of time to evaluate film efficacy for a similar purpose (figures 7.4 and 7.5).
Both tests showed reduced overall cell count on the surface from that experienced in tests with ‘regular’ non-woven which is caused by the lower surface area these materials possess for polymer and bacteria to bind to. Nevertheless, the trend of bacterial viability reduction was continued on these materials, with around a 50-70% decrease in *S. aureus* cell count observed on the treated materials versus controls. These assays provided information on the difference substrate composition makes to antimicrobial effects, possibly due to a combination of adhesion properties of the plasma film to the substrate, surface area and interaction of cells with the base substrate.

For both bacterial strains fewer cells were viable after exposure to pp-ZSB treated glass than polystyrene, which was counter to the result expected as plasma films
would be expected to bond more easily with the organic components in polypropylene than the inorganic components of glass and therefore contain more antimicrobial. It could the case that active zinc Schiff base was more available at the surface in the glass sample whereas it was able to bind more extensively to polypropylene which prevented release into bacterial cells.

These results were significant in determining antimicrobial efficacy after a very short period of time as similar efficacy was seen at two hours as for pp-ZSB treated non-woven material (fig. 7.6). Incubation for around four hours rather than two appeared to give greatest discrepancy in cell viability between control and treated fabrics as active material was accumulated in cells, but dead cells had not provided a platform for live cells to grow, whereas bacteria on control samples can proliferate unabated.

![Graph showing cell viability comparison](image)

**Figure 7.6:** JIS assay comparison of PAO1 on pp-ZSB treated squares of polystyrene after 2h and 4h

### 7.3 Filter work

A potential application outside of direct human contact with plasma films is that of filters. This could include drinking water or air conditioning systems (legionellas) or eye infections stemming from contamination of contact lens solution. For this purpose, an assay was conducted with filter strips supplied by an industrial partner.
The filter assay (fig. 7.7) was devised to test cell viability after passing through a treated filter.

### 7.3.1 Filter assay experimental

pp-ZSB was deposited onto a large rectangle of Vyon HP 2mm filter (Porvair) in the standard manner followed by pp-C$_2$F$_6$ adlayer. Filter was positioned vertically similarly to non-wovens through support with another filter strip. The filter was cut into frits of 5mm diameter along with identical non-treated filter, which was sterilised using UV/O$_3$ for five minutes per side.

![Figure 7.7: pp-ZSB deposited filter (l), cut frits (c) and assay schematic (r)](image)

Frits were loaded to sterile 1ml pipette tips aseptically. Gloves were sterilised using 70% ethanol before and after each sample was touched. Frits were loaded using tweezers and a 4mm cork borer, both of which were flamed in 100% ethanol and allowed to cool prior to each load. Frits were carefully loaded using the tweezers and once in position, were forced into the pipette tip using the cork borer. Treated frits were oriented face-on to flow. One to four frits were loaded to each pipette tip. 200µl of 10$^5$ CFU ml$^{-1}$ overnight culture of *P. aeruginosa* in LB (+ tween) inoculum was added to each pipette tip and a 1ml pipette applied to the tip. NB: the pipette was sterilised prior to each use by wiping with 70% ethanol and allowing drying. Bacterial solution was then slowly pushed through the filter tip over the course of one minute into an LB-agar plate and the resulting solution spread over the plate.
Each nutrient agar plate was incubated at 37ºC for 18h. A control 200µl of inoculum not passed through any filters was also plated.

7.3.2 Filter assay results

The results of this assay were graphically illustrated (fig. 7.8) and clearly demonstrated the activity of pp-ZSB films on bacteria passed through the filters. Compared to the control samples, virtually no viable cells were observed in solutions passed through the pp-ZSB films. The greatest count of cell viability was unsurprisingly observed in control solution directly plated on agar, with slight reductions in count seen as more filters were added between inoculums and solid growth media due to cells attaching to the control filter surface on the path through.

![Filter assay results](image)

**Figure 7.8**: Filter assay of PAO1 passed through filters containing pp-ZSB treated and non-treated Vyon HP 2mm filters

Viability was reduced by around two orders of magnitude on treated samples by comparison. This was perhaps due to a combination of contact-killing as bacteria were passed through the treated filter and any ZSB that was liberated from the filter surface was able to affect bacteria on the agar surface during incubation. Treated filters showed the same water-passing ability of non-treated and given the wide-pored filter chosen, observed effects are unlikely to be due to pore-blocking by pp-
ZSB films. This indicates that plasma treatment of filters with antimicrobials is another potential application. The use of these filters could easily be extended to air-conditioning units where Legionnaire’s disease is a common problem.

7.4 Wound dressings

Supplementary to use in items such as nappies and scrubs, non-woven materials are also used in wound dressings. Commercially available products containing antimicrobial are available including Acticoat™, discussed in chapter 1 and silver-loaded plasters. In order to ascertain the effectiveness of the coatings created here against such established products, wound dressings were deposited with pp-ZSB and pp-PTSM under standard conditions and a JIS assay conducted versus *P. aeruginosa* and *S. aureus* alongside Elastoplast™ silver dressings and Boots hydrocolloid dressings over four hours (fig. 7.9).
As can be seen above, close to 100% reduction in cell viability was observed in pp-PTSM films after four hours which was directly comparable to commercially available dressings and in fact slightly more effective versus MSSA than the Elastoplast\textsuperscript{TM} silver dressings. This could be explained as coordinated silver would be less susceptible to ion efflux mechanisms within the cell. The result for pp-PTSM clearly exceeds the efficacy of the highly hydrophobic hydrocolloid dressings and is
also much more active than pp-ZSB, which is to be expected when considering outright toxicity of silver and zinc. Nevertheless, pp-ZSB shows reduction in excess of 90% of all cells present in the dressing and there is a good chance this dressing in particular would be least inhibitive to wound healing. This result is hugely encouraging for potential use of these films in real-life application.

7.5 References

Chapter 8: Summary of process development

8.1 Method development

Over the course of this work the methods of compound preparation, film deposition and microbiological assessment of both compounds and films were constantly developed and improved to create materials which were viable for application. Improvements and the results of progress are discussed here.

8.1.1 Synthetic approach

The first novel complex produced here, PSSM, was designed in order to stabilise and increase the volatility of silver salts for plasma deposition. Many synthetic routes to the complex were attempted, from phosphine coordination to silver nitrate followed by complexation with maleimide; to a two step approach of silver salt synthesis and isolation followed by coordination by the Lewis base; with various solvent systems and silver precursors used. The preferred synthetic route eventually became apparent through high yielding and clean reaction products, which was subsequently adopted as a general procedure for production of silver-salts. Alteration of ligand systems through use of alternative methods (e.g. Schlenk lines) eventually resulted in the production of liquid silver complexes.

The synthesis of M$^{2+}$ ion complexes proved simpler on the whole due to the greater stability of zinc and copper, but design approaches were varied according to volatility of monomers, resulting in ZSB and CSB complexes of reasonably low melting point.

8.1.2 Plasma deposition

The initial plasma deposition of films was conducted on NaCl discs for FTIR analysis, which were subsequently replaced by KBr discs and finally by SAM-coated
gold chips which offered a big improvement in the sensitivity of measurement and removed the need for constant disc cleaning or preparation. Additionally, the FTIR measurement itself was greatly improved by use of an ATR-FTIR system in place of regular transmission FTIR.

The volatility of plasma monomers was improved by systematic alteration of ligand systems but precursor flow to the reactor chamber was also improved by the use of shorter-necked monomer chambers and external heating of the chamber. A second reactor was introduced with capability for heating the monomer inlet area of the reactor so improving monomer flow further and reducing monomer adhesion to the inlet.

Adhesion of plasma films to the substrate surface was improved by pre-treatment with oxygen plasma and hand-in-hand with microbiological assay results, affinity of liquids to the surface was reduced by formation of a fluorinated adlayer. Confocal microscopy of cell viability also offered insight to the ideal substrate position in the reactor for reduced microbiological growth. Film properties and functional group retention was improved by the systematic alteration in duty cycle.

For film characterisation, several approaches were altered throughout the process. FTIR was supplemented by deposition onto glass for mass spectrometry of dissolved films, which was subsequently replaced by surface-specific mass spectrometry in SIMS. XPS, electron microscopy and atomic absorption spectroscopy were also used to analyse films. Film thickness was initially measured by SPR through angle shift fitting which was replaced by the more accurate alpha-stepping. Thickness was also allied with weight measurements through QCM to deduce the density of films.

8.1.3 Microbiological assays

The antibacterial analysis of free complex and plasma deposited films also received a great deal of attention for method development. The initial bacterial strain used, *M. luteus*, was exchanged for more pathogenic strains and representatives of both Gram-positive and –negative bacteria in *S. aureus* and *P. aeruginosa*. The assays for
testing pure compound were improved from early methods of disc diffusion visualisation to the quantitative approach of minimum inhibition concentration, with standardisation of this assay achieved through the solubility of compound in non-toxic levels of DMSO. Cell counting through colony growth on solid media was supplemented by the use of optical density, which was calibrated using colony counts.

When testing plasma film efficacy, colony counting was also supplemented by the use of fluorescent tagging of bacterial cells. Initial experimental assays allowed the development of a standard assay for assessing viability of cells removed from the surface, while surface bound cells were also analysed using fluorescence and confocal microscopy.

A number of surfaces were tested in microbiological assays, ranging from Petri dishes in the first instance, to non-woven materials. The Japanese industry standard for testing antimicrobial efficacy of textiles was used to improve outright quantification of cell count from treated films and allowed comparison to commercial antimicrobial products. This assay was also used to test plasma films over a range of time periods to aid the understanding of the antimicrobial mechanism.

### 8.2 Antimicrobial quantification comparison

#### 8.2.1 Japanese industry standard processing

Alongside the quantification of antibacterial efficacy of films, the Japanese industry standard also defines a material as bacteriostatic or bactericidal and to what degree. This is achieved through measurement of bacterial cell counts on control at inoculation ($T_i$) and after incubation for a defined period of time – usually 18h ($T_f$). These numbers are processed to ascertain whether the test was effective for the control i.e. whether the control showed expected growth (equation 8.1).
Equation 8.1

\[ F = M_b - M_a \]

Assessment of effective JIS assay where \( F = \text{Growth value}, M_a = \log_{10} \text{CFU ml}^{-1} \text{on } T_i \text{ control}, M_b = \log_{10} \text{CFU ml}^{-1} \text{on } T_f \text{ control}. F > 1.5 = \text{effective test, } F < 1.5 = \text{non-effective test} \]

Once an effective test has been confirmed, cell numbers on treated material can easily be converted to bacteriostatic (S) or bactericidal (L) results (equation 8.2).

Equation 8.2

\[ \text{a) } S = M_b - M_c \text{ b) } L = M_a - M_c \]

Assessment of JIS assay where \( S = \text{bacteriostatic value}, L = \text{bactericidal value}, M_a = \log_{10} \text{CFU ml}^{-1} \text{on } T_i \text{ control}, M_b = \log_{10} \text{CFU ml}^{-1} \text{on } T_f \text{ control}, M_c = \log_{10} \text{CFU ml}^{-1} \text{on } T_f \text{ treated}. S > 2 = \text{bacteriostatic, } L > 0 = \text{bactericidal test} \]

Provided the bacterial growth on treated surfaces is reduced from the initial control i.e. \( L > 0 \), the surface is deemed bactericidal. If the bacterial growth is slowed by the treated surface but is greater than the initial control, the surface is bacteriostatic provided \( S > 2 \). These values are related to activity, so the more effective antimicrobial tests produce higher values of \( L \) or \( S \).

8.2.2 JIS film comparison

The best antimicrobial efficacy experienced for plasma deposited film system was compared using JIS quantification for the appropriate bacterial strain (fig. 8.1).
<table>
<thead>
<tr>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strain</strong></td>
<td><em>Pseudomonas aeruginosa PAO1</em></td>
<td><em>Staphylococcus aureus MSSA</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cell count</strong> (CFU ml⁻¹)</td>
<td>2.05 x 10⁴</td>
<td>1.33 x 10²</td>
<td>5.40 x 10²</td>
<td>3.84 x 10⁵</td>
<td>4.67 x 10²</td>
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<tr>
<td><strong>Growth value</strong> (F)</td>
<td>2.63</td>
<td>1.83</td>
<td>2.63</td>
<td>2.66</td>
<td>1.59</td>
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<td><strong>Bactericidal (L) / Bacteriostatic (S) value</strong></td>
<td>S = 1.20</td>
<td>L = 4.07</td>
<td>L = 3.76</td>
<td>S = 0.85</td>
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<td>Colony count</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Material</strong></td>
<td>Non-woven polypropylene</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Non-ionic surfactant</strong></td>
<td>Tween 20 (0.05% w/v)</td>
<td></td>
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</table>

**Figure 8.1:** Tabulated summary of the most effective JIS assay results for plasma films on non-woven material versus PAO1 and MSSA

The most effective surface from this data would appear to be pp-ZSB, although the assay was run over a slightly longer time than the silver films (4h versus 3h). Interestingly both pp-ZSB and pp-PTSM were strongly bactericidal from JIS quantification, with the general trend of greater effectiveness on PAO1 than MSSA followed. Despite the evidence of antibacterial activity in absolute cell count reduction, pp-PSSM films were determined as only weakly bacteriostatic.

The bactericidal result of the zinc-containing film could be considered slightly inaccurate however, as bacteriostatic behaviour has subsequently been observed on pp-ZSB treated films where the initial low colony counts observed after 24h agar
plate incubation were shown to increase slightly when plates were incubated for longer. Nevertheless, for the purposes of this test bacteria were suitably disabled to reduce viability when observed, so the result stands with this caveat.

All tests showed a growth factor of > 1.5 and were therefore deemed effective tests. It may be observed that the bactericidal/static value is dependent on the effectiveness of the test i.e. magnitude of growth value. If more bacteria is evidenced on the control material at $T_f$ relative to that at $T_i$ in a particular test, then it could be inferred that more viable cells are present in solution and could therefore colonise the surface more easily, overloading the antimicrobial.

### 8.3 Conclusions

The need for new antimicrobial coatings and surfaces is a vital and major challenge facing scientists across a multitude of disciplines encompassing the areas of biology, chemistry, materials science and engineering. Due to the broad scope of creating such materials, a wide range of techniques have been employed with varying results. The plasma deposition of metal-containing complexes was investigated for this purpose.

An initial organosilver complex, PSSM, was synthesised and used as a model system for deposition of plasma films with a great deal of method development. A variety of analytical and antimicrobial methods were used to explore the properties of these films, with promising results. Films were found to inhibit the growth of model bacterial organisms after suitable tailoring of film properties. Exploration of PSSM plasma films allowed progress onto monomer systems containing zinc and copper. The zinc system in particular being of higher volatility than PSSM allowed deposition of films which strongly resembled monomer structure through pulsed plasma and subsequently showed good activity against bacteria.

Method development in silver compound synthesis lead to highly volatile liquid silver complexes which in turn showed bactericidal activity against model microorganisms when plasma deposited. Like the zinc system, monomer
functionality was strongly retained in the resulting plasma films. Silver and zinc films were subjected to industrial-standard antimicrobial tests against commercially available wound dressings. Both films showed efficacy versus the model organisms of similar impact to the commercial products, which allows potential upscaling of these processes for industrial application. Low levels of observed metal content in films could indicate a need for a potentially antimicrobial ligand system to work in conjunction with the metal to show activity. Importantly, the films were shown to be non-harmful to human cells.

The work undertaken here presents the ability of plasma deposition as a route to simple one-step preparation of antimicrobial surfaces. Although the results of extensive testing here proved the effect of organometallic plasma films on microorganisms, it would appear many avenues present themselves for future development of these systems depending on the potential application. Such varied properties as blood-interaction, mechanical strength of films, scalability and substrate adhesion could be investigated ad infinitum. Thus, the work conducted here is but a starting point, yet the techniques and surfaces developed certainly show promise as a new method for antimicrobial surface generation.