

Modified Polymer Surfaces with Antimicrobial Properties

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Declaration of Originality

All work referred to in this thesis was carried out at the Department of Chemical Engineering, Department of Infectious Disease and the Department of Materials at Imperial College London; and at Procter and Gamble, Egham, UK between October 2017 and April 2022. Except where acknowledged, all content is original work of the author and no part of the thesis has been submitted for a degree at any other institution.

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Abstract

Biofilms are a multispecies community of bacterial cells that can establish over time on biotic and abiotic surfaces. Biofilm growth and maturation on industrial polymer surfaces poses a serious challenge to public health, industrial manufacturing, oil and gas industries, food production and healthcare. As a result, there has been increasing interest in the development of easily synthesised low-cost antimicrobial polymer surfaces and coatings to inhibit, and/or perturb bacterial attachment and biofilm viability.

This thesis was focused on modifying the surface of low cost commercial polymers, with high-density polyethylene (HDPE) being used as the model substrate. Surface modifications were performed using a range of approaches. These included different surface oxidative treatment methods in order to evaluate their intrinsic antimicrobial performance, and as an approach for improving the adhesion for other antimicrobial coatings being investigated. Tin-dioxide thin-films and low/medium molecular weight chitosan-zinc oxide nanocomposite coatings were also investigated and examined as possible antimicrobial surface coating technologies for polymer materials, primarily for HDPE. A key aim of this work was to evaluate microbial adhesion and biofilm viability at the initial stages of biofilm maturation (early-stage biofilm), with successful antimicrobial approaches then being further evaluated against a more mature biofilm system.

This study confirms that the sulfuric-chromic acid surface oxidation of HDPE and polypropylene surfaces showed marked reductions in bacterial adhesion and viability, with a decrease in attachment seen for both Gram-negative and positive bacterial species with increasing surface oxidation treatment time periods from 0 to 60 minutes. In particular, extended sulfuric-chromic acid oxidation treatment times of 30 and 60 minutes significantly compromised microbial viability, such that there was no agar growth of early-stage biofilm of Gram-negative organisms recovered from these surfaces when cultured, even after 3 months of aging this polymer material under standard laboratory conditions. The mechanism for this activity was ascribed to the formation of chromium esters which are known reactive intermediates in the sulfuric-

chromic acid oxidation of HDPE, and their surface presence was further confirmed using TOF-SIMS. Tin-dioxide nanoparticle coatings showed a reduction in the early-stage biofilm formation of *Pseudomonas aeruginosa*, irrespective of nanoparticle size of the tin-dioxide coating used, which was also observed when coatings were deployed on polypropylene and polycarbonate materials. A range of chitosan-zinc oxide composite thin-films were examined which exhibited minimal viability reduction against *Escherichia coli* early-stage biofilms. Therefore, this study could not affirm a significant antimicrobial performance for the chitosan-ZnO nanocomposite coatings on oxidised HDPE materials which was attributed to the loss of a key antimicrobial active functional group in chitosan, the primary amines. However, optimisation of the chitosan-ZnO formulations revealed some key trends, where increased concentrations of low molecular weight chitosan in the presence of zinc-oxide in 1% acetic acid, resulted in a 2.8-log reduction of *Escherichia coli* due to the presence of primary amine substituents, confirmed by a ninhydrin assay. Experiments performed against mature biofilms prepared in a 24-hour aqueous environment found no antimicrobial performance on all substrates and coatings tested.

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List of Symbols, Abbreviations and Physical Constants

A

A	Area of a surface
AFM	Atomic force microscopy
AMP	Antimicrobial polymer
AUG	Auger electron spectroscopy

B

<i>B. subtilis</i>	<i>Bacillus subtilis</i>
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C

CA	Contact angle
CFU	Colony forming unit
CI	Carbonyl Index
CLSM	Confocal laser scanning microscopy
CS	Chitosan

D

dd	Double distilled
DDA	Degree of deacetylation (in terms of chitosan)
DiBAC ₄ (3)	Bis-1,3-dibutylbarbituric Acid)trimethine oxonol
DIM	Diiodomethane
DLS	Dynamic light scattering
DLVO	Derjaguin, Landau, Verwey, and Overbeek

E

<i>E. coli</i>	<i>Escherichia coli</i>
EDS	Energy-dispersive X-ray spectroscopy
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
E-SEM	Environmental-scanning electron microscopy
ESKAPEE	<i>Enterococcus faecium</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> , <i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i> , and <i>Enterobacter</i> spp., and <i>Escherichia coli</i>

F

	FC	Flow cytometry
	FIB-SEM	Focused-ion-beam scanning electron microscopy
	FSC	Forward Scatter (in flow cytometry)
G		
	G	Free energy
H		
	h	Hour(s)
	HAI	Healthcare-associated infections
	HDPE	High-density polyethylene
	HI	Hydroxyl index
	HT	Hydrothermal treated
I		
	IAA	Initial attachment assay
	ICP-MS	Inductively coupled plasma-mass spectrometry
	ImageJ T	ImageJ threshold
	ISS	Ion scattering spectroscopy
J		
K		
L		
	LB	Lysogeny broth (microbial broth)
	LMW	Low molecular weight
	LPS	Lipopolysaccharides
	LVHR-SEM	Low-voltage high-resolution-scanning electron microscopy
M		
	MHSA	Mannose-sensitive hemagglutinin
	min	Minute(s)
	MMW	Medium molecular weight
	MW	Molecular weight
N		

	N	Number of item(s)/sample(s)
	NA	Not available
	NaOH HW	NaOH hot-wash (for chitosan film preparation)
O		
	OECD	Organisation for Economic Co-operation and Development
P		
	P&G	Procter and Gamble
	<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
	PBS	Phosphate buffered saline
	PC	Polycarbonate
	PDI	Polydispersive index
	PDMS	Polydimethylsiloxane
	PE	Polyethylene
	PEG	Polyethylene glycol
	PI	Propidium iodide
	PP	Polypropylene
	ppb	Parts per billion
	PS	Polystyrene
	PVC	Polyvinyl chloride
	PVP	Polyvinylpyrrolidone
Q		
	QAC	Quaternary ammonium compounds
R		
	r	Roughness factor (Wenzel's Model)
	RBS	Rutherford backscattering spectrometry
	ROS	Reactive oxygen species
	RI	Refractive Index
	RT	Room temperature
S		
	<i>S. aureus</i>	<i>Staphylococcus aureus</i>

	SD	<i>Standard deviation</i>
	SD*	<i>Sampling depth</i>
	SE	<i>Standard error</i>
	<i>S. epidermis</i>	<i>Staphylococcus epidermis</i>
	SFE	Surface free energy
	SI	Sulfonic Index
	SIMS	Secondary ion mass spectrometry
	S:N	Signal-to-noise ratio
	<i>spp.</i>	Species <i>pluralis</i> (multiple species belonging to the same genus)
	SSC	Side Scatter (in flow cytometry)
T		
	T20	Time duration of 20 minutes in saline (in biofilm assays)
	TCA	Tricarboxylic acid cycle
	TEM	Transmission Electron Microscopy
	TH	Threshold
	ToF-SIMS	Time-of-Flight Secondary Ion Mass Spectrometry
	TSB	Tryptic soy broth
U		
	UTI	Urinary tract infection
	UVO	UV/ozone surface oxidisation
	UVO-SA	UV/ozone and sulfuric-acid surface oxidisation
V		
	<i>V. cholerae</i>	<i>Vibrio cholerae</i>
W		
	W1, W2, W3	Wash 1, wash 2, wash 3
	WHO	World Health Organisation
X		
	XDLVO	Extended Derjaguin, Landau, Verwey, and Overbeek
	XPS	X-ray photoelectron spectroscopy

XRF	X-ray fluorescence spectroscopy
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

Y

Z

Symbols

γ_{SV}	Surface tension of the solid surface/ the surface free energy
γ_{LV}	Surface tension of a liquid
γ_{SV}	Surface tension between liquid and the solid
γ_{LV}^D	Dispersive component of the surface tension
γ_{SV}^D	Dispersive component of the surface energy
γ_{LV}^P	Polar component of the surface tension
γ_{SV}^P	Polar component of the surface energy
θ	Contact angle
θ^w	Apparent contact angle (Wenzel's Model)
θ^{CB}	Apparent Cassie-Baxter contact angle
f	Surface fraction
l	Sampling length

CHAPTER 1 Introduction

1.1 Background

Biofilms are a surface-bound community of bacterial cells that are embedded in a protective extracellular polymeric substance (EPS) of microbial origin that grows as the biofilm matures [1]. Evidence suggests that over 99% of microbes live in biofilm microecosystems [2], with biofilm microbes exhibiting up to 1000 times more tolerance towards both biotic and abiotic (e.g. antibiotics and disinfectants) stresses compared to their planktonic growth form [3].

The formation of biofilms is an area of great concern to public, commercial, and industrial sectors. For example, in healthcare and product manufacturing, biofilms can lead to product quality failures from damaged, blocked, eroded, or contaminated surfaces. Biofilms also cause an increased risk for the spread of infectious diseases, they are responsible for dental caries and nosocomial infections, as well as a variety of other infections [4,5]. The US National Institute of Health reported that an excess of 80% of microbial infections in the body are a result of infections caused by biofilms [6,7]. Some examples of nosocomial infections of microbial biofilms are listed in **Table 1.1** and these stem from contact with hospital surfaces including catheters and implants.

Devices	Common biofilm bacterial species associated with serious infection or disease found on corresponding surfaces/tools	Reference(s)
Sutures	<i>Staphylococcus epidermis</i> , <i>Staphylococcus aureus</i> and methicillin resistant <i>Staphylococcus aureus</i>	[8]
Contact Lenses	<i>Bacillus</i> spp, <i>Micrococci</i> , <i>Achromobacter</i> , <i>Stenotrophomonas</i> , <i>Escherichia coli</i> , <i>Staphylococcus aureus</i> , <i>Staphylococcus epidermis</i> , <i>Candida</i> , <i>Serratia</i> and <i>Proteus</i> .	[9][10][11]
Orthopaedic devices (e.g. heart valves, corneal implants)	<i>Staphylococcus epidermis</i> , <i>Staphylococcus aureus</i> , Gram-negative <i>Bacillus</i> , <i>Enterococcus</i> and <i>Candida</i> spp	[11]
Catheters	<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i> , <i>Enterococcus</i> spp., and <i>Candida</i> , <i>Klebsiella</i> , <i>Enterobacter</i> spp (Largely Gram-negative bacteria are prevalent as catheters)	[11] [12]
Hospital contact surfaces (e.g. telephones, keyboards)	<i>Klebsiella</i> , Vancomycin (antibiotic)-Resistant <i>Enterococcus</i> , <i>Proteus</i>	[13]

Table 1.1 Examples of common biofilm species causing nosocomial patient infections from biofilm contamination on hospital surfaces and tools, inspired from work presented in a review by Costerton et al. [14].

Acute infections originating from bacterial biofilms in a host organism are often difficult to eradicate when relying on the hosts adaptive and innate defence mechanism alone, therefore medical interventions are often required, for example in periodontitis (inflammation of gums) and otitis media (ear infection) [14]. Aside from the biohazards associated with biofilm formation and product quality failure, biofilms can also result in malodours, microbial induced corrosion and polymer degradation [15–17]. Biofilms possess intrinsic resilience, which includes a resilience against antimicrobial disinfectants and cleaning approaches [18,19]. Antibiotics, although highly effective, are limited to their application and longevity due to the resilience of bacteria, especially in a biofilm environment. Microbial resistance to antibiotics is a widespread issue across 22 countries, with 500,000 people annually exhibiting antibiotic resistance infections according to the World Health Organisation’s (WHO) recent report in 2018 [20]. In fact, scientists are looking into improving bacterial susceptibility to antibiotics due to the innate bacterial ability to develop antimicrobial resistance. For example, some studies

have focused on how to increase susceptibility of bacteria to antibiotics, as in the case of colistin, a last resort antibiotic with growing bacterial resistance. Colistin's antimicrobial activity is based on targeting the lipopolysaccharides in the cytoplasmic membrane, so bacterial susceptibility to colistin was improved by utilising a lipopolysaccharide transport inhibitor (murepavadin) which causes lipopolysaccharide accumulation in the cytoplasmic membrane [21].

Surfaces at high risk of microbial contamination include polymer materials which are extensively used and have largely replaced paper and other cellulose-based products over the years because of their lower costs, superior mechanical properties, and resistance to water degradation. Frequently found polymers include polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), and polycarbonate (PC) which are readily used for many applications, such as plastic implants, facemasks, personal protective clothing, catheters, pill casings, packaging, work-tops, instrument parts as well as disposable medical and laboratory components [22,23].

Although there have been decades of research into investigating the structure of biofilms, antifouling biofilm strategies and removal approaches, there are clear limitations to many of the solutions currently being used which largely pertain to the complexity of microbial organisms in a biofilm microecosystem and their resilience to environmental stresses. Polymers are typically sanitised by means of either heat or ionising radiation to remove biofilms, as well as topical chemical treatments including detergents, chelating ligands, surfactants, oxidising agents, and enzymes. The limitation is that these treatments are temporary and recontamination by microorganisms can occur when surfaces are re-introduced into the environment or the cleaning regime is not persistent. Furthermore, these methods may also cause increased levels of toxicity and contamination of surrounding product or the environment from the chemicals being used, and may also cause enhancement of the surface corrosion processes [17,24,25]. Another important factor to consider is the vast variety of bacterial species with different strains that are present. This issue in turn raises the problem of selectivity when looking to develop novel biocidal chemicals and materials. As a result, there is a significant need to develop a biocidal surface that is cost effective, durable, and able to inhibit the adhesion of pathogenic bacterial species of

different organisms. One approach to achieve this outcome is through the development of antimicrobial polymers (AMPs) which can be modulated and functionalised to minimise and inhibit biofilm formation by decreasing microbial attachment and/or preventing biofilm maturation. In light of the findings presented, the inhibition of biofilm formation on polymer surfaces is of great interest and this research project will focus on the surface modification of commercially relevant polymer materials and the inhibition of early-stage biofilm development. A particular focus will be to consider ways in which differing modified substrates can affect biofilm attachment and viability at the early-stage.

1.2 Thesis Outline

In this thesis a series of polymer surfaces have been characterised by assessing their physio-chemical attributes including surface energy, surface roughness and surface composition following a range of surface modification approaches. Investigations of the antimicrobial performance of these substrates have been focused largely towards early-stage biofilm adhesion of microbes and their viability using an array of novel and/or newly established assaying methods that includes the utilisation confocal laser scanning microscopy, cell enumeration of colony forming units on agar and to a lesser extent, flow cytometry and scanning electron microscopy imaging. A wide range of industrial polymer surfaces has been selected for surface modification, with HDPE being chosen as the model substrate used for all experiments due to its varied and widespread applications including disposable bottles, facemasks, pipes, recycling bins, personal protective equipment, disposable clinical components, and chemical containers. Also, HDPE is popular in industry, commercial and healthcare use due to its stiffness, durability, versatility, affordability, non-leaching (FDA-approved) and resistance to most chemicals. This thesis aims to evaluate the antimicrobial performance for a range of polymers that have been surface modified, including surface oxidation as well as metal oxide nanoparticles and chitosan thin-films, as possible antimicrobial treatments. Polymers are commonly surface modified by means of oxidation to achieve a more adhesive surface, however the evaluation of the antimicrobial performance of such treatment steps are often overlooked and not monitored. Therefore, a detailed study

of oxidation treatment on HDPE, as well as the oxidation of a range of different polymer materials, has been performed in order to provide a thorough overview of its intrinsic antimicrobial performance.

1.3 Project Management

The majority of experiments were carried out at Imperial College London under the supervision of Professor Daryl Williams in the Department of Chemical Engineering, the Department of Infectious Disease and the Department of Materials. The remaining experiments were conducted at Procter & Gamble's Egham research site under the supervision of Dr Kevin Wright and Dr Gareth Davies. Where experiments have been conducted by other personnel, or with other personnel, this assistance has been specifically listed in the thesis.

CHAPTER 2 Literature Review

2.1 Biofilms

2.1.1 Introduction to Biofilms

Most bacterial life on Earth takes place on surfaces and interfaces in the form of biofilms. Biofilms are a surface attached community of bacteria that can produce a protective EPS of microbial origin [1]. Biofilm proliferation can progress to EPS matrix development, the formation of complex microbial communities and structures, with variations in cell phenotype within the biofilm morphology to form a mature biofilm system. As a result, bacteria can exist as either 'planktonic' (free-floating in solution) or 'sessile' (anchored to a surface) species [26], where the sessile form represents an early-stage initial biofilm, before a mature biofilm forms. 'Biofilms' and 'biofouling' are commonly used terms and are often used interchangeably with each other to describe the surface build-up of organisms. However, biofilms are recognised to encompass both positive and negative implications, whereas biofouling is used to describe instances where the biofilm is only deleterious and harmful [27].

Biofilms have been found to exist as early as 3.3 to 3.4 billion years ago based on fossilised records [28–30], yet the earliest scientific observations of biofilms were reported in the 1680s, when Antonie van Leeuwenhoek investigated human teeth on a primitive microscope, which he described the biofilm as "animacules" [31]. Traction to further investigate these sessile organisms started to develop only 200 years later when scientists started interpreting these bacteria as "firmly adherent" to surfaces with the growth of an interfacial "film" of cells [32]. The first use of the word biofilm has commonly been reported to arise in 1981 by Bill Costerton [31,33] however in 1975 Mack and Ackerson used the term biofilm for trickling filter systems [34]. Nevertheless, Costerton is accepted by many as the father of the field of biofilms with his ground breaking discovery of the 'glycocalyx' biofilm matrix [1,35]. The glycocalyx are a network of polysaccharides surrounding the bacteria, and this breakthrough caused the re-definition of a biofilm to be sessile bacterial communities that are capable of self-

producing exopolysaccharide glycocalyx polymers, also known as the EPS, which have shown to aid in surface adhesion [3].

Many industrial sectors and domestic domains are massively impacted by biofilm proliferation which has accelerated interest into research on understanding biofilms; see **Figure 2.1**. However, biofilms have also offered a number of beneficial end uses for several applications, these include solvent manufacture, waste water treatment, removal of oil in sea or water systems and in the production of acetic, lactic, succinic and fumeric acid [36]. For instance, a single species biofilm of *Clostridium acetobutylicum* has been used in the commercial production of valuable solvents including acetone, butanol, and ethanol via fermentation processes, which has also been shown to be an efficacious source of high-value biofuel with the aid of palladium catalysis [37,38]. The application of biofilms in waste water treatment is also now well established, originating from their use in industrial trickling filters in the early 1870s by J. Bailey-Denton [39]. A trickle filter is a bed consisting of gravel, moss, biomass and support media where sewage and waste water can flow through, removing carbon- and nitrogen-containing compounds in the water, such as ammonia and acetate species [40–42]. Additionally biofilm uses include the production of acetic acid in the form of vinegar, where this chemical is largely developed from the oxidation of ethanol by bacterial biofilms of the *Acetobacter* genus [43,44], after the initial fermentation of fruits to form ethanol from sugars. Biofilms have also been used in industrial fuel cells for energy production, for example for producing electricity from acetic acid [45].

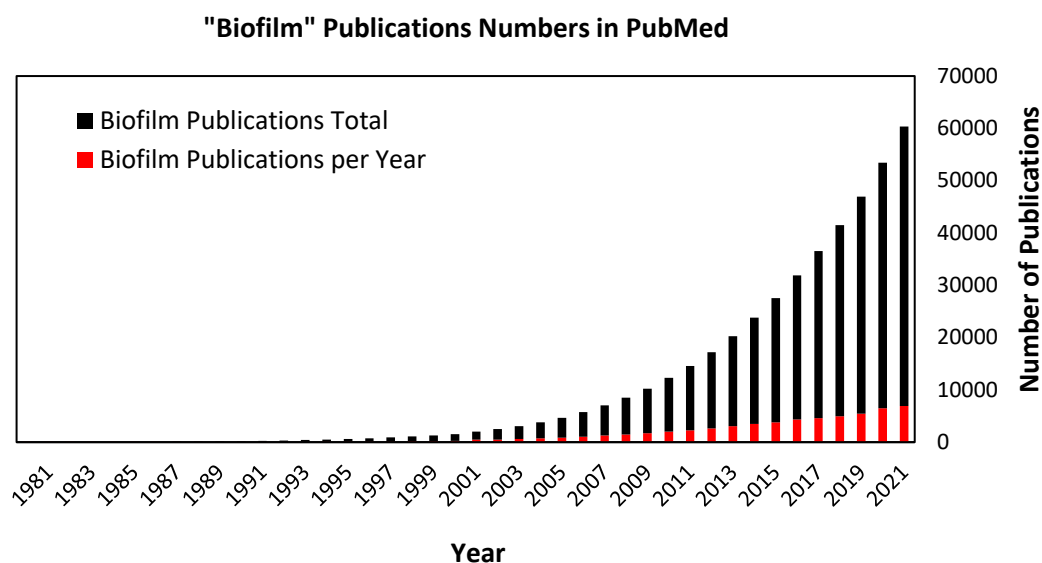


Figure 2.1 Accumulated publications containing the term 'biofilms' per year, obtained from PubMed, with the first use in 1975 ([https://pubmed.ncbi.nlm.nih.gov/?term="biofilm"](https://pubmed.ncbi.nlm.nih.gov/?term=)).

The complexity of the biofilm morphology is vast. Bacteria can communicate together in a biofilm via 'quorum sensing', a type of communication and decision-making process that determines their gene expression based on the local density of the population surrounding the bacterial cell. This effect is achieved by the transfer of small molecules, called autoinducers, that can be passed between adjacent bacterial cells [46]. The autoinducers accumulate with increasing cell density, and once a threshold level is reached this is detected by receptors in the cell membrane or cytoplasm which in turn activates gene expression that is involved with autoinducer production [47]. Some bacteria are activated to form 'persister cells' which remain as dormant variants that exhibit enhanced tolerance, and can become active once the local chemical treatment, external stress or therapy is terminated [48]. The emerging field of microbiology that investigates the group behaviour and social activity of bacteria is termed 'sociomicrobiology' [49]. Biofilms can also form complex internal channels and voids. Research by Kaplan *et al.* has found the existence of convection currents in the biofilm structure for different oral bacteria, which provides channels and voids that can allow the essential transport of planktonic bacteria to other sites [50]. The EPS matrix has been shown to encompass cell clusters fused inside that distributes connected channels

and voids to allow for the efficient supply of oxygen throughout the biofilm structure [51].

2.1.2 Stages to Biofilm Maturation

Many observations have shown a temporal step-wise progression in biofilm maturation and development in the presence of favourable environmental conditions, although the detailed molecular mechanism may differ from organism to organism [52]. In the late 1990s O'Toole and co-workers took the first steps to understand this progression by investigating mutant phenotypes of Gram-negative proteobacteria including *E. coli*, *P. aeruginosa*, *Pseudomonas fluorescens* and *Virio cholerae* [53–57]. This evolution has been summarised into five distinct phases of progression from (1) initial surface attachment, (2) monolayer formation, (3) migration to form multi-layered colonies, (4) significant production of EPS, and (5) biofilm maturation with 3-D architecture, followed by its dispersal [53,58]. These stages are illustrated in **Figure 2.2**, which is often viewed as a cycle rather than a linear process, for the continued spread of biofilm on surrounding surfaces [59]. This progression is believed to accommodate control points that allow for bacteria to regulate their gene expression for the different stages [58].

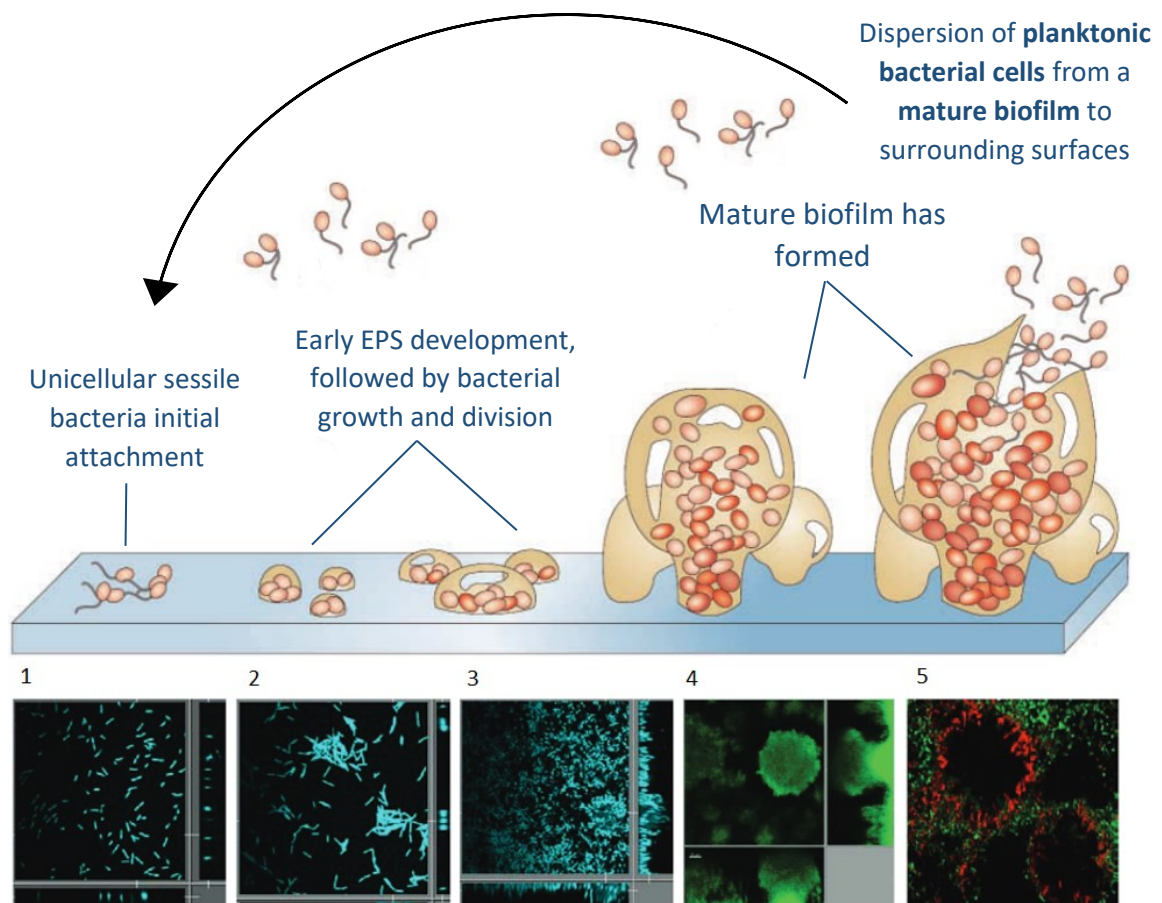


Figure 2.2 The evolution of unicellular microorganisms into a biofilm over time. Images 1-5 show the stages of biofilm growth using CLSM imaging of *P. aeruginosa* labelled with DAPI dye (image 1-3), chromosomal GFP (image 4) and LIVE/DEAD Viability BacLight kit (image 5); kindly sourced from Bordi and Bentzmann from an Open Access unrestricted review article [60].

These 5 stages of biofilm development are further discussed in the Sections below.

Stages 1-2: Initial surface attachment and monolayer formation

The growth of a biofilm starts with initial attachment of unicellular bacteria to the surface, where attached cells are able to upregulate specific adhesion genes within minutes of attachment to the surface [61]. For example, *E. coli* uses the BOLA transcription factor to regulate gene expression by influencing flagellar synthesis and

EPS (peptidoglycan cycle) production, as well as stimulation of the Krebs cycle (tricarboxylic acid cycle). Specifically the BolA protein represses the expression of flagellar genes and in turn reduces the cells swimming capacity [62]. Furthermore, many genes related to pyruvate metabolism and the Krebs cycle were induced with BolA, and the upregulation of several amino acid synthesis pathways [62]. The initial attachment of the unicellular microorganisms is a crucial step to biofilm development on surfaces. However, the cellular decisions to adhere to a surface are not absolute, it is a dynamic and reversible process whereby bacteria can detach and express the planktonic phenotype depending on several influencing factors, such as from local hydrodynamic forces [63]. Therefore, irreversible attachment of bacteria is often considered as sessile bacteria that can withstand shear forces in order to maintain their adhesion to the surface [64], therefore this attachment can be regarded as an early-stage biofilm. As a result, weak microbial adhesion to the surface at this initial stage is known as '*reversible attachment*' where the attached cells can be detached via low shear forces. It has been said that microbial surface attraction at this stage can be due to weak van der Waals interactions, as well as other intrinsic bacterial features such as bacterial flagella [65].

Efforts to interpret microbial adhesion has been made using the DLVO theory (Derjaguin, Landau, Verwey, and Overbeek) which treats the adhesive interactions of a bacterial cell as the same as adhesion interactions of a colloidal particle [66–68]. This theory suggests that weak attraction between a cell and a flat surface is based on a balance between attractive and repulsive molecular forces. The repulsive electrostatic forces are represented by bacterial cells due to their external negative surface charge at physiological pH values (between pH 5 and 7) [69]; such forces are long-range. The extended DLVO (XDLVO) theory considers hydrophobic/ hydrophilic forces coming from Lewis acid-base phenomenon as well as osmotic interactions [70], although it may not always corroborate with experimental findings [71]. The next phase after reversible attachment is '*irreversible attachment*' of the bacterial cells due to stronger chemical interactions that include both intermolecular hydrogen bonding, hydrophobic interactions, and intramolecular covalent linkages which then leads to monolayer formation and later the production of EPS [65].

Stage 3: Migration to form multi-layered colonies

Colonies originating from sessile bacteria initially develop as a flat monolayer of cells and, upon reaching a critical population, move into the third dimension forming multiple layers [72,73]. This phenomena has been recognised recently as a universal step in biofilm formation, and is considered as the stage where mechanical forces are influential [73]. Multi-layer biofilm development requires time and nutrient availability in order for adequate bacterial cell division to occur, which gradually leads to self-organised arrangements of bacterial colonies which favour nutrient availability, evacuation of waste, and protection from environmental and internal stresses [72].

There are exceptions to this cyclic phased progression of biofilm formation such as during initial surface colonisation events when bacterial cells bind to a bare surface. This exception is because in later subsequent stages, late colonisers can adhere to the sessile bacteria or the already-formed biofilm on a substratum. In these instances, it has been thought that the early stage colonisers express biochemical components suitable for initial attachment, with the later stage colonisers exhibiting different components that allow them to bind to the early colonisers [74]. This phenomenon is referred to as coaggregation, for example this process can be observed in fusobacteria, a genus of anaerobic, Gram-negative, non-spore-forming bacteria. Fusobacteria have the ability to become the dominant Gram-negative specie in biofilms associated with healthy gums and gingivitis plaque as it is able to readily coaggregate to a wide range of early and late stage colonisers [74,75].

Stages 4-5: Significant production of EPS and biofilm maturation with 3-D architecture

Once surface attached, bacteria must maintain contact in order to evolve to a mature biofilm and a key aspect to this happening is the production of EPS [76]. The EPS, also sometimes referred to as EPM (extracellular polymeric matrix), is formed during biofilm growth. It primarily consists of polysaccharides but may also include ions, lipids, nutrients and even DNA. The composition and structure of the EPS is largely determined by the polysaccharides present [77], these polysaccharide rich zones are often termed the glycocalyx, meaning “sugar coat”, which is also known as the slime layer [78]. Most

EPS consist of a backbone structure of rigid 1,3- or 1,4- β linked hexose residues that make the biofilm more rigid, and as a consequence provide mechanical support to the biofilm structure [77]. However, the EPS can differ from organism to organism, for example *P. aeruginosa* produces at least three known polysaccharides (alginate, Pel, and Psl) that provides structural stability to the *P. aeruginosa* biofilm. The alginate for example contributes to increased tolerance to antibiotic treatment, Pel offers an important role in cell interaction and structural scaffolding, and Psl contributing to initial attachment and microcolony formation [79]. Polymers in the EPS can form gels, both rigid and non-rigid weak gels that can be viscous in character, due to the presence of polysaccharides with high molecular masses. Electrostatic interaction between ions and exposed carboxylic acid moieties can also contribute to increased gelation [77]. The role of the EPS includes the anchoring and adhesion to a surface, providing mechanical stability to the film, allowing for the distribution and storage of nutrients, ions and trace metals, acting as a matrix for extracellular enzymes, as well as acting as a protective envelope for the bacteria within the biofilm [80]. Therefore, the fully matured biofilm resembles a complex 3D structure, containing channels for the transportation of nutrients, water and waste [81].

Stage 5: Biofilm Dispersal

Biofilm dispersal can be divided into passive and active processes, whereby passive dispersal refers to detachment mediated by external forces and active dispersal is initiated by the bacteria [82]. There are three distinct modes of biofilm dispersal, these are (a) erosion, (b) sloughing and (c) seeding, where erosion is the incessant release of single cells or small clusters; sloughing is the sudden detachment of larger portions of the biofilm; and seeding is the rapid release of a large number of single cells from cavities, also known as central hollowing [82]. Kaplan has regarded erosion and sloughing to be either passive or active, whilst seeding is always active in nature [82]. The dispersion of further bacteria from a mature biofilm encourages the spreading and expansion of the biofilm onto downstream and surrounding surfaces, refer to **Figure 2.2**, contributing to the cyclic nature of a biofilm's life cycle. Dispersal of biofilms can differ amongst organisms, for instance clumping dispersal (sloughing) where large

aggregates are detached from the biofilm has been seen in *Staphylococcus aureus* (*S. aureus*), in which the larger clumps were also highly tolerant towards oxacillin (an antibiotic treatment for infection) [83]; whereas when *P. aeruginosa* was examined, single-cell dispersal was observed more prominently [84]. Furthermore, as the thickness of the EPS increases, anaerobic conditions arise within the biofilm structure which can stimulate the sloughing off and detachment of biofilm segments [85].

2.1.3 Reasons for Initial Bacterial Adhesion to Surfaces

When a planktonic cell nears a surface from the bulk fluid or airborne cells, there are three cues and stimuli that can be experienced to stimulate irreversible surface attachment from a planktonic cell to a sessile cell; (a) changes in physiochemical properties compared to the bulk, (b) attachment of cell appendages and (c) envelope stress from the attachment of the cell body [86], see **Figure 2.3**. For example, the microenvironment surrounding a materials surface can differ from the bulk due to pH variations caused by physio-chemical properties present at the surface, which can stimulate irreversible bacterial attachment. Alternatively, the physical bacterial cell body can make contact with a substratum from envelope stress, for instance from unfolded proteins or from surface lipopolysaccharides (LPS) [86,87].

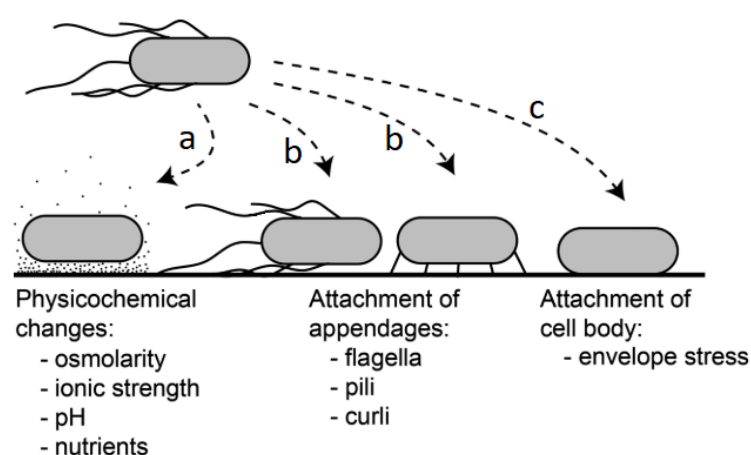


Figure 2.3 Schematic summary of the different signals that can trigger irreversible surface bacterial attachment to a substratum with some examples of causes, showing (a)

physiochemical changes, (b) attachment of appendages and (c) attachment of cell body. This list is not exhaustive. (Permission to publish this figure has been obtained using RightsLink®, licence number 5162491149682, in courtesy of Kimkes and co-workers).

Variations in the responses from these cues generally depend on three main variables at play; the liquid environment, the solid surface, and the bacterium itself [88]. For example, bacteria may have external appendages that aid in the adhesion process, like flagella and pili [88]. In some cases, this can be caused by an intrinsic chemotaxis response. For instance, *P. aeruginosa* has exhibited pili-based twitching motility to move towards a chemoattractant on a substratum, like dimethyl sulfoxide and succinate, with sub-micron precision [89]. This eventually resulted in a localised build-up of bacterial cells in specific regions that led to biofilm growth and formation within 20 h [89]. Bacterial cells can even interact with each other by chemotactic signalling through chemoreceptors, one example shows the selective growth of *E. coli* on semi-solid agar in the presence of succinate forming aspartate, with the *E. coli* cells sensing aspartate and forming symmetrical patterned biofilms on the agar surface [90].

Bacterial LPSs are bacterial endotoxins (capsules) that are amphiphilic and heterogenous in size and composition [91]. Approximately 3.5 million LPS molecules are found on the outer wall of Gram-negative bacteria which are commonly known for their ability to initiate inflammatory and immunomodulatory response in humans and animals [92,93]. LPS molecules have been suggested to function as adhesins that mediate binding to surfaces. These capsules are usually acidic polysaccharides secreted by bacteria that remain cell bound after secretion. In strains of *Staphylococcus epidermis* (*S. epidermis*) that were deficient in producing capsular polysaccharide adhesins, it was revealed that biofilm formation was significantly reduced in rabbit models to 1% compared to 75% of rabbits infected with non-deficient strains [76,94]. Furthermore, separate studies have shown that modification of the LPS to deacetylated LPS inhibited biofilm formation in several Gram-negative bacteria [95].

Irreversible cell attachment can also occur via mass transport through Brownian motion, sedimentation (gravitational forces), as well as from hydrodynamic forces of a bulk solution, which in turn can be affected by the substratum topography. Yang *et al.*

have demonstrated that convective mass transport directly towards a steel pipe can produce high cell attachment rates [65,96]. Additionally, *P. aeruginosa* and *E. coli* exhibit preferential attachment locations or 'hot spots' on tested curved surfaces due to local variations in the hydrodynamic environment, such as the leeward side of a pillar [97]. Overall it is understood that the influence of hydrodynamics on bacterial attachment is also largely dependent on cell morphology and their swimming behaviour [97]. Alternatively, bacteria can proliferate in cavities and corners of a substratum in order to survive, as well as increase their ability to adhere to a surface. Park *et al.* found that in a system of starved *E. coli* cells, the cells responded to chemo-attractants that are self-excreted, which in a varied topological environment, would favour small cavities for heightened survival [98]. On the other hand, Friedlander *et al.* has proven that *E. coli* can colonise crevices to access the enhanced surface area in order to reproduce and form a biofilm, with accelerated growth seen on rougher textured surfaces compared to smooth control samples [99]. Increases in surface average roughness (R_a) can result in an increase in bacterial adhesion, as with *S. epidermis* where heightened adhesion to rougher biomaterials was seen, like with titanium an increase of 2×10^4 CFU/mm² was observed with an increase in 9 nm R_a ; similar results were seen for all biomaterial surfaces examined in this study [100].

Bacterial adhesion is governed by the sum of attractive and repulsive forces between the cell and the surface, whilst being also mediated by possible substrate variables such as surface roughness and surface free energy [101,102]. Overall, this property is recognised as Gibbs energy barrier required for bacteria to adhere [65]. As a result, the surface energy of a substratum can affect bacterial attachment, particularly the initial binding of unicellular bacteria to a surface. Surface energy is the thermodynamic work needed to create a unit of surface and is given in units of mJ/m² [103]. It is the solid equivalent to the surface tension of a liquid. Research published on the influence of surface energy on bacterial attachment is substantial yet contradictory, with both hydrophobic (low surface energy) and hydrophilic (high surface energy) surfaces showing reduced bacterial attachment in different instances. For example, MacKintosh *et al.* observed biofilm growth of *S. epidermis* over 48 h on a surface modified PET with different surface energies. It was revealed that the hydrophilic surfaces caused a large

reduction in the initial adhesion of *S. epidermis* [100]. In another case, borosilicate glass beads were coated with polymers of varying surface energies and charge densities; it was established that there was a significant reduction in the initial bacterial adhesion of *Giardia lamblia* for the low surface energy (hydrophobic) polymer coatings used [104]. Largely, surface energy has been shown to be one of the key variables that can significantly influence bacterial attachment, although it is organism and substrate dependent. It has been argued that hydrophobic or rather, super-hydrophobic surfaces, exhibit very effective antibiofouling capabilities. For instance, novel research synthesising antiadhesive bacterial coating utilises this approach by Privette *et al.* where synthesised fluorinated nano-sized xerogels act as a super-hydrophobic surface coating, with bacterial adhesion of *P. aeruginosa* and *S. aureus* reduced by roughly 2 orders of magnitude [105].

Material surfaces are almost never found in a virgin uncontaminated state, usually there is a conditioning film present on the surface before bacterial attachment. The conditioning film usually consists of accumulated inorganic and organic molecules and can arise from the transport of molecules from the bulk to the surface via diffusion, sedimentation or turbulent flow from the bulk medium [65]. These films might, in other research disciplines, be described as a surface contaminant on the materials. A conditioning film can provide a nutrient source for biofilm growth and often contributes toward promoting the initial irreversible attachment of unicellular planktonic bacteria. However, this premise has also been found to be case-dependent [65]. For instance, a conditioned film of skimmed milk on stainless steel reduced the attachment of vegetative bacterial cells even at low concentrations of less than 1% skim milk [106]. Whereas Jain *et al.* demonstrated that the presence of a conditioning film on glass panels resulted in a substantial increase in bacterial attachment of marine bacteria even with varying bacterial cell surface hydrophobicity being considered, this adhesion increase was found to be caused by an increase in carbohydrates contents within the conditioning layer [107,108]. Conditioning films can also alter the substrates surface properties including interfacial surface free energy, hydrophobicity, and surface roughness. Lorite *et al.* established that a conditioning film on glass and silicon substrates produced an increase in hydrophobicity and a significant reduction in surface

roughness which did not correlate with microbe attachment, in fact the presence of polyphosphate moieties coincided with increased biofilm thickness. It is evident that the chemical composition of the conditioning film in this case was important in influencing bacterial adhesion [109]. A conditioning film is very difficult to eliminate from substrates. A study found that the presence of bovine serum albumin, a protein mixture commonly used as a nutrient source for bacterial culture preparation, increased the attachment of *S. aureus* on stainless steel compared to the negative control, absent of bovine serum albumin [110].

A summary of important variables discussed in Section 2.1.3 regarding bacterial cell attachment to a substratum has been provided below in **Table 2.1**.

Liquid Environment	Solid Surface	Bacterium
Hydrodynamics/flow/viscosity	Roughness	Species/strain
pH	Hydrophobicity/hydrophilicity	Flagella
Temperature	Surface free energy	Pili
Nutrients	Surface charge	EPS
Oxygen	Conditioning film	Cell adhesins (e.g. LPSs)
Electrolytes		Cell surface
		Charge/hydrophobicity

Table 2.1 Summary of key variables that can influence and induce bacterial cell attachment to a substratum.

2.1.4 Biofilm Formation Phenotype

Biofilm formation from a planktonic to sessile phenotype is regarded as a complex phenomena, and can be instigated by many external triggers that can induce the activation of a subset of genes required for biofilm formation [54,111]. Bacteria have developed multiple genetic pathways to respond to external triggers, which has led to their ability to adapt to readily form biofilms as their preferred and most prevalent natural form [2]. As a result, understanding and deconvoluting these genetic pathways is a complex, multifaceted and still not clearly understood process [54]. External triggers can differ amongst organisms and can also rely on several dependent

variables which include both environmental and other intrinsic factors. For instance, the O517:H7 strain of *E. coli* is able to form mature biofilms on stainless steel only in low nutrient media and low incubation temperatures of 12°C [112,113], whereas *E. coli* K-12 strain is able to form biofilms on abiotic surfaces in a variety of conditions [114–116]. Other influencing variables include pH, oxygen content, bacterial strain, moisture, conditioning film and fluid hydrodynamics which all play a varying role in inducing biofilm phenotype development [116–121], some are briefly discussed in the following section.

It has been argued that the presence of a suitable substrate is sufficient to trigger the biofilm phenotype. For example *P. aeruginosa* was able to express *algC*, a biosynthesis gene required for alginate synthesis, within minutes of attachment, although initial attachment to the surface seemed to be non-selective for *algC* promoter activity [122]. Therefore, bacteria can sense the solid surface once attached which leads to inducing a cascade of signalling responses or autoinducers that can trigger or heighten gene expression to start the process to biofilm maturation and growth.

The biofilm phenotype does not emerge homogeneously across a biofilm. Heterogeneous microenvironments are exhibited as a result of different local environments in the bacteria vicinity, such as pH, live-dead ratio of bacteria, EPS and water content [123]. Scientists have demonstrated this effect most prominently through the concentration gradients of oxygen, because oxygen fails to penetrate deep into a biofilm as it is actively respired by outer cells [123]. An example to this behaviour was demonstrated with *E. coli* by Beebout *et al.*, where an oxygen gradient led to spatially distinct expression of oxidases responsible for EPS production [124]. In fact, the development of microenvironments has been divided into three distinct localities based on oxygen and nutrient availability: (a) oxygen and substrate rich zone near the surface, (b) intermediate substrate rich and oxygen depleted zone, (c) substrate and oxygen depleted zone [125], see **Figure 2.4**. The latter microenvironment contains the persister cells, that are tolerant to antibiotics and biocides by transitioning to a metabolically

dormant state, which lack antimicrobial targets (without undertaking genetic modifications) [48,126].

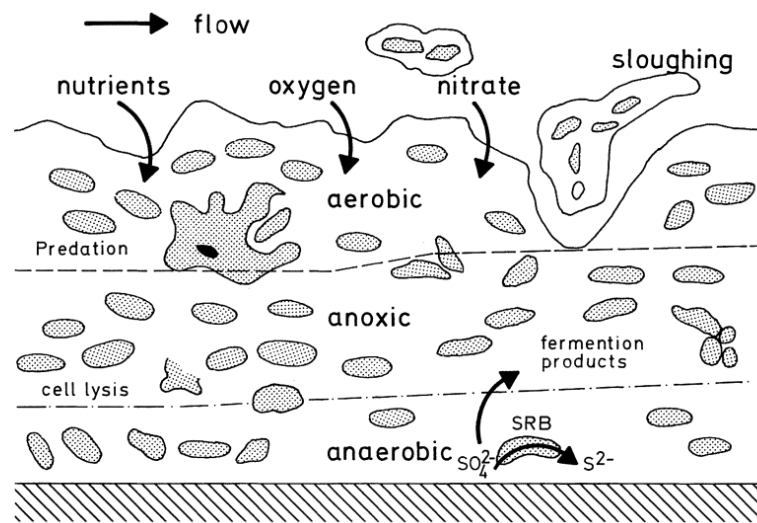


Figure 2.4 Schematic illustration of a mature biofilm and the three distinct communities, dependent on oxygen availability [127].

2.1.5 Types of Surfaces that can be Colonised

Bacteria can colonise many surfaces, given enough time, moisture and nutrient availability. This includes living or dead tissue (e.g. lung and skin) and any inert surfaces and anthropogenic materials (e.g. medical devices, industrial pipelines, ships, rocks) [128]. In natural aquatic ecosystems, sessile microorganisms greatly outnumber the organisms that are in suspension in the bulk [129]. In the food industry, biofilms are the major cause of food contamination leading to food poisoning, despite the use of disinfection procedures [130]. In medical environments, healthcare-associated infections (HAIs) which are infections caused by contact from hospital and healthcare facilities, are linked with an increase in morbidity and mortality, including device-associated infections like catheters and ventilators [5]. The existence of pathogenic microorganisms in drinking water is largely facilitated by their ability to form biofilms in the inner walls of pipes of distribution networks [131], which can lead to waterborne infections, biocorrosion, reduced heat exchange efficiency, cross-contamination and distribution system failures [130]. The prevalence of a conditioning film makes it very

difficult for any surface to evade biofilm contamination and development, particularly for materials that are in an aqueous medium where conditioning film formation is inevitable [132]. In fact, it has said to be impossible to develop a surface that cannot be colonised by bacteria, while at the same time be harmless to humans and the environment [86]. Biofilms can even exist at a gas-water interface, where hydrophobic microorganisms can accumulate, with the bacteria utilising hydrophobic substance as a nutrient source [127].

It has been suggested that when biofilm development on diverse synthetic material substrates are compared, different microbial communities are exhibited at early stages, however over time these communities become more similar, regardless of substrate variations [133]. Nevertheless, this early-stage phase of bacterial attachment is a key interest to scientists in order to modulate surfaces to inhibit, prevent or perturb the formation of sessile bacteria. It has been shown that the type of surface is significant in the genetic pathway used for biofilm formation. For example, bacterial attachment to biotic and abiotic surfaces can be carried out by different genetic paths, with some common features between both processes [76]. Watnick *et al.* discovered that *V. cholerae* (the El Tor wild-type strain) can form biofilms on borosilicate substrates (abiotic) using the mannose-sensitive hemagglutinin (MHSA) pilus which is not involved in pathogenicity. Yet when *V. cholerae* was exposed to chitin substrates (a component from the exoskeleton of crustaceans and one of the preferred niches for *V. cholerae*), biofilm formation followed an MHSA independent pathway [134]. Therefore, suggesting that the type of surface plays a significant role in determining biofilm formation pathways and their phenotype [48,126].

2.1.6 Strategies for the Prevention and Removal of Biofilms

Prevention and removal of biofilms is difficult as surfaces can become re-contaminated very easily. Nevertheless, there are four main antibiofilm approaches which summarise the strategies that are used to both prevent and remove (weaken, disrupt, and kill) biofilms, see **Figure 2.5**. Surface related technologies including coatings

and surface modification approaches for the prevention and removal of biofilms have been discussed in Section 2.2.

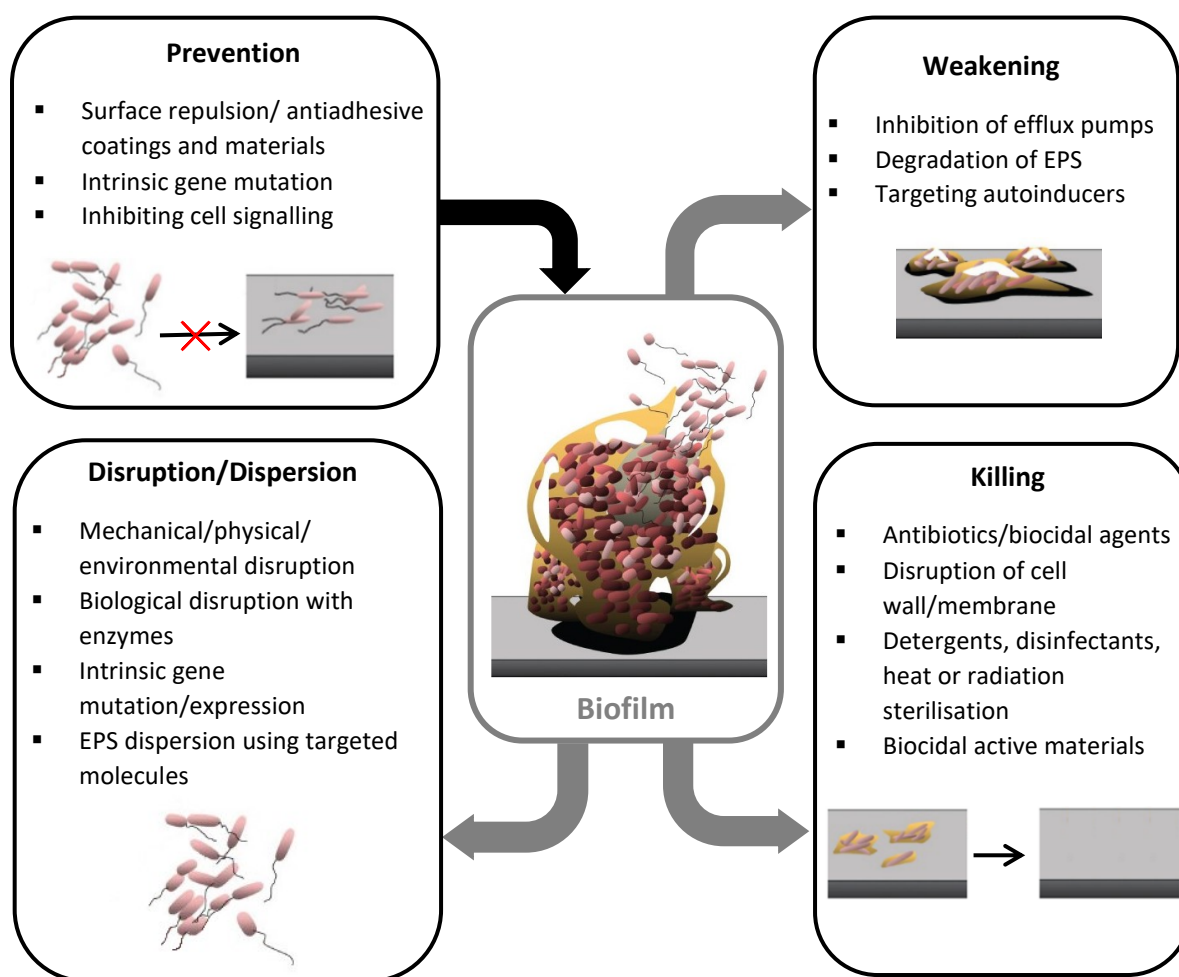


Figure 2.5 Antibiofilm strategies, summarised as prevention, weakening, disruption/dispersion and killing strategies, adapted from the work of Bjarnsholt et al. [135].

Prevention

Prevention can be achieved by various routes, one of which includes the use of small molecules to cause intrinsic changes to genes. For example, anti-virulence compounds have been shown to inhibit gene expression of virulence factors that were found to prevent the formation of *S. aureus* biofilms [136]. Some prevention mechanisms include interference with EPS formation, like *N*-acetylcysteine that has shown continual reduction of EPS in production of *S. epidermis* biofilms as the concentration of *N*-acetylcysteine

was increased [137] . Additionally, prevention strategies have also looked at using ultra-pure water as biofilm proliferation is rife in aqueous environments, particularly if carbon sources are present which is used by bacteria for growth. However, studies have shown that even the removal of carbonous materials from water in the form of ultra-pure water was unable to avert biofilm proliferation [138,139]. Several attempts have been made to prevent biofilm formation through modifying and modulating surface functional moieties to impart antiadhesive/antibiofouling properties, with varying degrees of success. This approach is further explored in Section 2.2 in greater detail.

Weakening

Biofilm weakening strategies are important approaches as they could be combined with other existing methods, like antibiotics, to eliminate infection and ultimately biofilm proliferation. Disruption of autoinducers is one way of achieving this outcome and this approach has been demonstrated in *P. aeruginosa* using a synthetic halogenated furanone compound [140]. This compound has specifically repressed AHL-mediated quorum sensing by reducing the production of important virulence factors and the expression of certain genes that caused a loss of bacterial biomass, however this chemical did not affect initial attachment [140]. Turbulent hydrodynamic conditions like high liquid velocities can also affect quorum sensing, although this effect is less prevalent in surfaces with crevices and cavities [141]. Efflux pump interference has shown to also weaken the biofilm; for example *S. aureus* NorA efflux pump contributes to resistance to many biocides, dyes, quaternary compounds and antiseptics [142]. Zimmerman *et al.* has established that nilotinib (a tyrosine kinase inhibitor) can inhibit NorA activity which, when used with ciprofloxacin (an antibiotic), is able to significantly reduce biofilm formation of *S. aureus* [142].

Disruption/Dispersion

Passive dispersal methods for biofilms include the use of dispersal agents such as the chelating EDTA (ethylenediaminetetraacetic acid), or nitric oxide, that can disperse *P.*

aeruginosa biofilms [143,144]. Alginate is the main component of the EPS for *P. aeruginosa* and Boyd *et al.* revealed that the increased expression of an alginate lyase enzyme substantially decreased alginate production, which in-turn resulted in an increase in the number of detached cells [145]. Environmental cues like oxygen tension has shown to cause sloughing of *P. putida* in an oxygen-limited environment [146]. Passive dispersal can be induced by mechanical triggers, such as ultrasound and sonication regimes [147], however these approaches are limited in practise due to operating costs. In dental care, fluid based shear is often used to remove biofilms which has shown to drastically remove the levels of dental plaque, the biofilm that grows on teeth, with increasing pressures and liquid velocities [148].

Killing

To kill cells in a biofilm, the biocide must penetrate the EPS of mature biofilms in order to access the microbial cell [149]. Regular cleaning and disinfecting are the main biocidal approaches used to prevent biofilm maturation and remove biofilms from surfaces, including the use of detergents, solvents, ozone and/or heat exposure [150]. Commonly utilised disinfectants in the food industry include peracetic acid and, quaternary ammonium compounds (QAC) like benzalkonium chloride. These disinfectants typically kill bacteria by the disruption of the cell membrane and/or interact with a number of cellular constituents [151,152]. Oxidising substances such as chlorine and peroxy-acetic acids are also often used, particularly in water treatments [149]. The WHO has advised that a residual concentration of free chlorine of 0.2 mg/L is needed for water treatment [153], though some reports have found that the use of chlorine in these systems is successful in removing planktonic bacteria, yet has a limited effect in reducing the concentration of biofilm at lower levels of chlorine. However Liu *et al.* has revealed that chlorine levels greater than 1 mg/L are required to inhibit microbial attachment, with lower chlorine levels in fact promotes bacterial attachment to surfaces [154]. Killing of biofilms using biocides with cleaning is enhanced by use of mechanical forces, such as using automatic scrubbers, high-pressure cleaners and hydrodynamic pressure through turbulent flow. Blanchard *et al.*, demonstrated a 99.7% increase in success of peroxy-acetic acid against biofilms in turbulent flow compared to

laminar for *P. aeruginosa* [155]. Also, electric fields have shown to enhance biofilm removal and increase the efficiency of biocides [156]. Surface based biocidal approaches are further discussed in Section 2.2.

2.2 Antimicrobial Surface Modification and Coating Strategies

Antimicrobial surface development is a contemporary approach for dealing with biofilms, which may be ‘antimicrobial’ and/or ‘antibiofouling’ [157,158], although the term antimicrobial is generally also used by some as a summary of both approaches. The antimicrobial activity of a material can be categorised further into two distinct pathways depending on the response exerted by the bacteria, these are: (i) bacteriostatic, which halt the growth and proliferation of bacterial cells; and (ii) bactericidal, which kill the cells. This latter process can also be further categorised in the form of lysis (bacteriolytic). On the other hand, antibiofouling refers to a materials innate ability to repel, inhibit or perturb biological adhesion; all of which are antibiofilm strategies, see **Figure 2.6**. The following section reviews a range of antimicrobial as well as antibiofouling surface and coating strategies that have displayed a significant impact on biofilm prevention and removal, as well as their limitations, applications and capabilities.

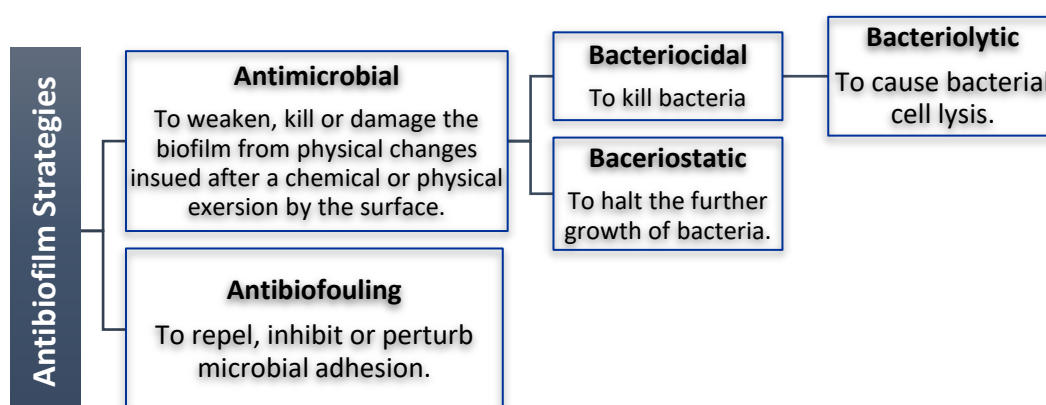


Figure 2.6 Graphical summary for the categorisation of antibiofilm strategies, and the key terms used in this thesis.

2.2.1 Antimicrobial Polymers

Though commodity polymers dominate the market, there is a growing interest in developing speciality polymers, such as those adapted, modified and developed for antimicrobial and antibiofouling applications, which are generally termed antimicrobial polymers (AMPs) [159]. AMP strategies are broad and can be categorised into naturally derived and synthetic polymers. There are two general approaches for the incorporation of antimicrobial agents into polymers that lack intrinsic antimicrobial activity. Firstly, antimicrobial activity can be achieved from the introduction of antimicrobial agents with monomers, followed by their polymerisation. The second approach is the coating or functionalising of antimicrobial agents onto the surface of the polymer after the polymer has been manufactured, which is beneficial for industries who outsource their polymer materials [160]. A brief summary of some of these strategies has been presented in **Figure 2.7**.

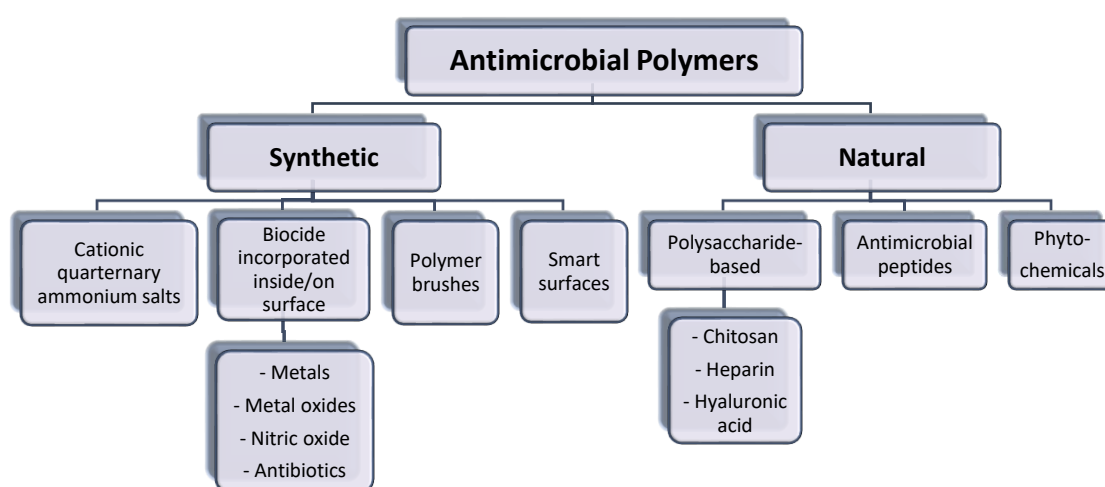


Figure 2.7 Hierarchal diagram of synthetic and natural antimicrobial polymer strategies.

AMPs have been evaluated since 1965, when Cornell and Dunraruma prepared polymers of 2-methacryloxytroponones and compared their bactericidal activity to the monomers they were derived from, although these were solution polymer studies [161]. In the 1970s Panarin and co-workers investigated materials containing

bactericidal quaternary ammonium compounds (QACs) which is now a widely explored approach in AMP development and can be used for multi-purpose surface functions [162–165], like antifogging and antimicrobial hydrophobic QACs [166]. Cationic QAC polymers may exhibit the ‘graveyard’ phenomena when a layer of dead microorganisms collect at the surface and act as a foundation for further microbial adsorption (similar to a conditioning film), which *in-vivo* can even trigger an immune response and/or inflammation [167]. Therefore, more recent QAC technologies have aimed to incorporate both antibiofouling and bactericidal capabilities to prevent adhesion and thus minimise the graveyard effect [168]. Another approach is incorporation of antibiotics with polymers, for example the copolymerization of methacrylate with modified Norfloxacin (an antibiotic used for urinary tract infections) with polyethylene glycol (PEG) to form an antibiotic PEG-methacrylate polymer. However, such polymeric conjugates come with a risk of facilitating the evolution of antibiotic resistance bacterial strains [169]. Interest in antimicrobial peptides are also increasing due to their apparent broad antimicrobial activity and recent studies having successfully demonstrated their application in preventing the formation of dental biofilm of *Streptococcus mutans* in rabbits after 24 h, following the grafting of the peptide onto the rabbits incisor surfaces [170].

Another AMP approach are smart surfaces, which includes stimuli-responsive polymers that have gathered attention for biomedical applications. These types of surfaces can be thermo-, pH-, chemo- or bio-responsive in order to be activated [159]. For instance, thermo-responsive polymers work by switching between a hydrophilic to hydrophobic phase upon changes in temperature. Poly(*N*-isopropylacrylamide) is the most studied polymer for this application which is able to switch phases at 32 °C in water [159]. A recent study showed a shrinkable hydrogel of this polymer coated onto silica glass slides was able to demonstrate varying adhesion capabilities to *Chlorella vulgaris* upon modulating the temperature from 15 °C to 35 °C [171]. Other smart surface AMPs include acid or enzyme utilisation for controlled bacterial attachment and/or death [172,173].

PEG and similar structures such as poly(glycidol) [174] have been well documented, including studies on their innate ability to exhibit antibiofouling properties against

biofilm attachment [175], functioning as an alternative method to combat biofilm growth by reducing bacterial adhesion during the early-stage of biofilm formation. One of the most studied natural polymers with antibiofouling properties is the polysaccharide hyaluronic acid [176]. In 1999 Morra and Cassineli coupled hyaluronic acid onto poly(ethyleneimine) coated polystyrene surfaces where hyaluronic acid exhibited a hydrophilic characteristic (22 °) which reduced the adhesion of *S. epidermis* and *E. coli* by several orders of magnitude [177].

Another natural polymer with well reported antimicrobial properties is chitosan, a polysaccharide derived from the *N*-deacetylation of chitin ($C_8H_{13}O_5N$)_n, see **Figure 2.8**. Chitin is the second most abundant biopolymer on earth, after cellulose [178]. The degree of *N*-deacetylation is used to distinguish between chitosan and chitin, with chitosan having *N*-acetyl-D-glucosamine units of less than 50% which also determines the solubility limit of the polymer in dilute acid [179]. Chitin can be obtained from a range of natural sources, crustaceans (shrimp, lobster, prawn, krill), insects (cockroach, butterfly, wax worm, silk worm), algae (diatoms, brown algae, green algae), fungi (*Mucor rouxii*, *Aspergillus niger*, *Penicillium notatum*) [180]. Chitosan and its derivatives have been heavily studied and are widely regarded as a highly effective class of AMPs against bacteria, fungi and viruses. Yet its effectiveness heavily relies on several key variables surrounding its preparation and synthesis including the degree of deacetylation (DDA), concentration, molecular weight (MW), chemical modifications, and the microorganism being tested. Some contradictory studies have been reported [181]. For example, the effect of MW on chitosan's antimicrobial properties is highly debated; in one study an increase in MW corresponded to a decrease in fungal growth of *Candida tropicalis* and *Candida parapsilosis* [182]; whereas, Tong and his co-workers have shown that *Cryonobacter malonaticus* was inhibited severely by both high and low MW chitosan [183].

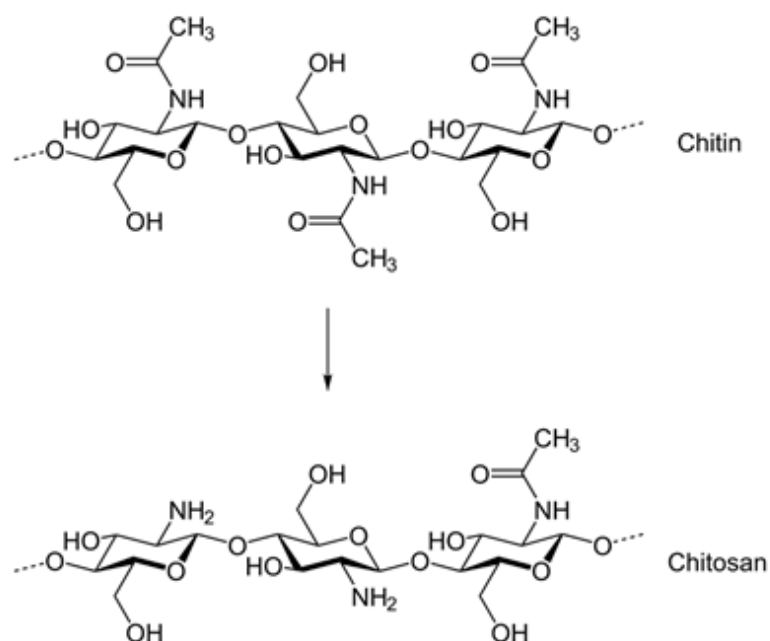


Figure 2.8 Molecular structure of chitin and chitosan derived from the deacetylation of chitin [184].

In terms of DDA, generally it is regarded that the antimicrobial activity is heightened with increased DDA levels. This behaviour is because more free amine groups are present at high DDA's which is generally considered to be chitosan's biocidal active agent [181]. This trend was also observed with biological cells (e.g. fibroblast and keratinocyte) adhering and developing on chitosan films [179]. It has been argued that the effect of MW is greater than DDA on the antimicrobial performance of chitosan [185]. The advantage of using chitosan is its broad spectrum antimicrobial activity with low toxicity for mammalian cells [179,186]. Chitosan can also be incorporated with other known antimicrobial species for enhanced antibacterial activity, such as silver or ZnO [187,188]. The mechanism of antibacterial activity of chitosan has been proposed by Chung and Chen to occur via a two-step sequential process. The first step involves an initial separation of the cell wall from the cell membrane, and the second step contains the destruction of the cell membrane [186]. This mechanism was determined by comparing the leakage of nucleotides during chitosan exposure in *E. coli* and *S.*

aureus to the known biocidal agents of EDTA and polymyxin B. EDTA has been known to destroy the cell wall (especially for Gram-negative bacteria) by chelating with Ca^{2+} and Mg^{2+} species in the wall, whereas cationic polymyxin B can react with anionic phosphate groups of phospholipids in the cell wall which destroys the cell membrane and increases permeability. For EDTA a smooth curve was seen for detected nucleotides released, whereas a linear increase was seen in polymyxin B; comparison of these trends with chitosan plots led to an undiscovered two-step antimicrobial mechanism of activity for chitosan, based on nucleotide leakage [186]. Other reports have also concluded chitosan interferes with the cell wall of microorganisms which leads to the leakage of internal electrolytes, including nucleic acids [185].

2.2.2 Metal and Metal Oxide Coatings

Metals have been used as antimicrobial materials for thousands of years. For instance Persian kings would only use silver cups as it was known to keep the water fresh for years, and Egyptians in 2000 BC (4000 years ago) used copper to sterilize water and wounds [189]. Also, ancient Egyptians, Indians, Persians, Phoenicians, Greeks and Romans have utilised copper and silver to disinfect water and preserve food [190]. The antimicrobial properties of metals and their oxide derivatives have been employed in many applications throughout history, yet their use in medicine diminished rapidly after the discovery of antibiotics in 1920 by Nobel laureate Sir Alexander Fleming [191]. The Organisation for Economic Co-operation and Development (OCED) has classed four metal and metal oxide nanoparticles to be of high interest due to their inherent properties and widespread use; cerium oxide, silver, zinc oxide and titanium dioxide [192]. Their applications range from glass polishers, purifiers, heat-resistant coatings, fuel additives, deodorants, paints, cosmetics, animal feeds, fertilisers and sunscreens to name a few [192], therefore their use in consumer facing products is already well established. In addition, many metals and metal oxide nanoparticles have shown to exhibit excellent antibiofilm properties against both Gram-positive and negative bacteria. The emergence of antibiotic resistance strains of bacteria from gene mutations has led to a renewed interest in metal and metal oxide nanoparticles as a

possible antimicrobial solution. Antibiotics attack bacteria using one mechanism, whereas metallic species can potentially utilise differing mechanisms. The antimicrobial mechanisms used by metals and metal oxide nanoparticles are well known, and follows one or more of the following mechanisms [193] listed in points (1), (2) and (3) below. The primary mechanism involves oxidative stress from reactive oxygen species (ROS) as a result of metal ion dissolution, which may lead to nucleic acid damage, protein oxidation or disruption and disarrangement of the bacterial cell membrane [192].

- (1) Mechanical damage to cell wall from electrostatic interaction
- (2) Oxidative stress from ROS
- (3) Disruption of protein and cell structures from metal cations

The synthesis of metal oxide nanoparticles can be achieved using a sol-gel method which involves the hydrolysis of metal organic compounds to produce a metal hydroxide network [194]. Employment of a sol-gel method is advantageous because the thin-film coating on a substrate can be attained through synthetically simple and low-cost means, such as dip-coating, spin-coating and laminar-flow coating; see **Figure 2.9**. The sol-gel method is a facile approach to obtain a coating because of its low cost in synthesis and preparation, no need of high temperatures, and is easy to deploy to obtain a uniform thin-film coating on complex substrate shapes [195].

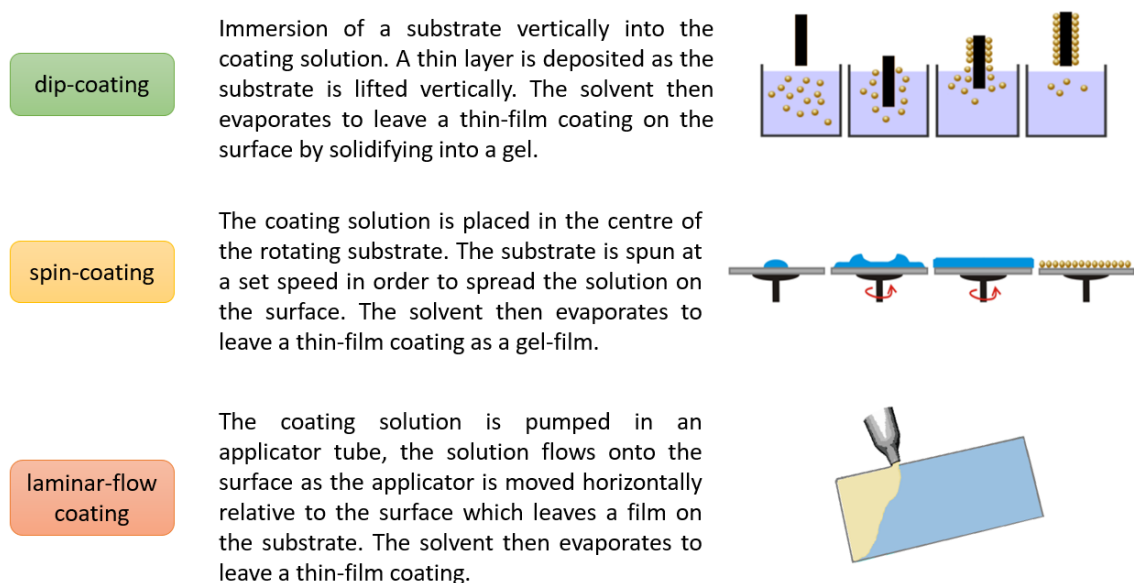


Figure 2.9 Illustration of spin-coating, dip-coating and laminar-flow coating methods [195].

Metal oxide nanoparticle coatings exhibit varying degrees of efficacy. Azam *et al.* has examined ZnO, CuO and Fe₂O₃ metal oxide films, reporting that ZnO possessed superior bactericidal properties against Gram-positive and negative bacteria with its bactericidal activity in the order of ZnO > CuO > Fe₂O₃ [196]. ZnO has also been examined as a coating on dental implants by deposition onto titanium disks, with a live/dead cell viability assay revealing cell death with an associated 74% reduction in biofilm biomass [197]. A combination of metal oxides can be used to achieve enhanced mechanical and antimicrobial properties, such as ZnO with TiO₂ which displayed excellent antimicrobial activity against *P. aeruginosa*. TiO₂ is a corrosion resistant material and ZnO provides a bactericidal impact with the release of Zn²⁺ ions [198]. Additionally, it has been found that ZnO nanoparticles can show enhanced antimicrobial performance when accompanied by light-activated antimicrobial agents like photo-sensitisers. These light-activated antimicrobials can damage bacteria through the production of harmful ROS via photodynamic therapy. This ultimately leads to oxidative damage and bacterial cell death. Commonly used photo-sensitizers include crystal violet, for instance Noimark *et al.* revealed that crystal violet and di(octyl)-phosphinic-acid-capped ZnO 3-4 nm nanoparticles can be incorporated into medical grade silicone and show antimicrobial

performance against both *S. aureus* and *E. coli*, with heightened results observed in the presence of light [199]. Optimisation of such a strategy showed that oleic acid-capped crystal violet-ZnO coated on polyurethane sheets to be highly effective in reducing *E. coli* biofilm viability even further after 4 h in the dark, as well as phosphinic acid-capped crystal violet-ZnO being highly efficacious against an epidemic strain of methicillin-resistant *S. aureus* under light [200].

Alternatively, SnO₂ demonstrated numerous examples of promising antibacterial capabilities [201–205]. It is an *n*-type semiconductor with a wide band gap that affords many uses, including conductive transparent electrode in solar cells, self-cleaning surfaces, rechargeable batteries and anti-reflection coatings [202]. SnO₂ nano-rods incorporated into polymers have afforded excellent bactericidal functionality against *S. aureus* and *E. coli*, which was enhanced with UV light exposure, whilst no cytotoxicity found to human skin cells (normal human dermal fibroblast cells) in the study [205]. Additionally, it was found that the SnO₂ nano-rods also reduced wear and friction rates as an added advantage to loading throughout the polymer material [205]. The activity of SnO₂ is thought to proceed via ROS production, which explains why it is photocatalytically enhanced through UV and visible light exposure [205]; further reports have also shown that the surface charge of SnO₂ can interact and disrupt the membrane of Gram-negative bacteria [206]. Additionally, composite SnO₂-ZnO sol-gel thin films coated onto glass substrates showed bactericidal activity against *E. coli* even in the dark [207]. The success of this metal oxide has crept through to industrial applications, such as for water treatment technologies. Aqua Metrology Systems Ltd (a company that provides water quality analysis and remediation) utilises SnO₂ as a foundation for one of its technologies (called SafeGuard™) which is an *in-situ* SnO₂ generator that acts as a biocide for drinking water and cooling systems against biofilms and remedies biocorrosion and biofilm proliferation. The SnO₂ also provides additional benefits of reducing Cr⁴⁺ to Cr²⁺ in drinking water, removing dissolved mercury, as well as providing selenium and lead recovery [208,209].

Silver nanoparticles can be loaded in polyelectrolyte multilayers [164], hydrogels [210,211], and polymers [211] to achieve antimicrobial coatings and films for AMP technology development. Aymonier *et al.* synthesised a hybrid system of silver

nanoparticles (1-2 nm) with amphiphilic modified poly(ethyleneimine) polymer, in which the silver nanoparticles were effective in reducing the number of *E. coli* cells by 98% [212]. Other examples include a uniform hydrogel-silver nanocomposite that revealed enhanced bactericidal activity against *E. coli* [210]. A separate study found that silver nanoparticles imbedded in a polymer sol-gel coating resulted in a 99.9% kill efficiency for *Lactobacillus plantarum* [211]. A mechanistic study found that silver species disrupted multiple cellular processes (e.g. TCA cycle) and was capable of disturbing Fe-S clusters through increased ROS levels, and silver also showed to affect membrane permeability of *E. coli* in this study [213]. Overall, silver infused coatings have shown to be very popular and beneficial, but large-scale use of silver for antimicrobial practises can be costly, particularly if the silver surface needs to be replenished or re-coated as the silver content diminishes. Nevertheless, the use of silver is the most commonly employed technique for the industrial manufacturing of antimicrobial materials in the real world, global company suppliers for public consumer needs include Microban®, SportingTex® and BioCote® to name but a few commercial examples.

The antimicrobial performance of metals and metal oxides is heavily influenced on the size, shape and even crystal structure of the nanoparticles [206]. For instance, Luna-Velasco *et al.* investigated the effect of SnO₂ nanoparticle size compared with bulk SnO₂ on the toxicity of *Bacillus Subtilis* (*B. Subtilis*), comparing 2 nm, 40 nm and a flower-like 800 nm SnO₂ species. It was observed that the smaller particle sizes offered greater hydrodynamic advantage and stability, it also afforded the best toxicity against *B. Subtilis*, although these were solution based studies [206]. Lin *et al.* observed that TiO₂ with an anatase crystal structure and a smaller particle size produced higher intracellular ROS species and malondialdehyde (stable end-product of lipid peroxidation and marker for oxidative stress), which afforded greater bactericidal results [214]. The toxicity of these nanoparticles may also differ with the microorganism tested, for instance due to varied membrane compositions [215]. Toxicity of metals and metal oxides based on bacterial cell membrane composition vary on a case by case basis. In some instances both Gram-positive and negative bacteria exhibited sensitivity, whereas

in other cases Gram-positive [216–218], or Gram-negative have shown superior sensitivity over the other [219]. Susceptibility of Gram-negative bacteria is also proposed to be due to the lipopolysaccharide molecules coating the bacteria which are negatively charged, and have a high affinity for most of the ions from the nanoparticle release (cations) which leads to increase ion uptake and intracellular damage [220]. Although both Gram-positive and negative bacteria are considered to have an overall negative surface charge, *E. coli* has been found to be more surface negatively charged and more rigid than *S. aureus* [220,221].

Use of metals and metal oxide coatings are also limited by their application scope, as once the concentrations of the active species/coating are reduced below a critical threshold level, the antibiofilm performance can be lost. This may be a problem for complicated and high shear systems, such as in water wells. For instance, field scale experiments of a copper coating for parts of a water wells were highly effective, but when coated on screens, casings and riser pipes in water wells in a real-world system, the coating eroded and oxidised, which rendered this approach ineffective [222].

Nevertheless, metal and metal oxide nanoparticle technologies can be incorporated and combined with other methods to achieve a synergistic and optimised antimicrobial surface with enhanced benefits. For example, the use of iron-based nano-structured surfaces [223] that incorporates topographical and chemical biocidal traits, or Au-chitosan coatings that contains bactericidal chitosan and Au components [224]. **Table 2.2** summarises metal and metal oxide nanotechnologies, inspired by the review from Mi *et al.* [225].

Nanotechnology	Mechanism of antimicrobial activity	Microorganisms	References
Ag	Release of Ag ⁺ ; ROS generation; membrane permeability; disruption of Fe-S clusters/Fe levels in cell	<i>E. Coli, Lactobacillus plantarum,</i>	[211,212,226]
Cu	ROS generation; membrane permeability; Cu ²⁺ release	<i>E. coli, Bacillus sp., Phaeodactylum tricornutum, Cholera sp.</i>	[226,227]
Au	Membrane permeability	<i>S. aureus</i>	[228]
ZnO	Release of Zn ²⁺ ; ROS generation	<i>P. aeruginosa</i>	[198]
SnO₂	ROS generation	<i>E. coli</i>	[201]
TiO₂	ROS generation; membrane permeability	<i>E. coli, Bacillus megaterium,</i>	[229]

Table 2.2 Summary of nanotechnology used for antibiofilm applications, including nanotechnologies incorporated on surfaces in the means of films, coatings, or composites.

2.2.3 Surface Energy

Fluorination as well as silicone based coatings and surface treatments are rising in popularity because they impart low surface energy properties to a material of interest. Many have suggested and found that low surface energy surfaces encourage reduced microbial and biological attachment, including Baier who discovered a negative correlation between bio-adhesion and surface free energy (SFE). This relationship is familiarised as the ‘Baier Curve’ which Baier originally proposed it in 1968 (**Figure 2.10**) [230,231], and has guided marine coating design for many years [232]. Baier defines a antibiofouling surface with critical surface tension between 20 – 30 mN/m as a “theta surface”, with its antiadhesive nature afforded from its specific surface physical hydrophobic parameters; see **Figure 2.10** [233]. This specific surface energy range for the theta surface is related to the critical surface tension to spread water on a substrate as 22 mN/m [233].

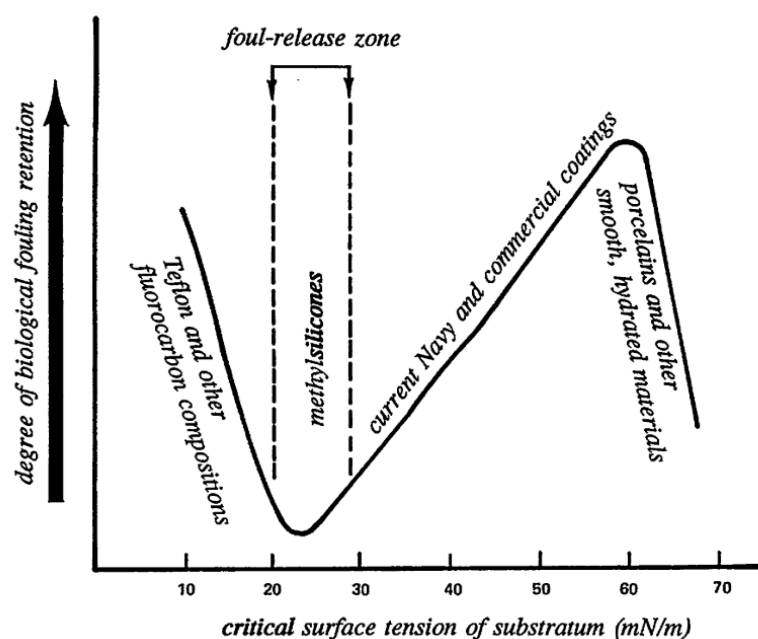


Figure 2.10 The Baier Curve, proposed by Robert E Baier showing the relationship between critical substratum surface tension and antibiofouling properties of a surface [233]. (Permission to publish this figure has been obtained using RightsLink®, License Number 5036471321739, in courtesy of Baier, Zimmerman and co-workers).

The observation of real-world scenarios have been used by Baier to support this hypothesis, like hydrophilic contact lenses that become surface contaminated by proteins [233], but has also been supported by other reports. For example, the fluorine functionalisation of PET showed a 98% inhibition of bacterial growth of *P. aeruginosa* [234], and a silicone infused polyurethane catheter significantly reduced *E. coli* and *S. epidermis* biofilm formation due to its low surface free energy properties [235]. However, studies have also compared the use of superhydrophobic surface to a slippery oil-coated surface and found that the adhesion of *E. coli* and *S. aureus* was ~98% lower for the slippery surface than the superhydrophobic surface [236]. Interestingly, the incorporation of crystal violet, a light-activated antimicrobial agent, to this slippery surface provided an added bactericidal performance for heightened antimicrobial activity in the presence of light which achieved a >3 log reduction, amounting to 99.9% kill for both *E. coli* and *S. aureus* [236].

Although in many instances antibiofouling has been exhibited by surfaces with low surface free energy, also supported by the Baier Curve, there are plenty of examples of hydrophilic coatings with no intrinsic bactericidal property that demonstrate reduced bacterial attachment, such as the previously introduced hyaluronic acid coating approach [177]. Hydrophilic materials that exhibit good resistance to bacterial adhesion are explained by the “Water Barrier” theory, which is often used to describe antiadhesive behaviour particularly for PEG-based polymers which are known for their antibiofouling properties. Chen and Zheng have reviewed the antibiofouling pathway of poly-hydrophilic and poly-zwitterionic materials where it is thought that the formation of a strongly bound surface hydration layer on the polymer surfaces can serve as a physical and energetic barrier to prevent the adhesion of bacteria and proteins [237,238].

The physical properties of surfaces, hydrophilicity and hydrophobicity, have also been shown to drastically affect bacterial morphology and community biofilm structure. The marine bacterium SW5 has found to grow in tightly packed multi-layered biofilms on hydrophobic surfaces, whereas on hydrophilic surfaces, fewer cells attach and cell division is influenced with the formation of longer cells that become loosely entangled and unstructured, and thus less densely packed biofilms form [239].

It can be concluded that the influence of SFE on biofilm proliferation is organism, substratum and environmental dependent, with often many variables in play. Therefore, it cannot be assumed that a low or high surface energy material is intrinsically sufficient to produce an antiadhesive AMP, with topography, surface chemistry, organism strain, pH, and the presence of biocidal active agents, being some of the additional factors needing consideration.

2.2.4 Surface Topography

The degree of surface roughness of a material is an important variable that affects bacterial attachment. Many natural surfaces such as insect wings and plant leaves contain intentional and distinct surface topographical features which impart antibacterial properties for self-protection and self-preservation. For example, the

Clanger cicada insect has wings with spiked nanopillars that have successfully shown to kill *P. aeruginosa* due to the topography of the surface alone, with most bacteria being found to be dead 5 min after contact with the insect wings. Bacterial viability analysis and scanning electron microscopy (SEM) imaging has confirmed that cell death was caused by the mechanical rupture of the cell wall, with the subsequent spreading of internal bacterial components outside of the cell. Ivanova and co-workers coated these wings with a very thin gold coating which increased the substrate's SFE but did not change the surface topography; yet the biocidal property of the surface was still preserved [240]. This demonstrates that the antimicrobial property exerted was solely topographical in nature.

The idea of self-cleaning surfaces was introduced by Bartholott and Neinhuis in 1997 whilst investigating the superhydrophobic lotus leaf. This leaf repels water due to its uniquely designed surface microstructures which includes trichomes, cuticular folds and wax crystals [241]. Bartholott and Neinhuis discovered that even after exposure of the lotus leaf to various microbial species, upon rinsing with water these organisms were completely removed and could not irreversibly bind to the leaf's surface [242]. Part of the microbial repelling ability was attributed to the reduced contact area between the cell and the surface, as droplets would sit at the top of epicuticular wax crystals [242]. It was established that the combination of surface topography and epicuticular wax with the hierarchal surface structure provided an optimised repellent and self-cleaning surface [243], see **Figure 2.11**. Other researchers have used the lotus leaf model to manufacture materials with comparable features in order to impart similar repellent and antibiofouling properties. Examples of this work include, nano-functionalised superhydrophobic coatings [244], etched and fluorinated polymer surfaces [245], and the world's first self-cleaning metal which has been developed from the TresClean project (funded by the European Commission) achieved through laser-processing for high-throughput applications, with scaling up currently underway [246].

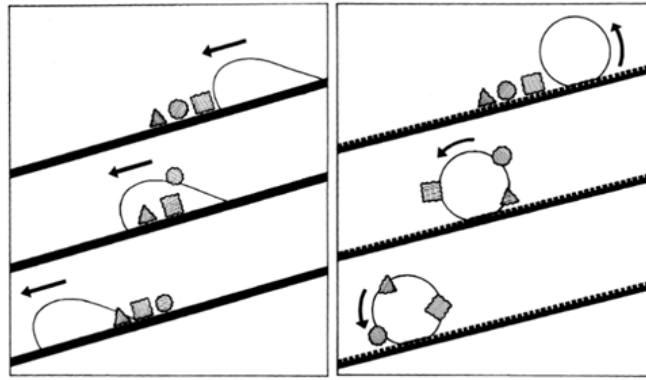


Figure 2.11 Summary of self-cleaning mechanism of the lotus leaf due to surface microstructures (right) upon exposure to water droplets/rinsing, compared to surface that does not have these features (left) [242]. (Permission to publish this figure has been obtained using RightsLink®, licence number 5162540828197, in courtesy of Bartholott and Neinhuis).

Surfaces inspired by the skin of fast-moving sharks (Sharklet AF™) is another well-known case of a topography driven antimicrobial surface. The 2 µm spacing circular pillars were engineered to enhance hydrophobicity with dimensions smaller than the *Ulva* spore, a eukaryotic alga cell, thus the Sharklet AF™ design has been a biomimetically inspired design rather than a being a biomimic material [247]. This material was the first reported study to reduce *Ulva* adhesion by 86% using a topography modulated surface by Carmen, Schumacher and co-workers [247,248]. The success of this material led to several extended publications by Schumacher *et al.* who revealed the success of Sharklet AF™ against adhesion of various algal zoospores/spores and *S. aureus*, [247–251], as well as other reports which showed the efficacy of Sharklet against *S. aureus* (MRSA and MSSA), *P. aeruginosa*, *E. coli*, *Klebsiella pneumonia* and *Acinetobacter baumannii* [252–254].

2.3 Bacteria and Biofilm Growth Curves

As prokaryotic organisms, bacteria replicate by binary fission which affords a rapid exponential growth rate under favourable conditions. This growth follows a predictable pattern of bacterial population growth that is well studied and accepted for a closed system [255], and is regarded as the bacterial growth curve that follows four distinct phases of progression: lag, exponential (log), stationary, and death phase, see **Figure 2.12**.

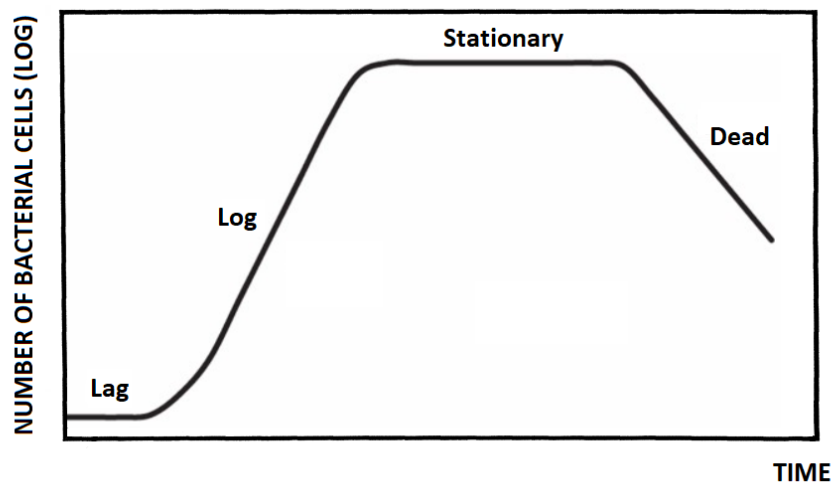


Figure 2.12 A representation of bacterial growth in a culture medium in a closed system, graphically depicting the four growth phases that describe the bacterial growth curve [255].

The initial lag phase corresponds to cellular activity without population growth. This analysis is based upon a small aliquot of concentrated cells having been introduced to a nutrient rich medium. Subsequently, the log growth phase follows, where bacteria are doubling in numbers from binary fission. During this phase metabolic activity is high and typical treatment methods like antibiotics are effective as species involved in bacterial growth and metabolism are antimicrobial targets. The stationary phase involves a decline in nutrient availability and increase in waste products, during this phase bacteria may adapt to generate virulence factors that can help them to survive in harsh conditions. Continuous decline of nutrient availability leads to the death phase which translates to the number of living and viable cells to decline and lysis. The dying and

lysed bacteria provide nutrients to surrounding cells to enable them to survive for a longer period.

Biofilm accumulation on a surface follows a sigmoid curve function, see **Figure 2.13**. The figure inset at the very beginning of the curve highlights that the accumulation process occurs immediately and results in a thin biofilm layer present during the early induction phase. This thin biofilm layer can also be termed as an early-stage biofilm. During log accumulation a mature biofilm begins to form and is fully perpetuated by the plateau phase.

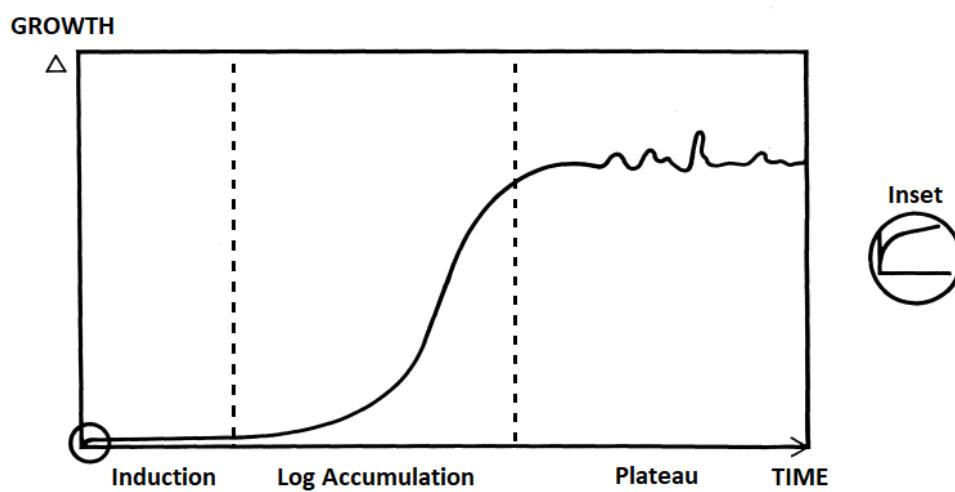


Figure 2.13 Progression of biofilm accumulation, Δ is the biofilm accumulation parameter and corresponds to film thickness, mass, cell number etc. The inset is emphasised to show that the accumulation process happens almost immediately. This graph was adapted from Flemming et al. [127].

2.4 Bacterial Selection

Most bacteria can be divided into two groups based on the Gram-staining reaction. This reaction is the crystal violet test, whereby Gram-positive bacteria can retain the dye and remain purple and for Gram-negative bacteria a pink/red stain is observed instead. The difference in staining is dependent on the composition of cell wall and outer membrane between the two. Gram-positive bacteria have thicker and a

more well-defined outer cell wall (peptidoglycan) with an underlying cell-limiting plasma membrane, the wall represents 15-30% of dry weight and constitutes part of its cellular rigidity. On the other hand, Gram-negative bacteria have a thin peptidoglycan layer between the inner-plasma membrane and outer-membrane, see **Figure 2.14** [256,257]. The peptidoglycan is a single covalently linked polymer network of cross-linked amino-sugars, it forms a protective mesh that defends bacteria from physical stresses, osmotic pressure and internal turgor [258].

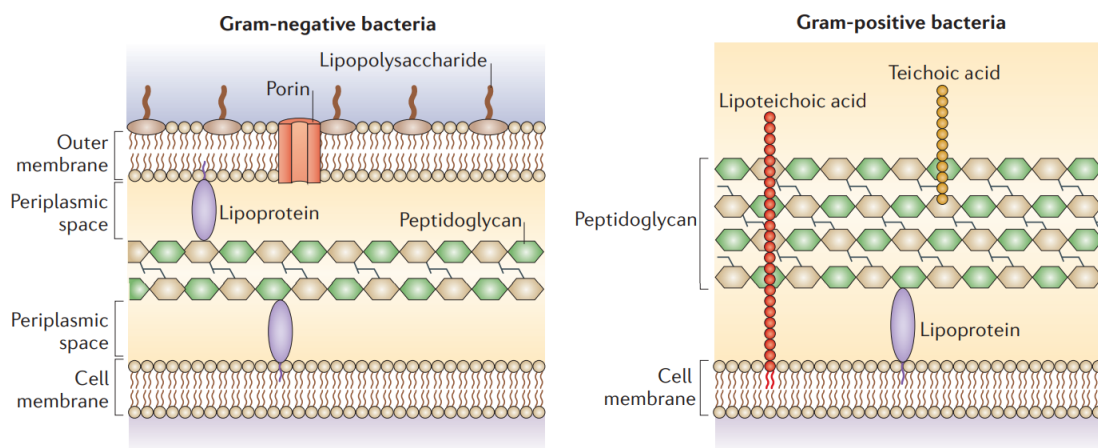


Figure 2.14 Diagram of the cell wall and membrane structure for Gram-negative and -positive bacteria [257].

Bacteria can also be categorised through their capacity to form spores; a spore is a dormant survival cell which can endure harsh and stressed conditions. For example, all fungi can produce spores, however not all bacteria are capable of forming spores. Bacterial spores are not for reproduction (unlike fungal spores) but are for survival. A spore-forming vegetative bacterial cell (actively growing cell) can produce a protective layer (spore) around its DNA until favourable conditions arise in which the spore coating is dissolved and vegetative cell functions resume; therefore, bacterial spores are extremely resilient and can survive draught, and even low pH environments. Examples of spore forming bacteria are *Bacillus* (aerobic) and *Clostridium* (anaerobic) species.

The most prevalent biofilm producers belong to the genera *Pseudomonas*, *Enterobacter*, *Flavobacterium*, *Algaligenes*, *Staphylococcus* and *Bacillus* [259].

ESKAPEE constitutes a collection of bacteria that are known to show resilience and resistance to commonly used products and treatments, they comprise of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* spp., and *E.coli* (ESKAPEE) [260]. Therefore, it is important to include an ESKAPEE organism for testing the efficacy of antimicrobial materials, due to their prevalence and resistant properties. The following segment (Section 2.4.1, 2.4.2 and 2.4.3) describes in more detail the three chosen microorganisms for this research project: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*.

2.4.1 *Pseudomonas aeruginosa*

P. aeruginosa is an opportunistic ubiquitous aerobic Gram-negative bacterium that is motile, rod shaped and non-spore forming, see **Figure 2.15**. It is the most researched microbe due its innate ability to readily form biofilms. It is known as one of the greatest biofilm producers and it can proliferate and exploit various environmental niches. *P. aeruginosa* largely affects immunocompromised individuals and is particularly prevalent in cystic fibrosis patients; it is also the second most common cause of ventilator-associated pneumonia [261]. Resistant forms of *P. aeruginosa* affects 100,000s of people every year including causing over 60,000 infections in Europe alone with about 4,000 attributable deaths annually [262]. Furthermore, *P. aeruginosa* biofilms are a major concern in water pipelines as a leading bacterial contaminant, and can even survive and proliferate in nutrient deficient conditions such as in distilled water for extended periods of up to 42 days [16,263–265]. *P. aeruginosa* is pleomorphic, meaning it is able to change its size and shape in response to environmental cues, though it cannot produce spores [266]. *P. aeruginosa* exhibits twitch motility to form biofilms. In fact, mutant forms of *P. aeruginosa* with defective flagellar motility were unable to produce even a monolayer of cells on a surface, whilst mutants that were deficient in IV pili formed monolayers but not cellular aggregates. This research suggests that flagellar motility is key for *P. aeruginosa* primary adhesion, and type IV pili are required for cellular aggregation processes to form a biofilm [56,129].

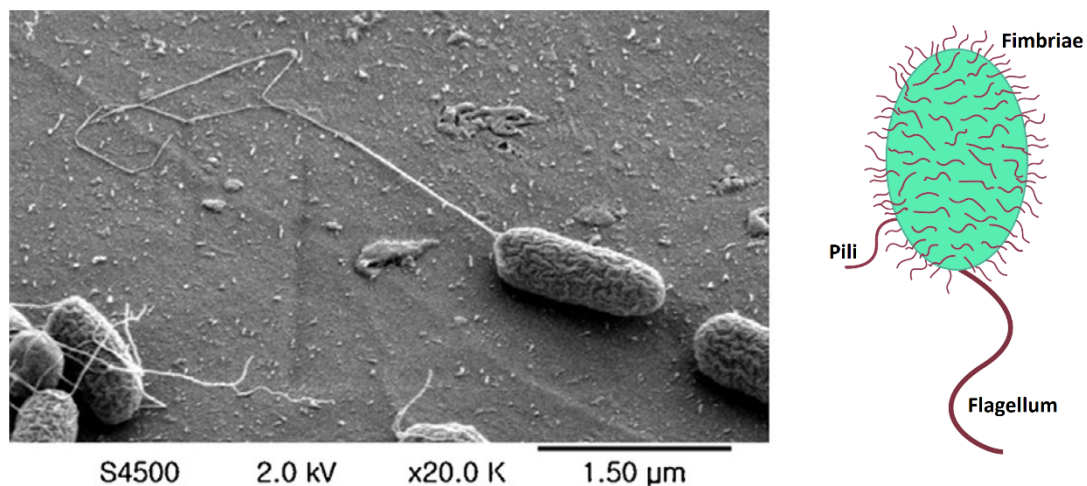


Figure 2.15 Left, *P. aeruginosa* PA01 SEM micrograph adherent on a polyethylene terephthalate polymer surface, courtesy of Bruzaud and co-workers, license number 5040770845609 [267]. Right, illustration depicting the three key surface appendages of a bacterial cell (fimbriae, pili, flagellum) that can aid in motility.

2.4.2 *Escherichia coli*

E. coli is another Gram-negative, rod-shaped, motile, non-spore forming facultative anaerobic bacteria that readily forms biofilms. SEM images of *E. coli* are shown in **Figure 2.16**. *E. coli* has become a model for understanding essential cellular processes, with a completed genome sequencing comprised. The formation of *E. coli* on catheters for example, has made catheter-associated urinary tract infections (UTI) one of the most frequent nosocomial infections, and has been recognised as the main cause of infection in 90% of all UTI cases in ambulatory patients [268]. *E. coli* is used universally as a marker microorganism to assess quality of drinking water as *E. coli* is rampant in faecal-contaminated water [269]. *E. coli* has multiple peritrichous flagella, fimbriae and pili which aids in its motility, surface adhesion and biofilm maturation [270].

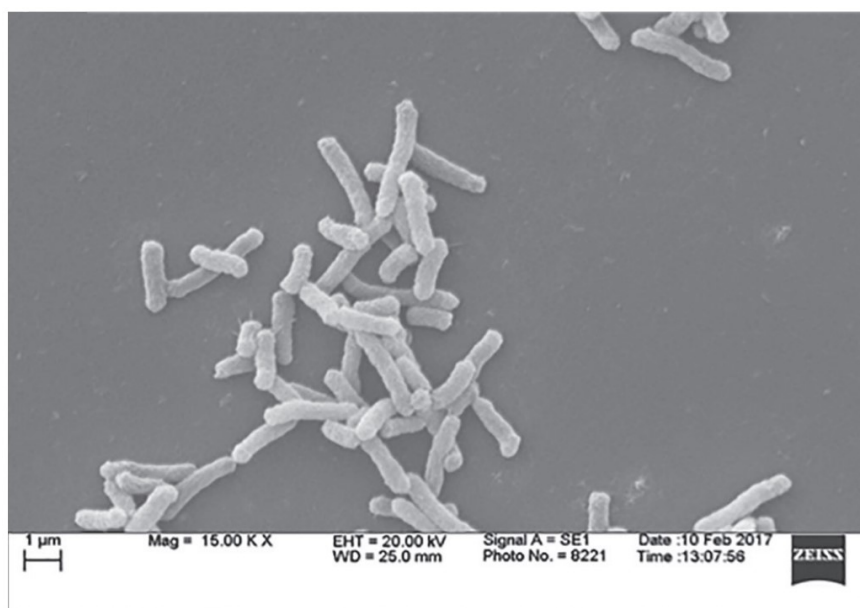


Figure 2.16 SEM micrograph of *E. coli*, provided by Ashmore and co-workers (open access document).

2.4.3 *Staphylococcus aureus*

S. aureus is a Gram-positive, non-motile, non-spore forming, spherical and facultative anaerobic bacterium. It has an extraordinary capacity to attach to surfaces such as medical devices by readily binding to proteins and conditioning films, see **Figure 2.17** [271,272]. It is a major cause of nosocomial and community-acquired infections, particularly playing a role in chronic infection from biofilm maturation on medical implants, where for instance *S. aureus* is the leading source of orthopaedic implant infection as well as nosocomial pneumonia, and the second leading cause of nosocomial bloodstream infection [273,274]. In 2017, approximately 120,000 cases of *S. aureus* bloodstream infections were reported, these led to 19,832 associated deaths in the USA alone [275]. *S. aureus* is also a major problem in the food and dairy industries as it can form biofilms on food contact surfaces. In fact, annually it was estimated that domestically acquired foodborne illnesses from *S. aureus* accounted for 241,188 illnesses, 1604 hospitalisations and 6 deaths in the US [276]. *S. aureus* is described as non-motile as it lacks obvious motility mechanisms such as flagella, and therefore

motility is limited to sliding, darting, carriage by fluid or Brownian motion which are considered as passive motility processes [277].

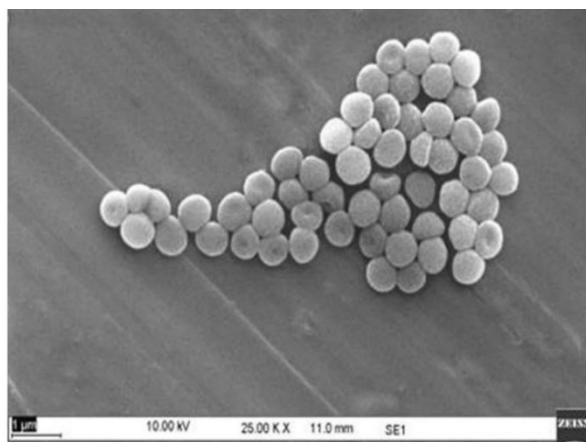


Figure 2.17 Scanning electron micrograph of *S. aureus* , kindly provided by Pandey et al. (open access document under Creative Commons CC BY license) [278].

2.5 Polymer Surface Selection and Treatments

2.5.1 Polymer Selection

The polymers selected for surface modification in this project to form antimicrobial materials has been based on two key attributes, firstly the chosen polymers must be common industrially used materials, and secondly, they must be compatible with chemical sterilisation methods such as ethanol cleaning. This factor is important, as autoclaving may destroy certain surface modifications performed, and some polymers also have low melting points. Below is a list of common industrial polymers and their suitable sterilisation methods, see **Table 2.3**.

Polymer	Common Derivatives	Sterilisation	Applications
Polyethylene	Very-low density (VLDPE), Low-density (LDPE), Medium density (MDPE), High density (HDPE), Ultra-high density (UHDPE)	Electron beam, gamma radiation, ethanol cleaning (can cross-link under high energy radiation)	Containers, packaging films, pouches, lid stock, breather patches, face masks, headers for bags.
Polypropylene	Homopolymer, copolymer	Autoclaved, ethanol cleaning (needs to be stabilised to survive sterilisation by radiation)	Homopolymers are used for thermos-forming, slit film and orientated fibres, high clarity, syringes, closures, sutures and gowns. Copolymers are used for food, household chemicals, beauty aid products, face masks, clear containers and hot fill applications.
Polystyrene	Expanded (EPS), Extruded (XPS), High Impact (HIPS)	Ethanol cleaning, gamma radiation, electron beam, UV sterilisation (steam and autoclave are not recommended)	Diagnostic instrument, disposable laboratory ware, petri dishes, tissue culture components, flasks, pipettes. High impact used for medical devices and laboratory ware.
Polyester	General purpose/crystal, high impact, syndiotactic, Polyethylene terephthalate (PET)	Ethanol cleaning (steam and high heat not recommended due to low T_g)	Dental tools, surgical instruments, surgical device cables and tubing, cannulae, packaging, syringe pump, high temperature caps
Polyvinyl chloride	Type I, Type II and CPVC	Gamma radiation, ethanol cleaning and autoclave.	Widely used in medical devices due to ease of processing and low cost; used for luer connectors, Y-sites, blister packs, packaging, bottles, fluid transport tubes, drip chambers, pull rings, oxygen facemask, gloves.
Polyacrylate	Homopolymer, copolymer and terpolymer	Gamma radiation (ethanol cleaning and steam not recommended)	Clear disposable plastics, chest drainage units, medical spikes, check valves, adaptors, catheter accessories.
Fluoropolymers	e.g. PTFE (linear fluoropolymer)	Ethanol cleaning (γ radiation can degrade PTFE)	Fittings, valves, pumps, tubing.
Polycarbonates	e.g. Bisphenol A-based	Ethanol cleaning, gamma irradiation, autoclave.	Safety glass, room divider, baby bottles, medical devices, refillable water bottles, automatic components, beverage containers.

Table 2.3 Summary of common industrial polymers, their derivatives, means of sterilisation and applications. Information largely sourced from McKeen [279].

2.5.2 Polymer Surface Treatments and Oxidisation Methods

Many common polymers are inert materials, and therefore it is not easy to graft, coat or bind antimicrobial agents and coatings on their surfaces. Furthermore, polymers generally contain additives that can migrate to the surface which can form a weak interfacial layer which can also reduce coating surface adhesion. Activation of a polymer surface prior to surface coating can be performed through a pre-treatment process. These can be classified into:

- Mechanical
- Thermal
- UV light, discharge/plasma/corona
- Solution based-chemical treatments
- Chemical additives

Pre-treatment through chemical oxidisation is a common strategy in industry to improve surface hydrophilicity, adhesion, printability, biocompatibility of polymers such as for medical textiles, electroplating of surfaces, and for the general enhancement of adhesion of materials or coatings to polymer substrates [280–283].

Early work by the Whitesides' group in 1977 has shown that successful PE oxidisation can be achieved by a combination of concentrated chromic acid treatment followed by a nitric acid exposure, producing a surface containing high yields of carboxylic functionality, with the film being referred to as 'poly-ethylene carboxylic acid', although aldehydes and ketones groups also were found on the surface at lower concentrations [284]. Vancso *et al.* has shown that such oxidised PE materials show a positive correlation between immersion time in the etching solution and consequent changes in substratum hydrophilicity, thus the surface functional groups formed [285]. This correlation levelled off rapidly after an initial steep increase in wettability, and was also mirrored in changes to surface roughness which increased significantly during the first 5-10 min of exposure with little change after 15 min [285]. Alternate chemical oxidisation solutions include sulfuric acid at elevated temperatures which after 8 h, was found to increase the roughness of a PE sample by 0.258 μm [286]. Nitric acid polymer oxidisation can also be performed but higher temperatures ($> 30\text{ }^{\circ}\text{C}$) are needed than

required for sulfuric acid etching. Also the procedure is more difficult and hazardous, with the release of hazardous brown NO_x fumes [287].

Ultraviolet/ozone (UVO) treatment is a gas phase approach that can oxidise polymer surfaces. This treatment is an effective method because it utilises an affordable and relatively low-cost instrument without the need for chemical preparation steps that may be hazardous such as strong acids. The UVO instrument works by feeding oxygen gas into a reactor where the sample is UV irradiated (at 184.9 nm) to produce excited state oxygen O_2^* from molecular oxygen that ultimately forms O_3 ozone. Ozone is re-irradiated (253.7 nm) to produce both molecular O_2 and atomic oxygen O^* , with the atomic oxygen being an ROS [288]. Polymers can be oxidised by atomic oxygen, molecular oxygen and ozone via abstraction of hydrogen to give radical carbon sites [288]. Sham *et al.* has prepared a detailed review that elaborates on UVO polymer oxidation processes [288]. There is a positive correlation between increasing UVO oxidation treatment time and an increase in the presence of surface oxidation functional groups and more highly oxidised species (i.e. more carboxylic acids and less ketones/ethers) [288]. UVO treatment has also been found to cause increased surface topographical roughness [288].

Another common method is corona discharge treatments. Corona treatment involves exposure to an atmospheric pressure air electric discharge. This discharge will accelerate electrons onto the polymer surface to cause hydrocarbon chain scission and in turn produce free radicals that form carbonyl moieties via reaction with ozone [289]. Generally, it is observed that polyesters are more easily oxidised with low power corona discharges, whereas PP requires significantly higher power densities to increase the SFE [289]. Corona treatment is widely used in industry and can introduce N and O containing species onto a surface following chain scission and subsequent oxidation, providing a surface rich in carbonyl and other oxygenated groups [290].

Plasma surface modification uses exposure to a high frequency plasma to produce free radical surface species that can react with surrounding polymer molecules and the plasma-phase species [289]. Low-pressure plasma processes requires a vacuum environment, whereas atmospheric pressure plasma treatments do not [289].

Oxidative treatment methods are heavily used in AMP coating strategies for improved thin-film adhesion or for functionalisation of an antimicrobial technology (coating) onto polymer materials. However, the antimicrobial performance of these pre-treatment oxidative approaches is not always included as controls in many studies, and thus their intrinsic performance as antibiofilm treatments are not fully understood. For example, Li and co-workers have utilised oxygen plasma treated polyurethane substrates and polyvinyl fluoride substrates to coat with quaternary chitosan or agarose (a neutral polysaccharide, a principal component of agar, shown to prevent marine fouling) [291]. The results observed a high antimicrobial performance against *S. aureus* and *P. aeruginosa*, but the plasma treatment of the substrates was not independently included in the study as controls. This exclusion of pre-treatment oxidative steps as controls has been reported by many others, and is common practise in many instances because the main interest of study is not the oxidising pre-treatment steps [292–294]. However, the introduction of oxidised polymer moieties at the polymer surface offers a quick and simple approach to incorporate active chemical groups and is a technique that is readily employed in various industrial settings. These oxidation treatments can also produce surface cavities and result in increased and varied surface topographies, which may in turn also influence bacterial attachment and/or viability of the biofilm [286,295].

2.6 Selection of Techniques for Surface Characterisation

2.6.1 Surface Chemical Analysis

Selection of relevant and sensitive surface chemical analysis is vital for monitoring the surface modification of a material. Investigating such parameters can be achieved using many instrument-based techniques. The general mechanism of these techniques involves surface molecules, or elements thereof, becoming excited by a radiation source (e.g. laser beam) which in turn causes a release of information on the surface species. Different techniques offer their own advantages and limitations, **Figure 2.18** provides a summary of some of the more popular experimental techniques, though some of these techniques are more bulk than surface sensitive [296].

• X-Ray sample excitation, determines chemical elements and relative abundance • Non-destructive • SD* < 5000 Å • Lacks sensitivity for trace and low atomic number elements, and thick samples	• X-Ray sample excitation, kinetic energy of ejected photo-electrons measured • Determines elements, chemical states, bonding and relative abundance • Non-destructive • SD* < 100 Å / 10 nm • ~1 nm depth resolution • Can be time-consuming to run • Very high vacuum required	• Noble gas ion beam sample excitation (He, Ne, Ar), the kinetic energy of scattered ions measured • Extremely sensitive, can identify first atomic layer (sample must be very clean), detects molecular and elemental species • SD* < 2 Å • Not suitable for nanomaterials, polymers and powders (as it is reactive), destructive method	• Infrared beam excites sample, vibrational modes are excited and absorb IR • Detects functional groups. • SD* < 2 µm • Some materials completely absorb IR radiation and cannot be tested; not sensitive for very thin-film coatings
EDS Energy-Dispersive X-Ray Spectroscopy	XPS X-Ray Photoelectron Spectroscopy	ISS Ion Scattering Spectroscopy	FTIR Fourier Transform Infrared Spectroscopy
• X-Ray sample excitation, determines chemical elements and relative abundance • Energy of Auger core electrons emitted from sample surface • Detects elemental composition, and chemical state of certain compounds. • SD* < 10 Å • High vacuum requirement	• Ion beam sample excitation, kinetic energy of scattered ions detected • Determines composition of surface • Quantitative non-destructive, measures depth distributions • Can test even insulating oxides. • Cannot resolve elements with same mass, limited for specimens with only a few elements	• Ion beam sample excitation, secondary ejected molecular ion fragments detected • SD* 1 nm, ToF-SIMS 1-3 monolayers • Can detect isotopes • Highly surface sensitive • Destructive technique, complex MS spectra output • Can be difficult to be quantitative • High vacuum requirement	• X-ray excitation of sample, fluorescent spectra measured • Measures film thickness and elemental composition • Non-destructive, low operating cost • SD* 100 µm • Not suitable for very light elements (H to Ne)
AUG Auger Electron Spectroscopy	RBS Rutherford Backscattering Spectrometry	SIMS Secondary Ion Mass Spectrometry	XRF X-Ray Fluorescence Spectroscopy

Figure 2.18 List of popular surface elemental and chemical characterisation techniques and their key features have been summarised (SD*= Sampling depth) [297,298].

XPS can detect all elements (except hydrogen and helium), their chemical state and electronic state, as well as empirical formula and bonding, with a high degree of sensitivity for surface species. XPS data can be collected using different modes, including a survey scan and a core level scan (wide scan) [299]. The survey scan provides a broad energy scan of the surface to detect all elemental species present, whereas the core level scan delivers detailed measurements of key peaks and allows for integration of these to give atomic abundance determination of the sample surface. Furthermore, a valence band scan can be performed which provides further information on the density and occupancy of electronic states in the valence band of a material.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a surface sensitive analytical technique that utilises a pulsed ion beam to remove molecules by ablation

from the outermost surface from atomic monolayers. The result is the release of neutral fragments, secondary ions fragments (+/-), as well as electrons from the surface. These secondary ions can be accelerated into a “flight-tube” for mass determinations relative to the time of detection. ToF-SIMS can be used for surface spectroscopy, surface imaging and depth profiling, and offers an extremely high detection sensitivity to shed light on detailed surface chemical information, including molecular formulae.

2.6.2 Determination of Surface Wettability

Surface tension γ_{LV} is one of the fundamental properties of liquids, and the surface energy γ_{SV} is the equivalent for solid interfaces. γ_{SV} can be described as the work or free energy (G) needed to increase the surface area (A) of a solid, thereby affording the units of mJ/m^2 [300], see **Equation 2.1**:

$$\gamma_{SV} = \frac{dG}{dA} = \frac{\text{free energy (mJ)}}{\text{unit area (m}^2\text{)}} \quad \text{Equation 2.1}$$

In 1805 Thomas Young established that a droplet of liquid on a surface presents a three-phase point at the liquid-vapour-solid interface, giving rise to a distinct contact angle θ which can be used to reveal the surface energy γ_{SV} , see **Figure 2.19**. This angle exists because the liquid molecules on the surface of the droplet have fewer neighbouring molecules than those in the bulk, and thus exhibit stronger attractive forces upon their nearest neighbours. Therefore, a net attractive force pulls the liquid molecules at the interface towards the bulk in order to minimise the number of liquid molecules at the droplet surface, this results in a minimal surface area of the droplet to form. Solid surfaces with specific surface functionalities and properties can influence the droplet shape by providing favourable intermolecular bonds with the surface liquid molecules, and in turn can result in changes of the area of a droplet and thus the contact angle (causing spreading/wetting). Consequently, a change in γ_{SV} is one way of determining

surface chemical modifications that have been made to a surface through contact angle (θ) measurements using liquid probes.

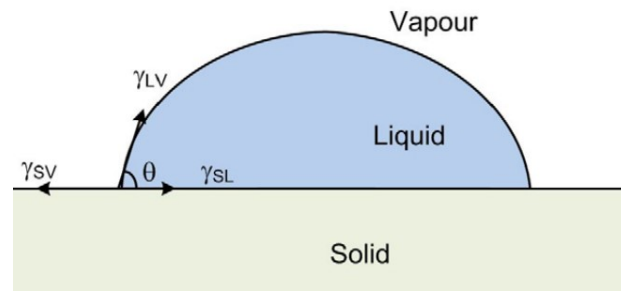


Figure 2.19 Schematic diagram presenting the contact angle (θ), and the interfacial surface tensions (γ_{SV} , γ_{LV} and γ_{SL}) between the three different phases. The three mechanical surface tensions are in equilibrium in the direction parallel to the solid surface [301].

Young's equation given below shows that there is a relationship between the surface energy of a solid γ_{SV} , the surface tension γ_{LV} of a liquid and the interfacial tension between the liquid and solid γ_{SL} , see **Equation 2.2** [301]. Where θ is the contact angle, γ_{LV} is the surface tension of a liquid, γ_{SV} is the surface tension of the solid surface, and γ_{SL} is the interfacial tension between the liquid and the solid:

$$\gamma_{SV} = \gamma_{SL} + \gamma_{LV} \cos (\theta) \quad \text{Equation 2.2}$$

The surface tension of a solid γ_{SV} is more commonly referred in this thesis as the SFE for the solids [103]. The conventional way to determine γ_{SV} of a flat surface is by measuring the contact angle θ using a contact angle goniometer, with well characterised liquid probes. A typical goniometer consists of a horizontal stage accompanied with a sample mount that is located between a light source and CCD camera, and a motorised liquid droplet dispenser that can be used to accurately dispense the probe liquid. To calculate the γ_{SV} of a substratum, different semi-empirical models can be used, which include different models for molecular interactions in their

calculation of the γ_{SV} . One popular approach is Fowke's Theory. This approach is a semi-empirical model derived from Young's equation (**Equation 2.3**) which relates the interfacial properties of liquids and solids to their polar and dispersive components. It assumes that γ_{SV} has two independent components, dispersive (γ_{SV}^D) and polar (γ_{SV}^P), see **Equation 2.4**, therefore the γ_{SV} can be calculated from the work of adhesion (W_{SL}) using the surface tension (γ_{SV}) values of the two liquid probes used and from the θ values obtained experimentally. The polar and dispersive surface tension (γ_{SV}^P and γ_{SV}^D) of liquid probes have been established and are already known. The work of adhesion (W_{SL}) has been described as the work needed to separate the two phases:

$$W_{SL} = 2\sqrt{\gamma_{LV}^D \gamma_{SV}^D} + 2\sqrt{\gamma_{LV}^P \gamma_{SV}^P} = \gamma_{LV} (\cos \theta + 1) \quad \text{Equation 2.3}$$

$$\gamma_{SV} = \gamma_{SV}^P + \gamma_{SV}^D \quad \text{Equation 2.4}$$

$$\gamma_{LV} = \gamma_{LV}^P + \gamma_{LV}^D \quad \text{Equation 2.5}$$

where; W_{SL} = work of adhesion, γ_{SV} = surface free energy of the solid, γ_{LV} = surface tension of the liquid probe, γ_{LV}^D = surface tension of the dispersive component, γ_{SV}^D = surface energy of the dispersive component, γ_{LV}^P = surface tension of the polar component, γ_{SV}^P = surface energy of the polar component, θ = contact angle.

Fowke's Theory is often used for its advantages in obtaining the γ_{SV} of a surface using only two liquid probes [302,303]. Typically, diiodomethane (DIM) and water are used as the probe liquids. The surface tension and their components γ_{LV} , γ_{LV}^P and γ_{LV}^D for pure water and DIM are provided below in **Table 2.4**. DIM has a high γ_{LV}^D of 50.8 mJ/m² and due to its lack of any dipole moments it has no polar component ($\gamma_{LV}^P = 0$), and therefore is an ideal probe to simplify the calculation for determining SFE (γ_{SV}) using **Equation 2.3** [304]. An example calculation using Fowke's Theory to determine γ_{SV} has been provided in the Appendix under the heading Chapter 2 [A].

Probe	γ_{LV}^P	γ_{LV}^D	γ_{LV}
DIM	0.0	50.8	50.8
Water	21.8	51.0	72.8

Table 2.4 Surface tension components (mJ/m^2) for pure water and DIM [305].

Fowke's approach uses Young's contact angle θ , but does not directly consider the surface roughness of a material in its model. The roughness of a surface can be important SFE (γ_{SV}) calculations because it may significantly influence the experimentally observed θ for some materials, and therefore introduces a degree of complication and error in values of γ_{SV} determined, though the overall forces present at the vapour-liquid-solid interface are unchanged [300]. To account for surface roughness contributions there are generally two models in use which look at roughness and relative changes to the θ measured. A liquid probe on a rough surface is thought to either penetrate the surface (Wenzel state) or sit on top of the surface (Cassie-Baxter state). Additionally, a droplet can switch between the two states due to external forces such as Laplace pressure (a pressure difference between a curved surface in a vapour-liquid phase boundary), see **Figure 2.20**.

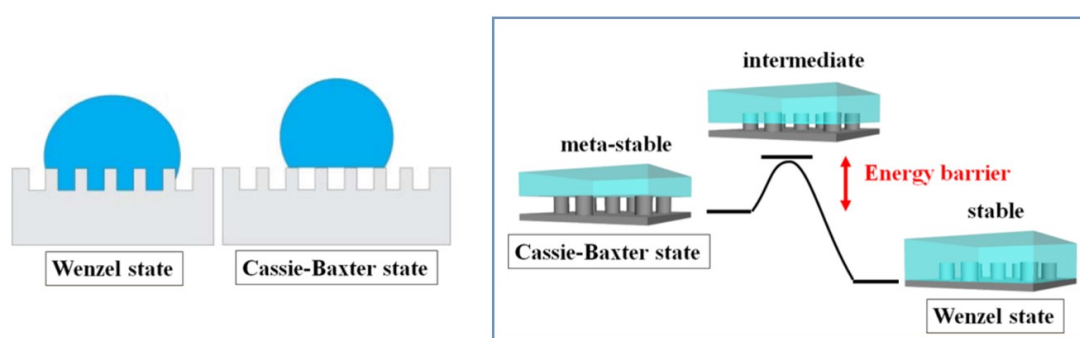


Figure 2.20 Diagram showing the Wenzel state of a droplet considered to penetrate into the topography of a surface and the Cassie-Baxter state where the droplet is suspended on top of a rough surface [306]. It is thought that the two states to be in equilibrium with each other, see diagram shown to the right [306,307].

The relationship between roughness and wettability was defined in 1936 by Wenzel [308] whose model proposes a relationship between the apparent (measured) contact angle (θ^w), and Young's contact angle (θ) of a flat surface, via the roughness factor (r). The roughness factor is defined as the ratio between the actual area of the solid-liquid interface and the nominally projected area, therefore for a smooth surface $r=1$ and for a rough surface $r>1$ [307], see **Equation 2.6**:

$$\cos \theta^w = r \cos \theta \quad \text{Equation 2.6}$$

In the Cassie-Baxter state the air is trapped in the grooves between the surface's topographical features which can result in a larger observed contact angle [307]. The Cassie-Baxter model extends on the Wenzel model and suggests that the apparent (measured) contact angle θ^{CB} should include contributions of different phases like heterogeneity and porosity, therefore this model considers the surface fraction of the solid and vapour phase; see **Equation 2.7**, where f is the surface fraction for a surface which is not heterogeneous:

$$\cos \theta^{CB} = f(1 + \cos \theta) - 1 \quad \text{Equation 2.7}$$

There are distinct roughness parameters and surface properties required to employ the Wenzel and Cassie-Baxter Models for calculating γ_{SV} . These include the surface area of liquid that is in contact with the solid surface, to be able to calculate the surface fraction (f), and the surface area of the projected rough surface in contact with the droplet to calculate the roughness factor (r). These parameters are difficult to obtain for a heterogenous rough surface and there can be large errors in their measurements/calculations, particularly for surfaces that are relatively smooth with random scratches and crevices that are not uniformly distributed on the surface. Therefore, in this study, roughness considerations in the measured θ s has not been corrected and considered.

In this project, the advancing contact angle has been utilised which is the maximum measured contact angle. The droplet volume is increased slowly stepwise on the horizontal surface under test, and the largest θ before the droplet collapses/spreads is defined as the advancing contact angle, see **Figure 2.21**.

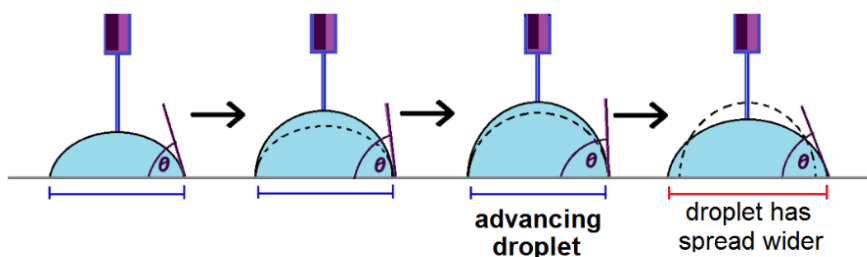


Figure 2.21 Advancing contact angle is the largest angle before the droplet spreads, the droplet is slowly increased in volume using a syringe that tip is maintained in the droplet.

2.6.3 Material Imaging Techniques

Surface imaging techniques are important to characterise modified surfaces such as thin-film coatings. Scanning and transmission electron microscopy (SEM/TEM), atomic force microscopy (AFM), scanning tunnelling microscopy (STM), and surface profilometry are common methods used to image the surface of materials. Two of the approaches relevant to the current studies have been described in more detail in this section.

AFM is an imaging technique primarily used for atomic level topographical analysis but can provide surface physio-chemical parameters such as the relative degree of adhesion and stiffness of the substrate, with high 3D spatial resolution. The instrument uses a cantilever which is an elastic beam with a sharp tip on one end that can interact with the surface in non-contact or contact mode by scanning the cantilever's tip over the region of interest. Usually, AFM instruments have vertical resolutions of less than 0.1 nm and X-Y resolutions of around 1 nm. The vibrational environment of the instrument can limit the resolution in the vertical direction, although resolution in the horizontal plane is also limited by the diameter of tip utilised for scanning.

White Light Interferometry (WLI) is an optical experimental technique which can obtain high quality 3D surface topography maps with 1 nm vertical resolution and X-Y resolution of ~ 100 nm. It is non-contact, therefore a non-invasive technique that works for opaque through to semi-opaque surfaces. 3D roughness can be obtained by imaging a series of areas of a surface, measuring the 2D roughness and averaging the values for all areas to obtain the 3D roughness data [309]. The technique uses a white light source that passes through a beam splitter and reaches the sample via a Mirau objective. The beam splitter splits the incident white light into two beams; one beam focuses the light onto the sample and the other is reflected by the beam splitter and focused on the reference mirror. The resultant interference images are then passed to the detector. For a full schematic diagram, see **Figure 2.22**.

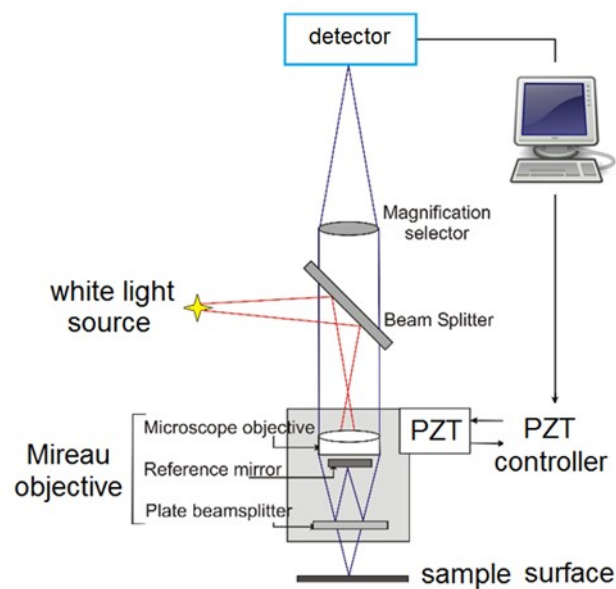


Figure 2.22 Schematic diagram showing the principle of white light interferometry.

Two common roughness parameters R_a and R_q were measured which are described as follows [309]:

- R_a is the arithmetic average roughness (or arithmetic mean deviation) from the mean line over the sample length (l) (see **Equation 2.8**). **Figure 2.23** demonstrates how R_a is calculated across a surface with length (l). This parameter is the most

commonly used roughness parameter for general quality control and is the average of how far each point on the surface deviates in height from the mean height:

$$R_a = \frac{1}{l} \int_0^l |y_i(x)| dx \quad \text{Equation 2.8}$$

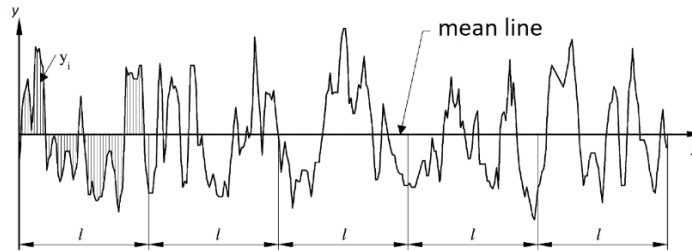


Figure 2.23 This graph presents the key variables used in the calculation of the average roughness R_a , where l is the sample length, and y_i is the height of the point being measured [309].

- R_q is the root mean square roughness, also known as RMS, and is the standard deviation of the distribution of surface heights (see **Equation 2.9**). This property is generally more sensitive to deviations in surface roughness than R_a :

$$R_q = \sqrt{\frac{1}{l} \int_0^l \{y(x)\}^2 dx} \quad \text{Equation 2.9}$$

2.7 Assessment of Biofilm Adhesion, Growth and Viability

2.7.1 Microscopy Methods

The human eye can distinguish between two points of 0.05 mm apart, without the need for any additional lenses for magnification. This distance is known as the resolving power or resolution of the eye. A microscope can use a lens or an assembly of various lenses to magnify this distance to a greater resolving power. The foundations of light

microscopy were established by Ernst Abbe in 1880s. Abbe found that the diffraction of light by a specimen and objective lens determined the image resolution, therefore he was able to establish the conditions needed to design a lens whose resolution was diffraction-limited [310,311]. At the start of biofilm research, light microscopy was used to examine cells grown on discs for imaging [312], and scanning/transmission electron microscopy (SEM/TEM) produced electron micrographs of biofilms and bacteria [313] as another approach. Microbial communities visualised by light microscopy were initially limited to resolving only individual cells from sessile organisms, nevertheless a translucent EPS matrix was still visible which occupied 3-6 μm spaces between cells [314]. TEM was unable to show real-life images as the EPS was severely affected by the dehydration due to the instrument's vacuum operating conditions, which could only be resolved with the aid of electron-dense ruthenium dyes [314,315]. Early SEM studies exhibited even more dehydration of microbial cells which caused greater image artefacts than in TEM [314], with vacuum driven dehydration rapidly condensing EPS components such as polysaccharides [316]. Over the years, these methods have developed into more advanced microscopy imaging techniques which are commonly used in the present day for analysing biofilms, sub-populations and EPS. Herein, the following section will explore commonly used modern microscopy developed techniques and their current application to assess biofilm adhesion, growth and viability.

Additional imaging techniques which have not been covered or mentioned here but could be used for biofilm imaging include white light interferometric microscopy [317] and magnetic resonance imaging [318].

Confocal Laser Scanning Microscopy

Confocal Laser Scanning Microscopy (CLSM) is an optical microscopic tool was first developed by Minsky in 1961 in order to better understand the human brain. It is an optical imaging tool that offers enhanced spatial resolution and depth selectivity to obtain 2D and 3D images of high quality. CLSM is able to view biofilms in their fully hydrated and living state using a range of distinct modulated optical wavelengths in

order to separate complex fluorescence signals [167]. The major advantage to CLSM is the ability to image hydrated and undisturbed microbial species directly bound to a surface. The diagram below shows the basic operational principle of CLSM, see **Figure 2.24**. The CLSM essentially works as follows; incident sample illumination is provided by a laser which provides monochromatic, coherent and collimated light. The laser beam, after passing through a pinhole aperture, is reflected by a dichromatic mirror, with the mirror only allowing light of a specific wavelength to pass through. The light is focused by the objective lens and scanned across the specimen in a defined focal plane. Secondary fluorescence emitted from points on the specimen pass back through the dichromatic mirror allowing certain wavelengths to pass and are focused as a confocal point at the detector pinhole aperture (confocal pinhole) by the objective. Secondary fluorescence emitted from points on the specimen pass back through the dichromatic mirror allowing certain wavelengths to pass and are focused as a confocal point at the detector pinhole aperture (confocal pinhole) by the objective.

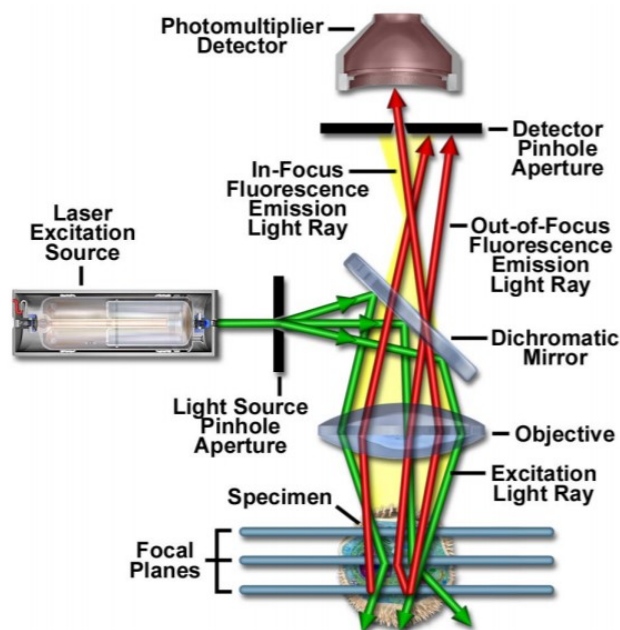


Figure 2.24 Schematic diagram showing how the CLSM functions in detail, this includes the focusing of the laser beam with the red arrow rays representing the reflected light and the green the laser beam rays, the sample being excited, and emission occurs which is detected [319].

The reflected light is received through the pinhole after a spot on the sample has been scanned in a raster fashion. This second pinhole aperture is positioned in front of the

detector in a plane conjugate to the image plane with a scanning point on the specimen; as this pinhole is **conjugate** to the **focal** point of the lens, it is therefore called the **confocal** pinhole, see **Figure 2.25**. The crucial design feature of a CLSM compared to a conventional fluorescent microscope is the confocal pinhole; by varying the pinhole diameter, the degree of confocality can be adapted to practical requirements, with the aperture fully open, the image is non-confocal. For enhanced spatial performance of the instrument the pinhole should be small as the pinhole restricts the sample plane from which light can reach the detector. High resolutions and high magnifications can limit field of view and as biofilms are spatially heterogenous, therefore looking at a narrow field of view may lead to erroneous imaging assessments, therefore careful confocal setting parameters should be assessed, considered and implemented.

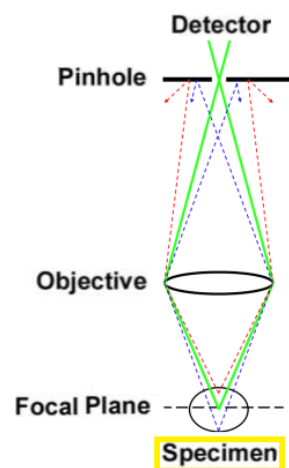


Figure 2.25 Schematic diagram showing the detector pinhole aperture placed in front of the detector at the confocal plane of the objective. Out of focus light (the red and blue lines) are eliminated as it does not pass through the pinhole to reach the detector, allowing for detection of specimen at a specific focal plane with a known depth.

A variable pinhole diameter allows for exclusively imaging a thin optical slice ($\sim 1\ \mu\text{m}$) out of a thick specimen (typically, up to $100\ \mu\text{m}$), a method known as optical sectioning. Optical sectioning eliminates the limitations of conventional fluorescence microscopy.

In conventional fluorescence microscopy, the image of a thick biological specimen will only be in focus if its Z dimension is not greater than the wave-optical depth of focus specified for the respective objective lens. The pinhole can suppress stray light, which further improves image contrast, therefore for enhanced images and performance the pinhole should be kept small; as it restricts the unwanted light that reaches the detector. As the pinhole diameter is constricted (decreased), depth discrimination improves (i.e. the optical slice thickness decreases), however this method should be done with caution as too small a pinhole and the desired signal value of importance may be excluded.

To visualise the bacterial cells using CLSM, specific fluorescent dyes are required. There are different approaches that can be considered, such as dye exclusion which is one of the oldest staining methods and is based on the physical exclusion of dyes by intact semi-permeable cell membranes. In addition to this, two-fluorochrome staining (dual-staining) can be used to differentiate between viable and non-viable bacteria. One commonly used dye pairing is Syto™9 with propidium iodide (PI). This method produces low levels of background fluorescence and can be used on a variety of different organisms. Overall CLSM can be utilised effectively to quantify and visualise biofilm structures, determine cell density, detect different EPS macromolecules using varying staining probes, with biofilms as thick as 100 µm capable of being visualised.

Atomic Force Microscopy

AFM was developed in 1986 [320], and has been used to investigate a number of biological materials and organisms, such as viruses, bacteria and DNA. AFM can be used to analyse biofilms by determining chemical and physical properties of surfaces as they interact with the AFM probe. AFM can investigate cellular mechanical properties (elastic moduli and turgor pressure) [321], adhesive properties [322], cell topography [323], as well as for surface imaging purposes. One study used AFM to understand changes in the formation of biofilms on various substrates, and observed that decreased substrate surface roughness correlated to loss of biofilm polysaccharide density, as well as a decrease in the number of cells attached [324]. Biofilm imaging using AFM can be

problematic because microbial cells are often attached to the surface via weak Lifshitz-Van der Waals forces. The scanning action of the AFM cantilever may damage or distort the biofilm sample, furthermore microbial cells are motile which can also result in a blurry image, or inaccurate images due to the time it takes to scan for imaging as well as larger dimensions visualised due to swelling [325]. Therefore, immobilization of the microbial cells prior to analysis using AFM is essential for obtainment of good quality images.

Electron Microscopy

Electron microscopy includes scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM is a tool commonly used to investigate early-stage microbial adhesion of single sessile bacteria that can form mature biofilms. It is capable of elucidating sub-micrometre scale appendages and features such as curli (proteinaceous surface structure), flagella and EPS which are known to play important roles in early-stage irreversible cell attachment of bacteria [326]. SEM images are generated by exposing the specimen surface to a high-energy beam of electrons under vacuum. The interaction of the incident electron beam with the specimen causes the emission of low energy secondary electrons and back-scattered electrons from the sample that produces a clear surface image signal which can be used to determine topographical features, with internal structures visualised by using various sample preparation methods [327]. For this purpose, the sample requires a conducting metal coating such as silver or gold in order to be visualised. The desire to visualise microorganisms and biological material has led to the advancement of new sample preparation techniques and instrument developments which provide superior images. Conventional biological sample preparation requires chemically fixing the sample (which may destroy and disrupt true structures and morphologies) and dehydrating the sample, which is essential as SEM is performed under vacuum. New preparation techniques have been developed which allow for enhanced contrast and reduced sample damage. These include the use of glutaraldehyde fixation, negative staining, the sputter–cryo technique, environmental-SEM, focused-Ion-Beam SEM, and coating with gold or osmium [328].

Cryo-SEM is effective when imaging samples containing moisture without causing drying artefacts. A moisture containing sample is rapidly frozen with liquid nitrogen (physical fixation), the sample can be cut physically in the SEM with a cold knife, and ice can be sublimed away using an internal heating system. The sample is then coated with gold for imaging. The clear advantage of cryo-SEM is the lack of pre-operational steps including dehydration and the investigation of time-based specimens ‘frozen in time’ with the total preparation time completed in minutes [329], although this approach may not work for all experiments. For example, one study found that cryo-SEM produced a frozen layer over the specimen which cracked when exposed to the electron beam [329,330]. In environmental-SEM (E-SEM), the sample can be imaged at moderate vacuum without the requirement of a conductive coating and in moist atmospheric environments which allows for the preservation of soft water sensitive components of the specimen [331,332]. Focused-Ion-Beam SEM (FIB-SEM) is a novel tool that allows for standard SEM viewing as well as FIB milling to remove sections for visualising subsurface structures. This milling is achieved by using a gallium source that can mill away segments of the specimen to a designated height, depth and width [330]. Alternatively, low-voltage high-resolution-SEM (LVHR-SEM) can achieve high spatial resolution of 2-5 nm, which was previously only possible with TEM.

2.7.2 Non-microscopy Methods

There is an array of non-microscopic approaches to examine biofilm viability, formation and growth. Viable plate counting methods of cells include: culturing, flow cytometry, direct viable counting, as well as determination of dry mass, total organic carbon content, and mass change from a quartz crystal micro-balance [333]. Some of the methods commonly used has been discussed in the below section.

Viable Plate Counting/Enumeration (Colony Forming Units/mL)

Viable cell enumeration or aerobic plate counting is a standard quantification method that is commonly used to determine the number of viable cells present in a solution.

Therefore, for studying biofilms, the bacteria in a biofilm must be released from the surface and transferred into a solution to perform this technique. Bacteria from biofilms can be obtained via scraping, vortex-mixing or sonicating to obtain a homogenised single-cell suspension that can be serially diluted and pipetted onto nutrient agar plates in order to allow for microbial colony growth of the collected cells [333]. The cells that are culturable and viable can grow after incubation (usually overnight) into distinct colonies on the plates which each represents a single colony forming unit (CFU). The colony total is enumerated to determine the CFU/mL of the test solution based on the dilution which allowed for distinct separation of colonies, and the volume of solution plated. Slow colony forming bacteria can also be considered by allowing for longer incubation times [333]. This method is not able to differentiate between viable cells that are non-culturable. Non-culturable cells were first discovered in 1982 where it was found that *E. coli* and *V. cholerae* living cells could enter a distinct state that showed their incapability to grow on routine media [334]. One key advantage of this technique is that it does not require advanced equipment and can be performed in most laboratories. However, it is time and labour intensive, also dissociating mature biofilm clumps into single-cell suspensions can be experimentally challenging [335].

Microtitre Plate Assays

Ammonium oxalate (crystal violet stain) has been used as early as 1930 for staining bacteria and was originally aimed for the detection and quantification of bacteria found in water systems for in-solution studies [336,337]. Early works by Fletcher in 1977 showed that attachment of bacteria and subsequent growth on surfaces can be measurable by staining the biofilm with crystal violet to allow subsequent quantification by microscopic counting [338]. This technique has been modified and adapted into a high-throughput screening system to measure bacterial attachment and biofilm growth using microtiter plates, formally known as the 'microtiter plate assay' or the '96-well plate assay'. Media inoculated with a small amount of bacteria in the plate wells leads to the formation of a biofilm on the sides and bottom of the well and on the air-liquid interface, and biomass build up can be quantified by staining with crystal violet [339]. Primitive accounts of this approach can date back to 1985, when studies by

Christensen *et al.* demonstrated that optical density can be used as a measure to see changes in *Staphylococci* attachment to 96-well plastic tissue culture plates [340]. This approach was popularised in the mid-to-late 90's by Mack and O'Toole with many variations of this assay being used for different organisms. For example, for the assessment of *P. aeruginosa* in the adhesion and formation of biofilms with PVC microtiter plates [56]. This mode of analysis is now regularly used alongside other comparable dyes like safranin for high-throughput screening experiments [54,341]. The dyes essentially rely on the accumulation of an intercalating pigment binding to negative charges in the EPS, as well as internal structures of some species such as nucleic acids, and therefore is generally regarded as a measure of total attached biomass. Alternatively tetrazolium salts like 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulphophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) can also be used in this system, which focuses on the cells ability to reduce tetrazolium salts to orange coloured formazan compounds, with increased concentration providing an amplified intensity in reading that is measurable using a microtiter plate reader [342]. However, different strains and bacterial species have shown markedly varied abilities to breakdown the tetrazolium salt and thus comparison of results from different organisms has proven to be challenging [343]. The Calgary system, also referred to as the MBEC experiment, is an extension to the 96-well plate assay but utilises a lid with 96 pegs attached that protrude down and suspend into the media [339]. This set-up allows for cells to adhere to the pegs, and the sides of the wells to proliferate and form a biofilm. The lid can then be removed and transferred to a new 96-microtiter plate containing fresh sterile media to measure attachment and growth.

Flow-Cytometry

Flow cytometry (FC) can be used to characterise suspensions of single cells or particles by analysing combinations of different fluorescent probes with different absorption and emission parameters. In the flow-cytometry instrument, cells flow through a narrow opening, which forces single-file mobility of the bacterial cells. A laser is used to detect the passing cells and determines optical characteristics of the cells from light scattering, absorbance or intrinsic and extrinsic fluorescence measurements [333], see **Figure 2.26**.

Several parameters can be measured simultaneously on thousands to millions of individual cells.

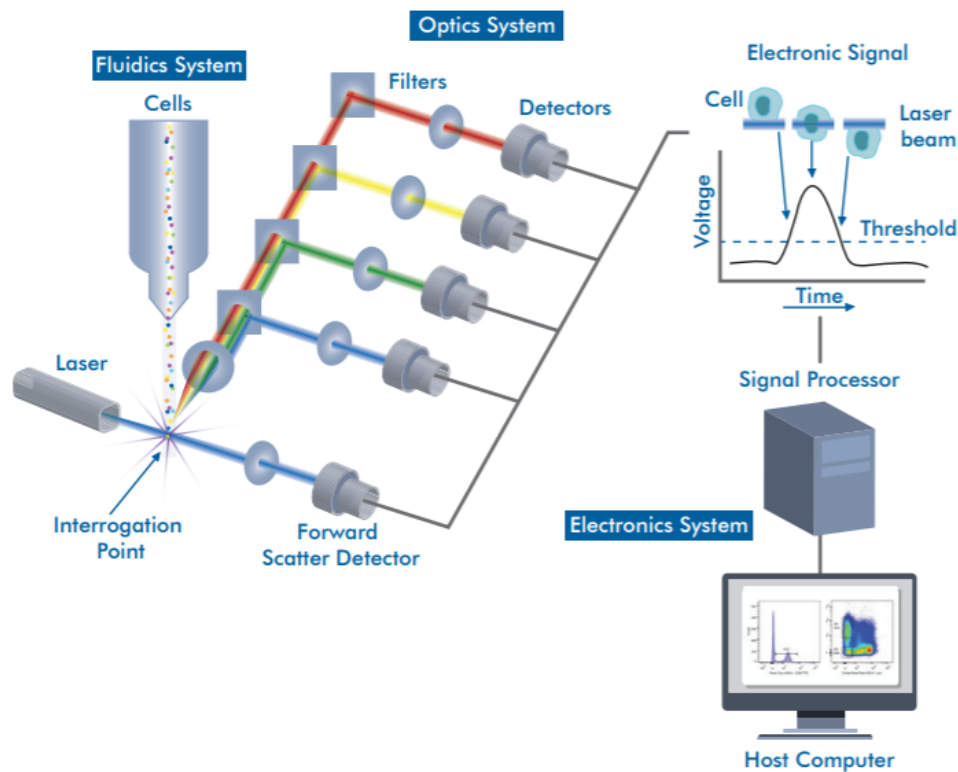


Figure 2.26 Schematic diagram showing the workings of a FC instrument. The fluidics system illustrates the entry of the cellular sample into the instrument, and the passing of cells through a narrow opening in front of the laser beam. A series of detectors, accompanied with filters (optics system) are used to distinguish between key emission information that will inform about a cells fluorescence dye uptake, the size and shape. The electronic signal graph demonstrates how an event (signal) is measured from the passing of a cell into passed the laser beam and recorded as an electronic signal in the FC.

In FC, each event represents a single cell interrogation, with forward scatter (FSC) and side scatter (SSC) signals being generated from the scattering or refraction of the laser light by the cell that is measured. FSC is measured in the forward direction as the laser passes through the cell and is refracted, whereas SSC is measured at a 90 ° angle, see **Figure 2.27**; both parameters provide important details about the size and shape of the

cell, therefore FSC and SSC are useful indicators to discriminate cell from cellular debris and detector noise.

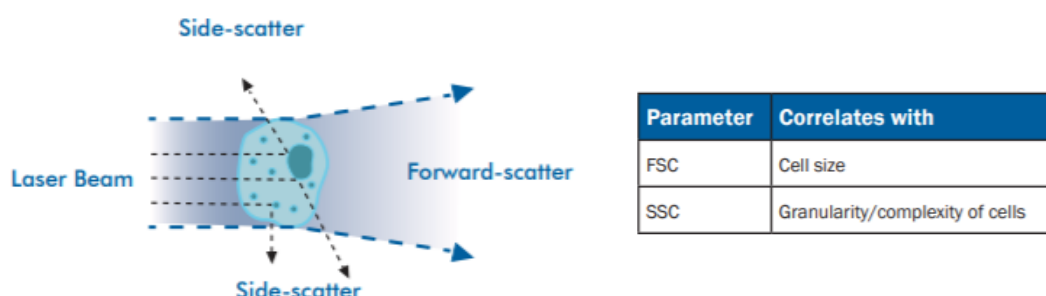


Figure 2.27 Forward and side scatter in FC to determine cell size and shape. The table on the right provides the key size parameters that is associated with FSC and SSC, whereby the complexity of a cell refers to internal component features of the cells.

Fluorochromes are fluorescent molecules that have distinctive excitation and emission spectra. FC uses lasers as excitation sources for fluorochromes, and by using varied laser wavelengths for different fluorochromes, multiple fluorochromes can be measured simultaneously. Different FC instruments have varying capabilities of what fluorochromes can be tested based on the laser options available. Once excited, the fluorescence from the stained cells are split into defined wavelengths and channels by a set of filters and mirrors within the FC's optics system. The fluorescent light is filtered by optical filters so that each detector will detect fluorescence only at a specified wavelength. These detectors are called photon-multiplier tubes and convert the incident photons into an electronic signal and record it as an event. The signal can be amplified by controlling voltage or gain settings on each detector. Each event is given a channel number depending on its measured intensity; the more intense the fluorescence, the higher the channel number the event is assigned. Overall, the different signal regions enable differentiation between the diverse fluorochromes in a system. There are various viability stains that can be employed to measure viability and microbial cell health, examples include TO-PRO[®]-3 iodide [344]; LIVE/DEAD BacLight kit containing Syto[™]9 with PI and SYBR[®] green with PI [345], all of which differentiates between membrane damaged and membrane intact cells. Alternatively, substitute dyes

can also be used which distinguish between membrane potential differences as healthy cells have polarised cytoplasmic membranes. Certain dyes can accumulate in the intracellular space of a cell with interior-positive membrane potential (a form of dead cell- depolarised cell) and binds to the intracellular lipid-containing elements. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄(3)) is one example of a membrane-potential dye [346]. It is an oxonol lipophilic probe that carries a delocalised negative charge, it has a low fluorescence intensity in aqueous solution, the intensity increases several fold in a milieu, such as the cytoplasm of a cell, therefore it can be used to detect intact non-fluorescent and depolarised fluorescent bacteria [347]. It is important to note that FC is restricted to solution studies, thus appropriate methods should be in place to effectively remove the biofilm bacteria from the substrate and re-suspend them in solution for FC analysis.

2.8 Summary

2.8.1 The Gap in Research

Biofilms are more difficult to remove and destroy than planktonic (free-floating) bacteria, primarily due to the presence of the EPS which forms upon biofilm maturation. Although there have been decades of research into investigating biofilm structure and behaviour including the examination of antibiofouling and biofilm removal approaches for polymer materials, there are limitations to many of the treatments currently in use. The common solutions employed include the use of detergents, chelating ligands, surfactants, oxidising agents and enzymes for biofilm removal and cleaning. However, these approaches tackle biofilm removal rather than prevention and have operational restrictions either due to pH, temperature, toxicity and contamination of surrounding products or the environment from the chemicals being used, or they may cause the enhancement of surface corrosion processes [17,24,25]. Another important factor to consider is the vast variety of bacterial species, each with different strains, that are present. This raises the problem of selectivity when looking to develop novel biocidal coatings for polymer materials. Furthermore, there is an interest in developing an approach for modifying common polymer materials, such as polyethylene and

polypropylene, using inexpensive and industrially feasible techniques using coatings or altering the polymers surface, so to prevent or perturb bacterial attachment. Overall, there is a significant need from both industry and healthcare to develop and evaluate cost-effective AMP approaches. Such a surface should be stable, durable, and able to inhibit adhesion of a wide range of pathogenic bacterial species from attaching to common polymer surfaces.

This research project will focus on the synthesis of novel surface chemistries for antiadhesive and antibiofilm capabilities. It is aimed for industrial applications involving common low-cost substrates, including polyolefin polymers. The research involves surface characterisation and understanding the surface physio-chemical properties of common polymers surfaces, and especially targeting bacterial initial attachment to these surfaces, looking at early-stage biofilm growth, viability and the modified surfaces bacterial killing capabilities. Novel hybrid surface chemistries previously not evaluated as AMPs are expected to be synthesised and tested against bacteria using a range of microbial assays that utilises confocal laser microscopy-based, viable cell enumeration on agar, and to lesser extent flow cytometry and SEM imaging.

2.8.2 Thesis Aims and Objectives

Given the information provided in this literature review, the aims of this research project will focus on:

- Modifying the surface of commercially sourced commonly used industrial polymers with HDPE as a model substrate, as well as other commodity polymers including PP, PVC, PS and PC. Modification of the polymers will include oxidative treatment approaches in order to improve thin-film adhesion to the polymer surface, as well as evaluating the efficacy of polymer surface oxidation as an antimicrobial treatment approach independently. Also, thin-film antimicrobial coatings will be evaluated as a possible strategies for the preparation of AMPs in this study, this will include tin-dioxide thin-films and low/medium molecular weight chitosan-zinc oxide nanocomposite coatings due to their already known and proven efficacious

antimicrobial capabilities, which have not been assessed as coating strategies for common industrial polymer materials.

- Different surface characterisation techniques will be utilised for the evaluation of the various polymer surface modifications, these techniques will probe physio-chemical properties, topographical variations, chemical composition, and functional group changes. In some studies, where the surface is particularly efficacious, stability studies will be performed to determine the application scope and operational limitations of these new materials.
- The antimicrobial performance of these materials will be examined using a range of microbial assays that can probe the attachment and viability of the biofilm during the early stages of microbial biofilm development. The study will focus on the utilisation of confocal laser scanning microscopy imaging (with the use of bacterial dyes), viable plate counting (of recovered biofilm), as well as flow cytometry studies, agar disk diffusion, and SEM imaging, where appropriate. In some cases, mature 24 h hydrated biofilm studies will be performed for comparison to early-stage biofilm studies, where relevant, in order to determine the limitations and applications of these materials.

CHAPTER 3 Experimental Methods and Materials

3.1 General Materials, Reagents and Formulas

3.1.1 General Excel Formulas and Statistical Analysis

Excel has been utilised extensively for data analysis in this project, commonly used Excel formulas have been used throughout this project during data analysis for averages, determination of standard deviation and standard error for data processing. Explanation of these formulas can be found in the Appendix under Chapter 3 [A].

Statistical analysis has been used, where relevant, to determine statistical significance between groups of samples, and to uncover whether the means are different from one another. In this project, a t-test has been performed to compare the means of two groups, and is reliant on assuming that the data being compared are independent, and normally distributed [348]. The test has been carried out using the Excel Formulae "TTEST" whilst assuming a 2-tailed test, and that the two samples being compared have unequal variance.

3.1.2 Bacterial Strains

- *P. aeruginosa* BioBall™ NCTC 12924 (ATCC equivalent strain 9027, provided from Procter & Gamble) originally sourced from Biomerieux, lot number B2286 and B2153; and *P. aeruginosa* PA01 www.pseudomonas.com/strain/show/107.
- *S. aureus* ATCC 6538 provided from Procter & Gamble, Reading, UK.
- *E. coli* ATCC 10536 provided from Procter & Gamble, Reading, UK; and *E. coli* MG1655 from the 'E. coli Genetic Stock Center'.

3.1.3 Polymer Materials

The polymers used in this research project were sourced from three suppliers, see **Table 3.1**. The polymers samples have been cut into various sizes dependent on the

experimental requirements. Generally, polymer sheets (films) were chosen due to their practicality and their ability to be readily cut, using scissors, to appropriate sizes. PS was cut using a laser cutter as this material is highly brittle, and the PVC was cut externally by the Mechanical Workshop at the Department of Chemical Engineering as these sheets were thicker.

Polymer Material	Colour	Thickness (mm)	Supplier
High-density polyethylene sheets	Natural (milky white)	1	Gilbert Curry Industrial Plastics Co Ltd.
Polycarbonate sheets	Clear	0.75	Gilbert Curry Industrial Plastics Co Ltd.
Polypropylene sheets copolymer	Natural (milky white)	1	Gilbert Curry Industrial Plastics Co Ltd.
Polyvinyl chloride sheets	White	2.55	RS Components
Polystyrene, laser cut from Sterilin™ petri dishes	Clear	1	Thermo Scientific™

Table 3.1 Material description of the polymers. Thickness dimensions have only been provided in this table because detailed size (2D) description used is experiment specific and therefore can be found in the experimental protocols.

3.1.4 Reagents and Chemicals

Reagents for microbial experiments:

- **Bis-(1,3-dibutylbarbituric acid) trimethine oxonol, DiBAC₄(3)** (Fischer Scientific, 2069785)
- **Cell scrapers, Bel-Art™ ProCulture cell life** (Fischer Scientific, 10385981)
- **Deionised water** was used for all biofilm related assays that was purified with the Milli-Q water purification (Millipore).
- **Glycerol**, for molecular biology ≥99.0% (Merck, G5516)
- **Syto™9** (Fischer Scientific, Invitrogen™, S34854) 5mM in DMSO
- **Syto™9 and propidium iodide** (Fischer Scientific, Invitrogen™, L7012) LIVE/DEAD™ BacLight™ Bacterial Viability Kit, Syto™9 with 3.34 mM in DMSO and PI with 20 mM in DMSO
- **Tryptic Soy Broth** (Sigma Aldrich., SLCD9263)
- **Lysogeny broth with agar lennox** (Sigma Aldrich., L2897)

- **Phosphate buffered saline tablets** (Sigma Aldrich., P4417)

The above reagents were sterilised where appropriate by autoclaving at 121-126 °C for 15 min prior to use.

Reagents and Chemicals for Surface Modification and Characterisation Experiments:

- **Acetic acid**, 99.8-100.5%, AnalR® NORMAPUR® ACS Reagent (VWR™, MFCD00036152)
- **Ammonia solution 28-30%** (Sigma Aldrich., Supelco®, 1054231000)
- **LMW Chitosan** (Sigma Aldrich., 448869) 75-85% deacetylated
- **MMW Chitosan** (Sigma Aldrich., 448877) 75-85% deacetylated
- **Chromium (III) nitrate nanohydrate** (Fischer Scientific, 4324488)
- **Deionised water** (18 Ω) using Centra ELGA system
- **Diiodomethane** (Sigma Aldrich., 158429) contains copper as a stabiliser
- **Dimethyl sulfoxide**, (Sigma Aldrich., 10103483)
- **Ethanol absolute ≥ 99.8%** (VWR, AnalR NORMAPUR® ACS Reagent, MFCD00003568)
- **Hydrindantin** (Sigma Aldrich., H17309)
- **Ninhydrin reagent** (Sigma Aldrich., BCCD6453)
- **pH 4.010 millesimal buffer standard** (HANNA® Instruments, HI-6004-01)
- **Lithium acetate dihydrate**, 99% (Thermo Scientific™, 11419046)
- **Nitric acid 68%** (VWR, GPR RECTAPUR®, MFCD00011349)
- **pH 10.010 millesimal buffer standard** (HANNA® Instruments, HI-6010)
- **Potassium dichromate** (Fisher Scientific, 10060310)
- **Stannous chloride dihydrate** (Sigma Aldrich., 96527)
- **Soap**, Evans Q'det unperfumed liquid detergent
- **Sulfuric acid ≥95%** (VWR, AnalR NORMAPUR® analytical reagent, MFCD00064589)
- **Tin(IV) chloride pentahydrate** (Fischer Scientific, 10428040)
- **Zinc oxide nanopowder** (Sigma Aldrich., 677450) <50 nm particle
- **Ultrapure spectrograde water** (Fischer Scientific, 4324518)

3.2 Synthesis, Treatment and Preparation of Materials and Solutions

3.2.1 Polymer Surface Oxidisation Treatments

Sulfuric-chromic acid oxidisation

The procedure was adopted from Sheng *et al.*, [295] where potassium dichromate (5 parts, 8.125 g, 3×10^{-2} mol) was dissolved in DI water (8 parts, 13 mL). 0.01 M concentrated sulfuric acid (100 parts, 88 mL) was slowly added with stirring and heated to 70 °C. The polymer materials were pre-cleaned sequentially with soap, acetone, ethanol and DI water then air-dried. The samples were submerged inside the heated sulfuric-chromic acid solution vertically for varied set periods of time ($T=0, 2, 10, 30, 45, 60$ min). The polymer surfaces were then rinsed in 4 beakers of DI water sequentially (80 mL) and left to dry overnight in a vacuum desiccator in the presence of silica, and further dried in an oven for 4 h at 65 °C.

Caution: The above experiment uses highly corrosive and oxidising chemicals and contact can cause severe skin and eye irritation and burns. Breathing sulfuric-chromic acid fumes can irritate the nose, throat and lungs. Do not handle until all safety precautions have been understood. The experiment must be performed outdoors or in a well-ventilated area, using personal protective equipment as required. To dispose, wait until solution has reached RT, dilute heavily with water by adding the solution to water, and discard as hazardous liquid waste for appropriate collection. Do not mix with other chemicals/liquid chemicals.

Sulfuric-acid oxidisation

This procedure was adopted from Zhang *et al.* [286]. Concentrated sulfuric acid (98%, AR) was heated to 95-100 °C. Polymer coupon surfaces were pre-cleaned sequentially with acetone, ethanol and DI water then air-dried, and placed inside the heated sulfuric acid solution for 15 min. The treated surfaces were then rinsed in 4 beakers of DI water sequentially (80 mL) and left to dry overnight in a vacuum desiccator in the presence of silica, and further dried in an oven for 4 h at 65 °C.

Caution: The above experiment uses a highly corrosive and oxidising chemical and contact can cause severe skin and eye irritation and burns. Breathing sulfuric acid fumes can irritate the nose, throat and lungs. Do not handle until all safety precautions have been understood. The experiment must be performed outdoors or in a well-ventilated area, using personal protective equipment as required. To dispose, wait until solution has reached RT, dilute heavily with water by adding the solution to water, and discard as hazardous liquid waste for appropriate collection.

UV/ozone oxidation (UVO)

Clean samples were placed inside a UV/ozone ProCleaner™ 220 Plus (BioForce NanoScience system) with UV intensity of 19.39 mW/cm² for 20 min.

UV/ozone - sulfuric acid oxidation (UVO-SA)

Clean samples were placed inside the UV/ozone a UV/ozone ProCleaner™ 220 Plus (BioForce NanoScience system) with UV intensity of 19.39 mW/cm² for 20 min, followed by submersion in concentrated sulfuric acid (98%, AR) at 95-100 °C for 15 min. The treated surfaces were then rinsed in 4 beakers of DI water sequentially (80 mL) and left to dry overnight in a vacuum desiccator in the presence of silica, and further dried in an oven for 4 h at 65 °C.

3.2.2 Cr-oxyhydroxide Synthesis and Film Preparation

The synthesis of the chromium-oxyhydroxide coatings were adopted from research investigations by Yang *et al.* [349]. Cr(NO₃)₃·9H₂O (15 g, 3.7x10⁻² mol) was dissolved in ultrapure water (75 mL), turning into a deep navy colour. Ammonia solution (28-30%) was diluted to 10% with ultrapure water (150 mL). At RT the diluted 10% ammonia solution was added dropwise to the chromium solution with continuous stirring, until pH 10 was reached. The resultant mixture gradually formed a deep green precipitate which was then stirred for 30 min. The mixture was centrifuged and rinsed three times

with ultrapure water to remove NO_3^- ions (8700 RPM, 10 min). Ultrapure water (75 mL) was added, and pH monitored to maintain a pH of 10. The solution was divided into two parts with one half placed in a sealed Teflon vessel and heated in a fan oven (Mettler Universal oven UF55) overnight at 200 °C for 15 h. This heat-treated solution is named the **hydrothermal treated (HT)** solution. HDPE was dip-coated into separate Cr-oxyhydroxide mixtures (100 mL) prepared (2 min vertical dipping/coating time) and placed in a vacuum-desiccator to dry (ultra-pure water was used to increase the volume to 100 mL, pH was maintained at pH 10).

3.2.3 Tin-dioxide Hydrosol Synthesis and Film Preparation

The synthesis of the tin-dioxide hydrosols has been adopted from Briscoe *et al.* [350]. $\text{SnCl}_4 \cdot 5\text{H}_2\text{O}$ (0.25 g, 7.1×10^{-4} mol) was dissolved in DI water (90 mL) and stirred. Further DI water was added (110 mL) and the solution was stirred, then $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (0.5 g, 2.6×10^{-3} mol) was added. The solution was heated and stirred for 1 h at 60 °C, a pale-yellow solution formed. $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (0.4 g, 2.1×10^{-3} mol) was added and left to age overnight for 16 h at 60 °C, a deep yellow solution formed. Surfaces were pre-cleaned in water followed by ethanol and left to air-dry. Half of the solution was taken prior to aging overnight; this solution was to examine the efficiency of aging based on the additional time and energy needed for the aging step. The pre-aged solution is named **tin-hydrosol A** and the post-aged **tin-hydrosol B**. The substrates were dip-coated by vertical submersion (2 min) and left to air dry horizontally on a flat surface, the top faced side drying is considered as the film coated surface, forming **Film A** and **Film B** coatings.

3.2.4 Chitosan/PVP-ZnO Nanocomposite Synthesis and Film Preparation

The synthetic procedure was obtained from Al-Naamani *et al.* [351]. A nanocomposite solution was prepared by the addition of commercial ZnO nanoparticles (varied amounts, see Chapter 6) into an acetic acid (1% or 10%, 100 mL) solution. The solution was mixed vigorously for 10 min and then chitosan powder was added (varied

amounts, see Chapter 6) and sonicated for 15 min to remove bubbles. NaOH was added (0.1 M) with vigorous stirring until pH 6 achieved, and the solution was kept at 60 °C overnight in an oven. HDPE surfaces (4.5 x 4.5 cm) were pre-cleaned using soap, ethanol and DI water, and oxidised using UVO (20 min) then sulfuric acid heat treatment 95-100 °C (15 min), UVO-SA. The polymer materials were dip-coated into all the nanocomposite chitosan-ZnO solutions prepared (2 min) and placed in a vacuum-desiccator to dry. After the coating was fully dried, selected samples were rinsed twice with NaOH (0.1 M) hot-wash at 85 °C and air-dried at RT.

3.3 Surface Analysis and Molecular Characterisation

3.3.1 Determination of pH

The Mettler Toledo pH meter was utilised with a 2-point calibration performed at pH 4 and 10. Calibration was conducted at the beginning of each use of this instrument using purchased buffer standards (HANAA® Instruments pH 4.010 millesimal buffer solution, and pH 10.010 Millesimal buffer solution). The probe tip was rinsed before and after use with filtered (0.22 µm) DI water and dried using a KIMTECH Science tissue. In between readings and during stand-by the pH probe was kept in a KCl solution 3 mol/L. All measurements were conducted under standard conditions at RT, and Fischerbrand™ pH strips (0-14, 0-6.0, 7.0-14) were used intermittently to confirm the results.

3.3.2 Dynamic Light Scattering

(a) Small colloidal particles less than 1000 nm in diameter

The Zetasizer µV Malvern Panalytical was utilised to determine the particle size of the tin-hydrosols. This instrument has a size measurement range of 0.15 nm to 1 µm and uses a 60 mW laser at 830 nm wavelength for scattering light off the particles. Disposable polystyrene cuvettes were used with 1 mL solution volume at room temperature (ca. 20 °C) and experiment duration of 1 min. This instrument divides

measurements into the number of runs, to allow for data filtering and processing with the quality of each run being assessed, and the poorer runs deleted. Instrument guidelines advises that as more runs are performed the assessed quality of the results improves, therefore the final run obtained has been used as the definitive data for analysis, with previous runs neglected. This final run obtained is also provided as an average value.

(b) Larger particles from 0.5 to 600 μm in diameter

A Mastersizer 2000 particle size analyser (Malvern) was employed to determine the particle size of the Cr-oxyhydroxide solutions prepared. This instrument has a maximum size range of 600 microns and uses a 633 nm wavelength for diffraction particle sizing. Accompanied with a dispersant tank for diluting and a pump stirrer (stirrer speed set at 2000 RPM) to allow a wide range of particle sizes and densities to be suspended. DI water is used for sample mixture dilution to the measurable range. Measurement time was set at 5 s, with 5 measurement cycles; the results are provided as a volume weighted distribution.

3.3.3 Refractometry

The Ceti Convex bench-top analogue refractometer was used to measure solution refractive indexes between 1.3-1.7200 nD, with a precision of 0.0005 nD. The wavelength used was the same as D line of sodium (589 nm). The instrument is equipped with a 30x focusable telescope and a thermometer.

3.3.4 Contact Angle Goniometry

Advancing contact angles using water and DIM were obtained using a Krüss Drop Shape Analyzer (DSA25) using the Advance Software with an automated liquid dispenser operating at 2.67 $\mu\text{L/s}$. An initial liquid droplet of 5 μL was placed on the coupon surface, and this droplet was increased by a series of 1 μL aliquots to allow measurement of the advancing contact angle. The advancing contact angles reported were an average of the right- and left-hand side contact angles using the tangent setting

from the software and noted as the largest angle before the drop collapses/recedes and consisted of a minimum of 3 different droplets each of DI water and DIM as probes ($N \geq 3$, +/- SE).

3.3.5 X-Ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) was performed with a Thermo Scientific K-Alpha⁺ instrument at 2×10^{-9} mbar base pressure. This instrument used a monochromated micro-focused Al K α X-ray source ($h\nu = 1486.6$ eV) and a 180° double-focusing hemispherical analyser accompanied by a 2D detector. The X-ray source operated at 6 mA emissions current and 12 kV anode bias thus obtaining a spot size of $400 \mu\text{m}^2$. An electron flood gun was used to minimize the sample charging that occurs when exposing an insulated sample to an X-ray beam. Charge neutralisation was deemed to have been achieved by monitoring the energy of the C1s signal of adventitious carbon present. All XPS spectra were recorded using the Advantage Data System software as was the quantification analysis ($N=1$ unless specified otherwise).

3.3.6 Atomic Force Microscopy

For atomic force microscopy experiments the Nanowizard 4 (JPK Instruments -now Bruker) was used. The sample was scanned with a silicon tip (PPP-NCHAuD from NANOSENSORSTM, serial number 96215F12L1344, resonance frequency 204-497 kHz and force constant 10-130 N/m), with a 2000 nm x 2000 nm scan area and a resolution of 256 x 256 pixel or 512 x 512 pixel in Quantitative ImagingTM-mode. The image was levelled, filtered using a median filter, analysed for roughness and adhesion images with JPKSPM data processing software (JPK system).

3.3.7 Attenuated total reflection Fourier-Transform Infrared Spectroscopy

ATR-FTIR data was obtained using a Perkin Elmer Spectrum 100 spectrometer accompanied with the Perkin Elmer Universal-ATR accessory. The spectra were taken in ATR mode in the wavenumber range between 500 to 4000 cm^{-1} , with a resolution of 2 cm^{-1} and scan number as 16, and experiment duration as 1 min.

3.3.8 Time-of-Flight Secondary Ion Mass Spectrometry

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) was conducted, and data analysed by Dr Sarah Fearn from the Department of Materials, Royal School of Mines, Imperial College London. ToF-SIMS was carried out using an IONTOF ToF-SIMS 5 instrument. High resolution mass spectra were collected using a 25 keV Bi^{3+} ion beam in high current bunched mode with a beam current of ca. 0.25 pA, and 100 μs cycle time. The area analysed was 500 x 500 μm^2 , and 50 scans were taken. Both positive and secondary ions mass spectra were collected, and an electron flood gun was used for charge compensation on the sample surface. Calibration of the mass spectra was performed using a series of simple hydrocarbons: $\text{C}_{2n}\text{H}_{2n-1}$ for the positive ions, and $\text{C}_{2n}\text{H}_{2n+1}$ for the negative ion spectra. Analysis of the mass spectra data, and peak identification was performed with the Surface Lab 7 software.

3.3.9 X-Ray Diffraction

Powders of Cr-oxyhydroxide prepared were dried overnight in an oven at 65 °C. X-ray diffraction patterns were collected using an X'Pert Pro X-ray diffractometer (Malvern Panalytical, UK) with the Cu-K α radiation at 40 kV and 20 mA, in a 2 θ scan range of 5°–90°, using a step size of 0.033.

3.3.10 Inductively Coupled Plasma-Mass Spectrometry

(a) Chromium Leaching Study

Samples were freshly prepared and cleaned in 80% ethanol and allowed to air dry. A droplet of DI water with volume the same as that used in the viable enumeration

assay was placed on to the surface for $T=1, 30$ min ($200\ \mu\text{L}$). The droplets were collected and added to $4.8\ \text{mL}$ of DI water with 2% nitric acid ($N=2-4$). The solution was filtered using a $0.2\ \mu\text{m}$ filter and analysed for Cr concentration using ICP-MS (Agilent Technologies 7900 System) equipped with the SPS 4 autosampler and Masshunter 4.4 software. N_2 gas flow rate was $15.0\ \text{L/min}$ and the auxiliary gas flow rate was $0.90\ \text{L/min}$. A 23-elemental component reference mixture (Aristar, Lot. 386065) was used for calibration of the Cr study; the calibration graph obtained from using the standards can be found in the Appendix under Chapter 3[B].

(b) Sn Leaching Study

Samples were freshly prepared and cleaned in 80% ethanol and allowed to air dry. Fresh samples were rinsed in three beakers of DI water ($20\ \text{mL}$), named as W1, W2 and W3. 2% nitric acid was added to $5\ \text{mL}$ of solution and measured under ICP-MS (Agilent Technologies 7900 System) equipped with the SPS 4 autosampler and Masshunter 4.4 software. N_2 gas flow rate was $15.0\ \text{L/min}$ and the auxiliary gas flow rate was $0.90\ \text{L/min}$. $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ was used to prepare the calibration standard of the Sn, the calibration graph obtained and used can be found in the Appendix under Chapter 3[C].

3.3.11 White light interferometry

A Zygo product Newview™ NX2 non-contact surface white light profilometer was used with the Mx software. Settings were optimised, and a $2.5\times$ lens utilised with a $0.5\times$ zoom which affords a field of view of $6.96\times 6.96\ \text{mm}$, in the CSI mode, and a scan length of $20\ \mu\text{m}$ with an acquisition time of $11\ \text{s}$. The longer the scan, the greater the height measuring capabilities and the longer the measurement time.

3.3.12 Ninhydrin Test for Primary Amines

The ninhydrin assay can react with primary amine groups to form a coloured product. The protocol used was adopted from Leane and co-workers [352]. Lithium

acetate buffer was prepared by dissolving lithium acetate dihydrate (8.16 g, 0.08 mol) in DI water (12 mL). The pH of this solution was adjusted to pH 5.2 using glacial acetic acid. Samples to be analysed were cut into 0.5 x 0.5 cm squares (x 3 of each type of sample). The ninhydrin solution was prepared by lithium acetate buffer (10 mL) being added to DMSO (30 mL) in a glass scintillation vial, then ninhydrin reagent (0.8 g, 4.9×10^{-3} mol) and hydrindantin (0.12 g, 3.7×10^{-3} mol) added. A sample to be analysed was immersed in the ninhydrin solution (1 mL) in a fresh glass scintillation vial and immediately capped. The vial was immediately shaken and heated in boiling water in a water bath for 30 min. After cooling a 1:1 ethanol-water (10 mL) was added to each vial and this was vortexed for 15 s at maximum speed to oxidise the excess hydrindantin. The absorbance of each solution was measured on a UV/vis spectrometer (UV-2600 Shimadzu System) between 300-700 nm.

3.4 Confocal Laser Scanning Microscopy: Specification, Software and Measurement

3.4.1 CLSM Instrument Set-up and Software Information

The Leica CLSM TCS SP2/SP8 was set up according to manufacturer's instructions. The instrument was equipped with a pulsed white light laser and hybrid detectors that allow for delayed detection of wavelengths of light emitted by the sample, tuneable from 0 to 10 ns. The laser intensity was set to 1 and the surface was brought into focus each time manually via the controller accessories using the z-height scale to focus. A minimum of 5 images were taken at random of three different samples for each type of material. Tuneable settings were set at gain 100, threshold 90 and optical z-slice of 10 μm , with 2 ns delay in gating. Detailed decision making of these settings and its optimisation can be found in the Appendix under Chapter 3[D] for the bacterial staining probes from the LIVE/DEAD™ *BacLight*™ Bacterial Viability Kit.

The images captured with the CLSM was processed using Comstat 2.1. This software is an analysis plugin tool for Image J which can interpret and analyse the images based on various user selected parameters. Image J is a java-based image processing software

that is freely available to the public, it was developed by National Institutes of Health and the Laboratory for Optical and Computational Instrumentation. Comstat 2.1 was created by Arne Heydorn and Professor Bjarne Esbøll in the form of a MATLAB® script, later Martin Vorregaard developed the software further in order to run via the public domain software Java® and Image J with the add on package LOCI tools [353–355]. COMSTAT was developed to analyse high-resolution CLSM images of biofilms. The COMSTAT script is used to simplify and reduce the processing time needed to interpret the confocal images for data analysis and quantification with high reproducibility, with the capability of comparing different properties derived from the biofilm images.

Digital images obtained comprise of voxels which are 3D image volume elements, the 3D analogue of a 2D pixel. This element is the smallest part of the picture which has a finite value that combines to make the full image. Higher resolution means more pixels or voxels, for example a 512 x 512 image comprises of 262,144 (512^2) pixels overall. In this study a set pixel resolution of 1024 x 1024 has been selected. The width (x) and length (y) of the voxel is the same as for the pixel, and the height (z) of the voxel is the distance between the slices. There are various selections and options that can be chosen when using Comstat 2.1 for image analysis, each of which looks at different desired substrate parameters. These parameters include for example, the biomass ($\mu\text{m}^3/\mu\text{m}^2$), maximum film thickness (μm), average biomass thickness (μm), roughness (R_q), fractal dimension and area occupied in layers (μm^2). Area occupied in layers measures the biomass pixel in each layer or confocal optical slice, therefore providing a percentage of area occupied from the image captured. The area occupied in layers was the chosen parameter used to analyse the data in our experiments, using Comstat 2.1 with the Image J plug-in [353–355]. This parameter was selected due to the reduced time needed for image capture of a slice, compared to imaging stacks with larger z-scale parameters. This time is important, as bacterial biofilms can deteriorate, multiply, and essentially change form with time.

3.4.2 Fluorescent Probe Selection and Spectra

To interpret and evaluate the presence and viability of bacteria, a fluorescent probe or marker is needed for identification, with the fluorescent emission being measured instead of reflection at a specific wavelength [356]. This marker can be expressed intrinsically to the cell (e.g. green fluorescent proteins) or fluorescent probes can be used at the time of the experiment. The use of tunable lasers with the CLSM allows for more precise excitation of molecules, and precise emission detection of data in the x-y-z scale. Probes selected to be used in this project with CLSM were:

- Syto™9
- Syto™9 and propidium iodide

Syto®9 is a green-fluorescent cyanine based nuclear and chromosome stain that has been shown to stain both live and dead Gram-positive and -negative bacteria, as well as mammalian cells. This dye is known to exhibit heightened fluorescence upon binding to cell nucleic acids. Excitation wavelength for Syto™9 was 488 nm and the detection bandwidths used were 495 to 510 nm, the excitation and fluorescence spectra has been provided in **Figure 3.1**. The use of Syto™9 staining of the biofilm allows surface bound cells to be directly imaged. The biofilm is also largely undisturbed as it is imaged under a fully hydrated environment using a liquid immersion lens.

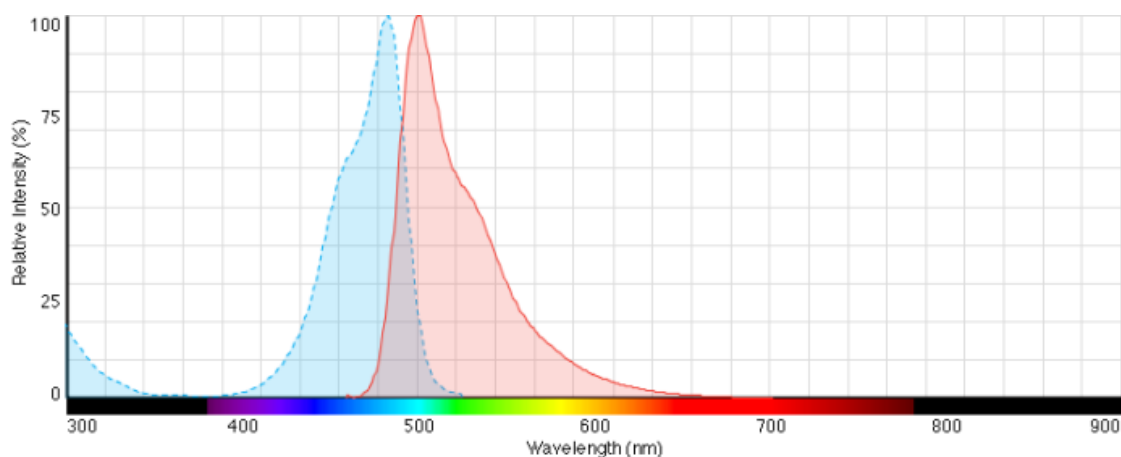


Figure 3.1 The excitation and emission fluorescence spectra of Syto™9 between 300 and 900 nm. Blue peak corresponding to excitation and the red peak corresponding to emission, spectra sourced from supplier website [357].

A combination of Syto™9 and PI are provided in the LIVE/DEAD BacLight Bacterial Viability Kits (L-7007, L-7012, L-13152). Excitation wavelength for PI was 538 nm and

the detection bandwidths used were 615 to 630 nm, the excitation and emission spectra has been provided in **Figure 3.2**. PI, a nucleic acid red-fluorescent counterstain that can be used to examine cell viability as a compromised cell membrane (dead cell) allows PI to penetrate the cell. This dye can be used in combination with other dyes like Syto™9 or acridine orange which stains both live and dead cells. The combination of the two stains provides a fast and reliable method of directly imaging the physiological state of the bacterial cell bound to a surface actively in real time, without the need to disturb the cells, remove the cells or manipulate the cells to obtain physiological information about membrane permeability [128].

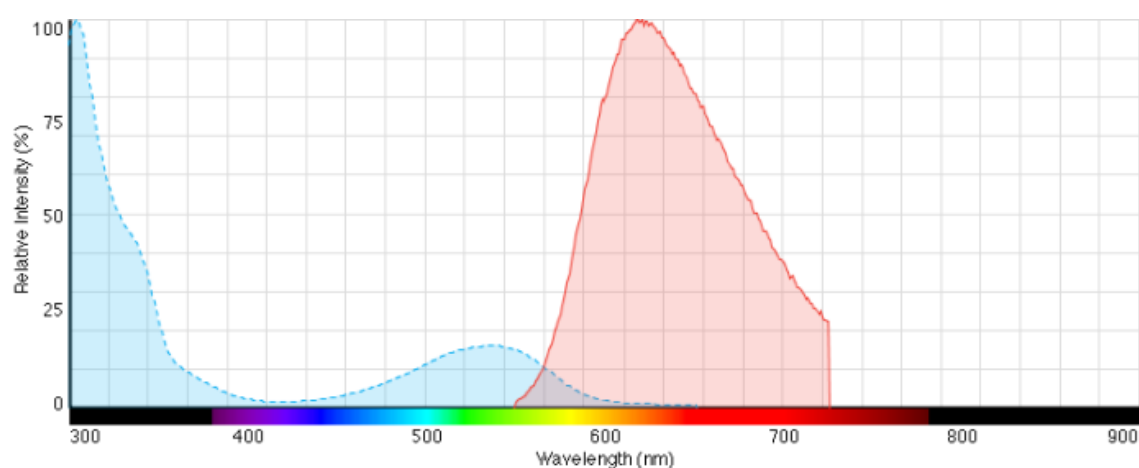


Figure 3.2 The excitation and emission fluorescence spectra of propidium iodide between 300 and 700 nm. Blue peak corresponding to excitation and the red peak to emission, spectra sourced from supplier website [358].

The use of suitable chemical probes to stain biologically active materials carries a degree of complexity and some limitations associated with certain selected dyes. There are many variables that can affect the fluorescent signal emitted and detected by the CLSM. These complications can include probe-conjugate availability, photobleaching, environmental sensitivity of fluorescence, background fluorescence, fluorescence resonance energy transfer, and overlapping excitation/emission profiles that limits the use of some fluorescence probes simultaneously.

A multicolour labelling experiment such as the LIVE/DEAD™ BacLight™ bacterial viability kit is a highly advantageous technique to monitor biological behaviour. Improved data capture can be achieved by signal isolation and maximising the spectral separation between multiple fluorescence emissions. Therefore, using dyes with narrow emission spectral bandwidths is highly beneficial, as well as emission spectra that are well separated. Below a detailed diagram (see **Figure 3.3**) of the spectral overlap using the bacterial viability kit with our settings shows that there is a significant separation between emission spectra. There is 0% overlap of the emission detection of PI in the Syto™9 detection emission (495-510 nm) bandwidth. Though there is a 3% overlap of the emission from Syto™9 in the emission bandwidth of PI, this overlap is small and is kept to a minimum to prevent false-positives and additional unwanted signal to be detected for the PI emission. This is important as Syto™9 has shown to enhance PI emissions than when PI is present on its own [359].

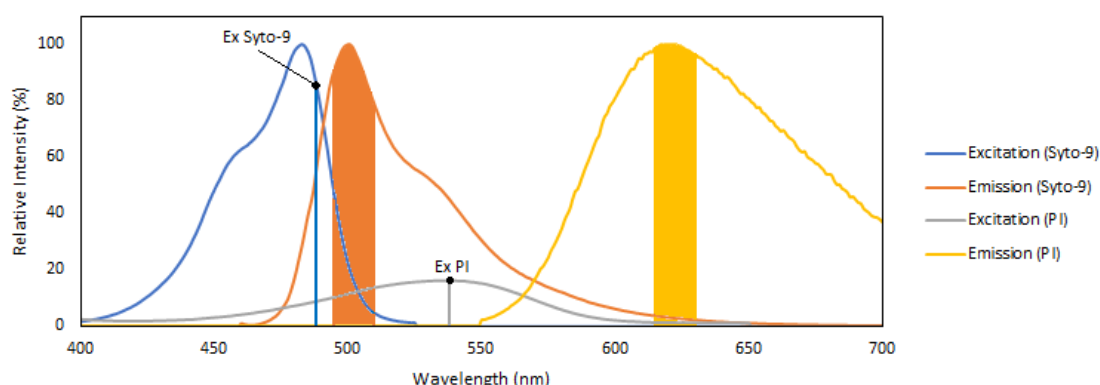


Figure 3.3 Mixed spectra of Syto™9 and PI provided alongside the annotation of the excitation wavelengths used, for Syto™9 is 488 nm and for PI is 538 nm. The emission bandwidths used have been highlighted by a block colour in the emission curve which for Syto™9 is between 495-510 nm and for PI is between 615-630 nm. The settings selected were automatically suggested by the Leica software on the CLSM TCS SP2 system as Syto™9 and PI are common dyes and the excitation/emission information is well-known.

PBS buffers, as well as liquid media components, have been shown to influence staining efficiency and hence CLSM imaging, therefore a water-probe solution has been selected for staining the bacteria in our experiments [359]. As recommended in the L7012 bacterial viability kit manual, the water-probe solution was prepared in a 1:1 mixture of

dyes (Syto™9 and PI) with a 3/1000 µL ratio of 'dye mixture/liquid' recommended; where for example for a 1000 µL microbial solution, 1.5 µL of Syto™9 and PI are each added. As biofilms are being investigated in this PhD research project, instead of in solution microbes, a water-probe solution has been prepared for staining the biofilm using the same aforementioned dilutions (3/1000 µL ratio of dye mixture/liquid with a 1:1 of each dye). The dilution of the probe in DI water is essential because a large area of surface contaminated with bacteria (biofilm) will require staining, and hence a large volume of probe solution is needed. The probe (as purchased) uses DMSO as the solvent system for the fluorescence probes. It has been shown that DMSO can influence bacterial behaviour, such as reducing *P. aeruginosa* pathogenesis and protecting *E. coli* from rapid antimicrobial-mediated killing [360,361], and so exposure to excess DMSO should be avoided. Furthermore, the probes are costly and larger than the required ratio for staining is not needed, consequently dilution of the probe in water helps address these issues.

The exposure of the water-probe solution over time has been examined for *P. aeruginosa* early-stage biofilm on the fluorescence intensity observed. Exact details of the protocol can be found in Section 3.4.5, with extended water-probe solution time periods of 5, 10, 30, 45 and 60 min. The results have been provided in **Figure 3.4**. It can be inferred that firstly, under these conditions, the early-stage biofilm is largely viable, and the intensity of the signal for Syto™9 is significantly reduced with time, even with 5 additional minutes from 5 to 10 min. The measured signal intensity for PI is gradually increasing over time. This increase may be due to the water-probe solution that is nutrient deficient. However, this increase is minimal compared to the decrease observed from Syto™9 over time. Overall, it can be concluded that the time the water-probe solution is exposed to the biofilm for staining must be consistent and constant throughout experimentation, as well as being kept to minimum with the exposure time of 5 min chosen in order to ensure sufficient time for staining with minimal loss in signal due to time effects. The reason behind this significant loss in signal intensity detected of Syto™9 over time may be due to bleaching, where the irreversible destruction of the excited fluorophore is observed. This behaviour can predominantly become the limiting

factor in fluorescence detectability over time. This observation has been confirmed by Steifel and co-workers when examining Syto™9 and PI against *P. aeruginosa* [362].

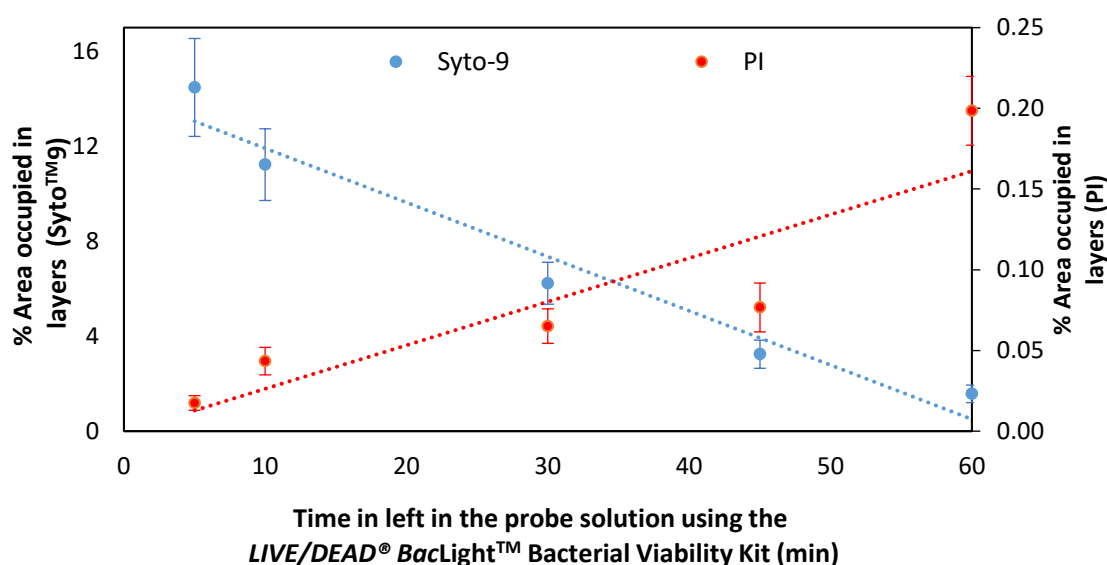


Figure 3.4 HDPE unmodified samples were exposed to early-stage biofilm of *P. aeruginosa* with $N=3$, minimum of 5 images taken per sample, $\pm$ SE. Linear trendline also provided for both.

3.4.4 Initial Attachment Assay (IAA) to Measure Bacterial Adhesion

The protocol has been inspired and adopted by work conducted by Jon Palmer where full credit is due for development of the IAA assay [363]. *P. aeruginosa* cells (NCTC 12924) were cultured overnight in 100% TSB and set to 0.2 OD (600 nm, 4.9×10^7 CFU/mL) using DI water. 200 μ L of 0.2 OD (600 nm) solution was pipetted onto the polymer coupon surface and left in static contact for 30 min for early-stage biofilm proliferation. The polymer coupon surface was then rinsed in 3 beakers of DI water (100 mL, 4.5cm²/s) to remove any planktonic cells. 200 μ L of Syto™9 (5 mM) probe solution (1/1000 in DI water) was pipetted onto the biofilm surface and left for 5 min in the dark. The probe solution was expunged using a tissue (avoiding surface contact) and replaced with DI water (200 μ L) for hydrated dip-lens CLSM imaging of the biofilm. Image-J (FIJI) and Comstat 2.1 software were used for the conversion to ome-tiff format and analysis of threshold and percentage surface area coverage of the cells in the images captured with optical imaging section set to 10 μ m in thickness, with a 1024 x 1024 pixel resolution using a 25x magnification liquid lens.

3.4.5 Viability Assay for Early-Stage Biofilm Viability Imaging

LIVE/DEAD™ *BacLight*™ Bacterial Viability Kit [364] was used with the CLSM TCS SP8 Leica Instrument with hybrid detectors with a pulsed white light laser. Bacterial cells (*P. aeruginosa* NCTC 12924, *S. aureus* ATCC 6538, *E. coli* ATCC 10536) were cultured overnight in 100% TSB, the solution was diluted to 0.2 OD (600 nm, 4.9×10^7 CFU/mL) using dd water. 200 µL of the suspension was pipetted onto a surface and left in place for 30 min, then rinsed in 3 beakers of DI water (100 mL, 4.5 cm²/s) to remove planktonic cells. 200 µL of probe solution containing 1:1 ratio of Syto™9 (3.34 mM) and propidium iodide (20 mM) in 3/1000 dilution in DI water was pipetted onto the biofilm and left in contact for 5 min in the dark. The probe solution was expunged using a tissue and replaced with DI water. A liquid lens (x25 magnification) on the CLSM was used to capture images at the surface. Image J (FIJI) and Comstat 2.1 was used for file conversion to ome-tiff, followed by an analysis of threshold and percentage surface area coverage from the biofilm, with the optical section set to 10 µm in thickness, 1024 x 1024 pixel resolution, 500 x 500 µm with a 25x dip-magnification lens [353–355]. As suggested by the probe supplier, the ratio of cells stained by Syto™9 and propidium iodide quantifies the proportion of live to dead cells present on the surface [364]. Calibration studies have been performed to validate the efficacy of the dye for this experiment; this data can be found in the Appendices Chapter 3[E].

3.4.6 Viability Assay for Early-Stage Biofilm Viability Imaging – extended T₂₀

The LIVE/DEAD™ *BacLight*™ Bacterial Viability Kit [364] was used with the CLSM TCS SP8 Leica Instrument with hybrid detectors with a pulsed white light laser. Bacterial cells (*P. aeruginosa* NCTC 12924) were cultured overnight in 100% TSB after taking a swab from the cell bank, the solution was diluted to 0.2 OD (600 nm, 4.9×10^7 CFU/mL) using DI water. 200 µL of the suspension was pipetted onto a surface and left in place for 30 mins, then rinsed in 3 beakers of DI water (100 mL, 4.5 cm²/s) to remove planktonic cells. Saline (0.85% NaCl, 200 µL) was added for 20 min (T₂₀), static and RT. The saline solution was removed by expunging with a tissue and replaced with 200 µL of probe solution

containing 1:1 ratio of SytoTM9 (3.34 mM) and propidium iodide (20 mM) in 3/1000 dilution in DI water was pipetted onto the biofilm and left in contact for 5 min in the dark. The probe solution was expunged using a tissue and replaced with DI water (200 µL). Image J (Fiji) and Comstat 2.1 was used for file conversion to ome-tiff, followed by an analysis of threshold and percentage surface area coverage from the biofilm, with optical section set to 10 µm in thickness, 1024 x 1024 pixel resolution, 500 x 500 µm with a 25x magnification dip-lens [353–355]. As suggested by the probe supplier, the ratio of cells stained by SytoTM9 and PI quantifies the proportion of live to dead *P. aeruginosa* cells present on the surface [364]. Calibration studies have been performed to validate the performance of the dye for this experiment; this work can be found in the Appendix Chapter 3[E].

3.5 Viable Plate Colony Counting Experiments

To corroborate the results obtained from the CLSM imaging with another microbial assay, a series of different assays were assessed for the removal the early-stage biofilm from the surface for subsequent CFU viable counting. These biofilm removal methods included:

- (i) Sonication of surfaces with biofilms in the presence of surfactants
- (ii) Agitation of surfaces with biofilms by shaking
- (iii) Agitation by shaking with glass beads of surfaces with biofilms

These three methods did not prove effective for various reasons which largely involved: ineffective dislodgement and lifting of the biofilm from the surface, and significant contamination from planktonic cells (that were not in contact with the surface). Details of these experiments, their results and conclusions can be found in the Appendix, under Chapter 3[F].

Subsequently the most effective method was found to be scraping of the early-stage biofilm, in the presence of PBS, for viable plate colony counting using re-usable PP cell scrapers. The Bel-ArtTM ProCulture cell lifter could be autoclaved and was thus also economical. Therefore, the following experiments for viable plate colony counting all

utilise this approach for recovery and detachment of the biofilm cells for subsequent plating out studies.

3.5.1 Viable Plate Counting and Flow Cytometry Experiment

P. aeruginosa was cultured overnight in 100% TSB, the solution was diluted to 0.2 OD (600 nm, 4.9×10^7 CFU/mL) with 100% TSB. 200 μ L of the suspension was pipetted onto a surface and left static for 30 min. The surface was then rinsed in 3 beakers of DI water (100 mL) to remove planktonic cells. 1 mL of PBS was placed on the surface, a cell-scraper was used to lift the biofilm off and re-suspend it into the PBS solution and the resultant solution was collected. This procedure was repeated twice for each polymer coupon to remove as much of the biofilm as possible (total volume 2 mL). 1 mL of the collected solution was stained with 0.625 μ g/mL of (bis-(1,3-Dibutylbarbituric Acid)Trimethine oxonol) (DiBAC₄(3)) and left in the dark for 10 min. The samples were run on a FACS BD Calibur Flow Cytometer System and data analysed using Flow-Jo software (N=1). The samples were kept in a cool room until plating out; the remaining 1 mL was serially diluted with PBS, plated out on agar plates and the colonies formed (CFU/mL) determined after 14-15 h overnight incubation at 37 °C. Duplicate coupons were tested using the same protocol resulting in a N=6 data set (2 samples, 3 repeats per sample).

3.5.2 Viable Plate Counting

Cells were cultured overnight in 100% TSB, the solution was diluted to 0.2 OD at 600 nm (corresponding to *P. aeruginosa* 600 nm, 4.9×10^7 CFU/mL; *E. coli* 1.8×10^8 CFU/mL and *S. aureus* 8.2×10^7 CFU/mL) using 100% TSB. 200 μ L of the suspension was pipetted onto a surface and left static for 30 min. The surface was then rinsed in 3 beakers of DI water (100 mL) to remove planktonic cells. 1 mL of PBS was placed on the surface, a cell-scraper was used to lift the biofilm off and re-suspend it into the PBS solution, this procedure was repeated once more, and the resultant solution was collected. The samples were kept in a cool room until plating out; the solution was serial

diluted using PBS, plated out on agar plates and the colonies formed (CFU/mL) determined after 14 -15 h overnight incubation at 37 °C. Duplicate coupons were tested using the same protocol resulting in a N=6 data set (2 samples, 3 repeats per sample).

3.5.3 Viability Plate Counting for Re-used Samples

The samples were sterilised in ethanol (80%) and allowed to air-dry near flame (Bunsen burner) before being used again. *P. aeruginosa* cells were cultured overnight in 100% TSB, the solution was diluted to 0.2 OD at 600 nm (corresponding to *P. aeruginosa* 600 nm, 4.9×10^7 CFU/mL) with 100% TSB. 200 µL of the suspension was pipetted onto a surface and left static for 30 min. The surface was then rinsed in 3 beakers of DI water (100 mL) to remove planktonic cells. 1 mL of PBS was placed on the surface, a cell-scraper was used to lift the biofilm off and re-suspend it into the PBS solution and the resultant solution was collected. This procedure was repeated to collect a total of 2 mL volume. The samples were kept in a cool room until plating out; the solution was serially diluted with PBS with three repeats, plated out on agar plates and the colonies formed (CFU/mL) determined after 14 -15 h overnight incubation at 37 °C. This procedure was performed within a period of 5 hr on the same day for a total of 4 uses, termed 'cycles'.

3.5.4 Viability Plate Counting for a Mature Biofilm

E. coli cells were cultured overnight in 100% TSB, the solution was diluted to 0.2 OD at 600 nm (*E. coli* 1.8×10^8 CFU/mL), with 1% glycerol (100 mL/2 samples) with 100% TSB. The samples were submerged inside (2 in one/back facing) and left for 24 h for biofilm maturation on the surface. Samples were removed with sterile metal tweezers and dipped in 3 x DI water (100 mL) to remove planktonic and weakly bound cells. PBS (1 mL) was placed on the surface, a cell-scraper was used to lift the biofilm off and re-suspend it into the PBS solution and the resultant solution was collected, this procedure was repeated once more (total volume 2 mL). All vials were kept in a cool room until

plated. The solution was then plated out onto agar from N to -12 and left to grow overnight in a static incubator at 37 °C.

3.6 Scanning Electron Microscopy

Samples were freshly prepared and cleaned in 80% ethanol and allowed to air dry. *P. aeruginosa* was cultured overnight in 100% TSB, the solution was diluted to 0.2 OD (600 nm, 4.9×10^7 CFU/mL) using 100% TSB. 200 µL of the suspension was pipetted onto a surface and left in place for 30 min, then rinsed in 3 beakers of DI water (100 mL) to remove the planktonic cells. Samples were fixed using 10% formaldehyde for 10 min and dehydrated at ambient temperature using serial ethanol gradient immersions (5 min each; 30, 50, 70, 85, 95, and 100% ethanol) and left to fully air dry for 24 h overnight. The prepared samples were coated with a 15 nm chromium with a Quorum system Q150T sputter coater, and imaged using ZEISS Gemini Sigma300 SEM, under the supervision and advice of Dr Mahmoud Ardakani, Department of Materials (Royal School of Mines, Imperial College London).

3.7 Agar Disk Diffusion Assay

E. coli (Mg1655) and *P. aeruginosa* (PA01) were cultured overnight in 100% TSB, samples were re-suspended the next day to reach mid-exponential phase (0.3-0.4 OD absorbance at 600 nm, 300 µL was added to 3.5 mL of soft agar, vortexed and plated out onto an agar plate to set. Test surfaces were placed onto the agar in triplicates (N=3) and put overnight in a static incubator at 37 °C.

Results and Discussion

CHAPTER 4

Surface Oxidisation of Industrial Polymers and their Antimicrobial Performance against Early-Stage Biofilms

4.1 Introduction

Synthetic industrial polymer materials generally lack any natural defences to inhibit microbial growth, biofilm proliferation, thereby allowing bacterial surface attachment and biofilm development to take place. Oxidative pre-treatments of polymeric materials are used frequently to improve the adhesion of coatings and surface treatments to polymers. This adhesion improvement was achieved by increasing the substrate surface free energy with the addition of oxygen moieties, though alterations to the surface morphology by increasing the surface area and surface roughness also facilitates better mechanical interlocking and chemical adhesion [292,365,366]. These treatment methods are often not explored independently as controls during evaluation of antimicrobial coating technologies in many cases. For example, in the use of vitamin C (ascorbic acid) to coat onto LDPE surfaces via plasma pre-treatment [292]; or the use of gelatin containing antimicrobial coatings (sodium octanoate and Auranta FV) to LDPE using cold plasma pre-treatment [293]; or for a chitosan coating on PP textile using plasma pre-treatment [294]. As a result, the intrinsic antimicrobial surface capabilities of oxidised polymers have not been fully explored in detail.

To the best of my knowledge there are minimal reported evaluations of oxidative pre-treatments of polymers and their antibiofilm/antimicrobial performance. However, the research presented to date has suggested a distinctive relationship between microbial attachment and surface oxidisation. Katsikohianni *et al.* have found that He and He/O₂ plasma treatment on polyethylene terephthalate (PET) provided a higher surface free energy that afforded reduced bacterial attachment, but afterwards the instability in the plasma-treated surfaces led to a reduction in surface free energy which favoured bacterial adhesion and aggregation against *S. epidermis* [367]. Recent work by Erdmann *et al.* has revealed that photo-oxidisation (UV irradiation) of HDPE reduced the initial bacterial attachment of *P. aeruginosa* and *B. subtilis*. This reduction was proposed to be from the increased number of hyperoxide and hydroxyl groups that may have led to

mutual repulsion of the cells [368]. However, it is important to note that contradictory findings have also been observed. For instance, a study by Que *et al.* revealed that ozone treatment of 96-well plates made from polystyrene was found to promote biofilm formation for *Staphylococcus capitis*, a bacterium that causes bloodstream infection. Que *et al.* proposed that the increased biofilm formation to be caused by the presence of the varied oxygen containing species introduced to the surface (C-O, C=O, O-C-O, and O-C=O) [369]; both of these latter studies mentioned are recent reports published in 2020.

A selection of polymer materials has been chosen for this study based on their ability to be easily sterilised by ethanol cleaning (70-80%), as well as their prevalent use in industrial applications as summarised in **Table 2.3**. HDPE was chosen as the model substrate to be treated and extensive studies were carried out using this material. HDPE was selected because it is one of the most widely used plastics globally, it is well-known for its excellent resistance to most solvents, low cost, very low water absorption and accepted use by regulated bodies such as the FDA [370]. The full list of polymer materials that have been evaluated in this thesis are provided in **Table 4.1** below. These are industrially sourced commercial materials, and their detailed preparation, synthesis, handling, chemical composition, and purity are not provided by suppliers. However, this is not a concern, as this project focused in modifying real-world commercial materials.

Polymers	Chemical Structure	Sheet Thickness (mm)
High-density polyethylene (HDPE)	$\left[\begin{array}{cc} \text{H} & \text{H} \\ & \\ -\text{C} & -\text{C}- \\ & \\ \text{H} & \text{H} \end{array} \right]_n$ HDPE is the more crystalline variant of PE based polymers, the lack of branching affords a more tightly packed structure.	1
Polypropylene (~94% PP and 6% PE) (PP)	$\left[\begin{array}{cc} \text{H} & \text{H} \\ & \\ -\text{C} & -\text{C}- \\ & \\ \text{H} & \text{CH}_3 \end{array} \right]_n$ Polypropylene is commonly synthesised with small % of polyethylene to provide a material with improved bulk properties.	1
Polystyrene (PS)	$\left[\begin{array}{cc} \text{H} & \text{H} \\ & \\ -\text{C} & -\text{C}- \\ & \\ \text{H} & \text{C}_6\text{H}_5 \end{array} \right]_n$ Premium grade of PS (Sterilin™) used in petri dishes.	Petri dishes 1
Polyvinyl chloride (PVC)	$\left[\begin{array}{cc} \text{H} & \text{H} \\ & \\ -\text{C} & -\text{C}- \\ & \\ \text{H} & \text{Cl} \end{array} \right]_n$	2.55
Polycarbonate (PC)	$\left[\text{O}-\text{C}_6\text{H}_4-\text{C}(\text{CH}_3)_2-\text{C}_6\text{H}_4-\text{O}-\text{C}(=\text{O}) \right]_n$	0.75

Table 4.1 Polymer materials utilised for this study.

Sulfuric-chromic acid surface polymer oxidation was originally chosen for investigation as a surface pre-treatment method to improve subsequent coating adhesion to the HDPE substratum. This reaction is a most popular and highly effective chemical method for achieving polymer surface oxidation at reduced temperatures, with a high degree of surface carboxylic acid moieties being formed [286]. The only reported antimicrobial evaluation of chromic-sulfuric acid etching of a polymer was by Choi *et al.* in 1999 [371]. In their study LDPE was chemically modified with sulfuric-chromic acid solution ($\text{K}_2\text{Cr}_2\text{O}_7:\text{H}_2\text{O}:\text{H}_2\text{SO}_4$ in 1:1.7:20.1 parts, 60 min, 65 °C) and tested against *Pseudomonas*

stutzeri. Polymer samples with the biofilm (after 24 h exposure) were heat-dried and weighted to determine the adhesion differences of the biofilm. No difference in adhesion was seen for this modification using this assay compared to the controls in this study [371]. Apart from this report, to the best of our knowledge, there has been no other reported evaluations of sulfuric-chromic oxidised polymer materials and their antimicrobial attributes. Therefore, in this Chapter the bactericidal and anti-biofouling properties of sulfuric-chromic acid oxidised polymer materials is fully explored. A range of oxidation reaction times as well as a range of different bacterial species will be assessed via different biofilm assays and imaging techniques for early-stage biofilm formation. HDPE as the reference substrate will be compared with a range of other industrial polymer surfaces including PP, PS, PVC and PC. Furthermore, the application scope and limitations of surface treatments and coatings on all of these polymers will also be assessed for possible real-world uses for these modified materials.

The performance of sulfuric-chromic acid oxidation will be compared with two other chemical surface oxidation methods. Therefore, this study will allow a direct comparison between the nature of all three oxidation treatments and specifically their influence on the antimicrobial properties against early-stage biofilms. The two other oxidation methods have been specifically chosen for key reasons as summarised in **Figure 4.1**. It will turn out that a specific factor considered in these oxidation choices is to identify how low residual amounts of certain elemental products, especially those based on S and Cr, may influence the antimicrobial properties of the surface.

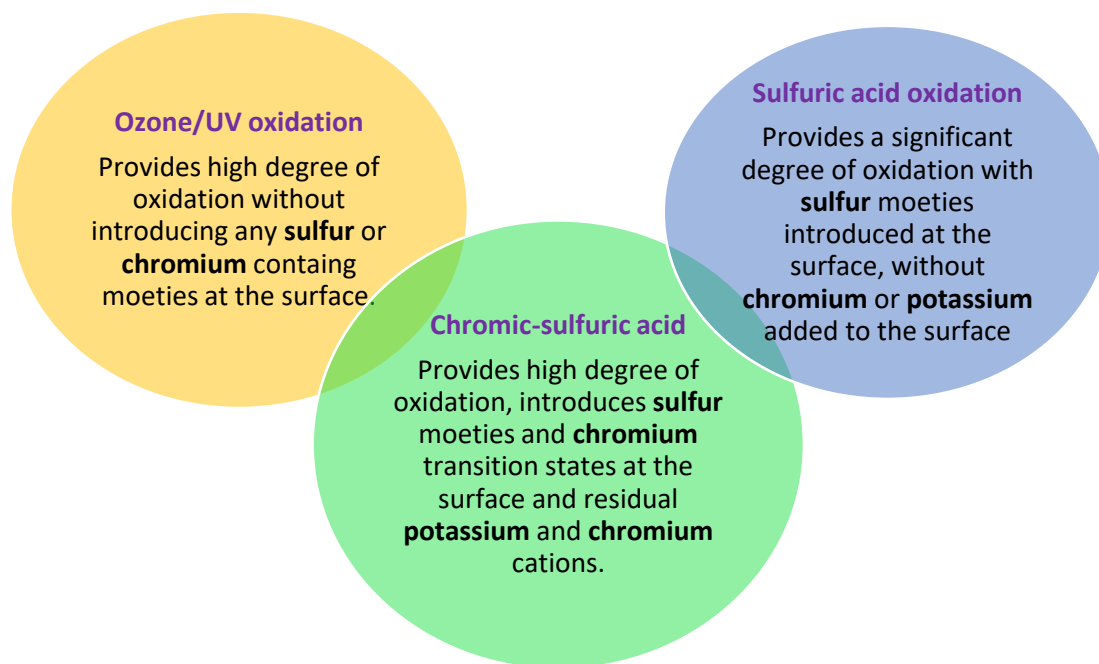


Figure 4.1 Summary of the selection reasons for the three different surface oxidation methods.

4.2 Results and Discussion

4.2.1 ATR-FTIR Spectroscopy of Sulfuric-Chromic Acid Modified HDPE

A range of surface characterisation techniques has been utilised to determine the degree of HDPE oxidation after surface modifications were performed with sulfuric-chromic acid treatment. The HDPE samples were chemically oxidised at various submersion time periods between 0-60 min. ATR-FTIR spectroscopy has been used to identify the major functional groups present and quantify the significant chemical changes that have occurred from sulfuric-chromic acid oxidation of HDPE. This characterisation technique is a common choice for surface oxidised polymers because of its efficacy and ease in identifying key oxygen containing functional groups such as carbonyl and sulfonic moieties, though it is not very surface sensitive with a typical depth of penetration of a few μm . **Figure 4.2** shows the ATR-FTIR spectra results gathered. There is a clear broad band present between $3550 - 3200 \text{ cm}^{-1}$ that is indicative of an -OH stretching band that suggests the presence of carboxylic groups and/or alcohol groups have formed, this is less significant for the 2 min oxidised surface

but increases with longer etchant submersion time periods. The appearance of new absorption bands between 1,400 and 800 cm^{-1} can be associated with sulfur bound oxygen stretching modes from a range of sulfur-containing oxidation products including $\text{O}_2=\text{S}-\text{OH}$, $\text{S}=\text{O}$, and $\text{S}-\text{O}$, as well as possible C-O stretching bands of primary alcohols. These results are similar to data obtained from literature on sulfuric-chromic acid oxidation of LDPE by Wang *et al.* [286]. The carbonyl peaks found between 1730-1680 cm^{-1} pertain to various carbonyl containing groups created from the successful oxidation of HDPE, with the formation of ketones, aldehydes, and carboxylic acids. It can be seen that the carbonyl peak intensity increases with increasing reaction time, this is in line with literature reports which have reported that a gradual increase in submersion with sulfuric-chromic acid results in a significant increase in the carbonyl FTIR peak intensity. It can be seen as a gradual sharpening of the C=O peak with increased submersion time in ATR-FTIR results and was similarly observed when LDPE was oxidised [286,372]. Blais *et al.* has produced one of the most extensive evaluations of sulfuric-chromic acid etching on the polyolefins PP, HDPE and LDPE. In their study, the HDPE did not show any apparent changes in the ATR-FTIR spectra upon oxidation. It was concluded that relative deep FTIR sampling depth of $1\text{ }\mu\text{m} = 10^4\text{ }\text{\AA}$ was too deep relative to the depth of the oxidation zone of 10^2 to $10^3\text{ }\text{\AA}$ [373,374]. This thesis successfully reports the ATR-FTIR spectra for HDPE using more sensitive equipment which does show a clear difference in the spectra from the control samples due to successful surface chemical oxidation.

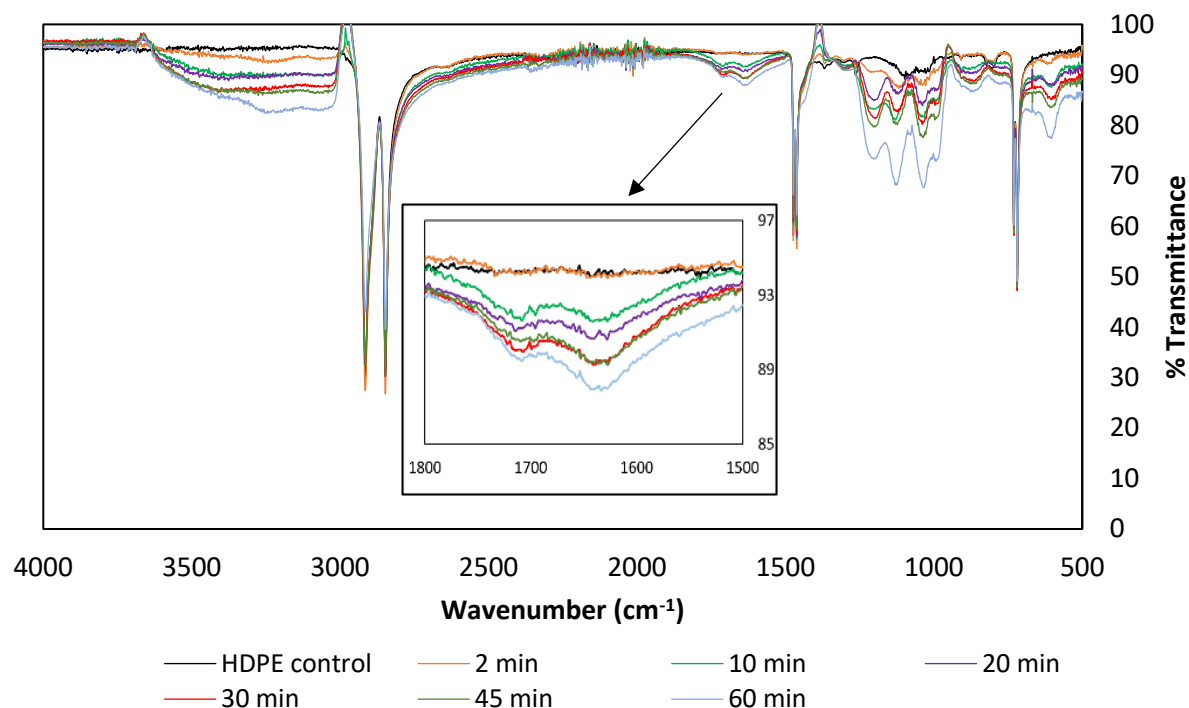


Figure 4.2 ATR-FTIR spectra of HDPE submerged in sulfuric-chromic acid for 0 to 60 min.

The carbonyl index (CI) based on FTIR data has been employed previously by many researchers to measure the degree of oxidation in polymers semi-quantitatively, and is one of the most common indicators used to evaluate the extent of polyolefin oxidation [286,375–377]. The CI considers absorption peaks of chemical groups including acids ($1712\text{--}1700\text{ cm}^{-1}$), ketones ($1723\text{--}1714\text{ cm}^{-1}$) and aldehydes/esters ($1733\text{--}1730\text{ cm}^{-1}$) that may have formed [377,378]. The CI is largely obtained from the integrated area of the carbonyl group. However, there are severe variations in how different researchers define these CI regions, which gives rise to incomparable CI values. Nevertheless, CI values have been calculated to further understand the changes made to the surface by means of sulfuric-chromic acid etching in this study using **Equation 4.1**. $A_{(1800\text{--}1500)}$ is the integrated areas of the absorption spectra of all carbonyl groups found between $1,800$ to $1,500\text{ cm}^{-1}$ and $A_{(760\text{--}660)}$ is the integrated areas of the absorption spectra of methylene groups that are expected to be found between 760 to 660 cm^{-1} . Division of $A_{(1800\text{--}1500)}$ by the integrated methylene peak areas $A_{(760\text{--}660)}$ has been performed to normalise the effect of film thickness across all samples. The ranges

used in this study were chosen based on the work by Wang and co-workers who also investigated sulfuric-chromic acid oxidation but for lower densities forms of PE; LDPE and LLDPE [286]. Furthermore, a newer method called the sulfonic index (SI) has also been developed by Wang *et al.* to also shed additional light on polyolefin oxidation which uses sulfuric acid based treatment methods [286]. This index was also calculated from the ATR-FTIR spectra based on $A_{(1250-840)}$ which is the integrated areas of the absorption spectra of sulfonic groups found between 1,250 to 840 cm^{-1} – see **Equation 4.2**. Finally, the hydroxyl index (HI) has been developed using this same methodology. Previous reports measuring the HI to analyse surface oxidation of polymers have done so without normalisation using the methylene/vinyl integrated peaks [368]. In this study however, the division by the methylene/vinyl region has been employed to keep this calculation comparable with the other two indexes (SI and CI). Thus, the HI has been calculated using $A_{(1250-840)}$ which is the integrated areas of the absorption spectra of hydroxyl containing groups (this includes acids like carboxylic acids) found between 3700 – 3100 cm^{-1} , see **Equation 4.3**.

$$\text{Carbonyl Index} = CI = \frac{A_{(1800-1500)}}{A_{(760-660)}} \quad \text{Equation 4.1}$$

$$\text{Sulfonic Index} = SI = \frac{A_{(1250-840)}}{A_{(760-660)}} \quad \text{Equation 4.2}$$

$$\text{Hydroxyl Index} = HI = \frac{A_{(3700-3100)}}{A_{(760-660)}} \quad \text{Equation 4.3}$$

The CI has not been previously reported for HDPE treated by sulfuric-chromic acid treatment, with previous reports having looked commonly at LDPE [286]. The results obtained indicated that although there was a general increase in the CI values with increasing etching reaction time, the levels fluctuate slightly but are consistent after 10 min of oxidation, with CI values of about 1.4 compared to CI of 1.1 for the control HDPE surface; see **Figure 4.3**.

The SI has increased more significantly upon oxidation reaction time, although not as significant as reported in literature when an LDPE was studied with sulfuric-chromic acid

oxidisation, which increased to 32.5 after a 60 min reaction [286]. It has already been established that sulfuric-chromic acid treatments preferably oxidises the amorphous domains for LDPE [285]. Actually this oxidant has been used as means to remove amorphous domains from semi-crystalline polymers [379]. LDPE has a lower crystallinity than HDPE, with LDPE comprising of more branched chains which restricts formation of regular crystalline structures and thus giving rise to greater extent of amorphous domains. Whereas, the higher density derivatives of PE such a HDPE have a lower degree of branching, therefore contain greater crystallinity [380]. As a result, the drastic increase in SI for LDPE shown in the literature of 32.5 can be associated with the greater number of amorphous domains present in LDPE compared to HDPE.

The HI index increases with increasing oxidation time, with the 60 min treatment time showing the highest index values. This increase can be associated with the higher number of polar functionalities added through oxidation, such as carboxylic and alcohol groups which provide increase in absorption in this region, see **Figure 4.3**. However, it is important to note that FTIR spectroscopy becomes increasingly noisier as substrate surface roughness increases. Surface roughness does generally increase and/or change with surface chemical oxidation as they can result in some etching effects, therefore giving rise to increased error levels in the index values calculated [381].

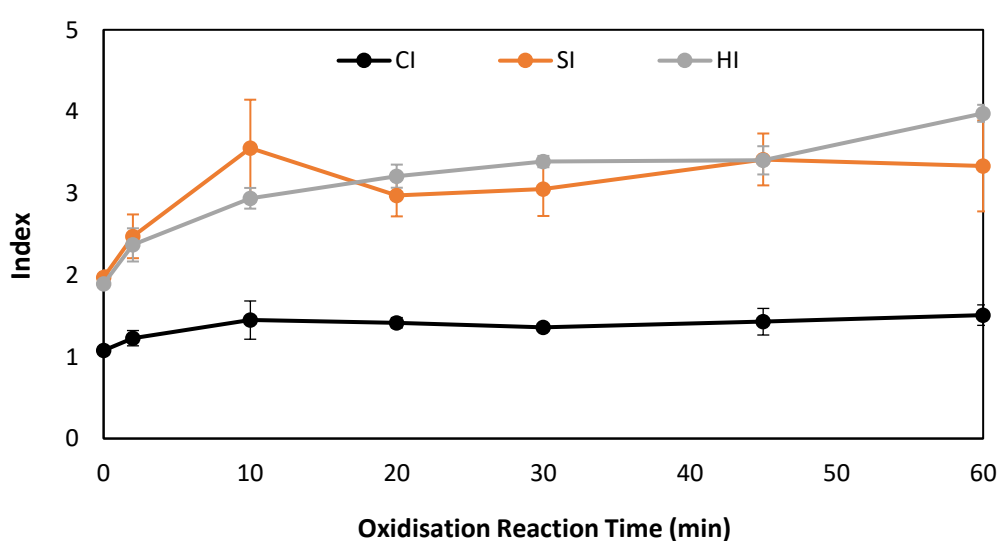


Figure 4.3 Carbonyl, hydroxyl and sulfonic indexes of sulfuric-chromic acid oxidised HDPE surfaces using ATR-FTIR integrated absorbance values, $N=4$.

4.2.2 Contact Angle Characterisation of Sulfuric-Chromic Acid Modified HDPE

Contact angle goniometry investigations on these samples has revealed a clear trend of increasing oxidation treatment time resulting in increased surface polarity as oxidation time increased from 0 to 30 min; see **Figure 4.4**. This result is not a surprise as Blais *et al.* and others have also observed the same trend when comparing water advancing contact angles for sulfuric-chromic acid oxidised PE under similar reaction conditions [285,373]. The use of increased reaction times results in an increased concentration of surface oxygen species found at the surface, specifically carbonyl and sulfonic surface species, which in turn increases the surface's chemical polarity.

When these samples were rinsed further in water (three sequential dips) and the water advancing θ was measured again, no changes in the contact angles, within experimental error, were observed (results in Appendix under Chapter 4[A]). This observation is important as the surface needs to be able to withstand short-term exposure to aqueous media for early-stage biofilm evaluation under a hydrated environment.

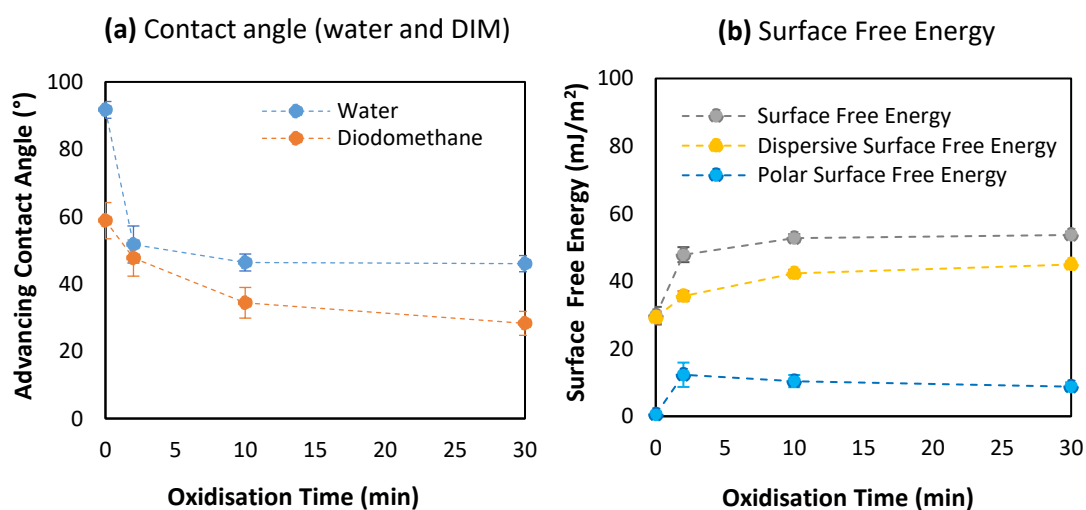


Figure 4.4 Surface wettability parameters shown, (a) water and DIM advancing angles θ and (b) surface free energy (polar, dispersive, total) of HDPE with increasing sulfuric-chromic acid oxidation time of 0, 2, 10 and 30 min (+/- SE).

4.2.3 XPS of Sulfuric-Chromic Acid Modified HDPE

The XPS percentage relative elemental abundance data shown in **Figure 4.5 (a)** confirms that a wide range of oxygen containing moieties are formed at the surface, especially after the initial 10 min of oxidation reaction time. This data also confirms the introduction of carbonyl and sulfonic surface groups due to the significant amount of atomic O and S on the surface, whilst the C percentage surface abundance has reduced. The presence of residual Cr at low levels also became more significant with increasing oxidation time, with some associated K cations also being present, see **Figure 4.5 (a)**. Analysis of the C1s core level XPS scan is often performed for oxidised polymers to determine the extent of bonding of carbon bound to heteroatoms, as this produces tailing and asymmetry in the C1s peak. The results obtained for C1s in this study highlights the oxidation of carbon with the tailing of the C-C peak 1s peak to higher binding energies, due to being covalently bound to an O atom(s). The peak that has formed at 289 eV is for -COOH groups; this peak gradually increases in concentration as the reaction time increases from 2 to 30 min see **Figure 4.5(b)**; with details on the C1s spectra for 20, 45, and 60 min reaction times can be found in the Appendix Chapter 4[B]. Increases in the peak intensities at 287 eV and 286 eV, which represents carbonyl (C=O) and alcohol (C-OH) moieties respectively, also follow with increased reaction times and comparable to those reported in the literature [282,374,382]. However, some reports suggests the presence of alcohol groups to be absent in these reactions [284].

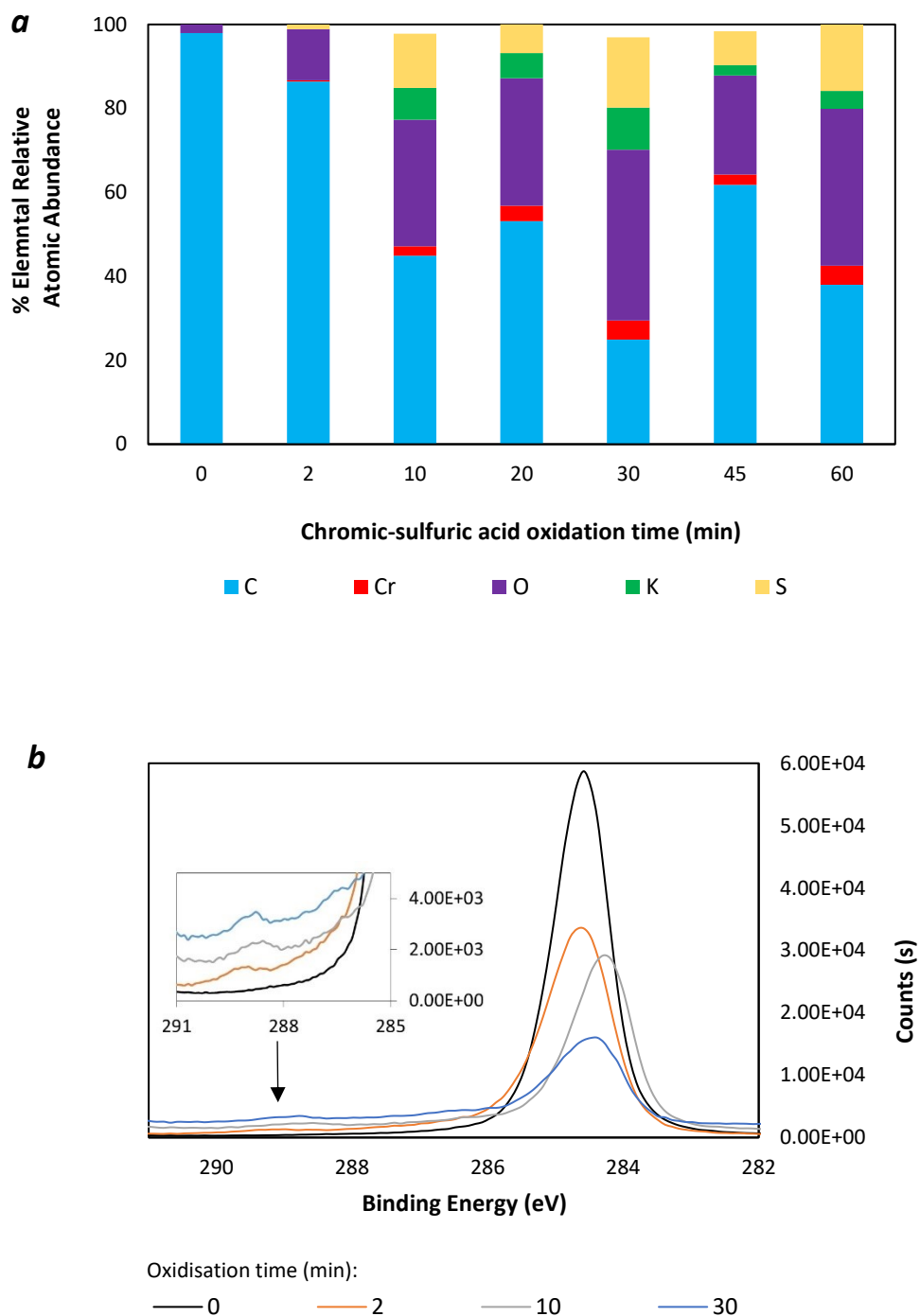


Figure 4.5 XPS results of HDPE surface oxidised with sulfuric-chromic acid treatment; (a) % elemental abundance of core species has been provided; (b) carbon 1s core level XPS scan.

The surface oxidation of PE using sulfuric-chromic acid is thought to proceed via a tetravalent Cr (IV) ester intermediate which hydrolyses to form an alcohol group [286,373]. The alcohol group can then rapidly form chain scission products such as aldehydes, alcohols and ketones, which can ultimately be further oxidised to carboxylic

acids, see **Figure 4.6**. The low level of Cr present in the XPS spectra, see **Figure 4.5 (a)**, is expected as it is said to be acting as a form of catalyst, with the carboxylic concentration increasing as Cr is lost from the surface, as stated by Briggs *et al.* [374].

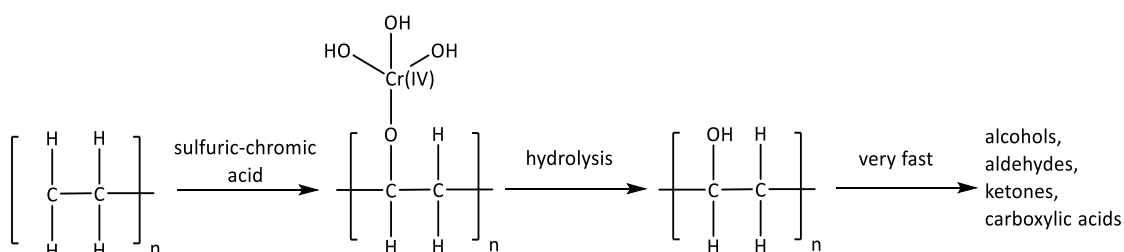


Figure 4.6 Proposed reaction pathway for sulfuric-chromic acid oxidation of PE [286,373].

4.2.4 Time-of-flight secondary ion mass spectrometry of Sulfuric-Chromic Acid Modified HDPE

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) was used to further understand the key functional groups present on the oxidised HDPE surface. Samples were run and analysed by Dr Sarah Fearn, instrument owner, Department of Materials, Imperial College London. ToF-SIMS is a highly sensitive surface analysis technique and can detect species at significantly lower levels than other traditional surface characterisation techniques, like XPS. This work was performed for untreated HDPE as a control and for HDPE that has been 30 min oxidised with sulfuric-chromic acid treatment. These results are shown in **Figure 4.7**. The data revealed for example that the “as received” HDPE contains a low residual surface contamination with polydimethylsiloxane (PDMS). Below in **Figure 4.7** the most abundance ToF-SIMS peaks present caused by sulfuric-chromic acid surface oxidation are shown.

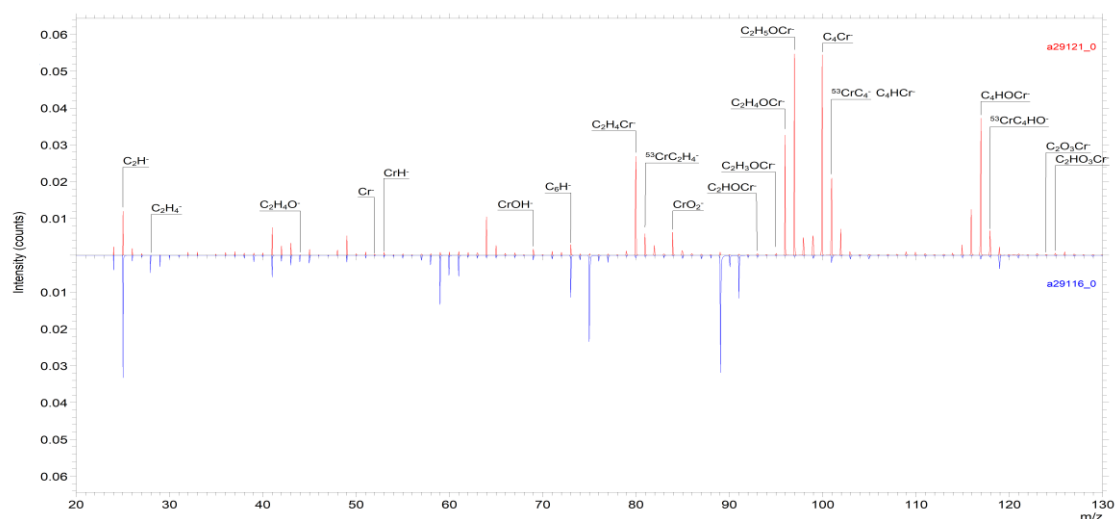


Figure 4.7 ToF-SIMS results showing comparison of most common peaks found by total ion intensity against m/z ratio for negative ions. The above (red) peaks are from functionalised 30 min sulfuric-chromic oxidised HDPE, and the bottom blue peaks are for the HDPE control (untreated sample).

The ToF-SIMS negative ion spectrum of the 30 min chromic acid oxidised HDPE provided revealed that there were significant organo-chromium species at m/z ratios of 95.9 and 96.9 corresponding to $C_2H_4OCr^-$ and $C_2H_5OCr^-$. Indeed the species $C_2H_5OCr^-$ is the most abundant negative ion fragment detected and is fully consistent with the chemical structure of the reactive intermediate proposed by Blais *et al.* [286,373] and shown in **Figure 4.6**. The control ToF-SIMS experiments conducted with untreated HDPE detected no organo-chromium species as expected. The presence of organo-chromium species, or rather more specifically chromate esters, has been suggested previously but not explicitly identified using surface characterisation instruments, due to the low levels of these species on the surface. Therefore this study is the first reported data to explicitly demonstrate the presence of these species and confirm the mechanism of this reaction [382].

4.2.5 Surface Roughness of Sulfuric-Chromic Acid Modified HDPE

In these studies, surface roughness has been monitored as it is known to change upon oxidation treatment significantly. This change may in turn influence early-stage biofilm activity and surface antimicrobial properties. Therefore, it is important to monitor surface roughness to ensure its influence and contribution in future experiments. Surface roughness data has been provided below along with example profilometry images, see **Figure 4.8**. Overall, the reported results are very low values for the average roughness (R_a) of between 1 and 2 μm , and root mean squared average roughness (R_q or RMS) during the 60 min of reaction time examined. Some variations are noted, but again the overall level of roughness is low. When Schönherr *et al.*, examined the R_a of LDPE between 0-15 min and at 60 min time points, a more gradual and progressive increase in R_a was observed, although measurements were not taken between 15 and 60 min [285]. Overall, it can be concluded that some changes in surface roughness of HDPE were observed, but overall, these surfaces are relatively smooth, with oxidation causing some small increases in R_a and RMS.

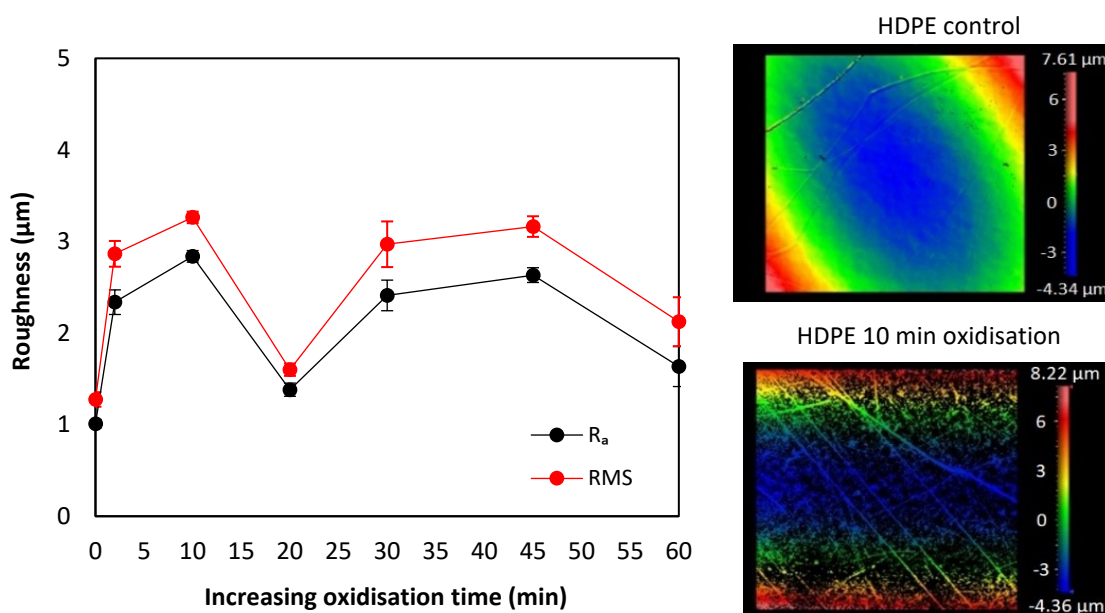


Figure 4.8 Surface roughness parameters (R_a and RMS) obtained from non-contact white light profilometry of HDPE etched in sulfuric-chromic acid solution at 70 °C with increasing oxidation time periods from 2 to 60 min. Two example 2D images have also been included.

4.2.5 Assessment of Early-stage Biofilm Attachment on Sulfuric-Chromic Acid Modified HDPE using CLSM

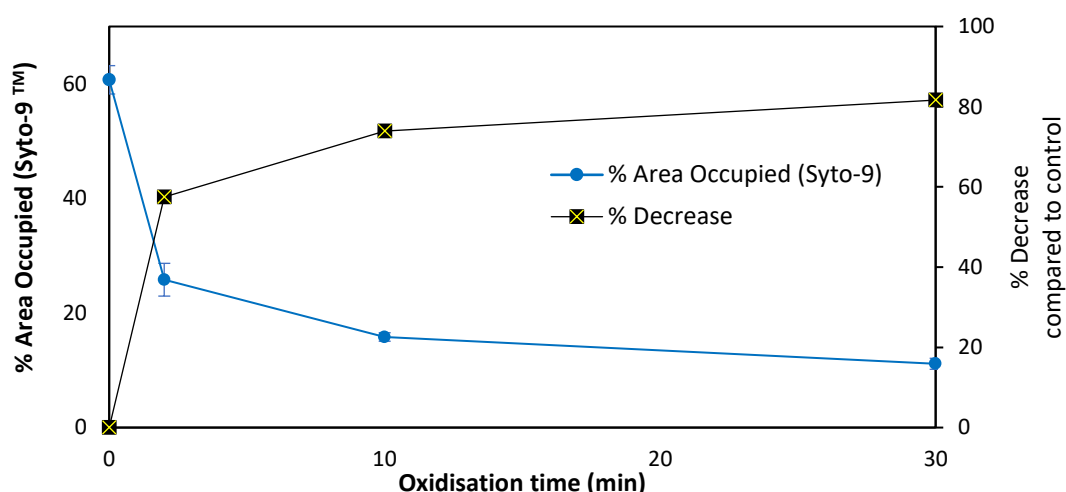
Having fully characterised the topographical, chemical and physical properties resulting from the sulfuric-chromic acid etching for HDPE, these samples were assessed against *P. aureuginosa*, our designated model organism, using CLSM to determine the early-attachment behaviour of the early-stage biofilm. Initial microbial attachment is a process that occurs within a few seconds to a few minutes, with the transition from reversible to irreversible attachment being dynamic and difficult to determine over time as there are many dependent variables involved [368]. The early-stage biofilm has been represented here based upon a 30 min bacterial exposure to the challenge surfaces, followed by sequential rinsing to remove the planktonic cells, as early-stage irreversible attachment of bacteria is often considered to represent the onset of biofilm formation and attachment processes [64,383]. At this early phase of bacterial attachment and initial biofilm development, research has shown that even at short-time periods, single and dispersed bacterial cells can bind to surfaces and this adherent behaviour is heavily governed by their ability to produce surface proteins that aid in surface attachment, which will later develop into mature EPS films [383]. It is well accepted that the EPS composition and quantity varies for different organisms, environmental conditions and biofilm age, [384] although at this very early stage of 30 min static droplet exposure, it is anticipated that EPS levels to be minimal, and potentially none. Therefore, the composition of EPS did not warrant detailed investigation in this study. Indeed, COVID-19 restrictions at the time has also prevented further experiments of this type.

Nucleic based dyes were used to assess the presence, and thus the attachment exhibited by *P. aureuginosa*, using Syto™9 with the adoption of an assay originally developed by Dr Palmer during his PhD [363]. CLSM is a useful method due to its ability to directly measure semi-quantitatively the amount and the physical state of the bacterial cells on the surface without the disruptive need to remove the cells from the surface for evaluation and quantification. A Comstat 2.1 plug-in with Image J was used to analyse the early-stage biofilm confocal images. The explicit function used was 'percentage area occupied in layers' from Comstat 2.1 which allows the number of set

pixels found using the known pixel size and the covered area as a percentage of the total slice size. In other words, it describes the area of the surface occupied by the *P. aureuginosa* cells in the captured image [355]. Detailed information on the CLSM setting and the experimental protocol can be found in Chapter 3 (Section 3.4).

These results reveal that surface sulfuric-chromic acid oxidation significantly decreases the number of *P. aureuginosa* cells that are surface bound, see **Figure 4.9**. In fact, increasing the surface oxidation time results in an exponential decrease in the surface coverage of *P. aureuginosa* observed, with an 82% decrease in the percentage surface area occupied by *P. aureuginosa* for the 30 min sulfuric-chromic acid oxidised HDPE surface compared to the untreated HDPE sample. **Figure 4.9** also provides example confocal images which visually highlights the significant effect on *P. aureuginosa* attachment from this treatment approach. Currently only one other report which was published recently in 2020 by Erdmann *et al.* reveals that oxidised HDPE to give reduced bacterial attachment at very early-stages of biofilm development. Their study looked at HDPE exposed to fuel (diesel), as HDPE is commonly used for diesel storage tanks, and some microorganisms are able to use diesel as an energy source and grow under these conditions in these tanks which is undesirable. In their study the extent of adhesion of *P. aeruginosa*, after 10 min exposure on HDPE that has been UV auto-oxidised, decreased upon increasing UV exposure, and thus increasing surface oxidation [368]. A similar result was reported by this same group when *B. Subtilis* (Gram-positive) was also investigated and compared to the untreated surface. This behaviour was directly attributed to the presence of oxidation products on the surface such as carbonyl and hydroxyl moieties. Furthermore, these observations were made across two different types of HDPE materials by Edmann and his team which suggests that this surface behaviour is irrespective of the specific HDPE sample used [368]. It is important to note that other studies have also shown the presence of terminal active oxidised groups, such as terminal exposed -OH groups, resulting in reduced protein and cellular adhesion [385]. Some researchers have reported and described this behaviour to be due to the 'Water Barrier' theory which was introduced in Chapter 2, particularly for PEG-based hydrophilic polymers [237,238]. Another possible explanation for reduced microbial attachment exhibited could be the detachment of bacterial cells due

to intrinsic cellular changes, such as the expression and integrity of surface appendages or membrane integrity induced from contact with the exposed oxidised surface containing hydroxyl and carboxylic groups. These chemical effects might result in subsequent detachment of sessile cells from the surface. These possible explanations will be explored further in this Chapter.



Oxidisation time (min):

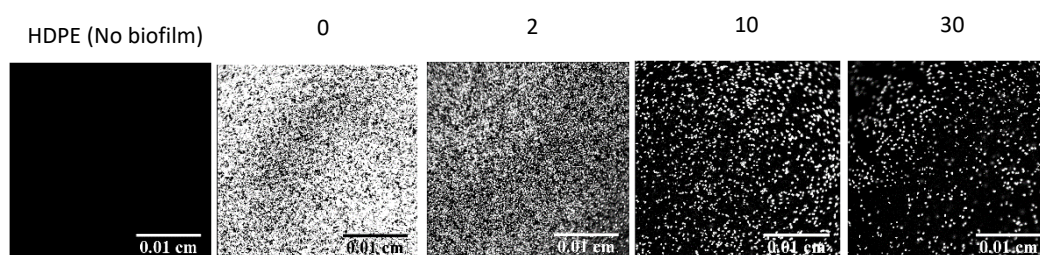


Figure 4.9 % Area occupied by *P. aureuginosa* early-stage biofilm on HDPE surfaces versus oxidation time as determined using CLSM ($N \geq 14$, $\pm$ SE), where 3 samples were examined with a minimum of four images taken of each sample. White spots are fluorescently labelled *P. aureuginosa* species, images also include a positive control without *P. aureuginosa* exposure but in the presence of the fluorescent Syto™9.

4.2.6 Assessment of Early-stage Biofilm Viability on Sulfuric-Chromic Acid Modified HDPE using CLSM

The viability of the early-stage biofilm formed on the various HDPE sulfuric-chromic acid oxidised surfaces with increasing oxidation times, was examined using four different approaches.

- CLSM imaging using fluorescence probes (Syto™9 and PI)
- Traditional agar culture techniques via viable plate colony enumeration of recovered cells after biofilm scraping
- FC using fluorescent probe, DiBAC₄(3) after biofilm scraping
- Scanning Electron Microscopy
- Agar disk diffusion assay

A LIVE/DEAD® BacLight™ Bacterial Viability Kit was utilised where the ratio of Syto™9/PI fluorescence intensities is used as an indicator of cellular viability compared to a control HDPE surface that has not been oxidised. Both dyes in the kit are intercalating nucleic acid probes resulting in an enhanced signal when cell-bound, however they differ in their permeability properties. The Syto™9 fluorophore stains all cells yet PI stains only membrane damaged and ruptured cells, with both dyes having a competitive binding for the same active sites. Calibration curves can be found in the Appendix Chapter 3[E] to show the activity of the dyes in our system.

These results suggest that although 2 min of reaction time may increase the number of viable cells initially, 10 and 30 min sulfuric-chromic acid treatment has significantly decreased cellular viability, particularly the 30 min treated surface which showed an 86% decrease when comparing the percentage area occupied in layers from the ratio of Syto™9/PI to an untreated HDPE surface, see **Figure 4.10**. When the data for cellular viability shown here in **Figure 4.10** is considered with the cell surface adhesion data shown in **Figure 4.9**, then both figures confirm a significant decrease in total attached cells with increasing etching reaction time relative to the untreated control HDPE surface. The 2 min HDPE oxidised surface shows an increase in the ratio of viable cells from fluorescent staining compared to the untreated surface, but overall adhesion amounts are still reduced. The viability ratio result for the 2 min oxidised sample, is

possibly due to the reduced number of viable cells seen after etching for 2 min, which causes reduction in the intensity of the signal in the ratio for the numerator. This change is because although there are less cells present for the 2 min sample, the percentage viable are higher.

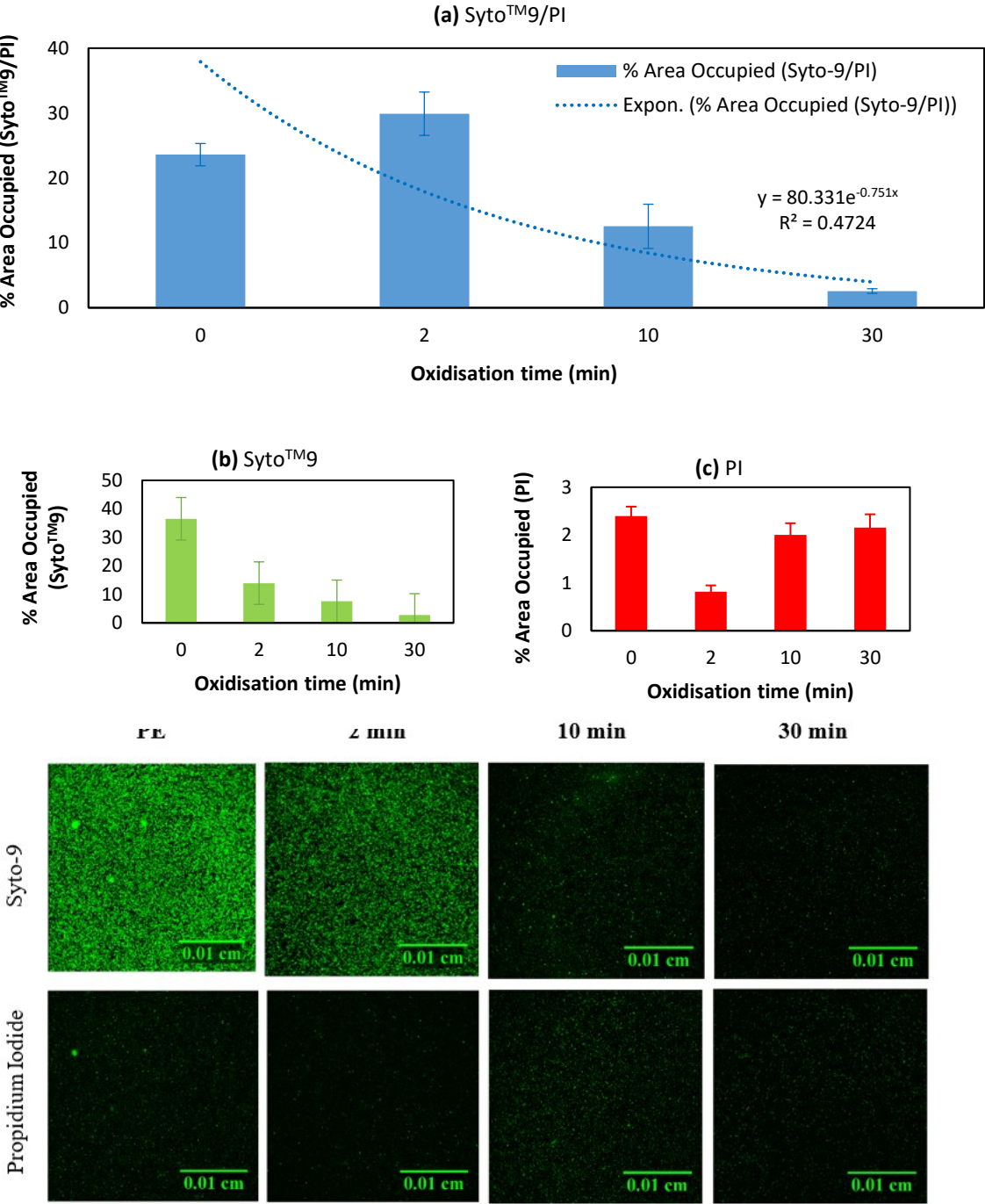


Figure 4.10 CLSM viability results of *P. aeruginosa* after sulfuric-chromic acid oxidisation of HDPE with treatment times of 2, 10 and 30 min, where (a) ratio of SytoTM9/PI, (b) SytoTM9 and (c) PI.

Example images of SytoTM9 stained cells have been provided in the upper row and the corresponding PI channel in the lower row (all cells stained are shown in green in the images), at 1024 x 1024 pixel resolution.

To investigate the influence of the surface on *P. aeruginosa* early-stage biofilm viability further, and to determine whether the biofilm is not only membrane damaged (PI stained) but also if it is culturable, the early-stage biofilm was re-prepared on new samples and examined for both membrane depolarisation using DiBAC₄(3) (bis-(1,3-dibutylbarbituric Acid) trimethine Oxonol), and for viability by plate cell counting after biofilm dispersal via scraping. DiBAC₄(3) is a membrane potential oxonol lipophilic fluorescent dye molecule. This dye accumulates in the intracellular space of a cell which has interior-positive membrane potential. Healthy bacteria maintain an electrical potential gradient with a negative interior membrane potential, and this gradient is vital for their function including ATP generation and substance transport, therefore such dyes have been used as a measure of membrane integrity and energy status, corresponding to a loss in viability [386]. DiBAC₄(3) binds to the intracellular lipid-containing elements and results in an increased fluorescence signal detected by FC [346].

There is a clear change in membrane depolarisation in the *P. aeruginosa* biofilm as DiBAC₄(3) staining is apparent from the FC histogram plots for the sulfuric-chromic acid oxidised HDPE samples, when compared to HDPE untreated surfaces where little to no cells were stained with DiBAC₄(3), see **Figure 4.11**. When *P. aeruginosa* were recovered by scraping using a cell-scraper from the prepared early-stage biofilm and cultured, the 10 and 30 min oxidised surfaces showed no colony growth after overnight culturing. This observation could be caused by a combination of both reduced microbial attachment and reduced viability which together achieve the significant decrease of 6-log reductions, with no growth observed in this experiment; see **Table 4.2**. A 6-log reduction was the maximum sensitivity range of our experimental protocol. Furthermore, FC indicated a reduction of membrane potential of *P. aeruginosa* recovered from scraping the early-stage biofilm, compared to the control surface, which suggests a loss in viability of the attached cells. Additionally, agar disk diffusion

experiments were performed. These samples did not form a ring of inhibition, which was expected as this antimicrobial treatment approach does not leach away substantial antimicrobial active agents from the polymer surface to cause a ring to form (see Appendix for full agar disk diffusion photos in Chapter 4[C]).

Finally, this study was also repeated for *P. aeruginosa* on treated HDPE samples that were aged (left untouched in a sealed vessel) for 1 month and 3 months at room temperature and humidity to determine the longevity of the antimicrobial surface properties under standard storage conditions. The environmental aging study data reported in **Table 4.2** showed that after 1 and 3 months of aging, both the 30- and 60-min treatments still showed a significant reduction of bacteria viability compared to the untreated surface. However, the 10 min treatment has lost nearly all of its efficacy and properties after 1 month of aging. This data infers that the 10 min sulfuric chromic acid treatment is not viable as a long-term antimicrobial treatment approach for real-world applications where long-term storage conditions are required.

	Fresh	1 month	3 months
HDPE sulfuric-chromic acid Treatment time (min)	<i>P. aeruginosa</i> CFU/mL (Log ₁₀)	<i>P. aeruginosa</i> CFU/mL (Log ₁₀)	<i>P. aeruginosa</i> CFU/mL (Log ₁₀)
0	6.7 +/- 0.007	5.9 +/- 0.4	6.6 +/- 0.1
2	6.0 +/- 0.07	6.3 +/- 0.1	6.5 +/- 0.2
10	No growth	5.3 +/- 0.8	4.8 +/- 0.5
30	No growth	1.4 +/- 1.4	No growth
60	NA	No growth	No growth

Table 4.2 Viable plate colony enumeration for oxidised HDPE surfaces obtained by culturing recovered cells after scraping the early-stage biofilm in PBS, and incubation of 15 h overnight growth for *P. aeruginosa* exposed to samples that are fresh, aged for 1 month and 3 months (NA=not available/not performed, +/- SD). T-test was not conducted as full data set was no longer available.

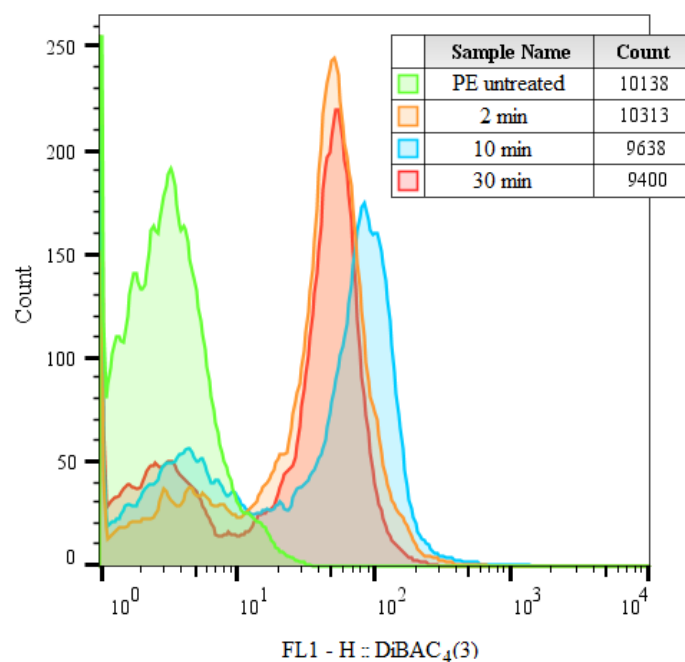


Figure 4.11 FC results showing a DiBAC₄(3) histogram of the early-stage biofilm recovered from scraping, the number of events has been given as counts in the gated area, which was set as 10,000. Calibration of the dye using FC and gating thresholds for this experiment has been summarised in the Appendix Section Chapter 4[D].

SEM was performed on two reference samples for further examination of the surface. Untreated HDPE surface and the 30 min sulfuric-chromic acid oxidised HDPE were imaged, see **Figure 4.12**. The images demonstrated both the strong oxidising and surface etching capabilities of this treatment created some surface topography changes in HDPE, but also a significant difference in the number and morphology of the *P. aeruginosa* cells attached, which can be seen clearly in the images (C) and (D) in **Figure 4.12**. It is important to note that SEM requires fixing of the cells as well as sequential ethanol dehydration and drying to be suitable for imaging under SEM. This extensive manipulation may have caused changes to the final image obtained as early-stage biofilm are more susceptible to experiencing reversible detachment without prominent EPS development. These SEM images also confirm that the EPS has not developed after 30 min static surface inoculation using a droplet (200 μ L) of 0.2 OD (600 nm) culture of *P. aeruginosa* in TSB.

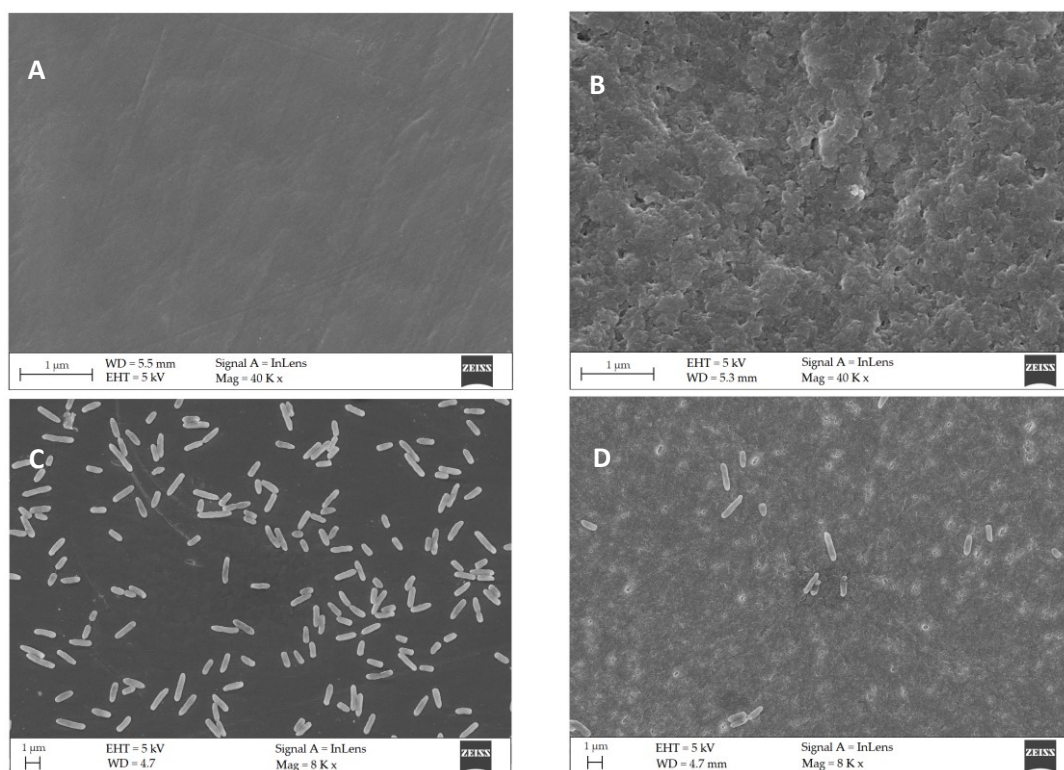


Figure 4.12 SEM images of (A) HDPE untreated, (B) HDPE 30 min sulfuric-chromic acid oxidised, (C) HDPE untreated exposed to *P. aeruginosa* early-stage biofilm, and (D) HDPE 30 min sulfuric-chromic acid oxidised exposed to *P. aeruginosa* early-stage biofilm.

The microbial study was also extended to *E. coli* and *S. aureus* to determine the antimicrobial efficacy of these HDPE surfaces against other model organisms which are known to readily form biofilms, and to see also how it would perform for a Gram-positive bacterium in the case of *S. aureus*. **Table 4.3** provides the viable plate colony counting results for *E. coli* and *S. aureus* early-stage biofilms tested. *E. coli*, like that of *P. aeruginosa*, showed no growth following 30 min oxidation, however the same was not observed for *S. aureus*. Therefore, 60 and 120 min HDPE oxidised surfaces were also prepared and also tested against *S. aureus* to see if longer oxidation treatment of HDPE would have any influence on the Gram-positive organism. But the growth and viability remained unchanged. This observation suggests that it is likely that the presence of the thicker cell walls of a Gram-positive species like *S. aureus* (20-80 nm), which is made up of mainly thick layers of peptidoglycan below the cell membrane, provides an added protection from environment and chemical stresses such as those

associated with surface-active oxidised materials [387,388]. The cell wall of bacteria are known to determine the cell shape, serve as a scaffold for anchoring other cell envelope components such as proteins, and provides resistance from the intracellular turgor of the cell [389–391]. In contrast, Gram-negative bacteria have a thin cell wall (<10 nm) [388]. These results have been seen in other reports. For example Prochnow *et al.* revealed that differences in bacterial outer cell walls and its effectiveness in self-protection has allowed *Bacillus subtilis* (Gram-positive bacteria with 55.4 nm cell wall) to resist significant viability losses from exposure to cold atmospheric-pressure plasma treated surfaces, whereas *P. aeruginosa* (2.4 nm cell wall) was almost completely eradicated with the same surface treatment [388]. Furthermore, it suggests that the activity of sulfuric-chromic acid oxidised HDPE materials are heavily reliant on their ability to interact and influence the bacterial cell wall to impart their antimicrobial surface impact on the early-stage biofilm.

HDPE sulfuric-chromic acid treatment time (min)	<i>E. coli</i> CFU/mL (Log ₁₀)	<i>S. aureus</i> CFU/mL (Log ₁₀)
0	6.4 +/- 0.10	6.3 +/- 0.14
2	6.3 +/- 0.20	6.4 +/- 0.01
10	6.3 +/- 0.14	6.2 +/- 0.08
30	No growth	6.4 +/- 0.19
60	NA	6.5 +/- 0.12
120	NA	6.6 +/- 0.05

Table 4.3 Viable plate colony counting for oxidised HDPE surfaces obtained by culturing recovered cells of *E. coli* and *S. aureus* after 15 h overnight growth for *E. coli* and *S. aureus* (NA=Not available/not performed, +/- SD).

The CLSM viability study was also performed for *S. aureus* and *E. coli*. In both cases it was found that the ratio of Syto™9/PI decreases as the etching time increases. For the Gram-positive bacteria though the change is less drastic than that observed for *P. aeruginosa* compared to *S. aureus*, see the example images provided in **Figure 4.13**. The reduction in this ratio is largely attributed to the total loss of cells adhered, which is

visibly apparent in the example images provided and obtained from the CLSM experiments. (The initial attachment assay using Syto-9™ solely were not conducted for these two organisms for comparison due to COVID-19 access limitations). These results are mirrored in the results obtained from *E. coli* studies in **Figure 4.14**. For *E. coli*, the values for Syto™9 and PI have been provided as an average for each etching time (0, 2, 10 and 30 min), the results shows that the Syto™9 percentage area occupied to decrease as etching time increases, with limited PI intensity changes. This data suggests that the antiadhesive property of the surface still impacts on both *E. coli* and *S. aureus*, even though *S. aureus* did not show a change in colony growth from studies reported in **Table 4.3**. The reason why the loss in viability from colony counting and culture growth experiments were not seen in *S. aureus* may be because there are still a significant amount of cells present on the 30 min sulfuric-chromic acid HDPE, which overtime, when cultured can grow and thrive, to reach values similar to that of an HDPE untreated sample, when left overnight. Nevertheless, the clear reduction in attached cells from this treatment method is visible visually in the example images provided in **Figure 4.13** and **Figure 4.14**. Although this reduction in attached cells of *S. aureus* may not be significant when the cells are recovered and cultured for colony enumeration assay on agar, this antiadhesive property may prove useful to help perturb biofilm formation if untouched. Reduced biofilm attachment has shown in itself to be influential in reducing biofilm formation in many instances and is a key approach by many technologies in combating biofilm proliferation [392,393].

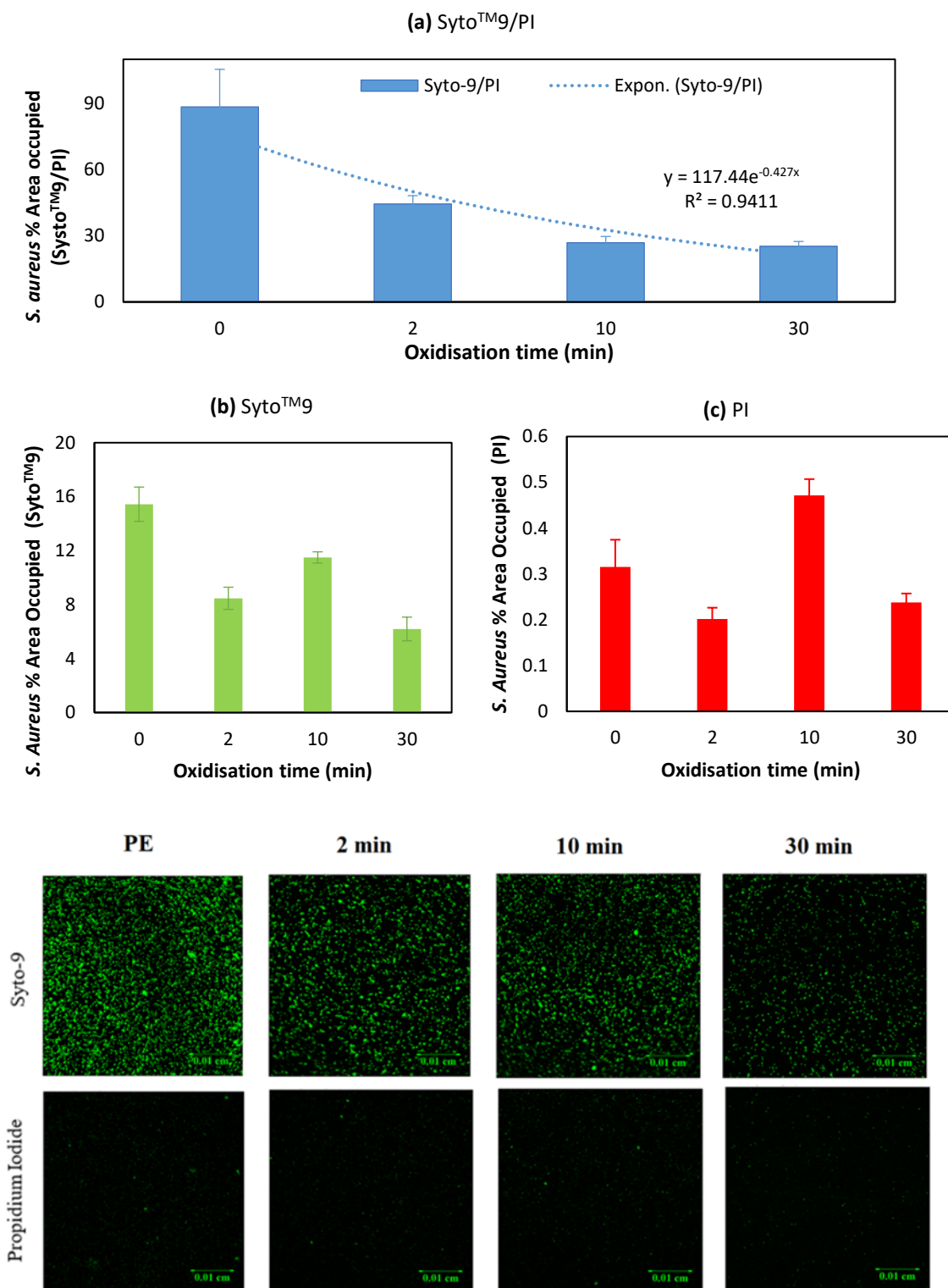


Figure 4.13 CLSM viability images of *S. aureus* upon exposure to sulfuric-chromic acid oxidation of HDPE with treatment times of 2, 10 and 30 min; where (a) the ratio of SytoTM9/PI, (b) SytoTM9 and (c) PI. SytoTM9 stained example images of cells have been provided in the upper row and the corresponding PI channel in the lower row (all cells are to be shown in green in the images), at 1024 x 1024 pixel resolution.

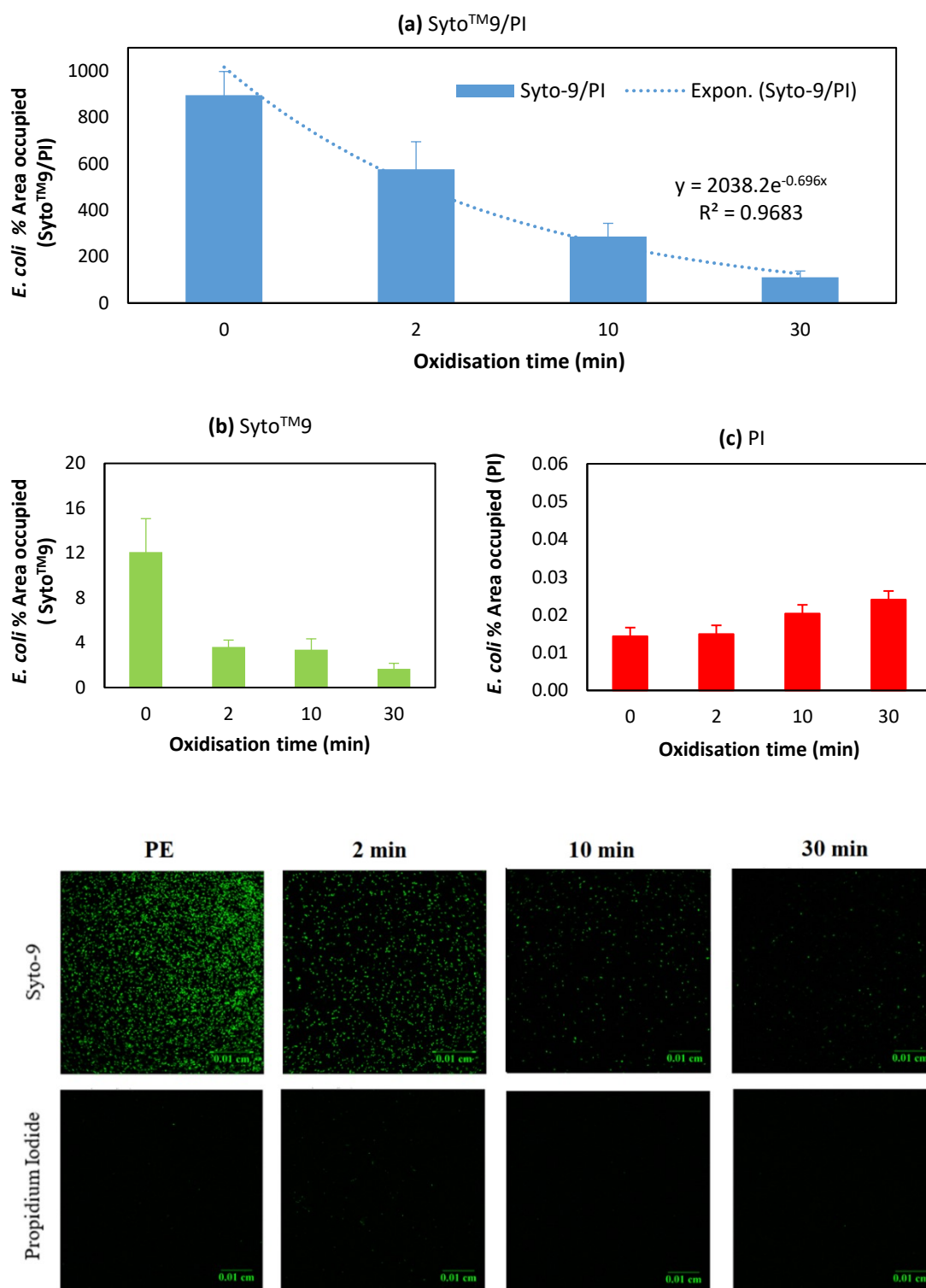


Figure 4.14 CLSM viability study of *E. coli* changes upon sulfuric-chromic acid oxidation of HDPE with increasing treatment times of 2, 10 and 30 min, (a) ratio of SytoTM/PI, (b) SytoTM9, and (c) PI channels. SytoTM9 stained example images of cells have been provided in the upper row and the corresponding PI channel in the lower row (all cells are to be shown in green in the images), at 1024 x 1024 pixel resolution.

These viability assays have demonstrated the substantial short-term killing capabilities of oxidised HDPE. There is a clear mode of action pertaining to loss of membrane integrity which was reflected in both FC and confocal microscopy imaging using two separate and independent fluorescent dye studies. The surface influence on reducing *P. aeruginosa* and *E. coli* viability may be attributed to the active surface chemistry of the oxidised HDPE. This chemistry could be from the presence of acid or alcohol exposed groups, surface chromium species, surface potassium residues, or sulfur oxidised functionalities present at the surface. It has already been shown that COOH groups are highly antibacterial. Gizdavic-Nikolaidis *et al.* have patented emeraldine salt forms of copolymers due to their antibacterial efficacy as it was discovered that the acidic groups on the molecular chains were essential for bacterial cell death via cell membrane breakage [394]. Chromium has also been extensively examined for its biofilm reducing abilities and has shown to be successful in many different forms, including CrO₃ nanotechnology, Cr(III) and Cr(VI) complexes [395–397]. The primary mode of the antibiofouling performance of these oxidised HDPE surfaces may be attributed to inducing loss of bacterial cell membrane integrity and potential as these are known variables that have previously shown to effect bacterial initial cell adhesion significantly against *P. aeruginosa*, such as effecting EPS production and cell to cell signalling [398]. Further investigation of these possible reasons will be investigated later in this Chapter.

4.2.7 Evaluation of Alternate Oxidative treatments against Sulfuric-Chromic Acid Oxidisation on HDPE

To understand how the surface modifications that have caused the large antiadhesive properties and the accompanying antimicrobial result, it was decided to utilise two other surface oxidisation approaches for comparison to see if oxidisation alone will suffice in producing an antimicrobial HDPE surface, or are the surface antimicrobial results caused by specific features of the sulfuric-chromic acid treatment alone. **Figure 4.15** outlines the two alternative oxidisation methods chosen in more detail, comprising of UVO oxidisation and sulfuric acid heat treatment.

The oxidation treatment times for these two new methods have been decided using ATR-FTIR spectroscopy and further validated with XPS analysis data. This validation is because it was important to create a surface with specific chemical properties to compare with the sulfuric-chromic treatment. These new oxidation treatments were chosen to be deficient in sulfur moieties, carboxylic moieties or chromic containing residues; our three most likely antimicrobial chemical surface attributes. Below the ATR-FTIR spectra of HDPE oxidised with the new treatment periods of sulfuric acid and UVO are shown. Time-exposure progression of sulfuric acid and UVO oxidation using ATR-FTIR can be found in the Appendix Chapter 4[E].

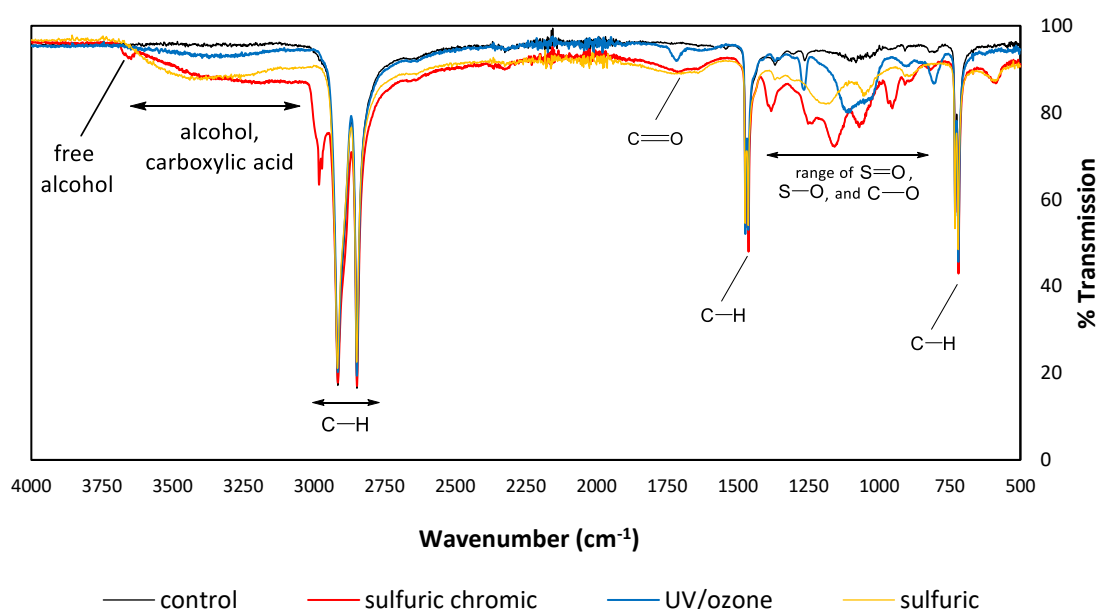


Figure 4.15 ATR-FTIR of HDPE oxidised with sulfuric-chromic acid for 30 min, and sulfuric acid for 15 min and UVO for 20 min; the spectra has been annotated with key functional groups that have formed following these oxidation treatments.

The ATR-FTIR spectra of the three oxidised materials (**Figure 4.15**) show significant levels of oxidised species from the different treatments, with sulfuric-chromic acid being the most oxidised. UVO does not show a significant broad band in the region of 3500-3700 cm^{-1} compared to the other two approaches, however this deficiency can be explained in terms of practicality. Sulfuric and sulfuric-chromic acid treatments are aqueous approaches which can be very difficult to fully dry and remove residual water

contaminants as the surface has become significantly hydrophilic, even with extensive vacuum drying in the presence of a desiccant (silica) and drying in the oven (65 °C, 4 h minimum). When XPS was performed on these surfaces, it was found that the C1s core level scans show clearly that oxidation by UVO and chromic-sulfuric acid introduces significant surface concentrations of carboxylic acid moieties, whereas the sulfuric acid treatment alone largely introduces ketones and alcohols groups, see **Figure 4.16 (a-d)** [399]. Sulfuric acid hot treatment of HDPE (15 min) did not result in significant enough oxidation to produce substantial carboxylic acid species, as the expected carboxylic peaks at 288.8-289.0 eV [399] were not prominent in the C1s spectrum scan, see **Figure 4.16 (c)**. Therefore sulfuric-acid treatment (15 min) provides a surface with high sulfonic moiety concentrations but lacks carboxylic surface groups. This treatment is beneficial in order to compare the antimicrobial properties of sulfonic groups independently. Furthermore, this approach does not introduce chromium and potassium at all to the surface, which is advantageous to also assess the influence of the deficiency of these species in the antimicrobial performance of these materials, in comparison to other materials in this study. It was found that both the sulfuric acid hot treatment and the sulfuric-chromic acid etching methods introduced 7.7-10.0% sulfur to the surface of HDPE, see **Figure 4.5 (a)**. On the other hand, UVO treatment introduced significant amounts of carboxylic moieties to the surface, see **Figure 4.16 (b)**, and does not introduce sulfonic surface groups, therefore allowing the evaluation of the antimicrobial performance of carboxylic groups independently. The ability for UVO treatment to provide significant carboxylic groups to the surface has been seen and supported in other literature studies using this oxidation approach on PE [400].

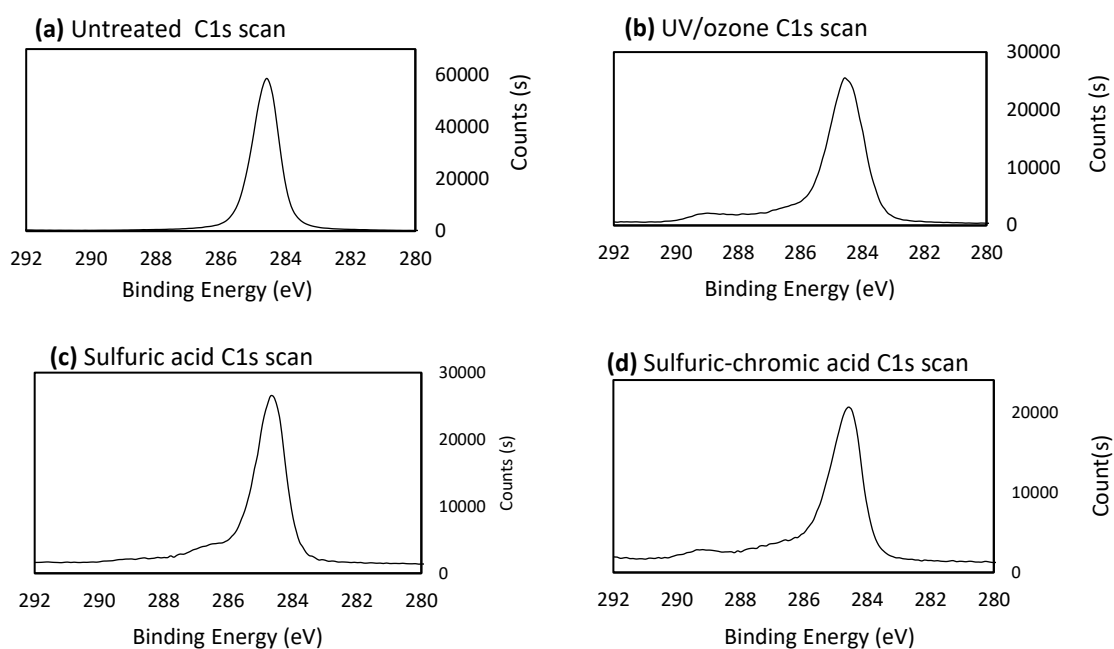


Figure 4.16 XPS carbon 1s core level scan with a 400 μm spot size of HDPE oxidised surfaces for (a) untreated, (b) UVO treated, (c) sulfuric acid treated and (d) sulfuric-chromic acid treated.

Contact angles studies with water and DIM have been performed and Fowke's Theory was used to calculate γ_{SV} of the HDPE modified substrates, see **Table 4.4**. The untreated HDPE surfaces did not exhibit a significant polar contribution to its surface free energy as would be expected for a hydrocarbon-based polymer like HDPE. However, after oxidation, a lot more of the surface free energy contribution can be attributed to polar surface properties. UVO HDPE treatment offered the highest degree of hydrophilicity and translated to a SFE γ_{SV} of 59.8 mJ/m^2 , with a water advancing θ decrease of $\sim 52^\circ$ from $\sim 90^\circ$.

HDPE Surfaces	Water θ (°)	DIM θ (°)	γ_{sv}^p (mJ/m ²)	γ_{sv}^d (mJ/m ²)	γ_{sv} (mJ/m ²)
Untreated	88.7 +/- 0.5	43.7 +/- 2.7	2.0 +/- 0.7	37.7 +/- 1.4	39.7 +/- 2.1
UVO	36.8 +/- 0.5	47.9 +/- 1.4	24.4 +/- 1.4	35.4 +/- 0.7	59.8 +/- 0.6
Sulfuric acid	41.6 +/- 1.3	41.9 +/- 1.3	17.0 +/- 1.7	38.6 +/- 0.7	55.6 +/- 1.0
Sulfuric-chromic acid	51.8 +/- 0.2	40.4 +/- 3.3	9.1 +/- 1.5	39.4 +/- 1.7	48.5 +/- 0.6

Table 4.4 Advancing water and DIM contact angles θ for HDPE surfaces; untreated surface, UVO, sulfuric acid and sulfuric-chromic acid oxidised HDPE surfaces, γ_{sv}^d and γ_{sv}^p are the dispersive and polar components respectively of the surface free energies.

XPS has shown that the C surface content has reduced by half, and there is an average of 33 +/- 5% O relative atomic abundance which increased following the use of the three surface oxidation approaches, compared to the 2% of O found in the control, see **Figure 4.17**. The 2% of O on the control untreated surface is due to the presence of a low amount of PDMS contamination. Only the sulfuric-chromic acid oxidised HDPE contained residual Cr and K which was found to be at 1.7% and 1.1% respectively. Surprisingly, UVO treatment introduced the least amount of O to the surface, see **Figure 4.17**. However, the UVO HDPE oxidised sample did contain significant silicon contaminants of about 11% which was seen. This contamination was later found to be due to variations of levels of silicon added from industrial manufacturing factors which resulted in a PDMS layer on the HDPE being found.

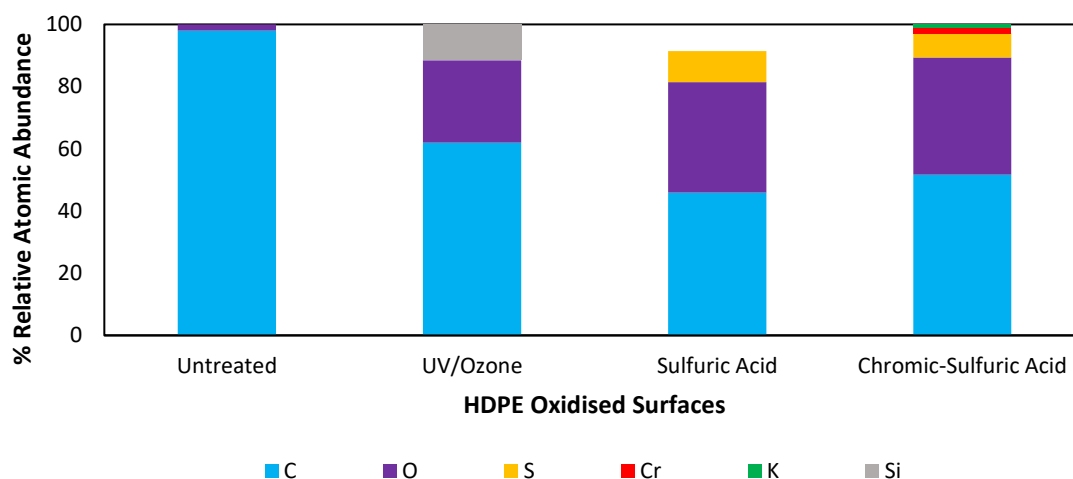


Figure 4.17 XPS percentage elemental surface relative atomic abundance of key elements (C, O, S, Cr and K) of HDPE oxidised surfaces.

Surface roughness determinations were obtained from white light profilometry studies, see **Figure 4.18**. The results revealed increases in surface R_a of about $1 \pm 0.3 \mu\text{m}$ for all three oxidation approaches. Sulfuric-chromic acid oxidation did not show a significant difference in the R_a and RMS values which suggests limited numbers of random surface cracks and changes in the overall topography have occurred, compared to the other two approaches.

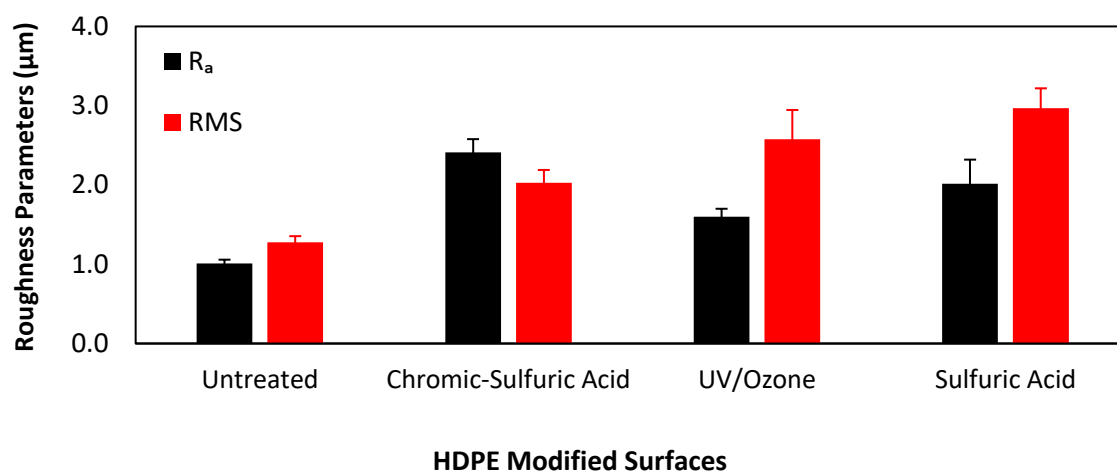


Figure 4.18 Profilometry results from HDPE oxidation using three different approaches (sulfuric-chromic acid etching, UV/ozone, and sulfuric acid; with average roughness (R_a) and the root mean squared average roughness (RMS) provided, $N=3$).

These prepared surfaces have been exposed to *P. aeruginosa* early-stage biofilm using the cell colony assay of the recovered biofilm bacteria. The results show that sulfuric-chromic acid oxidation caused a 5.2-log reduction in *P. aeruginosa* when re-tested in this experiment, when compared to the untreated HDPE surface, with no reduction seen using sulfuric acid and UVO treatments for HDPE, see **Table 4.5**. Both the sulfuric acid and the chromic-sulfuric acid introduced similar amounts of sulfonic moieties to the surface, and therefore it is evident that these sulfonic groups are not the antimicrobial active species because of the lack of antimicrobial performance in the sulfuric acid oxidised HDPE samples. In addition UVO treatment of HDPE produced surface oxidation with significant levels of -COOH groups being introduced, and this group also did not confer any biocidal impact. This result implies that the antibiofilm properties seen from sulfuric-chromic acid oxidised HDPE must be attributed to surface species that are not associated with the other two oxidation methods. Therefore, the residual levels of K and/or Cr that has been found present on the surface after sulfuric-chromic acid oxidation may be the key antimicrobial species. The presence of organochromium species from the ToF-SIMS of HDPE oxidised with sulfuric-chromic acid shows the presence of reactive Cr ester intermediates such as C_2H_5OCr , formed from the oxidation process. These species are known to hydrolyse and liberate Cr species which can possibly affect the bacterial cell membrane, its permeability and membrane potential. There are significant reports in the literature to confirm the solution antimicrobial credentials of Cr species. Jaber *et al.*, prepared protein nanoparticles loaded with Cr^{3+} and found an antibacterial effect against two strains of *E. coli* with varying antibiotic resistance profiles. Without the Cr^{3+} being present there was no antibacterial effect. These nanoparticles were able to inhibit biofilm growth and formation [401]. The antimicrobial activity of Cr^{3+} complexes with different ligands is well established and has been demonstrated and explored in various reports [402,403]. The activity of Cr^{3+} may be attributed to its high chelating ability as it is regarded as electron-deficient and can therefore participate in electron transfer free energy reactions (donor-acceptor mechanism) resulting in the oxidation of susceptible molecules [404]. Surface roughness is not thought to be a contributing factor as the other two oxidation approaches used also introduced a similar level of surface roughness to HDPE. Surface profilometry data shows that, within error, the sulfuric and

chromic-sulfuric oxidised surfaces have similar average roughness levels; therefore, substratum surface roughness is not seen as a factor in the antimicrobial activity.

HDPE Surfaces	<i>P.aeruginosa</i> CFU/mL (Log ₁₀)
Untreated	6.6 +/- 0.09
UV/ozone	6.5 +/- 0.03 {p=0.01}
Sulfuric acid	6.4 +/- 0.02 {p=0.004}
Sulfuric-chromic Acid	1.4 +/- 1.4 {p=0.0003}

Table 4.5 Early-stage biofilm viability from cell colony enumeration after exposure to various oxidised HDPE surfaces for 30 min static *P. aeruginosa* exposure. {p-value} determined by t-test and compared to HDPE untreated results.

4.2.8 Chromic-Sulfuric Acid Oxidisation on Alternative Polymers including (PC, PP, PS and PVC)

As the sulfuric-chromic acid treatment of HDPE led to a substantial reduction *P.aeruginosa* and *E. coli* viability, it was decided to apply this treatment method to a wider range of commonly used industrial polymers including polycarbonate (PC), polypropylene (PP), polystyrene (PS) and poly vinyl chloride (PVC) to determine whether this treatment could oxidise these polymers and impart antimicrobial functionality against early-stage Gram-negative bacterial biofilms. These surface modifications were conducted using 30 min treatment time as this reaction time has proven highly effective in the study with HDPE. It is important to note that there are no reported investigations of sulfuric-chromic acid oxidisation and its influence on antimicrobial properties on the additional polymers studied here.

Characterisation of these newly modified polymer surfaces was performed with XPS and contact angle goniometry. XPS surface analysis of 30 min sulfuric-chromic acid oxidised polymers has shown that the treatment causes varying degrees of oxidisation which is polymer dependent, see **Figure 4.19 (a-d)**. For example, oxidisation of PP has resulted in a 12.4% increase in surface O content with the introduction of carboxylic moieties,

whereas PVC showed no change in oxygen content, although there was an increase in possible alcohol/ester moieties at the surface. It was found that sulfuric-chromic acid oxidation was not effective in oxidising PVC, but instead caused a migration of Cl species to the surface. PS and PP saw significant oxidation, whereas with PC there were only a 7% increase in O surface abundance with a loss of surface carboxylic acid moieties. PS oxidation resulted in a significant ketone introduction at the surface, though few carboxylic functionalities were introduced. However, PP shows the presence of carboxylic groups [399]. Full survey scans can be found in the Appendix under Chapter 4[F].

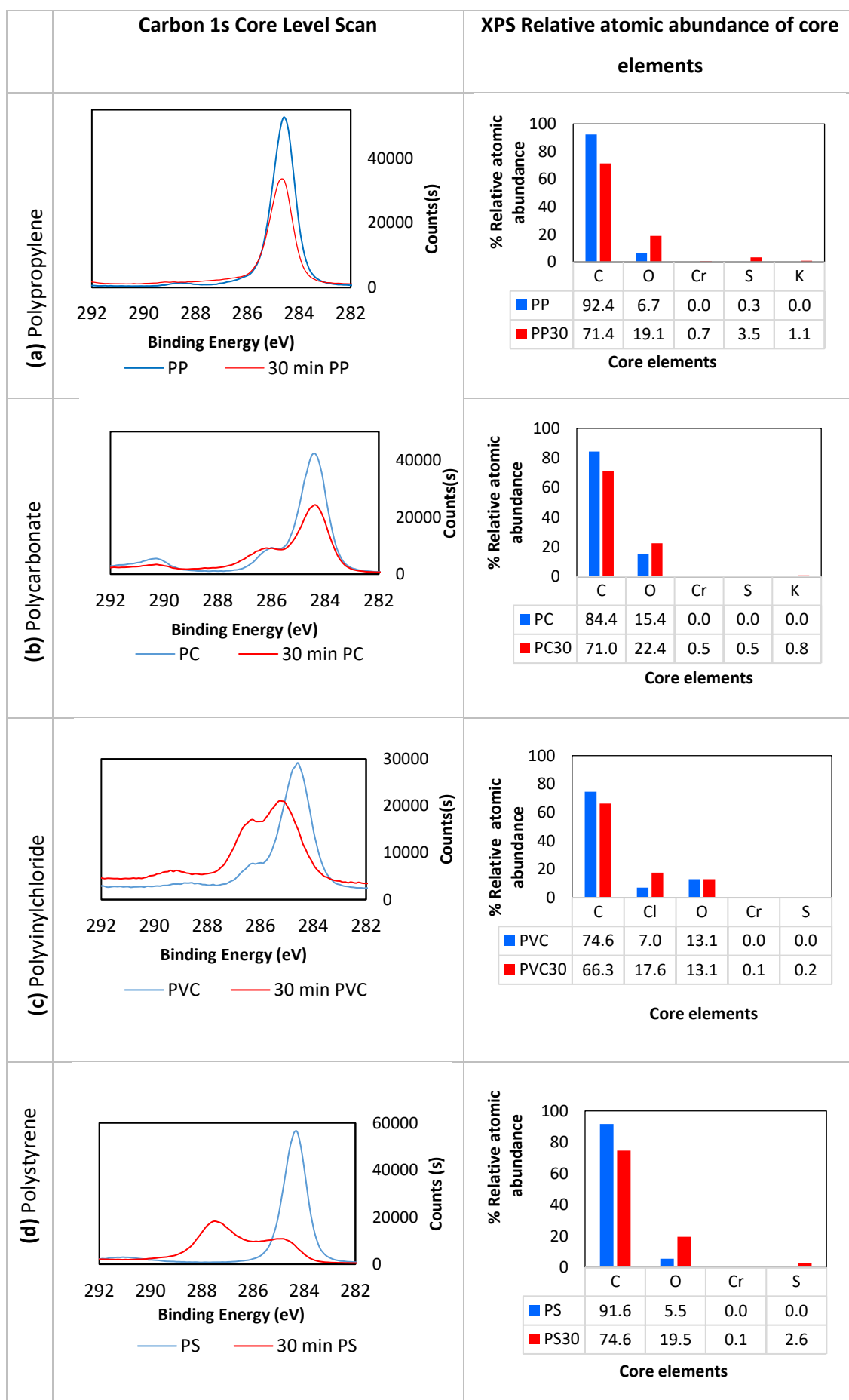


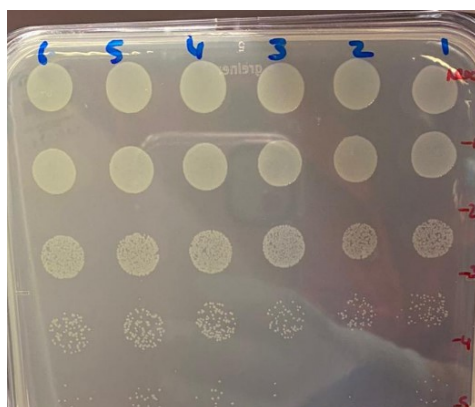
Figure 4.19 Carbon XPS 1s core level scans of untreated and 30 min sulfuric-chromic acid surface oxidised polyolefins: (a) PP, (b) PC, (c) PVC, and (d) PS, as well as percentage relative atomic surface composition. K has been included where its presence has been found in XPS.

Contact angle goniometry measurements revealed that PP exhibited the largest increase in surface hydrophilicity of all the polymers examined. This change corresponded to a 75 ° decrease in the advancing contact angle θ for water, see **Table 4.6**. This change can be largely attributed to the O content increasing. There is an overall positive correlation in increasing the percentage of relative atomic abundance of O from XPS measurements with reductions in the water advancing θ for most materials, although PVC is an exception to this trend. PVC has shown no significant change in the percentage of relative atomic abundance in O content from XPS measurements, however there was an observed increase in hydrophilicity from the contact angle studies. This increase can be attributed to the migration of chlorine which added 10% additional chlorine at the surface following the oxidation procedure **Figure 4.19 (c)**. These surfaces were then exposed to *P.aeruginosa* early-stage biofilm for assaying. Overall, colony cell enumeration of the scraped early-stage biofilm only revealed PP to have shown a reduction in biofilm viability, even though PS was also significantly oxidised by sulfuric-chromic acid oxidation, see **Table 4.6**. The oxidised PP result is not as large as the 5.2-log reduction from the HDPE sulfuric-chromic acid oxidised materials. Nevertheless, the PP images of the *P. aeruginosa* biofilm recovered demonstrates a significant 3-log reduction.

Surfaces	Water θ (°)	DIM θ (°)	γ_{sv}^P (mJ/m ²)	γ_{sv}^D (mJ/m ²)	γ_{sv} (mJ/m ²)	XPS Ratio C:O % Abundance	<i>P. aeruginosa</i> CFU/mL Log ₁₀
HDPE	88.7 +/- 0.5	43.7 +/- 2.7	2.0	37.7	39.7	48.0	6.6 +/- 0.1
Ox-HDPE	51.8 +/- 0.2	40.4 +/- 3.3	9.1	39.4	48.5	1.4	1.4 +/- 1.4 {p=0.0003}
PP	95.4 +/- 0.5	71.0 +/- 2.8	0.03	22.3	22.3	13.8	6.5 +/- 0.1
Ox-PP	20.5 +/- 4.1	45.3 +/- 4.6	33.8	36.8	70.6	3.7	3.5 +/- 0.2 {p=1.4x10 ⁻¹⁰ }
PVC	86.4 +/- 0.3	40.9 +/- 1.7	1.7	39.2	40.9	5.7	6.4 +/- 0.1
Ox-PVC	64.6 +/- 0.9	17.6 +/- 4.2	0.2	48.4	48.6	5.1	6.2 +/- 0.2 {p= 0.1}
PC	85.9 +/- 0.4	18.9 +/- 0.6	5.1	48.1	53.2	5.5	6.4 +/- 0.1
Ox-PC	52.5 +/- 1.7	12.9 +/- 1.4	3.2	49.5	52.7	3.2	6.3 +/- 0.2 {p= 0.2}
PS	93.5 +/- 0.4	NA*				16.7	6.4 +/- 0.2
Ox-PS	48.0 +/- 1.5	NA*				3.8	6.4 +/- 0.1 {p= 0.9}

* DIM could not be used for PS as material dissolves and degrades in this solvent

PP-untreated (N=6)



PP- 30 min chromic-sulfuric (N=6)



Table 4.6 Water and DIM advancing θ for the polymers examined, and the calculated surface free energies (Fowke's Theory), XPS elemental surface % C:O, and *P. aeruginosa* viability results following 30-min surface exposure to form an early-stage biofilm. PP and sulfuric-chromic treated PP colonies images shown. T-test were conducted relative to respective polymer controls, {p=value}.

PE and PP have a heterogenous polymer morphology comprising of a mixture of amorphous and crystalline components. It has already been demonstrated that chromic acid oxidation occurs preferentially within the amorphous components, with the resultant surface after oxidation containing increased crystallinity [285]. The

diminished antimicrobial activity in PP can be explained by its lower fraction of secondary carbon groups, which results in a lower concentration of chromate ester species. XPS revealed that the 30 min sulfuric-chromic acid oxidised PP to contain only 0.7% Cr, see **Figure 4.19 (a)**, whereas 30 min sulfuric-chromic acid oxidised HDPE contained 1.7% Cr, see **Figure 4.17**.

4.2.9 Recyclability and Water Stability of Sulfuric-Chromic Acid Modified HDPE

It has been established thus far that sulfuric-chromic acid oxidation is able to reduce early-stage biofilm adhesion and viability for HDPE and PP, with evidence highlighting the 3 month sample durability for HDPE when left under standard room conditions (25 °C and 40%RH). To determine the limitations of this modification further, sulfuric-chromic acid oxidised HDPE was sterilised (using 80% ethanol) and re-used to determine the stability limitations of these coated materials after repeat cycle useage. This test will be important in determining possible applications for these materials and also understanding the surface reactivity towards antimicrobial solutions. The experiment was performed over a period of 5 h, the samples were used as normal following a 30 min *P.aeruginosa* static droplet exposure for early-stage biofilm development, with the resultant biofilm then being scraped for agar overnight colony counting. After this first use (first cycle), the samples were sterilised in the same format, dried near a flame, and re-used up to four times following the same protocol for early-stage biofilm assessment with *P. aeruginosa*. Furthermore, a set of freshly pre-prepared sulfuric-chromic acid oxidised HDPE samples were exposed to 24 h in DI water, and their antimicrobial performance assessed against an early-stage biofilm of *P. aeruginosa* to see how extended exposure to water alone would influence the biocidal surface properties.

The data showed that the anti-biofilm properties were completely lost after the first use (cycle 1) which corresponds to the first water immersion event, see **Table 4.7**. The *P. aurgionsa* colonies formed after overnight culturation remained consistent and were unchanged from cycles 2-4, and was similar to the untreated HDPE surface. Exposure of

the 30 min HDPE surfaces to a complete 24 h submersion in DI water against *P. aeruginosa* early-stage biofilm also resulted in the complete inactivation of the surface, see **Table 4.7**.

Runs	Untreated HDPE CFU/mL (Log₁₀)	30 min sulfuric-chromic treated HDPE CFU/mL (Log₁₀)
Cycle 1	6.4 +/- 0.2	No growth { $p=7.7 \times 10^{-16}$ }
Cycle 2	6.2 +/- 0.1	6.4 +/- 0.3 { $p=0.2$ }
Cycle 3	6.2 +/- 0.1	6.6 +/- 0.2 { $p=1.8 \times 10^{-5}$ }
Cycle 4	6.5 +/- 0.2	6.5 +/- 0.1 { $p=0.7$ }
24 hr water immersion	6.3 +/- 0.4	6.3 +/- 0.1 { $p=0.9$ }

Table 4.7 HDPE sulfuric-chromic acid treated surfaces and their antimicrobial performance on *P. aeruginosa* early-stage biofilm viability following multiple washing and use cycles, as well as 24 h water immersion study to evaluate the influence of excessive water exposure, {*p*-values} determined by a *t*-test and compared to samples of each according cycle.

The overall results from the aqueous stability study suggest that the antimicrobial active species has been lost under these conditions. There may be three possible reasons:

- Physical scraping to remove the early-stage biofilm for assaying has resulted in the loss of antimicrobial functionalities
- Loss of antimicrobial functionality due to take up of antimicrobial agent by biofilm after cycle 1
- Sequential dipping in water to remove planktonic cells and/or the 80% ethanol cleaning has caused removal of the antimicrobial surface species after cycle 1

Therefore, to determine the outcome, the chemical composition of surfaces after the first cycle (cycle 1) were investigated to further decipher these chemical changes. As a result, XPS was performed on the HDPE samples exposed to 30 min of sulfuric chromic treatment after and before cycle 1, see **Table 4.8**. These samples had to be sterilised before XPS analysis with ethanol (80%) cleaning. The center of the square coupon which had biofilm contact was compared with the coupon edge to determine if any significant

surface elemental compositional differences were present due to biofilm contact. The results indicate again that there were no differences in chemical composition from the center versus the outside of the coupon, within error. Also, upon use, O, Cr and S content has significantly been reduced with a ca. 23% reduction in O content as well as a ~1.3% reduction in Cr compared to controls.

	% Relative Atomic Abundance			
Abundant Elements	After Cycle 1 (Centre)	After Cycle 1 (Outside)	Unused/fresh samples (Centre)	Unused/fresh samples (Outside)
Carbon	51.7	50.4	85.3 +/- 1.5	82.8 +/- 1.2
Oxygen	37.7	36.0	12.5 +/- 1.3	14.9 +/- 0.7
Chromium	1.7	2.0	0.5 +/- 0.07	0.7 +/- 0.1
Sulfur	7.7	7.7	0.8 +/- 0.2	0.8 +/- 0.1
Potassium	1.4	3.9	0.0	0.0

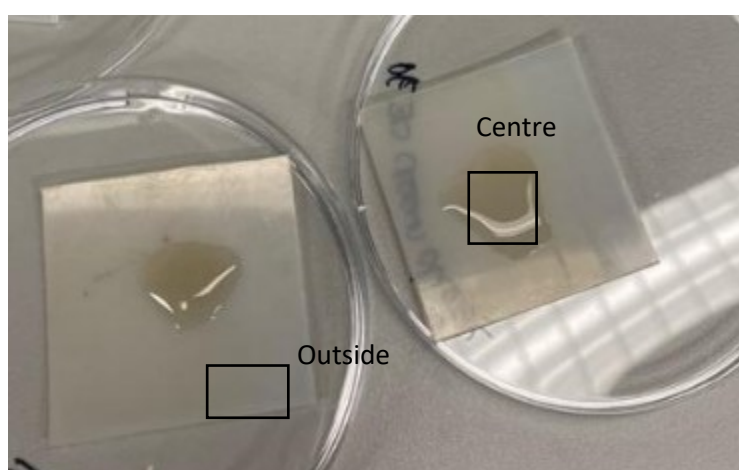


Table 4.8 XPS percentage relative atomic abundance of 30 min oxidised HDPE sulfuric-chromic acid treated samples, tested against *P. aeruginosa* early-stage biofilm. Controls (unused/fresh samples) have been provided for each for comparison.

Furthermore, samples submerged in DI water for 24 h and 7 days under standard conditions and RT, were examined under XPS to further corroborate how water alone influences the surface composition over time. It was found that after 24 h there was a substantial loss of surface O, amounting to a 1/3 decrease; this decrease remains

constant even after 7 days with about 14-15% atomic O found. The C content saw a 42% increase after 24 h, which also remained constant even after 7 days submersion in DI water, see **Figure 4.20 (a)**. Analysis of the core level C1s shows loss of carboxylic acid groups from the surface due to hydration, see **Figure 4.20 (b)**. The Cr surface content decreased from 2.4% to 0.6% for the fresh sample to the 24 h hydrated samples.

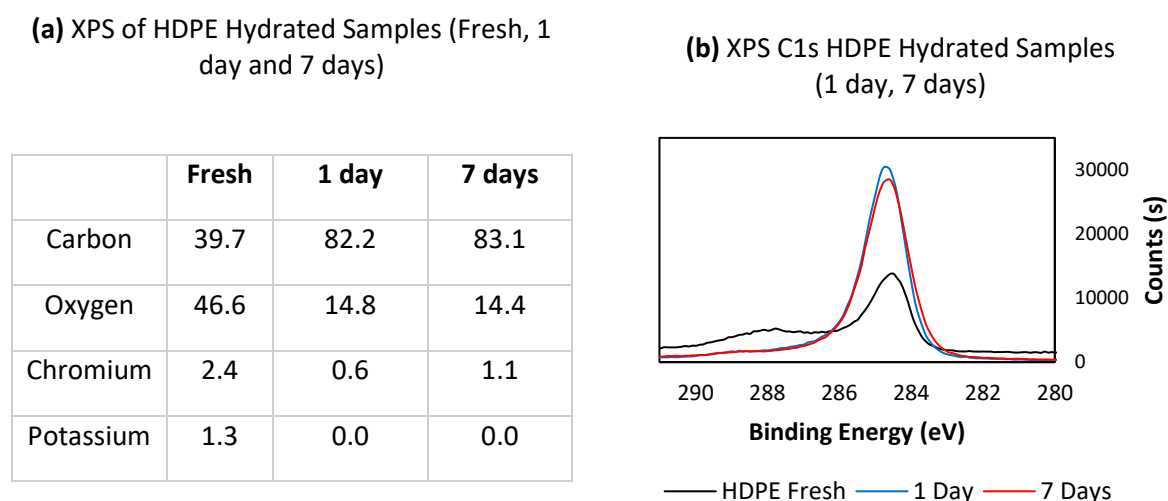


Figure 4.20 XPS measurements of surfaces exposed to prolonged water submersion, (a) % relative atomic abundance of core elements found at the surface; (b) carbon 1s core level scan of fresh (not hydrated), 24 h hydrated and 7 days hydrated HDPE sulfuric-chromic acid oxidised surfaces.

CLSM was used to assess *P.aeruginosa* early-stage biofilm on 24 h hydrated samples, compared with fresh non-hydrated samples, see **Figure 4.21**. These results confirm that sample immersion in water for 24 h has caused a loss in the antimicrobial effect. Interestingly the 2 min oxidation treatment time with sulfuric-chromic acid on HDPE only resulted in a small increase in viability (ratio of Syto™9/PI) after submersion in DI water, **Figure 4.21 (a)**. However, when examining the CLSM results for the separate channels of Syto™9 and PI in **Figure 4.21 (b)** as an average for each oxidation reaction time (0-30 min), a gradual reduction in the amount of Syto™9 signal intensity is observed, with increasing oxidation time; although this does not translate to a reduction in viability from the colony counting experiment. This suggests that the antiadhesive properties of these materials are still prominent, however to a lesser

extent as there is still a lot of attached cells from submersion in water for 24 h. After 24 h there is a significant loss of the biocidal species, therefore the antiadhesive properties of the material can be attributed to the oxidation and introduction of polar O moieties at the surface. This hypothesis has been proposed by others, and has been discussed and mentioned in the introduction of this Chapter. Furthermore, this also further highlights the importance of using different microbiological assays to examine and assess the antimicrobial performance to determine the full extent and biocidal property of a surface in greater detail.

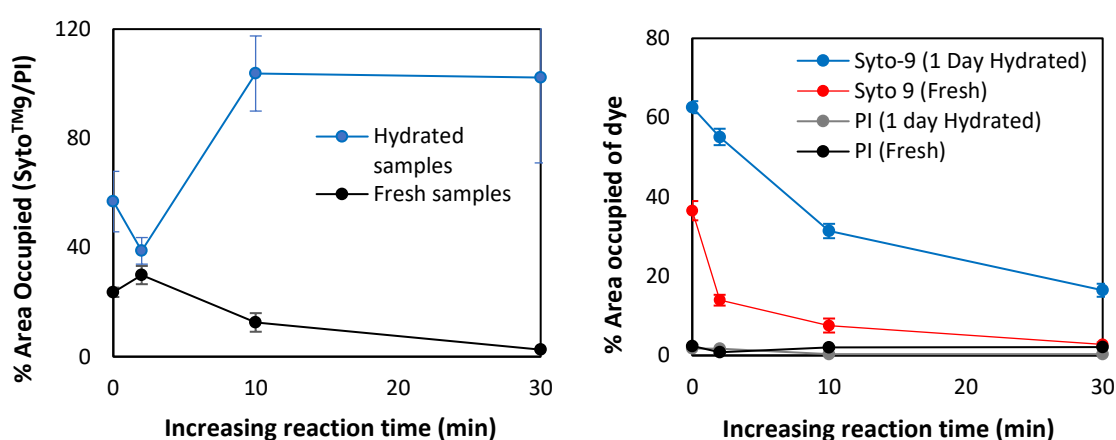


Figure 4.21 (a) *P.auruginosa* initial biofilm exposed to non-hydrated and 24 h hydrated samples; (b) CLSM of *P.auruginosa* initial biofilm on sulfuric-chromic acid oxidation HDPE and the changes in Syto™9 and PI channels summarised versus increasing oxidation time.

4.2.10 Comparison of Cr-oxyhydroxide coating to Sulfuric-Chromic Acid Treatment on HDPE

A key question to be answered is what is the critical water-soluble surface species present which confers these antimicrobial properties? Complex pH-dependent equilibria are known to occur in ‘chromic-sulfuric acid’ solution systems, and it is known that the sulphate complex $\text{CrO}_3(\text{OSO}_3)^{2-}$ can be formed [405]. Another possibility are chromate esters which are well established intermediates in the oxidation of hydrocarbons [18]. So, it is possible that intermediate or insoluble products of this chemistry are present on the treated surfaces. Chromic acid has a strong preference for

reacting with secondary carbons, making amorphous PE a suitable substrate for reactions producing chromate esters. To understand this topic further a separate small study was conducted to compare the leaching of Cr species using ICP-MS, compared to a model Cr containing surface thin-film to determine the importance of Cr leaching in the antibacterial behavior.

Cr-oxyhydroxide materials were prepared, and dip-coated onto HDPE which had been oxidised with UVO and sulfuric acid treatments (UVO-SA). The synthesis procedure was adopted by Yang *et al.* to prepare $\text{CrO}(\text{OH})\cdot 2\text{H}_2\text{O}$ at pH 10 overnight and involved a hydrothermal processing step to increase crystallization, however crystallisation of the materials in this study may not be advantageous, therefore samples with and without hydrothermal treatment (HT) were prepared [349]. XRD confirmed the successful synthesis of the Cr-oxyhydroxide colloids when compared with XRD by Yang *et al.* [349], see **Figure 4.22**. The HT process does show increase in crystallinity, with the Cr-oxyhydroxide prepared being comparable in peaks with data by Yang and co-workers.

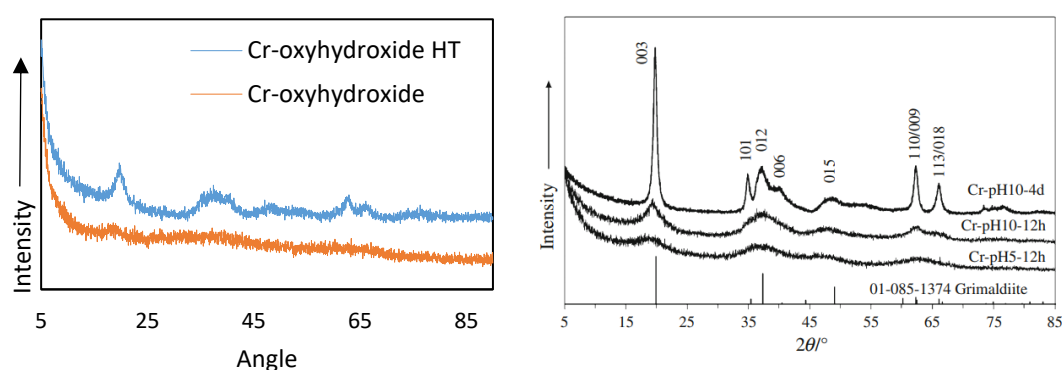


Figure 4.22 XRD pattern of Cr-oxyhydroxide materials obtained with and without hydrothermal (HT) processing on the left, preparation involved pH 10 conditions and 15 h overnight HT treatment for HT treated sample. XRD of samples prepared by Yang and co-workers have been provided on the right for comparison.

XPS was performed on the Cr-oxyhydroxide/HT powders and films on HDPE to determine the success of the coating procedure. The % elemental abundance of key elements obtained from XPS has been provided in **Table 4.9**. The use of carbon tape for

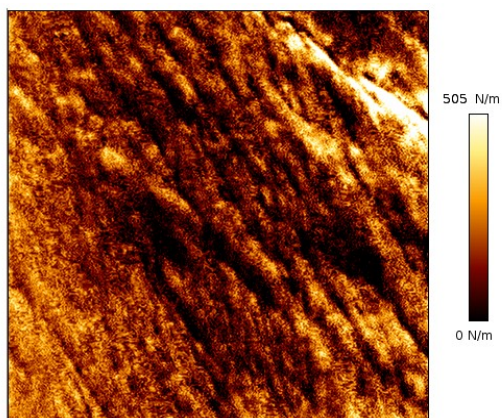
the measurement of the Cr-oxyhydroxide/HT powders results in a carbon elemental abundance of 6-12%. The importance of surface polymer oxidation and the preparation of coatings has been demonstrated here, as the surfaces without oxidation showed no Cr to be present at all.

HDPE Modified Materials	C	O	Cr	S
Cr-oxyhydroxide powder	17.6	63.4	19.0	0.0
Cr-oxyhydroxide powder HT	6.9	67.2	25.9	0.0
HDPE Cr-oxyhydroxide	94.5	3.7	0.0	0.0
HDPE Cr-oxyhydroxide HT	91.6	5.6	0.0	0.0
HDPE ox-Cr-oxyhydroxide	50.1	38.4	8.1	2.6
HDPE ox-Cr-oxyhydroxide HT	27.4	53.3	11.4	4.4
HDPE ox	63.8	23.9	0.0	6.1
HDPE untreated	98.0	2.0	0.0	0.0

Table 4.9 XPS based % surface elemental abundance. The Cr-oxyhydroxide/HT powder was measured using an adherent carbon tape. HT: hydrothermal treatment, ox: UV/ozone oxidised (20 min) then sulfuric acid oxidised (15 min, 90-100 °C). Sulfur was included as oxidation using sulfuric acid introduces sulfur surface moieties. Full XPS survey scans can be found Appendix Chapter 4[G].

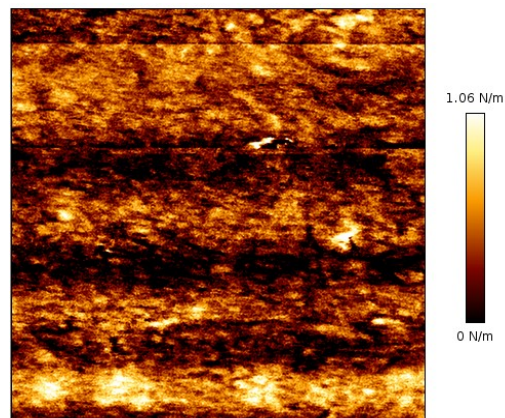
AFM imaging was performed on the successfully coated Cr-oxyhydroxide/HT HDPE samples (HDPE oxidised samples), and the images are shown in **Figure 4.23**. HT treatment shows clear variation in the film coating prepared with more globular surface aggregates present in the HT film, and larger repetitive structures in the non-HT film. DLS was performed on the solutions for the Cr-oxyhydroxide/HT prepared, and this test revealed that agglomeration of the coating solution had occurred with these samples, where the average volume weighted mean particle size was 23.3 μm for the HT solution, and 58.8 μm for the non-HT solution. Although the film prepared contains agglomerate clusters, the significant presence of Cr is apparent and key in our intention to evaluate the antimicrobial property of this element.

HDPE untreated



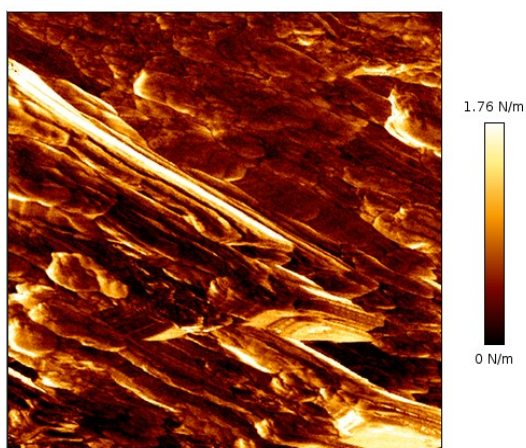
2 μm x 2 μm , slope fit, QI™ imaging mode, 512 x 512 pixel

HDPE oxidised (UVO-SA)



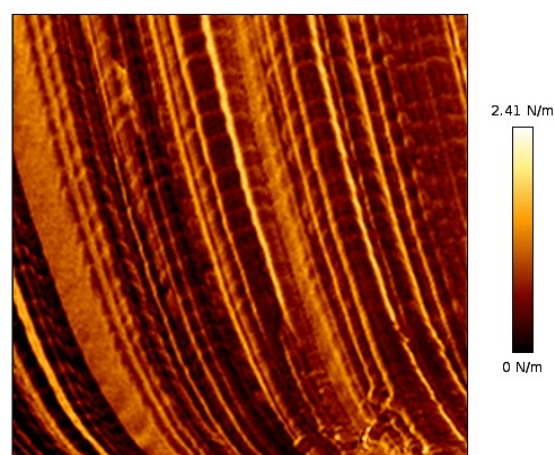
2 μm x 2 μm , slope fit, QI™ imaging mode, 512 x 512 pixel

HDPE ox-Cr-oxyhydroxide HT



2 μm x 2 μm , slope fit, QI™ imaging mode, 512 x 512 pixel

HDPE ox-Cr-oxyhydroxide



2 μm x 2 μm , slope fit, QI™ imaging mode, 256 x 256 pixel

Figure 4.23 AFM imaging of oxidised (ox) HDPE with UVO-SA treatment, coated with Cr-oxyhydroxide films and the relevant controls. A slope fit has been provided as affords the clearest images.

The colony counting experiment via scraping of the early-stage biofilm was performed on these samples against *E. coli* and *S. aureus* to determine if a Cr containing coating is sufficient alone in producing an antimicrobial effect. Here a surface was prepared with a higher Cr surface concentration present than the sulfuric-chromic acid oxidised HDPE

surfaces. These Cr-oxyhydroxide films contained 8.1-11.4 % Cr when HDPE is pre-treated (oxidised) before coating with the Cr-oxyhydroxide film. The viability results have been provided below in **Table 4.10**. The results show that there was no antimicrobial effect observed with these materials against the early-stage biofilm of *E. coli* and *S. aureus*.

HDPE Modified Samples	<i>E. coli</i> CFU/mL Log₁₀	<i>S. aureus</i> CFU/mL Log₁₀
Untreated	6.55 +/- 0.03	5.97 +/- 0.14
Oxidised (UVO-SA)	6.39 +/- 0.16 {p=0.07}	6.17 +/- 0.11 {p= 0.2}
Cr-oxyhydroxide	6.55 +/- 0.05 {p=0.8}	6.01 +/- 0.13 {p=0.9}
Cr-oxyhydroxide HT	6.58 +/- 0.07 {p=0.3}	5.97 +/- 0.10 {p=0.7}
Oxidised Cr-oxyhydroxide	6.39 +/- 0.10 {p=0.004}	6.08 +/- 0.24 {p=0.2}
Oxidised Cr-oxyhydroxide HT	6.17 +/- 0.44 {p=0.008}	5.50 +/- 0.06 {p=0.01}

Table 4.10 Colony enumeration results from the early-stage biofilm assay of *S. aureus* and *E. coli* on Cr-oxyhydroxide/HT containing samples on HDPE modified and unmodified polymers (+/- SD). Herein, 'oxidised' refers to UVO-SA surface oxidation. A t-test was performed relative to untreated HDPE control, {p=value}.

To understand these results further, an experiment was conducted to investigate the leaching of Cr from these coated surfaces when exposed to 200 µL of aqueous media (DI water); 200 µL is the volume used in the colony counting assay for a period of 30 min exposure. It is important to find out how much Cr is being leached from these two types of Cr containing surfaces; the Cr-oxyhydroxide coated surfaces and the sulfuric-chromic acid treated surfaces. ICP-MS is a highly sensitive technique to measure trace elemental levels of metals at very low concentrations. Indeed 1000 ppb (µg/L) is the upper concentration limit of this approach. The 200 µL collected was added to DI water to get a total volume of 5 mL. This solution dilution had to be performed so that there was enough volume to run the samples using ICP-MS as this volume is the minimum

quantity required for the analysis. A calibration standard solution used was provided by the Departmental Analytical Laboratory whose staff who also ran this specific experiment; Kaho Cheung and Patricia Carry. The Cr calibration graph can be found in the Appendix under Chapter 4[I]. The samples were also exposed to leaching for a period of 1 min only, instead of the 30 min to see if that influenced the leaching results obtained.

Samples	1 min (µg/L)	30 min (µg/L)
Untreated	-2.0 +/- 6.5	-11.5 +/- 0.8
Oxidised	-11.7 +/- 0.2	- 11.2 +/- 0.1
Oxidised Cr-oxyhydroxide	41.8 +/- 54.2	73.2 +/- 4.3
Oxidised Cr-oxyhydroxide HT	182.9 +/- 33.3	77.8 +/- 9.9
2 min sulfuric-chromic acid	311.9 +/- 81.2	470.0 +/- 179.1
10 min sulfuric-chromic acid	>1000	>1000
30 min sulfuric chromic acid	>1000	>1000

Table 4.11 ICP-MS determination of chromium leaching concentration from a range of substrates after contact with 200 µL of DI water for 1 and 30 min. The 200 µL was diluted to 5.152 mL for measurement using ICP-MS (+/- SE, N=2).

The 10 and 30 min sulfuric-chromic acid oxidised HDPE polymers exhibited a Cr concentration of >1000 µg/L which suggests that the leaching of Cr is substantial and significant compared to the very low Cr levels that is found leaching from the Cr-oxyhydroxide/HT films. This data suggests the importance of a Cr labile active species for antimicrobial effects. However, this hypothesis raises the key questions of Cr cytotoxicity to humans, marine organisms, and non-target species which has not been investigated in this PhD research project.

4.2.11 Mature Biofilm Performance of Cr-Oxide Coating and Sulfuric-Chromic Acid Modified HDPE

To understand the application scope of these materials more broadly, not only was an early-stage biofilm formation investigated, but in this section a preliminary study on mature biofilms was conducted. One method was to prepare a mature biofilm which involves sample hydration in a media rich environment in the presence of bacteria for an extended period; overnight. Details for this experimental protocol can be found in Chapter 3, Section 3.9 for *E. coli*. In brief, samples were immersed in TSB media with 1% glucose with *E. coli* cells at 0.2 OD (600 nm) for 24 h at 37 °C static incubation, followed by biofilm removal using a surface scraping, followed by a plating out for viable enumeration of CFU's from neat to log -12. These results are provided in **Table 4.12**. The mature biofilm study ultimately showed no difference between any modified HDPE coating under these conditions. These results are not a surprise considering the leaching attribute to these materials after exposure to a hydrated environment, as well as the loss of antimicrobial activity after exposure to water for long periods of time for sulfuric-chromic acid oxidised materials. Furthermore, it can be observed that there is an increase of 2-log scales roughly across the board for the quantity of mature biofilm present compared to an early-stage biofilm examined previously.

Samples	CFU/mL Log ₁₀ <i>E. coli</i>		Samples	CFU/mL Log ₁₀ <i>E. coli</i>
HDPE control	7.99 +/- 0.16		HDPE control	7.9 +/- 0.4
HDPE 2 min oxidised	7.89 +/- 0.39		HDPE oxidised	7.9 +/- 0.3
HDPE 10 min oxidised	8.03 +/- 0.10		HDPE oxidised Cr-oxyhydroxide HT	8.0 +/- 0.3
HDPE 30 min oxidised	7.97 +/- 0.26		HDPE oxidised Cr-oxyhydroxide	8.0 +/- 0.2
			HDPE Cr-oxyhydroxide HT	8.2 +/- 0.1
			HDPE Cr-oxyhydroxide	7.6 +/- 0.2

Table 4.12 Mature biofilm 24 hr exposure of *E. coli* to sulfuric-chromic acid oxidised HDPE and viable enumeration of and Cr-oxyhydroxide coated HDPE oxidised samples and the CFU results

obtained (+/- SD). The two experiments were conducted on separate occasions and therefore two control values for HDPE has been provided.

4.3 Conclusion

The surface of HDPE has been successfully oxidised using sulfuric-chromic acid oxidation, which was analysed using a range of surface characterisation techniques providing chemical, topographical, and physical parameters before and after modification. Increasing the sulfuric-chromic acid oxidation time has shown a direct decrease in microbial attachment and thus reduced the development of biofilm formation, when freshly prepared samples were examined. The sulfuric-chromic acid treated HDPE effected the membrane of Gram-negative bacteria, by causing membrane depolarisation, and to a lesser extent, membrane damage, which translated to a 5.2- to 6-log reduction with the early-stage biofilm of *P. aeruginosa*. This antimicrobial performance was not seen when the Gram-positive bacteria *S. aureus* was tested. However, the reduced microbial attachment of *S. aureus* was observed with increasing oxidation reaction time using sulfuric-chromic acid treatment, though to a lesser extent than *E. coli* and *P. aeruginosa*. This work supports findings by other researchers that oxidation of a polymer like HDPE can effectively reduce microbial attachment prior the onset of biofilm formation for both Gram-positive and negative organisms.

The antimicrobial property of sulfuric-chromic acid treatment was compared with hot sulfuric acid and UV/ozone oxidation approaches on HDPE to determine if oxidation alone will suffice in producing an antimicrobial polymer. Only the sulfuric-chromic acid oxidation treatment provided a reduction in biofilm viability when cultured. Therefore, the antimicrobial active species must not be associated with generic oxidised moieties, but with species which are associated specifically with the sulfuric-chromic acid treatment. Detailed comparison of a model Cr-oxyhydroxide coatings with sulfuric-chromic acid treatment approach on HDPE revealed that the extensive loss of labile Cr species, specific to the sulfuric-chromic acid treatment, is essential in producing an antimicrobial result.

The use of sulfuric-chromic acid etching on alternate polymers PP, PC, PVC and PS was investigated. This treatment was not effective in oxidising PC and PVC. Between PP and PS, only PP showed an antimicrobial result when tested against the early-stage biofilm of *P. aeruginosa*, showing a 3-log reduction. The reduced anti-biofilm performance observed with PP can be attributed to the reduced levels of Cr found at the surface of PP, relative to HDPE.

Immersion of oxidised HDPE samples in water, resulted in the loss of key antimicrobial Cr species which suggests that this polymer treatment is limited to single use polymer applications, perhaps such as disposable products used heavily in the medical field. Evaluation of attached *P. aeruginosa* cells after exposure of samples to 25 h water still showed a trend of reduced microbial attachment with increasing etching reaction time, which confirms that the anti-adhesive and anti-biofouling property of sulfuric-chromic acid oxidised surfaces is due to the oxygen containing functional groups which are still present at about 15% relative elemental atomic abundance, whilst other residual species (such as Cr) are lost or reduced. Mature biofilm studies under a 24 h hydrated environment for maturation has confirmed the limitations of this AMP approach, as no antimicrobial performance was obtained when compared to the control materials. This was thought to be from the loss of antimicrobial active residual Cr from the surface under these hydrated conditions.

CHAPTER 5

Tin-dioxide hydrosol coating and its Antimicrobial Performance

5.1 Introduction

Tin and tin-based compounds have been examined for their antimicrobial performance extensively. Notably, in the 1960s the use of organotin(IV) complexes (e.g. tributyltin and triphenyltin) have shown potency as antifoulant paints coated onto ship hulls with 18-24 month protection [406]. However, these organotin compounds provided exceptional toxicity to non-target marine organisms by interfering with biological processes, causing the abnormal growth and decrease in population of species like mussels and oysters which resulted in their ban in 2008 [406]. Examination of pure tin metal has been performed by Yasuyuki *et al.* and compared to 8 other metals. In fact, it was discovered that tin was one of the pure metals that did not exhibit any antimicrobial activity against *S. aureus* and *E. coli* which was thought to be attributed to the low solubility of tin in test solutions at 24 h and 72 h [407]. This suggests that not all tin compounds and species exhibit antimicrobial performances, and their performance may vary depending on the composition of the tin material and its solubility.

Oxides of tin such as SnO₂ have shown promising antimicrobial properties as coatings and in solution as nanoparticles in different experimental systems. For example SnO₂ nano-rods have been incorporated into polymers to give excellent bactericidal properties against both Gram-positive and -negative bacteria, with no cytotoxicity found [205]. In a separate study, Luna-Velasco *et al.* investigated the effect of the size of SnO₂ nanoparticles versus bulk SnO₂ on the toxicity of *B. Subtilis*, comparing 2 nm, 40 nm and a flower-like 800 nm SnO₂ particles. It was observed that the smaller size particles offered greater hydrodynamic advantage and stability, and also afforded the best toxicity against *B. Subtilis* [206]. Many SnO₂ sol-gel coatings have been found to be very successful in reducing bacterial viability with high-durability and good thin-film stability. Examples include, composite SnO₂-ZnO sol-gel thin films coated onto glass substrates showing antibacterial activity against *E. coli* even in the dark [207].

There are various methods of coating SnO₂ nanoparticles on surfaces, such as sputtering [408,409], spray pyrolysis [410], sol-gel [411], and chemical vapour deposition [412,413]. Of these, the sol-gel coating technique is capable of producing thin-films that are uniform, and are low cost to prepare because the process does not require expensive and sophisticated equipment [414–416].

Sol-gel coated SnO₂ thin-films on polymer materials such as HDPE has not been tested previously for its antimicrobial activity. Therefore, this Chapter will study the limitations and capabilities of coating SnO₂ onto industrial polymers using a simple synthesis and coating procedure that could be replicated on an industrial scale with ease. However, surfaces such as HDPE are renowned for the chemical inertness, making the direct application of coatings challenging. There are a plethora of approaches that can be employed to chemically modify the surface of HDPE to transform it into a more readily adhesive backbone for the subsequent sol-gel coating procedure. These approaches include electron and ion beam irradiation, gas discharge plasma, ultraviolet-irradiation and surface chemical oxidation [417]. Surface pre-treatment to improve metal oxides sol coating adhesion to polymers are common and often essential to help maximise coating longevity, and hence its performance [350]. Chemical surface oxidation has proven to be a successful approach and sulfuric-chromic acid etching has shown to reduce antimicrobial adhesion and viability against HDPE and PP when investigated in this project (see Chapter 4). Therefore, this method of polymer pre-treatment has been utilised here to oxidise the HDPE surface in preparation for coating with tin-dioxide nanoparticles. However, careful attention has been taken to include controls so that sulfuric-chromic acid oxidation pre-treatment did not complicate evaluating the effectiveness of the tin-oxide nanoparticle coating.

5.2 Results and Discussion

5.2.1 Tin hydrosol Synthesis and Coating

Kenney *et al.* developed a method to synthesise a tin-dioxide hydrosol from tin chloride precursors, $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{SnCl}_4 \cdot 5\text{H}_2\text{O}$, where hydrolysing the salts resulted in the formation of tin hydrous oxide sols (tin-hydrosols), for coating onto PE [418]. The procedure for tin-hydrosol synthesis in this method includes an aging overnight step. However, for this task, two different hydrosol solutions were prepared, one overnight aged and one not. They are termed **tin-hydrosol A** and **tin-hydrosol B**, where A is the non-aged solution, and B is the aged overnight solution. The procedure for the tin-hydrosol used can be found in Chapter 3 under Section 3.2.3.

Kenney *et al.* reported that a considerable amount of tin-hydrosol is required to be coated on the surface of PE to enhance the surface's wetting. This observation was found to be dependent on the '*wetting time*'; this time is the minimum amount of time the surface is submerged in the colloidal solution, which was found to be 15 s minimum in this study [418]. The strength and stability of film adhesion was determined by the amount of colloidal thin-film remaining after ultrasonic rinsing, air/water jet spray and exposure to adhesive tape [418]. The tin-hydrosol thin-films were prepared in this project with coating time of 2 min and a pre-oxidative treatment step used to improve tin-hydrosol coating to HDPE which was the sulfuric-chromic acid chemical oxidation.

The size of the tin-hydrosols was measured using dynamic light scattering (DLS). The refractive index (RI) for these solutions were found to be 1.3 and the absorption properties of the solutions, measured through UV/vis spectroscopy, at 830 nm were 0.001 for tin-hydrosol A and 0.1 for tin-hydrosol B. These two parameters are required for an accurate DLS calculation of the particle size (see Appendix for RI and absorption data Chapter 5[A-B]). Therefore, with these known parameters, a study on the particle size of the colloids in the tin-hydrosol solution could be examined through DLS of both freshly prepared hydrosol samples.

The DLS experiment provides information on a number of particle parameters, including the volume distribution, number distribution, polydispersity index (PDI) and Z-average.

Where volume distribution relates to the volume taken up by a particular size of particles. The number distribution relates directly to the number of particles at a certain size. The PDI is a measure of size heterogeneity, which can occur in a sample due to variations in the synthetic methods employed, by sample agglomeration or aggregation. Additionally, Z-average also known as the cumulant mean, is an intensity mean calculated by the signal intensity relating to the Z-average size of the particle. The Z-average increases as particle size increases (further explanation of these parameters for this instrument has been given in the Appendix Chapter 5[C]).

Two batches of each tin-hydrosol, A and B, were prepared and examined through DLS, see **Table 5.1** (full DLS data in Appendix Chapter 5[D-E]). The number distribution shows that 100% of particles have averaged diameters at 13.8-14.3 nm for tin-hydrosol A, and 100% particles are averaged at 21.8 – 23.6 nm for tin-hydrosol B. The larger size for tin-hydrosol B was expected as this solution is the aged variant and results in larger particles being formed. The volume distribution depicts a different particle size attribute and informs us about the volume taken up by a particle of a certain size. It was observed that 99.9% of volume occupied by colloids in tin-hydrosol A were averaged at 18.8 – 19.8 nm +/- 9; and 99.3-4% of volume occupied in tin-hydrosol B were of 30.6-31.6 nm +/- 14. These values are slightly larger than the number distribution, this increase is because these two values are calculated separately from the intensity distribution. Transformation of the intensity to a number or volume distribution may cause discrepancies and variations as these two distributions are calculated differently. For example, number distribution reports the smallest size measured therefore noise and impurities may be misleading and amplified. As a result, both volume and number distributions have been provided and compared against. In this case the data correlates closely with one another, where the number distribution on average provides values that are 6.8 nm less than the volume distribution, see **Table 5.1**.

Solutions		Particle Size Diameter Distributions (nm)			Polydispersity Index (PDI)
		Z-Average	Volume Average	Number Average	
Tin-hydrosol A	Batch 1	32.34	19.77 +/- 9.40	14.27 +/- 4.37	0.257
	Batch 2	32.51	18.81 +/- 9.07	13.80 +/- 4.09	0.328
Tin-hydrosol B	Batch 1	50.39	31.57 +/- 14.39	23.66 +/- 6.86	0.264
	Batch 2	50.36	30.59 +/- 14.96	21.86 +/- 6.73	0.252

Table 5.1 DLS results of tin-hydrosol A and tin-hydrosol B with the number, volume, Z-average distributions as well as the polydispersity indexes.

The Z-average was measured as 32 nm for tin-hydrosol A and 50 nm for tin-hydrosol B. These values are calculated directly from the signal intensity and becomes more inaccurate if the distribution is broad, such as with a sample that contains high polydispersity levels. In this case the polydispersity index (PDI) is not very high and ranges between 0.2-0.3 which is considered moderate. In conclusion this data shows that both tin-hydrosols are in the nanoscale diameter range which is a desirable property due to smaller nanoparticles of SnO₂ being previously reported as highly effective antimicrobial agents [206].

5.2.2 Surface Characterisation of SnO₂ Coating on HDPE

Tin-hydrosol A and B were dip-coated onto both untreated and sulfuric-chromic treated HDPE to form **Film A** and **Film B** respectively. The sulfuric-chromic oxidised HDPE samples were treated for 2, 10 and 30 min. These thin-films have been denoted as 2-, 10-and 30- Film A/B, depending on the tin-hydrosol used. Characterisation of the surface of all thin-films were performed through contact angle goniometry whilst the degree of SnO₂ nanoparticle leaching was also determined through ICP-MS analysis. The initial water advancing contact angle θ was measured and the surfaces were then

washed sequentially in three beakers of DI water (W1, W2, W3). Further water advancing contact angle θ measurement were obtained after W3 to note any changes to the surface properties from the sequential washing. ICP-MS measurements on the rinsed washings collected were used to evaluate the Sn concentrations found leaching off the polymers following the washing steps, see **Figure 5.1**.

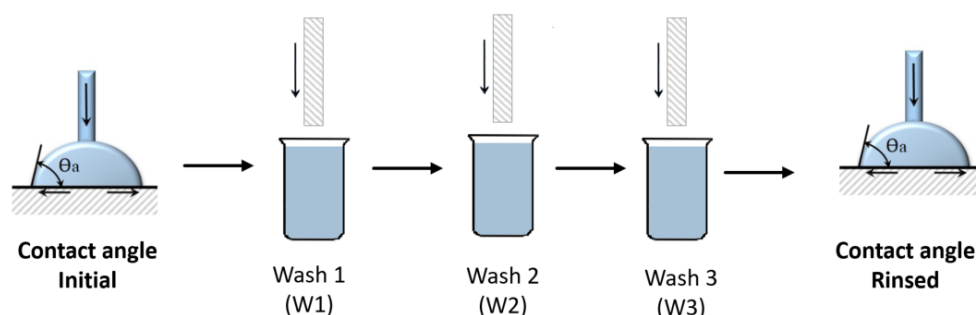


Figure 5.1 Procedure for evaluating leaching of Sn by submersion in three beakers of DI water with initial and rinsed water advancing contact angle measurements being taken. Full experimental details can be found in Chapter 3.

The water advancing contact angle θ after rinsing three times was shown to remain consistent across all samples after the three washes (W1, W2 and W3). It was observed that for first rinse, residual unbound SnO₂ nanoparticles that are weakly attached at the surface, were removed for both coatings. But after the third wash (W3), the washing collected contained only 34.2 µg/L for Film A coatings and 12.9 µg/L for Film B of Sn, in comparison to analysis of Sn in the first wash (W1) which showed 790.6 µg/L for Film A and 246.8 µg/L for Film B, see **Figure 5.2 (b)** and **(c)**. Therefore, it was concluded that surface leaching is negligible after the initial rinsing step (W1) which removed the unbound SnO₂ residues that had been left from the dip-coating procedure. Furthermore, this level of leaching after W3 is not considered as hazardous given that Sn oxides are commonly used in various applications where exposure levels are high. For example Sn oxides are used in cosmetics as an abrasive, bulking, and opacifying agent which is then applied to the hair or skin, and also used as colorants in food-contact polymers [419]. Additionally, the FDA advises that tin-dioxide concentrations in food

should not exceed more than 1% of the weight of the food [420]. This 1% level of consumption is a lot higher level of exposure compared to the potential leaching and exposure from Films A and B coatings found. Furthermore, it can be observed that sulfuric-chromic acid etching of HDPE has shown to reduce SnO₂ nanoparticle leaching, specifically for 10-Film B even after the initial rinse, see **Figure 5.2 (b)**. The observations are supported by previous research, where oxidation of PE improves SnO₂ sol adhesion as a coating [350].

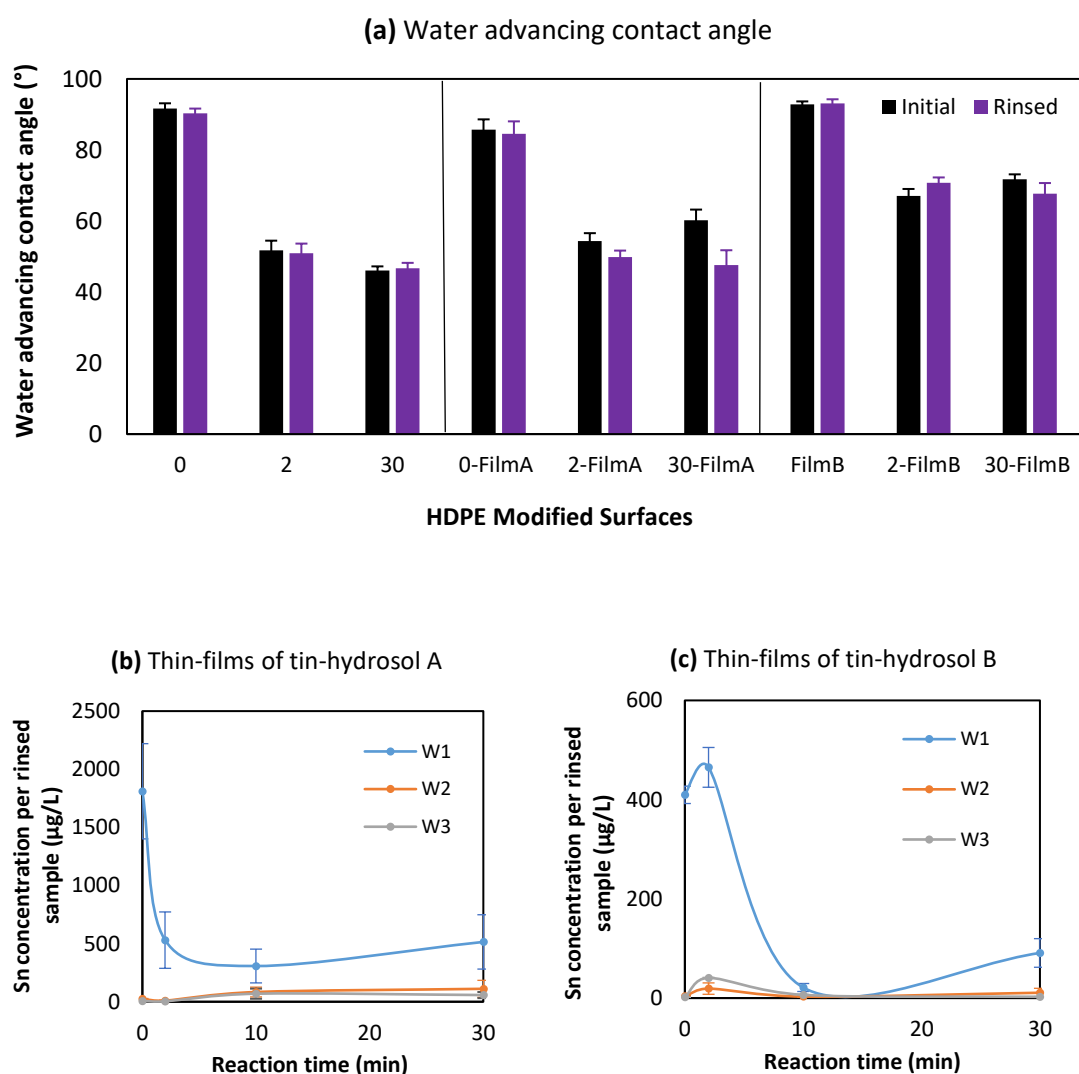


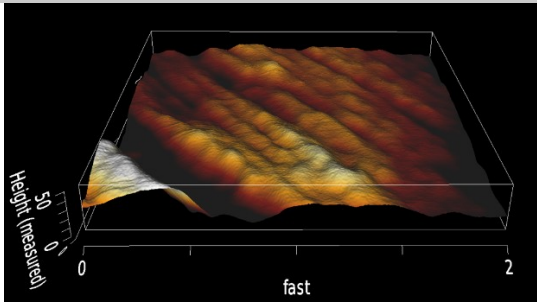
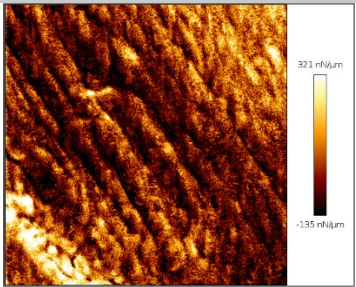
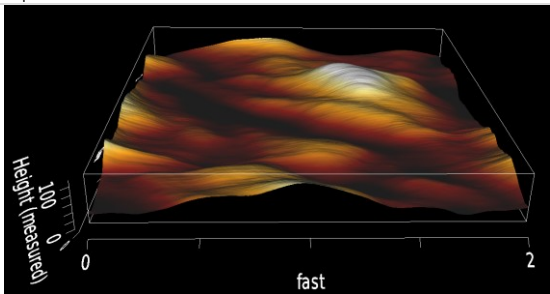
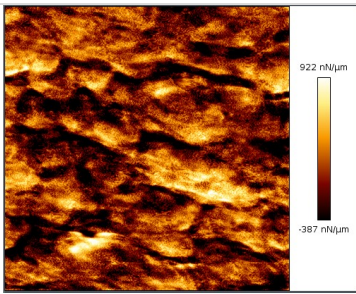
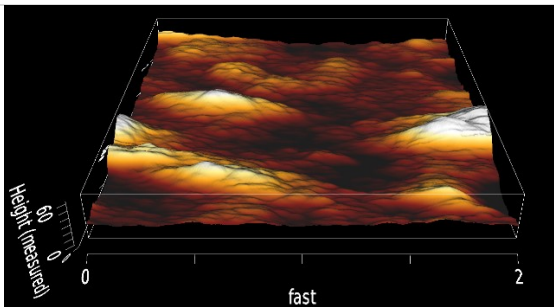
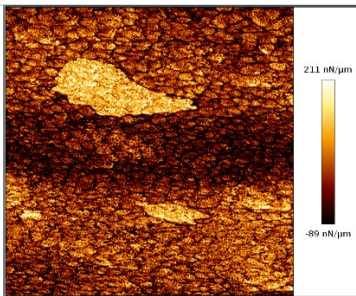
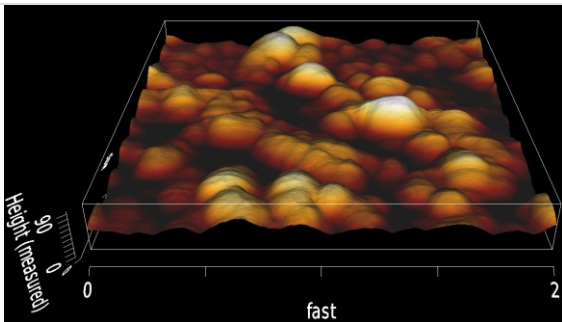
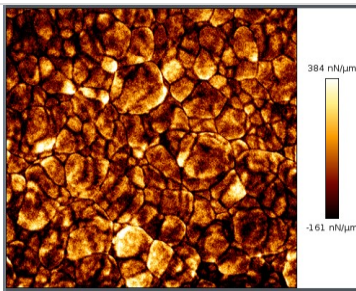
Figure 5.2 (a) Water advancing contact angle θ measurements of surfaces before and after rinsing in DI water whilst (b) and (c) show ICP-MS results of Sn concentration found from three DI water rinsing for Film A and Film B coatings respectively.

The water advancing contact angle θ does not significantly change after rinsing in DI water suggesting that overall film surface chemistry and stability is maintained during the short-term exposure to an aqueous environment, see **Figure 5.2 (a)**. This property is vital to monitor as early-stage biofilm studies performed on these samples are in a hydrated environment and therefore film stability is required in the short-term aqueous condition. It is notable that Film A, Film B and the untreated HDPE have similar water advancing θ for non-oxidised surfaces. Therefore, the coating with SnO₂ does not alter the hydrophobicity/hydrophilicity of the original HDPE polymer significantly as anticipated. This observation is contrary to previous studies reporting SnO₂ films to exhibit strong hydrophilic surface properties using the same synthetic procedure, after 60 s of dip-coating with PE [350,418]. In fact, the tin-hydrosols prepared by Briscoe *et al.*, where the synthetic procedure has been adopted from, has shown water advancing contact angle θ to decrease by 39 ° when coated onto PE. A significant decrease transforming the non-wetting PE surface into a more wetting surface. The lack of a clear reduction of surface hydrophilicity in this system following tin-hydrosol treatment may be attributed to differences in the polymer surface material backbone selected or based on efficacy of coating the untreated HDPE surface. Evaluation of the film's composition will be further explored later in this Chapter.

AFM images were obtained to visualise the surface coverage and evaluate the particle coatings on HDPE and sulfuric-chromic acid oxidised HDPE, see **Figure 5.3**. The AFM images were obtained for the slope (stiffness) and topography (height measured) automatically from each force-distance curve. **Figure 5.3** shows the 3D roughness (2 x 2 μ m scale) and 2D stiffness (slope), as well as the R_a and R_q roughness parameters for the height (measured) image. AFM imaging was important to confirm the presence and size of the SnO₂ particles on the HDPE surfaces with and without HDPE 30 min sulfuric-chromic acid surface oxidation. It was found that the nanoparticles are randomly distributed on the surface with an average particle size for Film A (without HDPE oxidation) of around 2.1 nm. However, in the presence of 30 min sulfuric-chromic oxidised HDPE, 30-Film A, gave a particle size of 10.1 nm. On the other hand, Film B and 30-Film B had SnO₂ nanoparticle sizes of 28.1 and 21.2 nm respectively. The nanoparticle size/grain size was determined using the cross-sectional analysis from AFM

height imaging of the thin-films (see Appendix under Chapter 5[F]). It can be concluded that overnight aging to prepare tin-hydrosol B from tin-hydrosol A has doubled the nanoparticle size due to increased agglomeration events, based on both AFM and DLS data sets.

The AFM results from 30-Film A, 0-Film B, 30-Film B largely supports the number distribution results from the DLS measurements. This view is supported by the literature where the number distribution from DLS measurements of metal oxide nanoparticles has shown to fit well for monodispersed samples when compared to other methods for particle size determination such as TEM [421]. Additionally, the number distribution has been most commonly used to determine SnO₂ particle size using DLS by other researchers [422,423]. Film B shows a very smooth surface with an R_a of 0.5 nm. However visually, the nanoparticle size from Film A and 30-Film A images look very similar (**Figure 5.3**), and therefore the variation in height in 30-Film A is also contributed by the oxidised HDPE surface beneath. Surface coverage is largely uniform with the 2 mins dip-coating submersion approach for the oxidised materials.

	3D Roughness	2D Stiffness
Control (HDPE)	 2000 nm x 2000 nm $R_a = 5.6$ nm $R_q = 7.4$ nm	
HDPE 30 min oxidised	 2000 nm x 2000 nm $R_a = 14.78$ nm $R_q = 19.17$ nm	
30-Film A	 2000 nm x 2000 nm $R_a = 6.0$ nm $R_q = 8.1$ nm	
30-Film B	 2000 nm x 2000 nm $R_a = 10.5$ nm $R_q = 13.3$ nm	

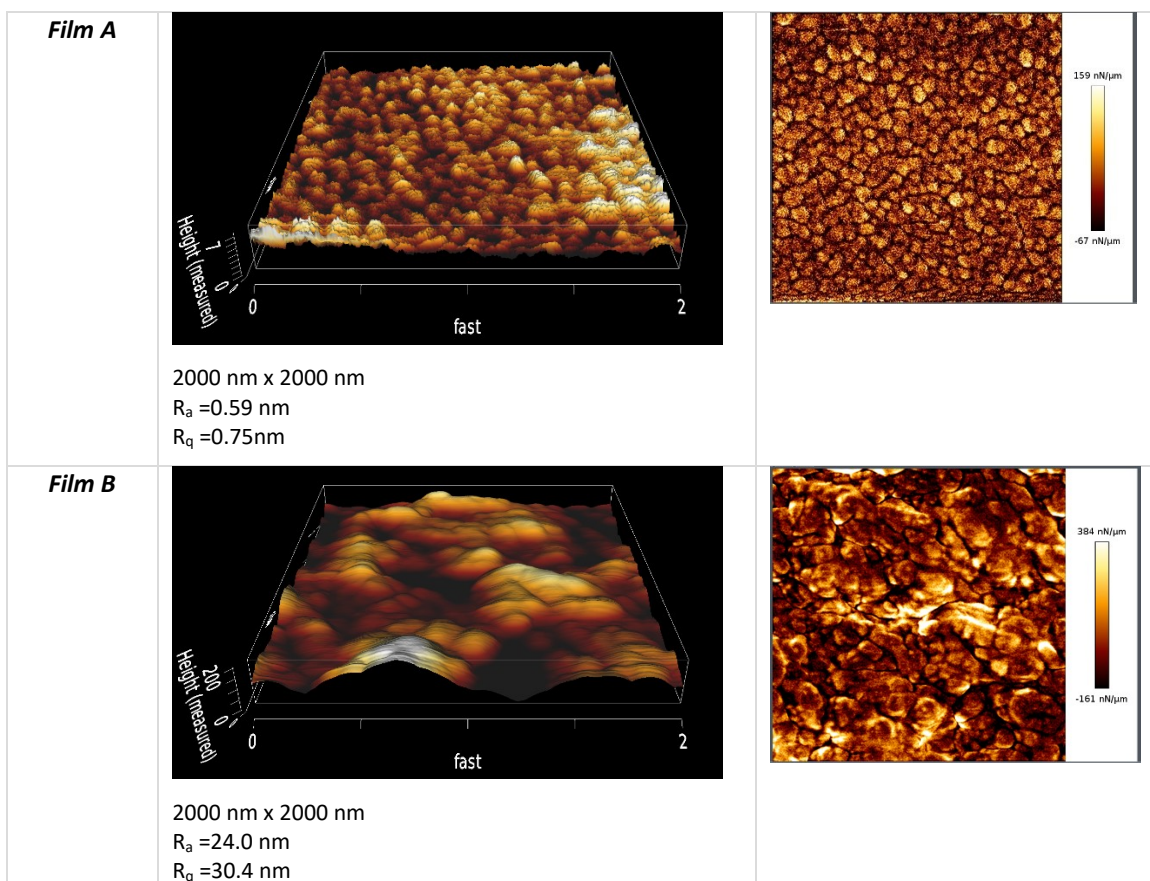


Figure 5.3 AFM images scaled to 2000 nm x 2000 nm with resolution 512 x 512 for surface stiffness and 3D height corresponding images captured are presented using JPK AFM system in QI™ mode. Oxidisation treatment method used refers to 30 min sulfuric-chromic acid chemical treatment at 70 °C.

Surface free energy γ_{SV} calculations were performed to quantify the changes made to the various HDPE surfaces using advancing contact angle data for water and DIM. Fowke's Theory has been utilised to calculate the surface free energy γ_{SV} at the substratum, see **Figure 5.2 (a)**. In Chapter 4 it was observed that an increase in surface polymer hydrophilicity is seen upon increasing surface oxidation with sulfuric-chromic acid etching of HDPE and these results are presented again in **Table 5.2** for comparison. The presence of the SnO₂ thin-films on the oxidised HDPE materials has caused an increase the hydrophobicity of these surfaces. This behaviour is expected as SnO₂ has previously been reported to exhibit water advancing contact angle θ of about 60 ° for materials prepared using this sol-gel method [350]. Furthermore, 2-Film A has shown

to not significantly change in its contact angle θ values which suggests that 2 min oxidisation may not be sufficient in aiding surface adhesion of Film A, **Table 5.2 (b)**.

HDPE Modified Samples <i>Oxidisation time (min)</i>	Advancing Water θ (°)	Advancing DIM θ (°)	γ_{sv}^D (mJ/m ²)	γ_{sv}^P (mJ/m ²)	γ_{sv} (mJ/m ²)
0	91.7 +/- 1.4	58.8 +/- 3.1	29.3 +/- 1.8	0.5 +/- 0.8	29.8 +/- 2.6
2	51.7 +/- 2.7	47.7 +/- 2.7	35.6 +/- 1.5	12.3 +/- 3.6	47.8 +/- 2.2
10	46.4 +/- 1.5	34.4 +/- 2.3	42.3 +/- 1.1	10.4 +/- 1.8	52.7 +/- 1.2
30	46.0 +/- 1.2	28.3 +/- 2.1	44.9 +/- 0.8	8.8 +/- 1.3	53.7 +/- 1.0
0-Film A	85.7 +/- 2.9	67.9 +/- 3.1	24.1 +/- 1.8	0.8 +/- 1.6	24.8 +/- 2.0
2-Film A	54.4 +/- 2.2	43.8 +/- 1.9	37.7 +/- 1.0	8.7 +/- 2.3	46.4 +/- 1.7
10-Film A	68.6 +/- 2.7	46.6 +/- 2.3	36.1 +/- 1.2	2.1 +/- 1.7	38.2 +/- 1.8
30-Film A	60.2 +/- 3.0	50.0 +/- 1.7	34.3 +/- 0.9	7.4 +/- 2.8	41.7 +/- 2.2
0-Film B	92.9 +/- 0.8	62.0 +/- 2.1	27.4 +/- 1.2	0.4 +/- 0.4	27.8 +/- 1.7
2-Film B	67.1 +/- 1.9	45.3 +/- 1.6	36.9 +/- 0.9	2.4 +/- 1.2	39.3 +/- 1.3
10-Film B	72.6 +/- 3.8	47.7 +/- 1.1	35.6 +/- 0.6	1.0 +/- 1.4	36.6 +/- 1.6
30-Film B	71.7 +/- 1.4	47.1 +/- 1.8	35.9 +/- 1.0	1.2 +/- 0.8	37.1 +/- 1.1

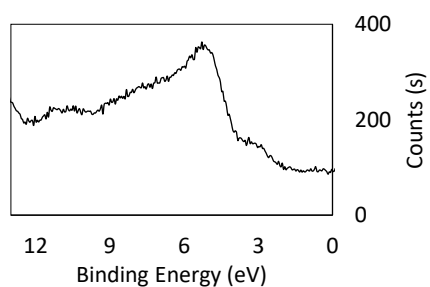
Table 5.2 Surface wettability and Fowke's surface free energy measurements versus surface modification against increasing surface oxidisation time of 0, 2, 10 and 30 min and coating with Film A and Film B.

Samples were analysed under XPS to examine the elemental composition present at the surface, see **Table 5.3** (full survey scans in Appendix under Chapter 5[G]). A significant surface presence of Sn species is observed for all coated samples. Valence XPS scan were conducted for both Film A and B coatings showing that tin dioxide is present rather than the mono-oxide form, due to the distinctive three peak pattern that appears between 0-13 eV that is descriptive of the dioxide specie, see **Figure 5.4**. Literature valence scans from XPS of SnO and SnO₂ can be found in **Figure 5.5** [424]. Furthermore, the Sn:O ratio shows that there is approximate but consistent 1:2 ratio which corroborates with the presence of the di-oxide species. Comparison of the untreated control HDPE with the Sn coated surfaces shows that there is clear evidence suggesting the coating procedure is successful with the addition of significant SnO₂ at the surface.

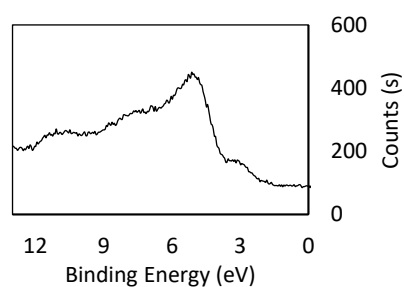
	% Atomic Composition					
	control	30 min	0-Film A	30-Film A	0-Film B	30-Film B
C	98	28.6	71.2	44.0	54.1	33.1
O	2	47.5	16.4	37.1	30.6	43.0
Sn	0.0	0.0	8.3	16.7	12.4	22.5
S	0.0	11.0	0.0	0.0	0.0	0.0
Cr	0.0	2.6	0.0	0.0	0.0	0.0
K	0.0	6.7	0.0	0.0	0.0	0.0
Sn:O ratio	NA	NA	1:98	1:2.2	1:2.5	1:1.91

Table 5.3 % Relative atomic abundance from XPS measurements taken for SnO₂ coated and uncoated HDPE samples (N=1, 400 µm spot size). The 30 min refers to the time of sulfuric-chromic acid oxidation pre-treatment performed on the HDPE prior to coating with tin-hydrosol solutions A and B.

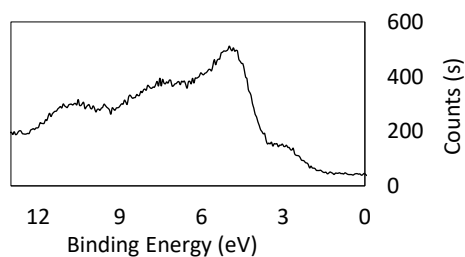
(a) 0-Film A



(b) 0-Film B



(c) 30-Film A



(d) 30-Film B

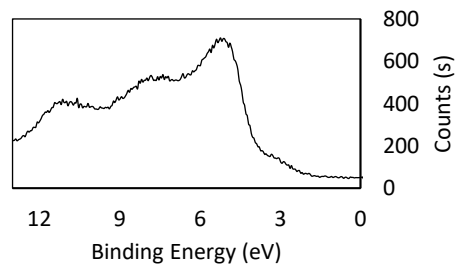


Figure 5.4 XPS valence scan obtained for HDPE with Film A and Film B for 0 min and 30 min oxidised HDPE samples, showing the distinctive peaks associated with the presence of SnO₂.

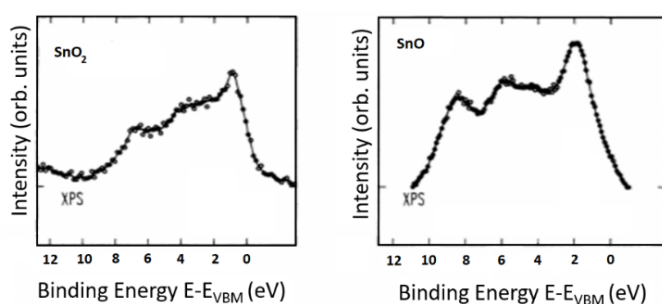


Figure 5.5 Experimental valence-band XPS spectra of SnO_2 and SnO by Themlin *et al.*, 1992 [424].

5.2.3 Antimicrobial Performance of SnO_2 Coated HDPE: Initial Attachment

An investigation of bacterial attachment to form an early-stage biofilm following a 30 min static inoculant exposure to challenge surfaces was performed and evaluated using CLSM imaging. The results obtained revealed that the more hydrophilic surfaces (sulfuric-chromic acid etched) show reduced adhesion of *P. aeruginosa*, see **Figure 5.6**. However, similar studies performed by Palmer *et al.*, using a variant of this assay, has discovered that hydrophobic materials on inert surfaces such as polymers and stainless steel, show reduced *P. aeruginosa* adhesion which is SFE γ_{SV} dependent [363]. The observation of hydrophilic sulfuric-chromic acid etched HDPE showing reduced adhesion of *P. aeruginosa* was reported in Chapter 4. This same performance is also observed here when there are SnO_2 coatings present, such as Film A and Film B, with increased oxidation times preceding the SnO_2 coating, generally leading to reduced early-stage *P. aeruginosa* biofilm development. Therefore, surface chemical oxidation is essential in reducing attachment of *P. aeruginosa*, as without, the total amount of early-stage biofilm does not significantly decrease. These observations are in line with those reported in Chapter 4, however, there is also some reduction in attachment when there is no oxidation of HDPE, 0-Film A or 0-Film B. Example CLSM images of this phenomena has been provided for visual aid, see **Figure 5.6**, where the white dots represent the *P. aeruginosa* surface-bound cells to the polymer substrate.

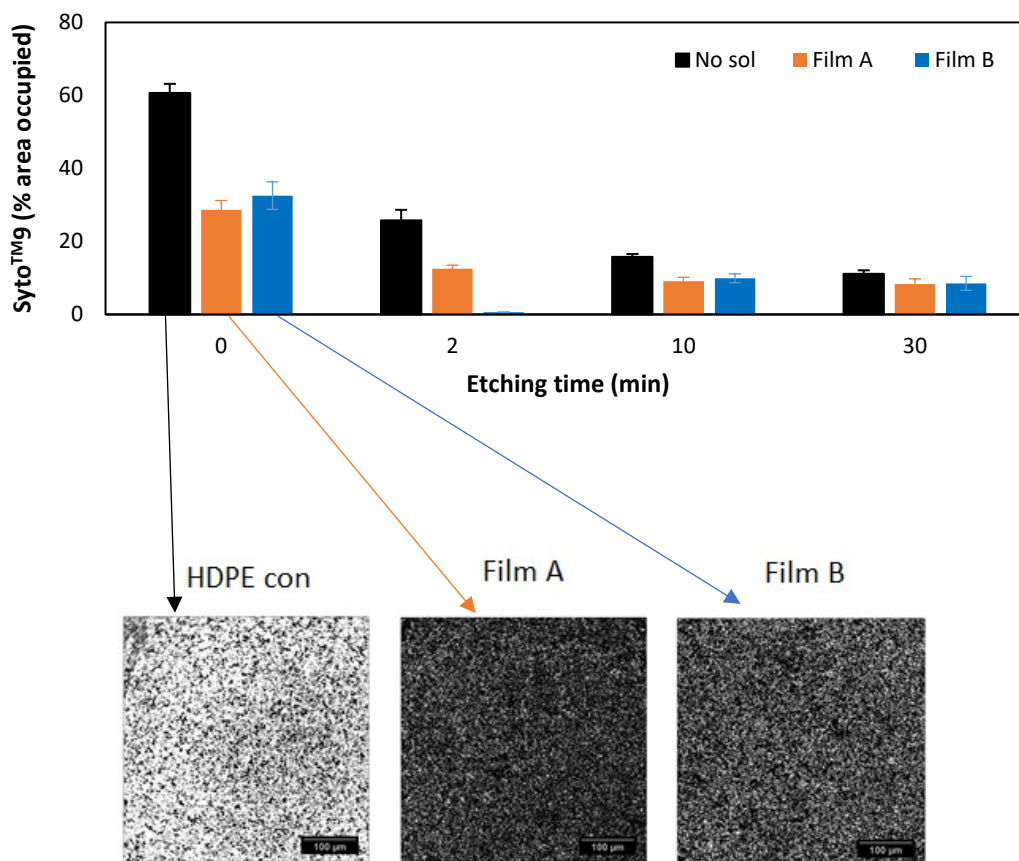


Figure 5.6 CLSM assessment of bacterial early-stage biofilm adhesion of *P. aeruginosa* present on the surface from SytoTM9 stained cells imaged upon 30 min surface inoculant exposure for a range of oxidised and SnO₂ coated HDPE materials. Number of images taken of different positions N=15-65, with a minimum of two biological repeats performed. Sample images have been provided for the HDPE control, as well as Film A and B coated samples, all without surface oxidation treatment, scale bar is set to 100 µm.

5.2.4 Antimicrobial Performance of SnO₂ Coated HDPE: Viability

A viability assay, combining SytoTM9 and PI as part of the LIVE/DEADTM BacLightTM Bacterial Viability Kit from InvitrogenTM, against *P. aeruginosa* was used to determine the viability of the early-stage biofilm using CLSM. This approach is beneficial due to the direct visualisation of the physiological state of the biofilm without disrupting the surface. In addition, a hydrated environment is achieved using a dip-lens, so that the biofilm is not disturbed due to dehydration. The results obtained are shown in **Figure 5.7**. The sulfuric-chromic oxidised HDPE results analysed in Chapter 4 have also been

included in this figure for comparison, which also serve as controls for this experiment. The CLSM experimental results using the Syto™9 and PI found that 0-Film-A and 0-Film-B alone, exhibit a high reduction in *P. aeruginosa* viability. This effect is then enhanced in the presence of surface oxidation using sulfuric-chromic acid from comparing the ratio of these stains using Comstat 2.1 and image-J analysis of the images. In this study it is also important to fully interpret the Syto™9 and PI channels separately, as the ratios from **Figure 5.7** can be misleading because the surfaces show different trends for PI and Syto™9 and are sample dependent, see **Figure 5.8**.

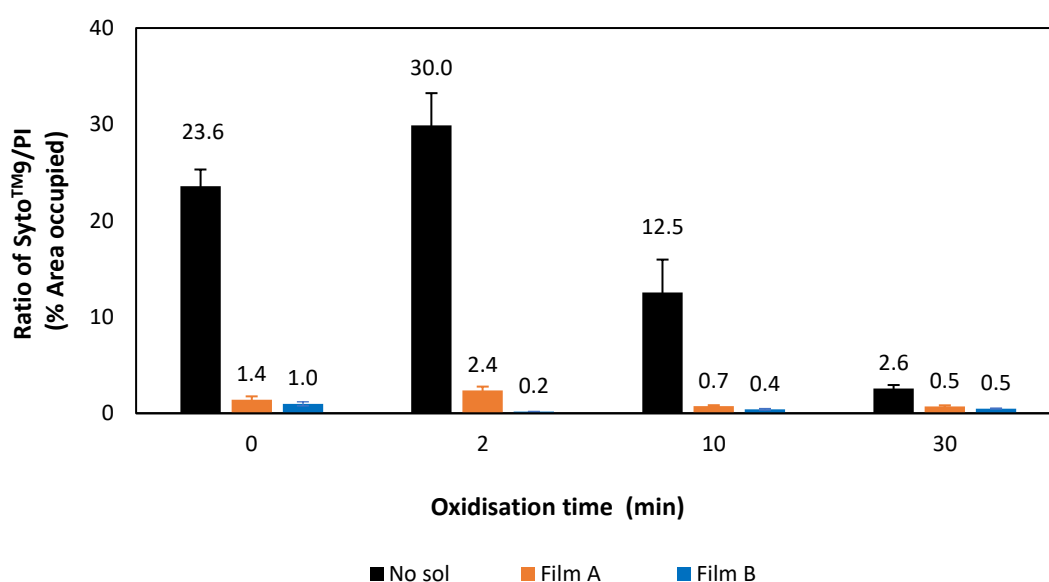


Figure 5.7 Cellular viability assay results, showing Syto™9/PI ratio which is the % area coverage occupied by stains Syto™9 compared with PI. Number of different samples tested, N=3-6 with 6-10 images taken per sample using 4 different bacterial cultures on different days.

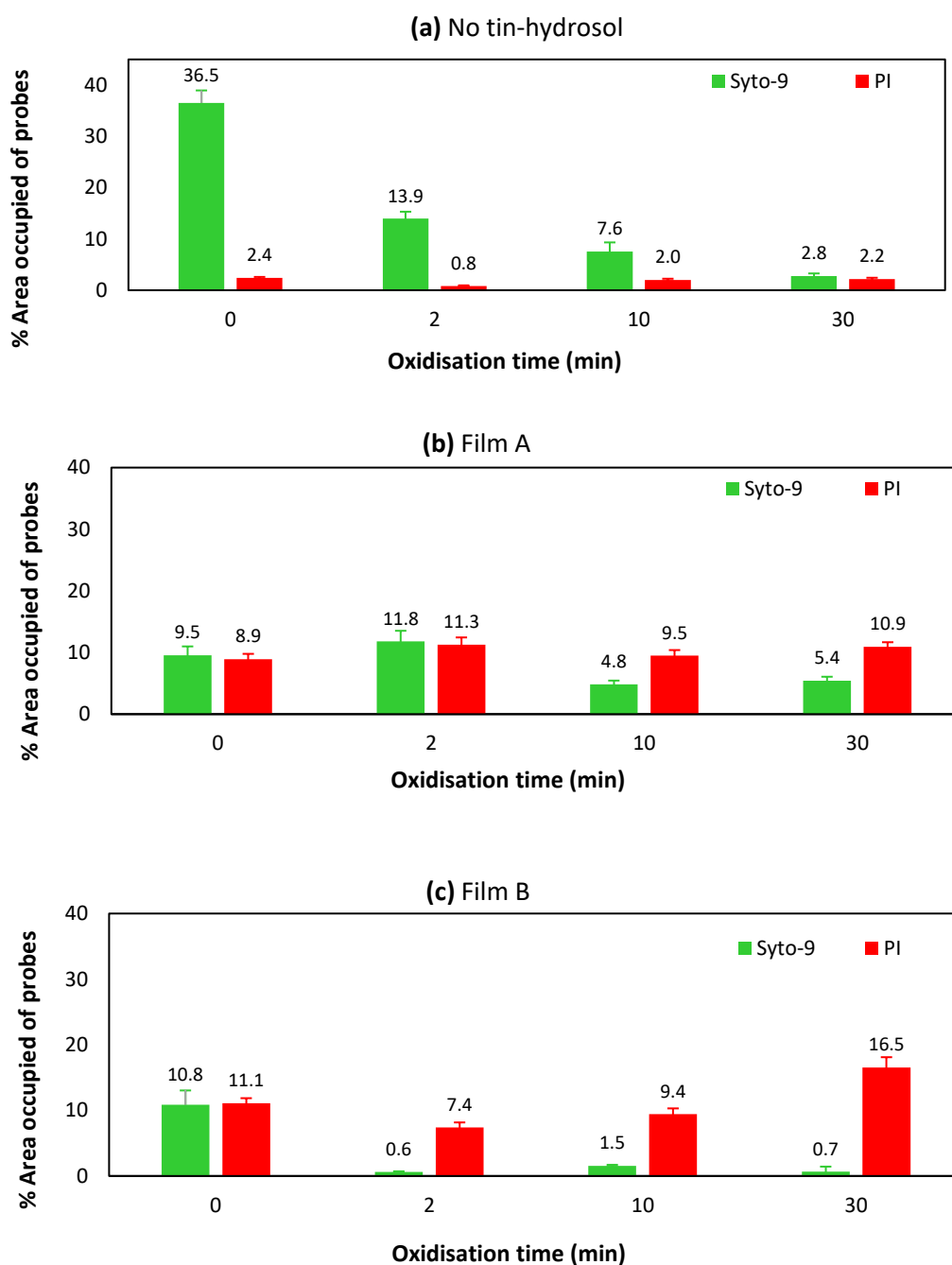


Figure 5.8 SytoTM9 and PI results with the SytoTM9 and PI channels values averaged separately for the % area occupied in layers from Comstat 2.1 plug-in for ImageJ for HDPE samples (a) without tin-hydrosol coating, (b) Film A, and (c) Film B, with increasing sulfuric-chromic acid oxidation time from 0 to 30 min.

The main advantage of interpreting and displaying the data as shown in **Figure 5.8** is that observations are clearer, as opposed to those in **Figure 5.7** which is the

recommended method by the kit supplier and reported by researchers. This method of data visualisation reveals that the sulfuric-chromic acid treatment of HDPE specifically influences adhesion significantly when looking at the SytoTM9 channel, however the level of PI remains constant, see **Figure 5.7 (a)**. It is worth noting that Chapter 4 also revealed that sulfuric-chromic acid oxidation influenced viability as the remaining cells on the surface were shown to be membrane depolarised. Films of tin-hydrosol A showed there to be a significant increase in the PI signals compared to the non-coated surfaces, confirming much higher levels of non-viable cells directly present on the surface. The SytoTM9 signal of films of tin-hydrosol A show a reduction in the intensity compared to the untreated HDPE sample; with the 10- and 30-Film A leading to more reduced SytoTM9 signals due to the lower number of adherent cells, see **Figure 5.7**.

On the other hand, Film B exhibits markedly reduced SytoTM9 signal, and the PI channel increased significantly, especially for 30-Film B, see **Figure 5.7 (c)**. This data would imply that there are reduced numbers of cells adhered, and increased numbers of non-viable cells. To understand this further, example images have been provided of a selection of samples in **Figure 5.9**. These images suggest that the control surface, untreated HDPE, has almost no PI signal and a large quantity of SytoTM9 stained cells present in the SytoTM9 channel, which can be attributed to the early-stage biofilm being highly viable under these conditions. Film A shows the quantity of SytoTM9 and PI stained cells to be comparable to one another. The example images provided for Film B shows local variations in the early-stage biofilm viability where the right side is more membrane damaged, from an increase in PI staining. This difference may be due to variations caused by manual dip-coating. It is important to note that SytoTM9 and PI are competitive for the same receptors, although SytoTM9 technically stains live and dead cells. In the presence of PI the intensity of SytoTM9 reduces as PI is taken up by these receptor sites. This behaviour is a known phenomenon and has also been confirmed in previous calibration studies for these experiments (see Appendix Chapter 4[D] for calibration results). This behaviour also explains the results from Film B, where there is a shift in probe absorption based on the region where cells have exhibited a loss in viability (membrane damage) as these stains are competitive with each other.

Therefore, it can be considered that when Syto™9 is used with PI that Syto™9 can be considered as a live stain and PI as a dead stain.

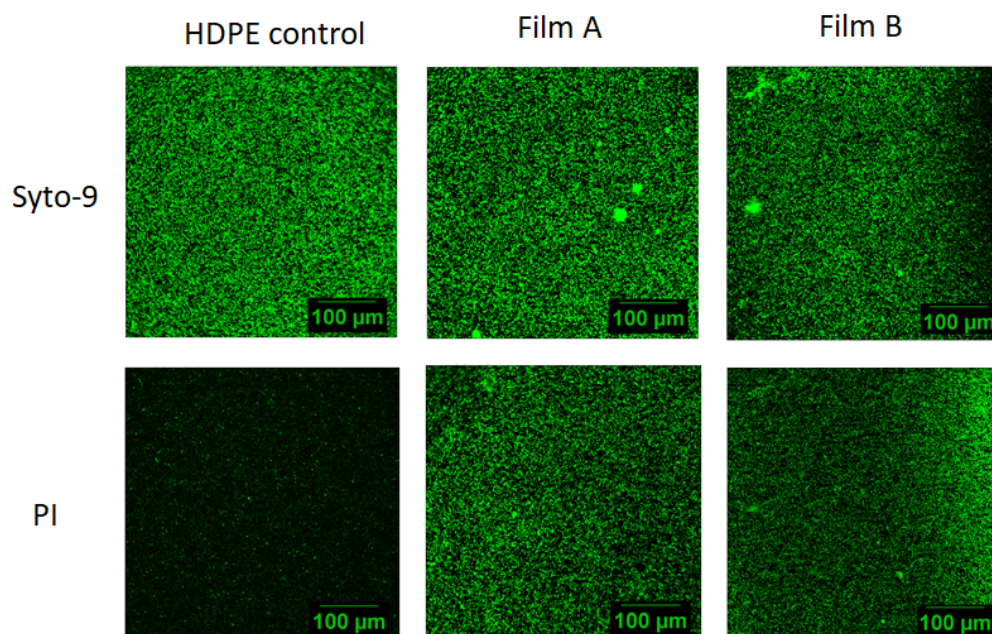


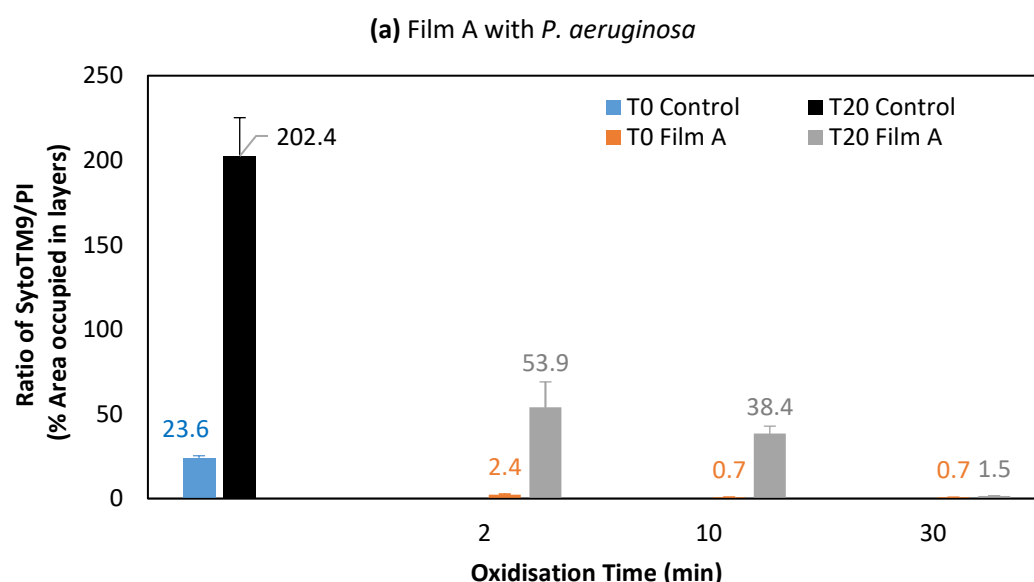
Figure 5.9 Selected sample CLSM images of HDPE samples without any pre-treatment (sulfuric-chromic oxidation). This shows the effect of the SnO₂ films on the viability of the early-stage biofilm.

5.2.5 Antimicrobial Performance of SnO₂ Coated HDPE: Extended 20 min Study

An extended study was performed which investigated the effect of leaving the early-stage biofilm for an additional 20 min under saline conditions (termed T₂₀) to examine the effect of these surfaces on antimicrobial properties. This test was also evaluated using CLSM viability imaging using Syto™9 and PI stains from the viability BacLight kit from Invitrogen. The aim of this was to study how the early-stage biofilm would develop under an extended time of 20 min (T₂₀) and to evaluate a different environment on the viability. The experiment was similar to that performed as outlined in Chapter 3, Section 3.4.5, but modified by the addition of a saline solution for 20 min

prior to the dye exposure stage. Detailed explanation of the procedure can be found in the methods and materials section under Chapter 3 Section 3.4.6 (a method flow chart is provided in the Appendix under Chapter 5[H]).

These experiments were performed for SnO₂ coated HDPE samples with sulfuric-chromic acid oxidation (2, 10 and 30 min), and compared to HDPE untreated (control) surface which has no tin-hydrosol present, see **Figure 5.10**. The longer time provided with saline allowed the biofilm to mature and grow which is represented by the increase in signal intensity for the ratio of Syto™9:PI, when compared to the untreated HDPE. However, the presence of the SnO₂ coatings has significantly reduced growth, even with the extended 20 min in saline, particularly for 30-Film B and 10-Film A samples.



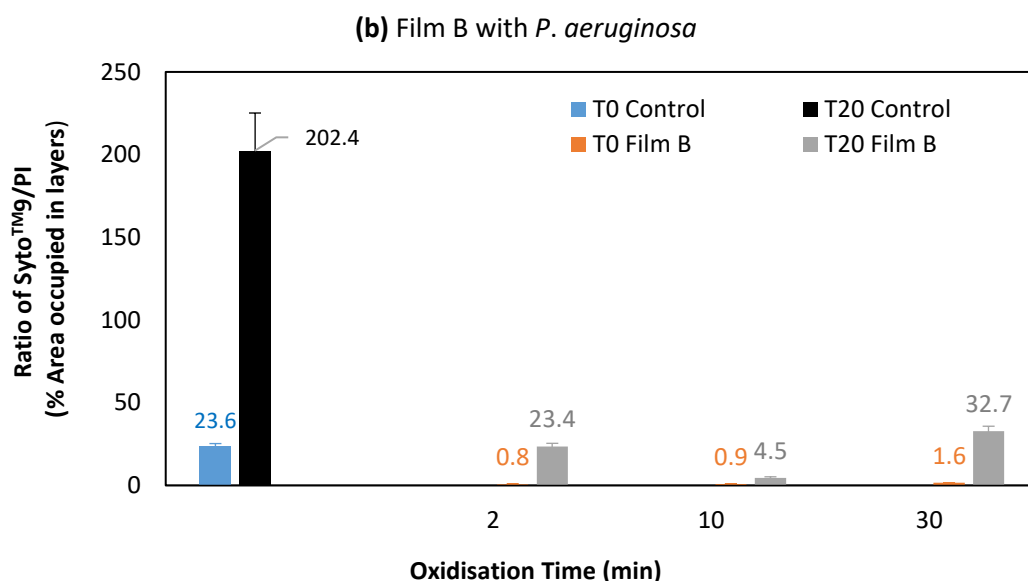


Figure 5.10 T_{20} saline delay experiment of Film A (a) and Film B (b) coated onto HDPE surfaces with sulfuric chromic acid oxidation. The controls data are on the two left hand columns representing untreated HDPE with no coating or oxidation. The ratio has been obtained from Syto[™]9/PI from CLSM imaging, $N=3$ with a minimum of 4 images taken of each sample for *P. aeruginosa*.

Evaluation of the separate Syto[™]9 and PI channels reveals that the control untreated HDPE surface shows little PI signal at T_0 and this level is further reduced at T_{20} **Figure 5.11 (c-d)**, whilst the Syto[™]9 intensity increases from T_0 to T_{20} . This suggests that the extended 20 min in saline has allowed *P. aeruginosa* species to grow in numbers and thus viability. Whereas the oxidised HDPE with the SnO₂ coatings have provided reduced Syto[™]9 intensity at T_{20} compared to the untreated HDPE, with increased PI signal intensity, particularly for 30-Film A where the level of PI has maintained after 20 min in saline at about 11%.

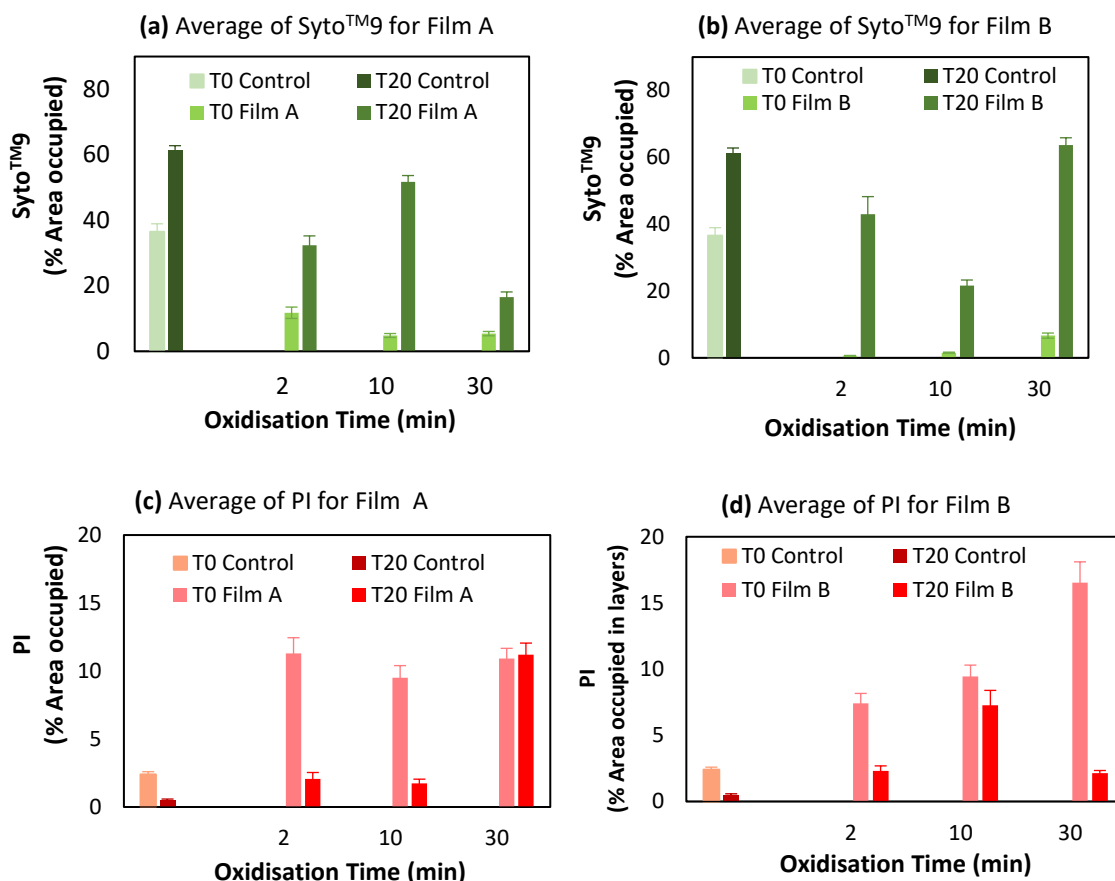


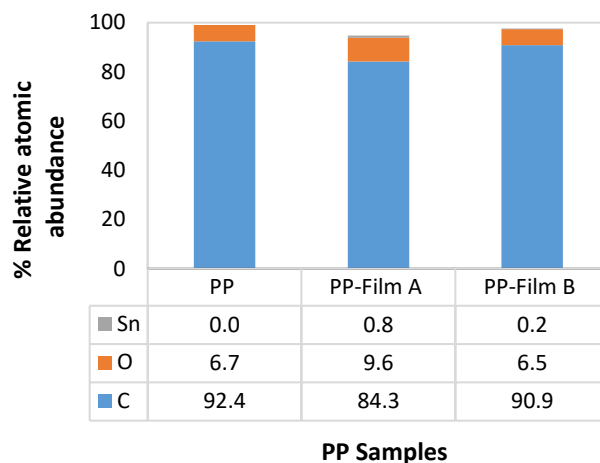
Figure 5.11 Average CLSM Syto™9 and PI channel values for Film A and Film B on untreated and sulfuric-chromic oxidised HDPE materials, where (a) Syto™9 values for Film A, (b) Syto™9 values for Film B, (c) PI values for Film A, and (d) Syto™9 values for Film B.

5.2.6 Antimicrobial Performance of SnO₂ on Other Polymers

The performance of coating tin-hydrosol A and B onto a range of other industrial polymers was also investigated. The polymers used were PP and PC as these two polymers have not been surface oxidised before coating with the tin-hydrosols and are compared with HDPE untreated and 30 min sulfuric-chromic acid oxidised HDPE samples. The materials were analysed under XPS to determine the composition of Film A and Film B formed on these polymers. The XPS results for HDPE and 30-HDPE surfaces coated with the SnO₂ sols (Film A and Film B) are shown previously in **Table 5.3**, Section 5.2.2. The XPS results obtained for PP and PC show that the Sn content is not significant for these materials, although it is important to note that N=1 for this study with a 400

μm spot size examined. PC contains a significant O content because of its carbonate repeating units.

PP



PC

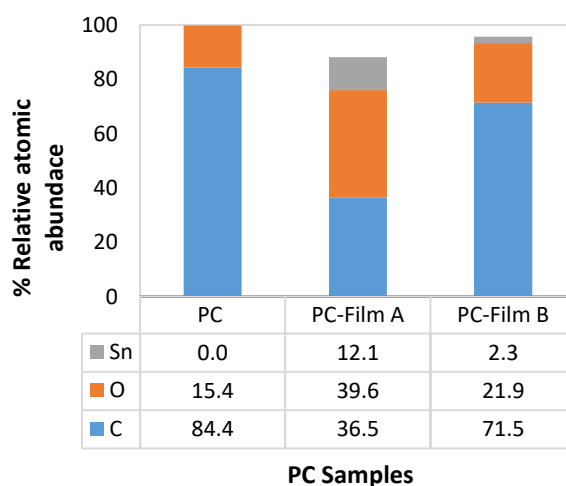


Figure 5.12 XPS results from Film A and Film B formed on untreated PP and PC polymers, as well as the uncoated control materials provided, in terms of % relative atomic elemental abundance.

AFM was performed on these materials as shown in **Figure 5.13**. A distinctive nanoparticle size could not be clearly determined from these AFM images.

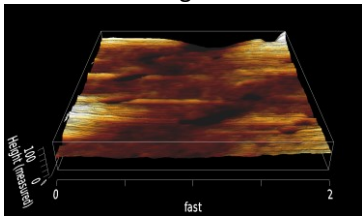
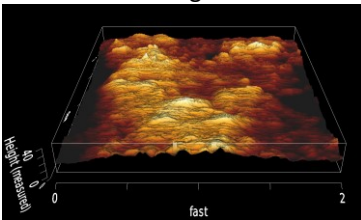
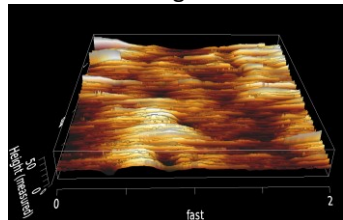
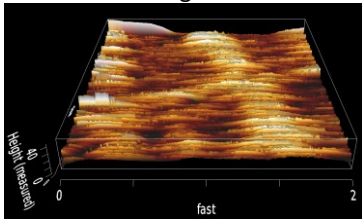
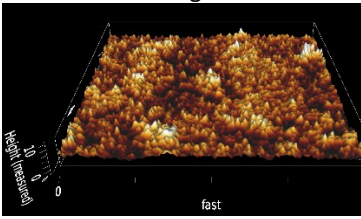
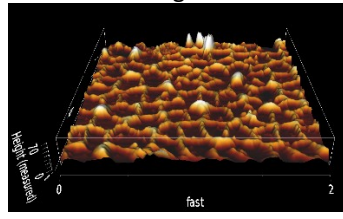
PP 3D Roughness  $R_a = 4.7$ nm $R_q = 6.7$ nm 512 x 512	PP-Film A 3D Roughness  $R_a = 4.5$ nm $R_q = 5.6$ nm 512 x 512	PP Film B 3D Roughness  $R_a = 3.7$ nm $R_q = 4.8$ nm 512 x 512
PC 3D Roughness  $R_a = 2.6$ nm $R_q = 3.4$ nm 512 x 512	PC-Film A 3D Roughness  $R_a = 0.77$ nm $R_q = 1.0$ nm 256 x 256	PC-Film B 3D Roughness  $R_a = 5.1$ nm $R_q = 7.2$ nm 256 x 256

Figure 5.13 AFM images of PP and PC samples coated with tin-hydrosol A and tin-hydrosol B to form PP-Film A/B and PC-Film A/B with 2000 x 2000 nm scan area.

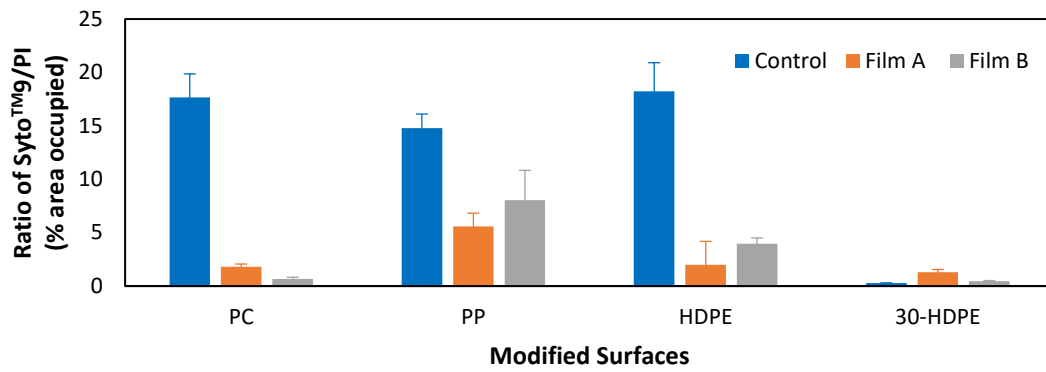
Advancing contact angle measurements were obtained using DIM and water. The samples were further rinsed and cleaned under aqueous conditions, and the contact angle measurements were repeated to determine the stability of these materials under short-term aqueous conditions. The surface free energies for these surfaces was determined using Fowke's Theory and are shown in **Table 5.4**. The surface free energies do not change significantly for PP following coating to form PP-Film A/B. However, for PC the thin-film has caused the surface to be more hydrophobic. The results obtained show that rinsing in water does not affect the water advancing θ which implies that film stability is maintained under these solution conditions.

Samples	DIM θ (°)	Water θ (°) Fresh	Water θ (°) Rinsed	Total Surface Free Energy (mJ/m ²)
PP	71.0 +/- 2.8	94.7 +/- 0.3	95.4 +/- 0.4	52.3 +/- 0.5
PP-Film A	71.4 +/- 2.1	90.2 +/- 1.0	88.6 +/- 1.2	27.0 +/- 1.1
PP-Film B	74.1 +/- 1.9	85.9 +/- 1.6	84.4 +/- 1.7	20.1 +/- 0.8
PC	18.9 +/- 0.6	84.5 +/- 0.4	85.9 +/- 0.4	22.3 +/- 1.7
PC-Film A	72.6 +/- 3.3	77.9 +/- 1.0	73.3 +/- 2.3	22.4 +/- 1.1
PC-Film B	75.1 +/- 1.3	96.1 +/- 1.4	101.0 +/- 0.5	22.6 +/- 1.2

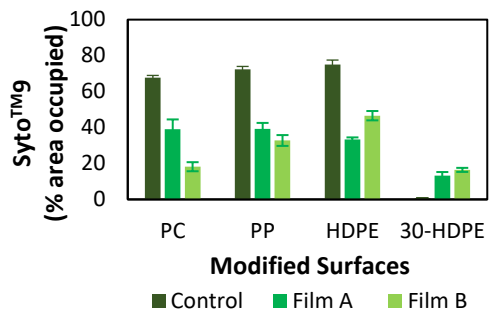
Table 5.4 Advancing contact angle results from PP and PC coated with tin-hydrosol A and tin-hydrosol B to form PP-Film A/B and PC Film A/B, (+/- SE), n=2 samples tested, with 6-9 advancing droplets evaluated. Fresh and rinsed water advancing θ have also been provided, the surface free energy has been calculated from the fresh water θ values.

Images were obtained using the CLSM microscopy using the LIVE/DEAD BacLight kit with the viability assay outlined in Chapter 3 under Section 3.4.5. This assay was performed on freshly prepared samples as well as samples that were exposed to ethanol overnight cleaning (80% EtOH). This was performed in order to evaluate the durability of these materials against ethanol cleaning (80% EtOH) as this is a common approach utilised for the sterilisation and sanitisation of surfaces. The data obtained is provided in **Figure 5.14**. This figure shows that the presence of the SnO₂ films has resulted in the reduction of viability, which is less prominent for the PP materials for the fresh samples. Furthermore, ethanol cleaning did not have a significant effect in the efficacy of these materials as antimicrobial surfaces against *P. aeruginosa*.

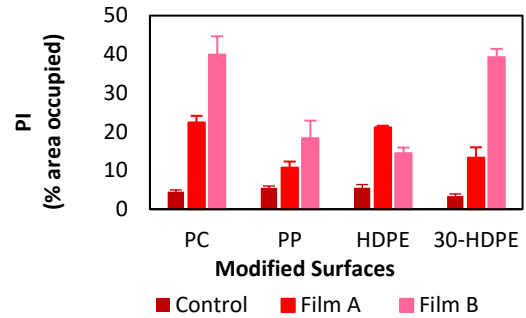
(a) Fresh samples



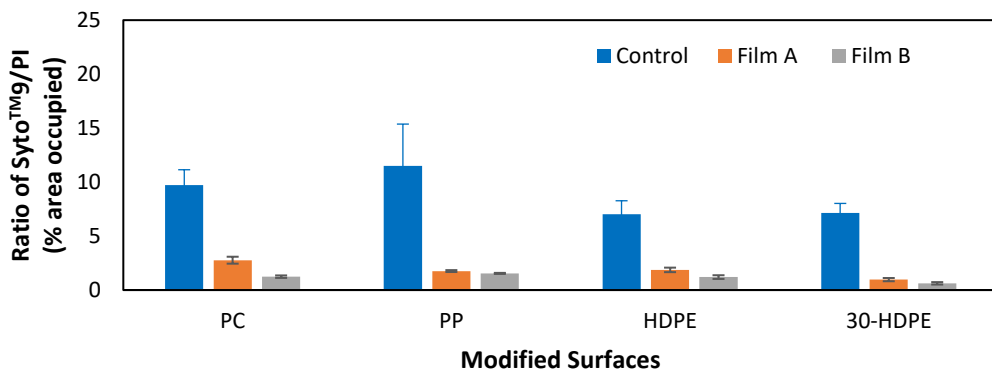
Fresh samples (Syto™9)



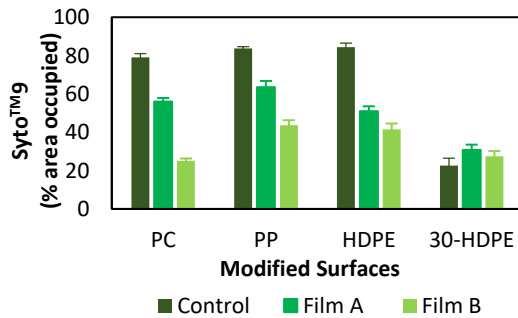
Fresh samples (PI)



(b) EtOH 24 h overnight results



EtOH 24 h (Syto™9)



EtOH 24 h (PI)

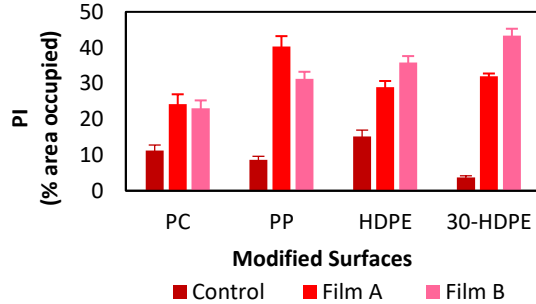


Figure 5.14 CLSM results on *P. aeruginosa* obtained from (a) fresh samples, and (b) EtOH 24 h rinsed samples of PP Film A/B and PC Film A/B samples. The data has been provided as a ratio of Syto™9/PI from using Comstat 2.1 plug-in for Image-J.

5.2.7 Viable Enumeration Assay of SnO₂ Coated HDPE Surfaces

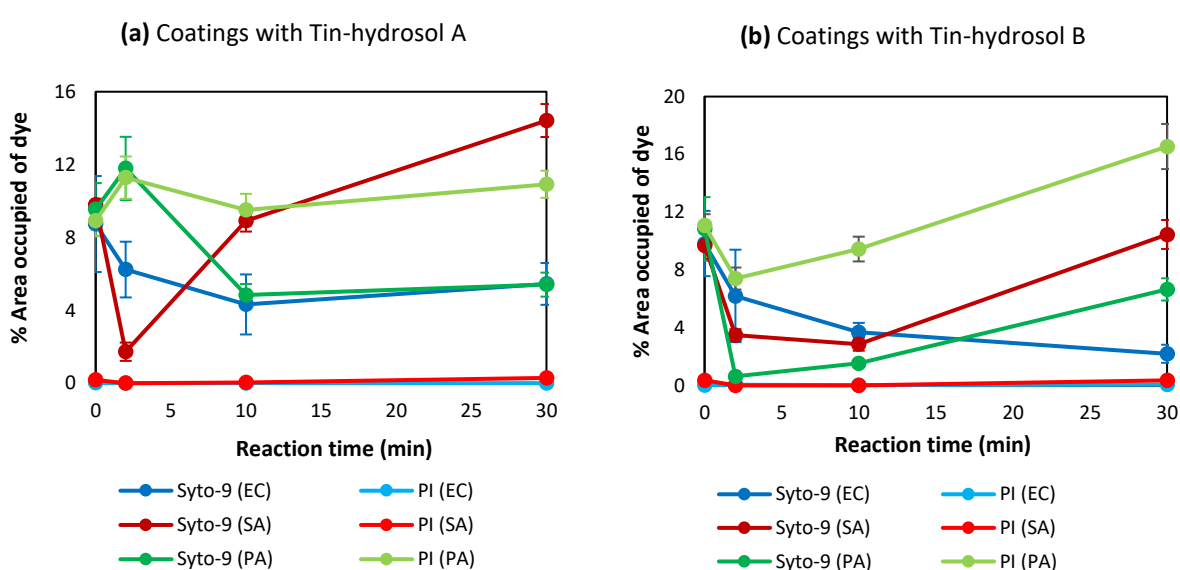
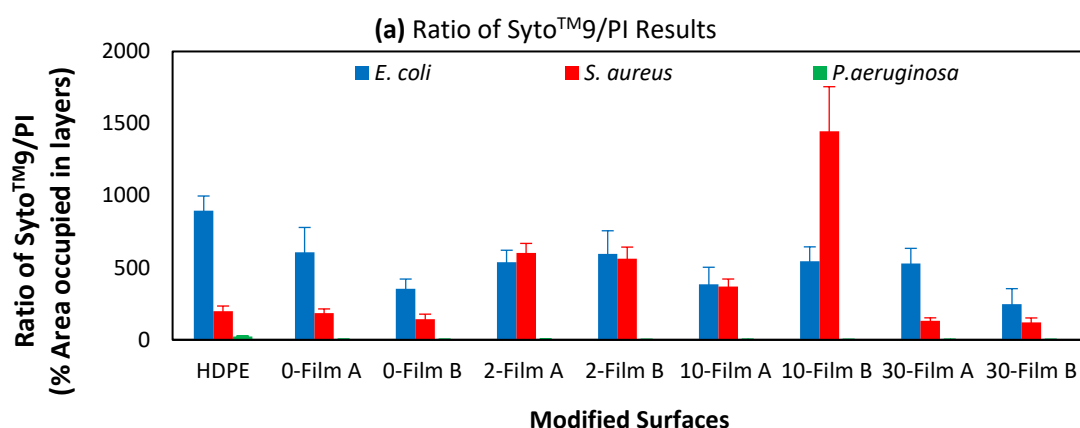
To corroborate and evaluate the findings from the CLSM experiments conducted the SnO₂ thin-film coated polymers, agar colony counting of the scraped early-stage biofilm was performed to determine the culturability of the recovered biofilm on these surfaces. Details for this method can be found in Chapter 3.6.2. It was important to compare the CLSM viability assay with other techniques because there are limitations of using CLSM and staining of bacteria alone. For example, it has been found that Syto™9 in the presence of PI can exhibit an over estimation of live cells when the dyes are combined in one system using *P. aeruginosa* [362]. This phenomenon was explained by the incomplete displacement of Syto™9 by PI in the dead cells. The results of these colony counting experiment are shown in **Table 5.5**. It was found that some of the Film A and Film B can reduce the bacteria growth by up to a 4-log reduction, however with the 2 min etched (2-Film A/B) surfaces no improvement was noted. The 10- and 30- Film A/B maintain a high level of antimicrobial loss of culturability and thus viability.

HDPE surfaces	<i>P. aeruginosa</i> CFU/mL Log ₁₀
HDPE	6.8 +/- 0.02
0-Film A	2.5 +/- 0.1
0-Film B	2.7 +/- 0.1
2-FilmA	6.3 +/- 0.1 {p=4x10 ⁻⁵ }
2-FilmB	6.4 +/- 0.04 {p=3x10 ⁻⁵ }
10-FilmA	3.6 +/- 0.1 {p=1.7x10 ⁻³ }
10-FilmB	3.6 +/- 0.1 {p=1.3x10 ⁻³ }
30-FilmA	4.2 +/- 0.1 {p=3.7x10 ⁻⁴ }
30-FilmB	4.3 +/- 0.1 {p=1.7x10 ⁻⁴ }

Table 5.5 Colony counting viability experiment for *P. aeruginosa* on Film A and Film B coated samples in log-scale, N=2 samples three repeats were performed. T-test was performed relative to respective controls, i.e. for 2-FilmA compared to 0-FilmA, {p=value}.

5.2.8 Evaluation of Other Bacterial Species on SnO₂ Coated HDPE

A CLSM early-stage viability experiment using LIVE/DEAD BacLight kit against *E. coli* and *S. aureus* organisms was conducted. *P. aeruginosa* CLMS viability results using the same kit which data was previously presented in **Figure 5.7** and **Figure 5.8** which are also included for comparison in **Figure 5.15**. The Syto™9/PI ratios calculated are extremely and abnormally high in the 500-1000 region. To fully interpret the Syto™9 and PI channels separately and comprehend the abnormal ratios, the results were translated to graphs as presented in **Figure 5.15 (b)** and **(c)**. Here the Syto™9 intensity for % area occupied is only between 4-10% with a very low PI intensities of < 1%. Given that the ratios are a division of the Syto™9 by PI, this data then leads to an extremely high and misleading viability value, hence 500-1000 region. This represents the limitations of using the Syto™9/PI ratios therefore it is advantageous to also evaluate the raw data of Syto™9 and PI data separately. Overall the results obtained suggest that the PI channels do not change upon increasing sulfuric-chromic acid oxidation for coatings with tin-hydrosol A and B. For the Syto™9 channels *E. coli* shows to tendentially reduce with increasing sulfuric-chromic acid oxidation time, for both tin-hydrosol A and B films. This result is not a surprise considering *E. coli*, like *P. aeruginosa* is a Gram-negative organism with often similar behaviour. However, *S. aureus* shows reduced Syto™9 signal for 2-Film A/B only, compared to the untreated HDPE control.



HDPE untreated SytoTM9 and PI values:

- *E. coli* (SytoTM9 12.1 +/- 2.98, PI 0.01 +/- 0.003)
- *S. aureus* (SytoTM9 10.7 +/- 0.9, PI 0.4 +/- 0.07)
- *P. aeruginosa* (SytoTM9 36.5 +/- 2.4, PI 2.4 +/- 0.2)

Figure 5.15 *E. coli* (EC), *S. aureus* (SA) and *P. aeruginosa* (PA) CLSM viability results using SytoTM9 and PI, (a) ratio of SytoTM9/PI; (b) the SytoTM9 and PI channels averaged for each oxidation time period for coatings with tin-hydrosol A; and (c) the SytoTM9 and PI channels averaged for each etching time period for coatings with tin-hydrosol B.

A colony counting experiment was also conducted for *E. coli* and *S. aureus* against a selection of samples, with the results provided in **Table 5.6**. Interestingly both films (A and B) found to have no negative effect on the culturability and hence viability of the early-stage biofilm for these organisms under agar growth. However, with *P. aeruginosa* reduction in viability/culturability has been seen which is more significant when HDPE sulfuric-chromic acid pre-treatment was not conducted, view **Table 5.6**.

Surfaces	<i>E. coli</i> CFU/mL (Log ₁₀)	<i>S. Aureus</i> CFU/mL (Log ₁₀)	<i>P. aeruginosa</i> CFU/mL (Log ₁₀)
HDPE	6.1 +/- 0.1	6.3 +/- 0.3	6.7 +/- 0.02
0-Film A	6.4 +/- 0.04	6.2 +/- 0.1	2.5 +/- 0.1
0-Film B	6.3 +/- 0.1	6.1 +/- 0.1	2.7 +/- 0.1
30-FilmA	6.4 +/- 0.05 {p=0.1}	6.1 +/- 0.2 {p=0.4}	4.2 +/- 0.1 {p=3.7x10 ⁻⁴ }
30-FilmB	6.6 +/- 0.1 {p=0.03}	6.2 +/- 0.2 {p=0.08}	4.3 +/- 0.1 {p=1.7x10 ⁻⁴ }

Table 5.6 Colony counting results of early-stage biofilm with *E. coli*, *S. aureus* and *P. aeruginosa* for tin-dioxide coated samples. T-tests were performed and compared to respective controls, i.e. for 30-FilmA results were compared to 0-FilmA, {p=value}.

A mature biofilm study was also performed on these samples against *P. aeruginosa* presented as **Table 5.7**. The results suggest that under hydrated environments these modified surfaces does not translate to a change in CFU or any related antimicrobial activity when cultured overnight. This loss of performance may be understood in terms of the leaching mechanism where antimicrobial materials have the capability of leaching significant amounts of antimicrobial active agents and chemicals over time. Leaching leads to the inhibition and reduction of the biofilm that can form in a hydrated environment. However, when the materials being investigated, as in this project, contain very limited amounts of antimicrobial species at the surface, these concentrations are unable to deliver an antimicrobial impact at the scale necessary to effect more mature biofilms.

HDPE Modified Samples	Mature Biofilm <i>P.aeruginosa</i> CFU/mL (Log ₁₀)
Untreated	7.9 +/- 0.4
30 mins sulfuric-chromic acid	8.2 +/- 0.3
30-Film A	8.6 +/- 0.3
30 min-Film B	8.7 +/- 0.2
Film A	8.1 +/- 0.3
Film B	7.9 +/- 0.1

Table 5.9 Mature biofilm study 24 h hydrated media rich environment with *E. coli* against various sulfuric chromic acid etched HDPE surfaces coated with Film A and Film B.

5.3 Conclusion

Tin-hydrosols were successfully synthesised using a sol-gel method to produce 2 solutions (tin-hydrosol A/B) with different particle sizes as confirmed by DLS and AFM. The tin-hydrosol B particles were approximately double the diameter of the tin-hydrosol A particles. Coating of these hydrosol particles onto various untreated control polymers and sulfuric-chromic acid oxidised HDPE has been performed successfully, with XPS confirming the distinctive valence scan for the presence of SnO₂ surface species. Sulfuric-chromic acid pre-treatment of HDPE has reduced SnO₂ surface leaching when these surfaces were rinsed, although levels of leaching overall are negligible and minimal after three rinses/washes under aqueous conditions.

Contact angle goniometry has confirmed that the hydrophilic effect of oxidising HDPE is still seen at the surface in the presence of the SnO₂ coatings (Film A/B), with an increase in oxidation reaction time translating to an increase in hydrophilicity. Furthermore, this oxidative pre-treatment has resulted in reduced microbial attachment of *P. aeruginosa* early-stage biofilm with increasing oxidation treatment time, whose effects were extended when in the presence of the SnO₂ coatings especially for Film A. CLSM has allowed the direct analysis of the viability of the early-stage biofilm of *P. aeruginosa* using fluorescent probes Syto™9 in conjunction with PI. These tests

have shown that sulfuric-chromic acid pre-treated HDPE when coated with Film A and Film B produced enhanced levels of viability loss, with higher PI signal intensity found. This behaviour was also maintained even after 20 min additional exposure of the biofilm to the surface under a saline environment. However, with the viable enumeration assay, the oxidised HDPE with Film A/B showed reduced viability growth only for 10 and 30 min oxidised samples, whereas the HDPE samples without pre-treatment that had been coated with Film A/B showed the largest reduction in colony counts compared to the HDPE untreated control. This suggests that different microbial assays can probe and reveal underlying aspects of bacterial behaviour, each with their own assumption, advantages and limitations. Herein, it can be concluded that the SnO₂ thin-films cause *P. aeruginosa* membrane damage, which in the presence of sulfuric-chromic acid oxidised HDPE, also caused a loss of microbial attachment, thus producing a more antibiofilm surface.

The application and performance of SnO₂ thin-films has been evaluated on PP and PC surfaces using a CLSM viability study, which revealed sustained antimicrobial properties for PC in particular, and only minimal activity with PP. This may be because these polymers were not sulfuric-chromic acid oxidised for heightened biocidal and antiadhesive properties to be seen, which is an area that could warrant investigation in the future. Furthermore, these samples were also investigated under a stressed environment, with overnight exposure to ethanol which showed sustained antimicrobial activity with high PI signal intensities seen.

S. aureus and *E. coli* were also investigated for HDPE coated tin-hydrosol samples, though these studies have shown no antimicrobial performance against these species using Film A and Film B coatings. Furthermore, a mature biofilm 24 h study was also performed, and it was found that under this extreme environment that such SnO₂ thin-films are unable to provide a reduction in viability when examined against *P. aeruginosa*.

CHAPTER 6

Antimicrobial Performance of Chitosan-ZnO nanocomposite coatings

6.1 Introduction

Ever since the broad antimicrobial capabilities of chitosan was proposed by Allan and Hardwiger in 1979, chitosan has attracted attention by many researchers [425]. Chitosan is a biodegradable, biocompatible, linear aminopolysaccharide, a copolymer of *D*-glucosamine and *N*-acetyl-*D*-glucosamine which is derived from the deacetylation of chitin. Chitin production has been estimated at approximately 10^{11} tonnes annually and is the second most abundant natural biopolymer [426]. The antimicrobial performance of chitosan has been evaluated against planktonic bacteria and biofilms of varying organisms extensively, including Gram-positive and -negative bacteria and fungi [427,428], as well as multi-species biofilm populations [429]. The antimicrobial activity of chitosan is highly dependent on several intrinsic factors based on the preparation of a chitosan film, including the MW, the degree of acetylation (DA) and the specific microorganism being tested [179,430] (refer to Chapter 2 Section 2.2.1). Chitosan's antimicrobial activity has been suggested to be driven by its influence on cell wall integrity. More specifically, previous studies have stated that the anti-biofilm property of chitosan is largely derived from its polycationic nature involving the functional amino groups ($-NH_2$) from the *N*-acetyl amino moiety of the glucosamine units [188,431,432], however alternative theories have also been proposed. Proposed theories include the interaction of diffused hydrolysis products with microbial DNA, the potential chelation of chitosan with microbial nutrients and essential metals, whilst others propose that chitosan acts as a blocking species on the cell membrane preventing nutrients from entering the microbe [433]. It has also been proposed that chitosan's carboxylic groups allowing the film to redissolve when exposed to a microbial solution [434]. Chitosan has been mainly investigated as an in-solution and bulk antimicrobial agent [186], however there are various reports of it being coated onto materials, forming chitosan-films, with high antimicrobial efficacy being exhibited [435,436]. Most studies evaluating chitosan-films primarily investigate mature biofilms

and do not examine the anti-biofilm capabilities against an early-stage biofilm environment.

ZnO nanoparticles have also been recognised as effective antimicrobial agents, particularly their biocidal properties which have been found to improve with a reduction in nanoparticles size [437]. The microbial activity of ZnO has been related to its ability to increase membrane permeability that eventually leads to accumulation of ZnO nanoparticles in the membrane as well as internalisation of these nanoparticles in the cell, ultimately resulting in cell death [216].

Chitosan is a biopolymer and has been combined with other antimicrobial agents including metal and metal oxide species [438,439]. Specifically, chitosan-ZnO nanocomposites have shown promising antimicrobial properties as coatings and are seen as a less toxic option compared to other antimicrobial surface technologies due to chitosan being a natural polymer. Chitosan has been reported as one of the most investigated polysaccharides for antimicrobial edible films and coatings [440]. Concurrently ZnO nanoparticles are commonly used in food processing and agricultural applications due to their biocompatibility and ZnO is classed by the FDA as “generally recognised as safe” (GRAS) [441,442]. Al-Naamani *et al.* demonstrated that chitosan-ZnO hybrid coatings inhibit the bacterial growth of *Pseudoalteromonas nigrifaciens*, a marine bacterium commonly found in biofilms, when compared to a pure chitosan coating. Interestingly, these observations occurred in the presence of light, as in the absence of light no significant antibacterial activity was detected, whilst the nanocomposite performed better than the chitosan coating alone [351]. Other studies have synthesised varied forms of a chitosan-ZnO composite coating and have observed promising antimicrobial results [188,443,444]. The applications of chitosan-ZnO nanocomposite coatings have not been explored as a coating for HDPE however. In this thesis the use chitosan-ZnO nanocomposite coatings will be explored for HDPE. This study will specifically examine the efficacy of this composite coating against a common biofilm forming bacteria, *E. coli*, which has shown large prevalence in industry, using early-stage biofilm assays.

6.2 Results and Discussion

6.2.1 Modulation of Coating Formulations

Chitosan-ZnO nanocomposite coatings were prepared as outlined in Chapter 3 and adapted from the synthetic protocol of Al-Naamani *et al.* [351]. This protocol was chosen because it affords a smooth film coating using a simple dip-coating approach which is commonly used to prepare chitosan films [445,446]. There are several formulation variables that have been adjusted in this Chapter in order to optimise an effective antimicrobial chitosan-ZnO thin-film to coat HDPE.

Firstly, the film preparation method involves dissolving ZnO and chitosan in an aqueous acidic medium, noting that chitosan's solubility is limited by the solution pH. Chitosan can contain various degrees of deacetylation (DDA) of the amine groups, and when the degree of acetylation of chitosan is lower than 50%, chitosan becomes soluble in aqueous acidic solutions due to the protonation of the $-NH_2$ group [433]. A range of variables taken from the Al-Naamani *et al.* procedure to synthesise a chitosan-ZnO composite film on HDPE were varied in order to find an optimal formulation for antimicrobial activity for the coating on HDPE. Firstly, given chitosan's limited solubility, a 1% acetic acid solution was used as the solvent system which is consistent with that reported by Al-Naamani *et al.* Lower solvent pH's are required for improved chitosan solubility because when the pH increases above chitosan's pKa (approximately 6.5), chitosan loses its charge and coils up into an insoluble morphology. The solubility is important for forming a uniform thin-film. Naturally, chitosan is positively charged with a pKa of 6.3 to 7.0 [447]. Therefore, the use of 1% acetic acid solvent system was used, as well 10% acetic acid solution in this study.

A final NaOH-hot wash (NaOH-HW) was applied after surface dip-coating. This wash has also been reported to reduce film solubility by ensuring deprotonation of the cationic amino groups. The base used to neutralise chitosan can influence film properties including crystallinity, surface chemistry, nanotopography, and mechanical properties. In this study NaOH was used which is the most commonly used base for neutralisation of chitosan [448]. However, other studies have revealed that chitosan films exhibit improved antimicrobial activity in more acidic conditions due to the poor solubility of

chitosan when exposed to higher pH's [433]. Therefore, despite the NaOH-HW strengthening the coating stability by reducing solubility, this step may also have a negative effect on the antimicrobial efficacy as a coating. Therefore, this washing was also investigated to determine its relevance and importance in achieving an antimicrobial chitosan-ZnO nanocomposite coating on HDPE, against an early-stage biofilm of *E. coli*. Therefore, selected films prepared were exposed to NaOH-HW for comparison, (see Chapter 3, Section 3.2.4 for the experimental protocol.)

The effect of chitosan concentration was investigated to see if this was a limiting factor, including batches that reached the saturation limit of the solution in order to evaluate very high concentrations of chitosan exposure. It was found that chitosan becomes insoluble in 1% acetic acid after 1.75 g/100 mL in the presence of 0.2 g ZnO.

Furthermore, it was important to evaluate the effect of varying ZnO concentration on anti-biofilm behaviour; ZnO concentrations of 0, 0.2 and 0.75 g were studied. Similarly, the maximum quantity of ZnO soluble was 0.75 g/100 mL in 1% acetic acid. Beyond this point only partial solubility was observed (images of the solubility trials for ZnO can be seen in the Appendix under Chapter 6[A]). As higher concentrations of ZnO may be desirable, to increase ZnO solubility, lower solvent pH environments were considered. Richardson *et al.* has established that ZnO solubility improves as the pH reduces [449]. The pH's of 1%, 5% and 10% acetic acid (100 mL) was measured and were found to have pH's of 2.62, 2.24 and 1.86 respectively. As a result, 10% was chosen as a lower pH aids in both chitosan and ZnO solubility. Additionally, the higher amounts of ZnO being added will likely increase the pH which is unfavourable for the solubility of chitosan and ZnO, further supporting the use of a higher acetic acid concentration. Consequently, to determine the maximum concentration of ZnO able to dissolve in 10% acetic acid, a small study was performed where ZnO was added to 100 mL of 10% acetic acid until maximum saturation was achieved and the pH was also monitored, the results for this test can be seen in **Figure 6.1**. It was found that a maximum of 1.5 g of ZnO could be dissolved in 10% acetic acid, however this afforded a solution with pH 3.8, a nearly 2 pH increase from the addition of ZnO was found.

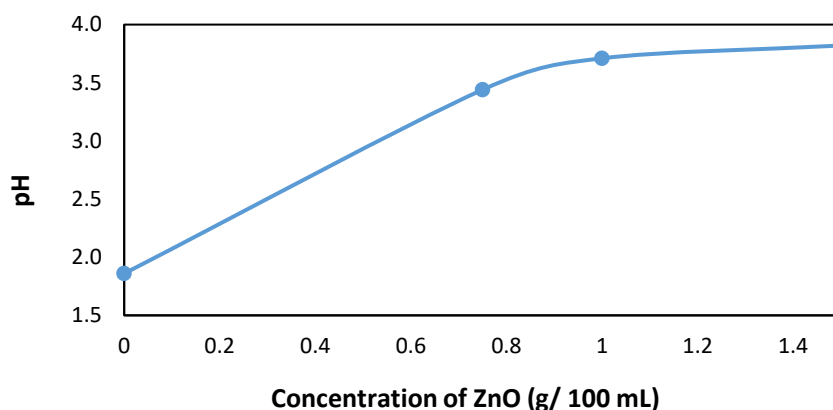


Figure 6.1 Effect of ZnO concentration on pH to achieve maximum saturation in 10% acetic acid.

Lastly, to determine if ZnO is independently acting as an antimicrobial agent, an alternative polymer was also selected and used for film casting for comparison with chitosan nanocomposite films. Herein, medium molecular weight polyvinylpyrrolidone (PVP), also known as Povidone, was selected at 5%v for comparison. PVP is a water and organic solvent soluble polymer made from the radical polymerisation of the monomer *N*-vinylpyrrolidone. PVP is a commonly used in the pharmaceutical and cosmetic industries, offers good film formation, and adhesive to various substrates. It is non-toxic, non-ionic, inert, temperature resistant, pH-stable and biocompatible [450].

As a final note, preliminary coating of the chitosan-ZnO nanocomposite films revealed poor adhesion onto those HDPE substrates without any surface oxidation. The lack of film adhesion on the polymer was visually apparent during the microbial assessment stage; where the surface scraping step to dislodge and remove the early-stage biofilm resulted in also removing the chitosan-ZnO nanocomposite films. Therefore, surface oxidation was performed using UVO and sulfuric-acid oxidative treatment (UVO-SA) methods which improved film adhesion sufficiently to withstand the microbial scraping stage. Herein all coatings were applied onto UVO-SA treated HDPE surfaces.

A summary of the different preparation conditions relative to the work of Al-Naamani and co-workers [351] has been provided below. ZnO nanoparticle <50 nm nanoparticle size which is a similar size to that used by Al-Naamani *et al.* was used. Furthermore, the DDA has been kept constant for LMW and MMW chitosan used, purchased at 75-85%

deacetylation for both forms. All samples prepared have been examined against an early-stage biofilm of *E. coli* (ATCC 10536), *P. aeruginosa* was not used in this study as the batch in the lab had some issues during this time and more was needed to be provided from sponsors.

Based on the above-mentioned variables, the final experimental reagent concentrations have been tabulated below:

	Polymer (x/100 mL solvent)	ZnO (g/100 mL solvent)	Solvent (100mL)	NaOH-HW Yes/No
1	LMW 0.1 g chitosan	0.2	1% acetic acid	No
2	MMW 0.1 g chitosan	0.2	1% acetic acid	No
3	LMW 0.1 g chitosan	0.2	1% acetic acid	Yes
4	MMW 0.1 g chitosan	0.2	1% acetic acid	Yes
5	LMW 1.5 g chitosan	0.2	1% acetic acid	No
6	MMW 1.5 g chitosan	0.2	1% acetic acid	No
7	LMW 1.5 g chitosan	0.2	1% acetic acid	Yes
8	MMW 1.5 g chitosan	0.2	1% acetic acid	Yes
9	LMW 1.75 g chitosan	0	1% acetic acid	No
10	MMW 1.75 g chitosan	0	1% acetic acid	No
11	LMW 1.75 g chitosan	0.75	1% acetic acid	No
12	MMW 1.75 g chitosan	0.75	1% acetic acid	No
13	LMW 0.5 g chitosan	0	10% acetic acid	No
14	LMW 0.5 g chitosan	1.5	10% acetic acid	No
15	LMW 1 g chitosan	0	10% acetic acid	No
16	LMW 1 g chitosan	1.5	10% acetic acid	No
17	MMW 5%v PVP	0	10% acetic acid	No
18	MMW 5%v PVP	1.5	10% acetic acid	No

Table 6.1 Experimental matrix performed, film casting maintained at pH 6 using 0.1 M NaOH in all cases. Where LMW and MMW refer to low and medium molecular weight of the polymers.

6.2.2 Evaluation of Chitosan-ZnO Coating on Oxidised HDPE

The composition and presence of the chitosan-ZnO coatings were examined using a range of techniques, including XPS, ATR-FTIR spectroscopy and AFM. Contact angle goniometry was performed but did not show any meaningful trends or information, therefore this data can be found in the Appendix, under Chapter 6[B].

XPS was performed on a selection of these samples (due to instrument access and time constraints) to determine the surface composition ratios for C:O:N:Zn present. The data has been provided as a ratio relative to N because N is a key element present in all polymers used for the film (chitosan and PVP), see XPS results in **Table 6.2** (full survey scans of these results can be found in the Appendix for Chapter 6[C]).

The data revealed that an increase from 0.1 g to 1.5 g for both LMW and MMW formulations has caused an increase in the content of chitosan on the surface by 3.8 times when comparing the ratio of N to C elements found. This is expected as more chitosan is being added to the formulation. It can be observed that the NaOH-HW has shown to reduce the amount of N relative abundance overall that was present on the surface, the NaOH-HW has also reduced the amount of ZnO which may be due to its dissolution by hydrolysis. Kinetic evaluation of ZnO has proven that a sufficiently large pH increase can lead to a metastable solubility stage with a higher degree of stable Zn^{2+} forming, proposed to be due to the formation of a hydroxide interfacial layer [451]. The percentage of N drops in both cases for 1.75 g concentrations MMW and LMW chitosan in the presence of 0.75 g ZnO (formulations 11 and 12 in **Table 6.2**). Furthermore the 1.75 g LMW sample (formulation 11) shows very high levels of ZnO present relative to N at the surface. This decrease may be because the addition of greater ZnO increases the alkalinity of the system [449], which in turn leads to reduce chitosan solubility effectively in solution.

	Coating Formulations	N	C	O	Zn	S
1	LMW 0.1 g chitosan – 0.2 g ZnO – 1% acetic acid	1	31.1	10.7	2.8	0.0
2	MMW 0.1 g chitosan – 0.2 g ZnO – 1% acetic acid	1	31.4	13.9	1.1	0.0
5	LMW 1.5 g chitosan – 0.2 g ZnO – 1% acetic acid	1	8.2	4.7	0.3	0.04
6	MMW 1.5 g chitosan – 0.2 g ZnO – 1% acetic acid	1	7.8	4.6	0.3	0.06
7	LMW 1.5 g chitosan – 0.2 g ZnO – 1% acetic acid – NaOH HW	1	11.2	6.1	0.0	0.0
8	MMW 1.5 g chitosan – 0.2 g ZnO – 1% acetic acid – NaOH HW	1	16.6	16.4	0.3	0.2
9	LMW 1.75 g chitosan – 1% acetic acid	1	8.2	5.8	0.0	0.2
10	MMW 1.75 g chitosan – 1% acetic acid	1	7.3	4.4	0.0	0.04
11	LMW 1.75 g chitosan – 0.75 g ZnO – 1% acetic acid	1	36.7	55.7	41.6	0.0
12	MMW 1.75 g chitosan – 0.75 g ZnO – 1% acetic acid	1	40.7	19.5	7.9	1.0
17	PVP – 10% acetic acid	1	12.7	3.7	0.0	0.1
18	PVP – 1.5 g ZnO – 10% acetic acid	1	33.2	10.4	0.2	0.5

Table 6.2 XPS ratio of C:O:N:Zn:S relative to nitrogen. Full survey scans can be found in the Appendix under Chapter 6[B].

ATR-FTIR is a useful tool to characterise the presence of key functional groups because different covalent bonds in these functional groups provide different characteristic absorption frequencies from their vibrational modes. The intensity of absorption primarily depends on the polarity of bond and the number of bonds responsible for the absorption, so with more of the same bond offering higher intensities. Although it is important to note that the intensity and sharpness of the peaks may also depend on other experimental factors such as moisture content of film, how much load the ATR crystal puts on the surface, and how soft/hard the material is which affects the contact area of the crystal with the surface for measurements. Nevertheless, ATR-FTIR is a common technique used to evaluate chitosan films and is a technique utilised in this study to identify key structural functional groups found within chitosan and examine if there were chemical group changes associated with the different formulations prepared.

The reference samples used were HDPE untreated, HDPE UVO-SA surface oxidised and the chitosan commercial powder (starting material). These have been compared with chitosan-ZnO films prepared using 0.1 and 1.5 g/100 mL LMW/MMW chitosan with 0.2 g/100 mL ZnO nanoparticles, without NaOH-HW treatment, refer to **Table 6.1** and **Table 6.2**. The ATR-FTIR results gathered show a broad strong band in the region of 3600-3000 cm^{-1} which corresponds to -OH and -NH stretching modes. Chitosan contains both primary and secondary alcohol functionalities which translates to broad bands in this IR band region. This viewpoint is supported by reports investigating the FTIR of chitosan [452], and is associated with the internal variations in hydrogen bonding from these functional groups. The HDPE UVO-SA oxidised sample revealed a broad and strong -OH band arising from extensive oxidation treatments, as expected. Peaks associated for C-O from secondary and primary alcohol groups can be found in the spectra between 1140-1030 cm^{-1} for both LMW and MMW films, see **Figure 6.2**, which are weak bands compared to the chitosan powder [452]. The N-H bending amide II peak was shown between 1567-1550 cm^{-1} for MMW chitosan-ZnO film and 1563-1661 cm^{-1} for LMW which corroborates well with literature findings [435,445,452]. The C=O stretching from the amide I group presents was found at 1643 cm^{-1} for LMW and 1646-1643 cm^{-1} for MMW, this result is also seen in chitosan films prepared by other researchers [435]. There is also a small but sharp peak for the OCO bending mode from acetate that's from the solvent system which is found present at around 613-612 cm^{-1} found in all films of LMW and MMW chitosan-ZnO nanocomposite materials prepared [453], which is not found in the starting material.

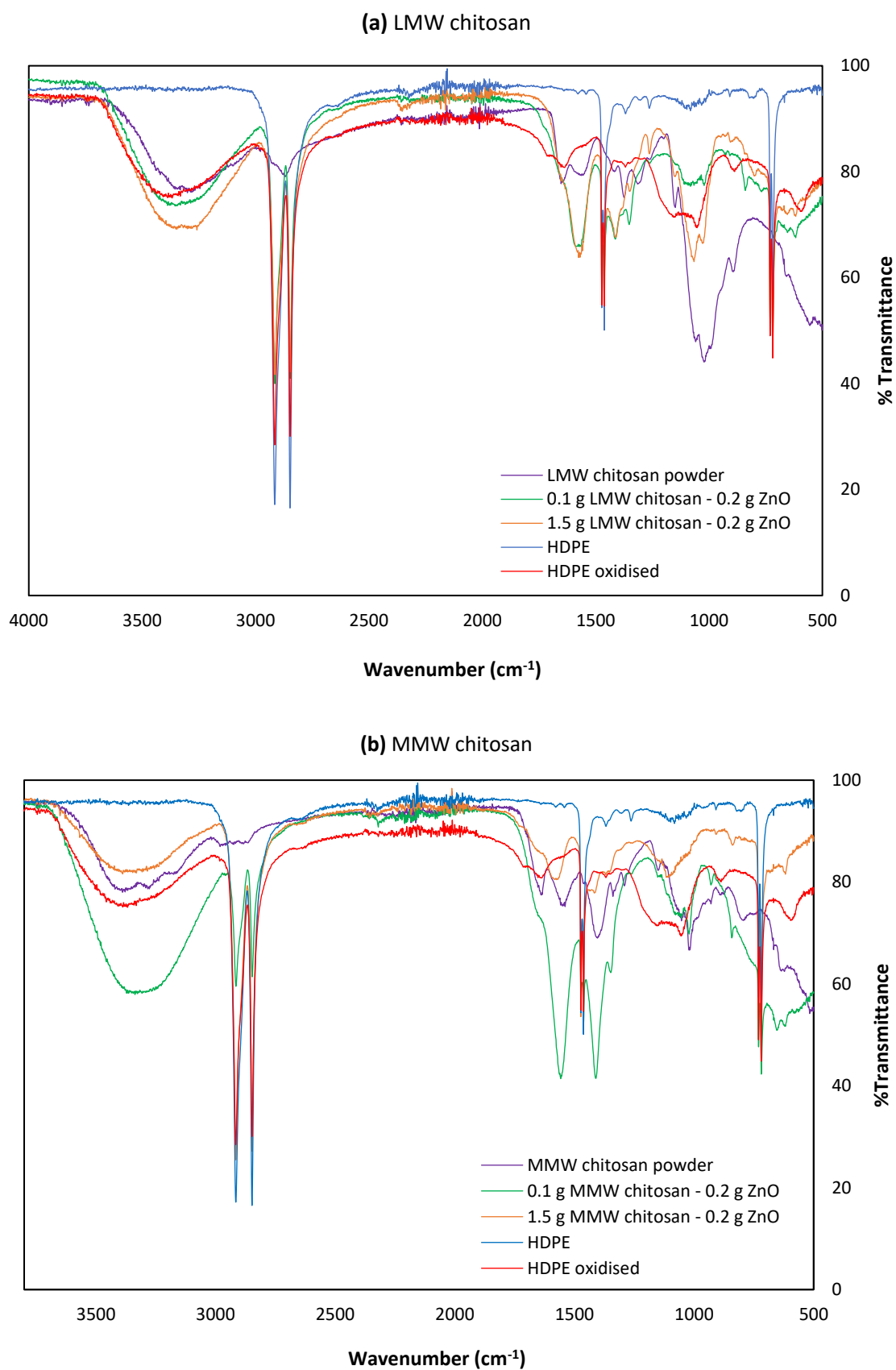


Figure 6.2 LMW (a) and MMW (b) chitosan-ZnO nanocomposite coatings compared to LMW chitosan powder, HDPE untreated and HDPE oxidised control materials.

The results showed that overall, the increase in the ZnO concentration from 0 g to 0.75 g has reduced the intensity of key peaks for % transmittance. This result is seen in nearly all concentrations of LMW and MMW chitosan tested in this matrix, which is more prominent for MMW chitosan containing films, see below **Figure 6.3**.

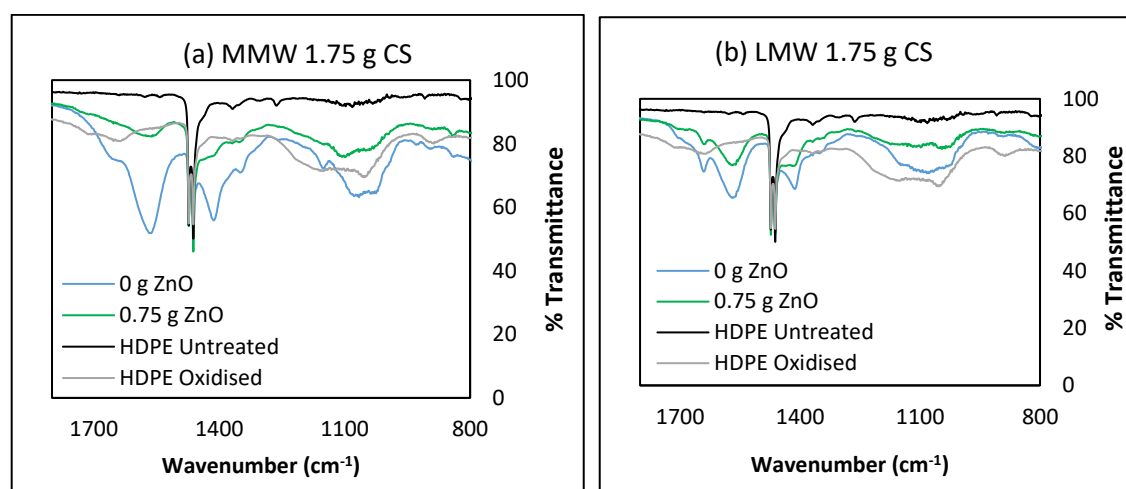


Figure 6.3 ATR-FTIR of MMW and LMW chitosan dip-coated at 1.75 g /100 mL at varying ZnO nanoparticle concentrations.

ATR-FTIR on these samples has found that the strong double peak present between 2915-2850 cm^{-1} for HDPE is due to the asymmetrical $-\text{CH}_2$ stretching of the methylene group is not prominent and sharp which suggest that coating using 10% acetic acid is highly efficacious in forming a thick film that is able to mask the intensity of these peaks, see **Figure 6.4**. A sharp NH amide II peak is present in all films at around 1550 cm^{-1} found in chitosan, which is less prominent in the chitosan powder control. Furthermore, peaks for the presence of C-O from alcohols was seen at around 1120 cm^{-1} for all films found from chitosan. Sharp peaks from the carboxylate groups due to the presence of acetic acid in the system is also spotted in the spectra, at 1400 cm^{-1} as anticipated, which is not found in the LMW chitosan powder.

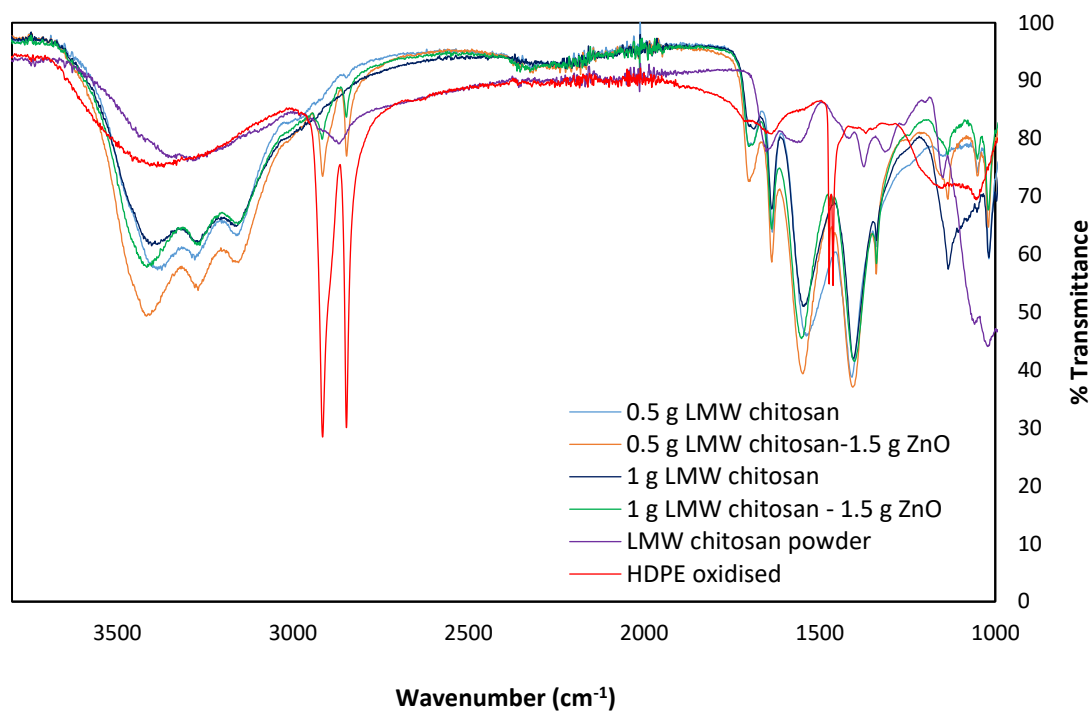


Figure 6.4 ATR-FTIR of LMW chitosan films and nanocomposite films containing ZnO nanoparticles, using 10% acetic acid.

The formation of the PVP films has been examined also using ATR-FTIR, the C=O stretching peak is found at 1629 cm^{-1} for the PVP film, and 1638 cm^{-1} for PVP-ZnO composite film; C-N stretching is shown at $1292\text{--}1293\text{ cm}^{-1}$ for both PVP films, and corroborate with findings from literature of PVP coatings [454], see **Figure 6.5**.

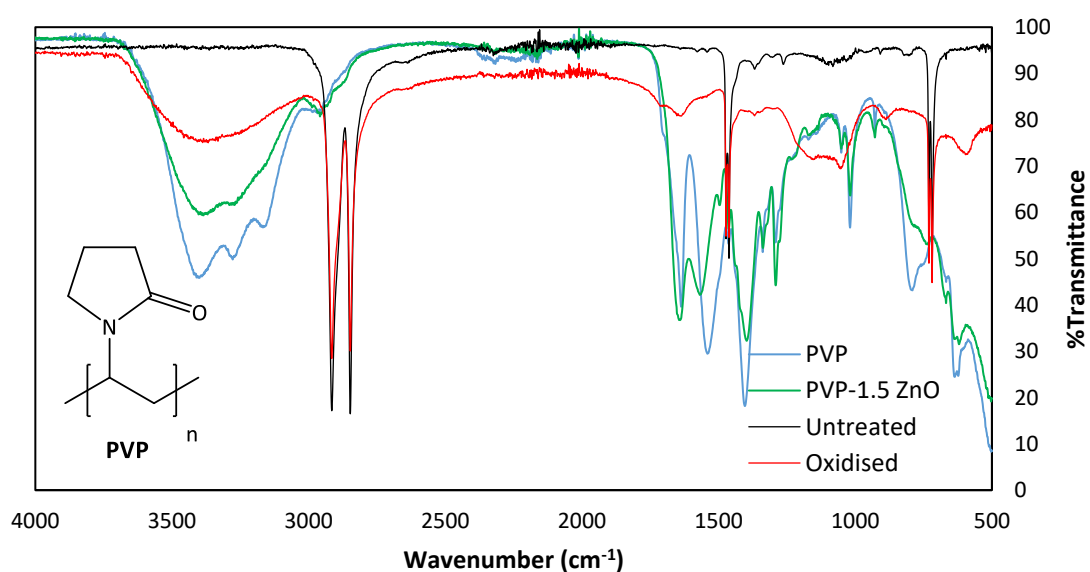


Figure 6.5 ATR-FTIR of films on UVO-SA HDPE of PVP and PVP-ZnO nanocomposite (1.5g ZnO).

AFM images for a range of chitosan-ZnO nanocomposite films are shown in **Figure 6.6**. These results show that there is a great variation in film uniformity depending on the synthesis conditions. The roughness parameters obtained (R_a and R_q) depict a smooth surface to have been prepared using these procedures. For example, LMW CS 1.5g-0.2g ZnO (with NaOH-HW) shows clear depiction of the ZnO nanoparticle uniformly distributed on the surface of the coating, whereas the same formulation, without the NaOH-HW, shows no such uniformity. This may be due to various factors, which may involve variations in manual dip-coating, drying, sample formulation, and film heterogeneity.

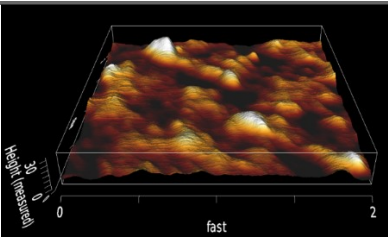
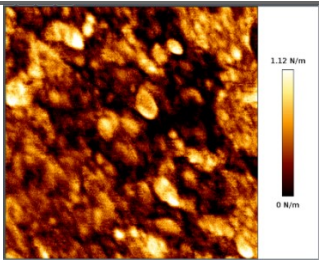
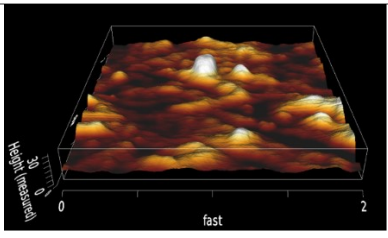
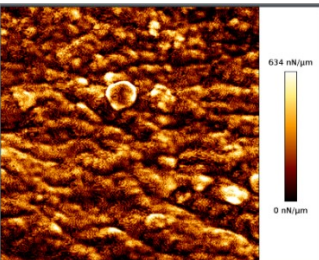
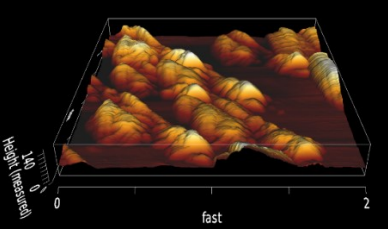
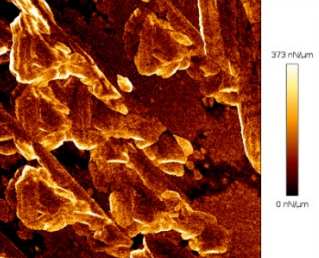
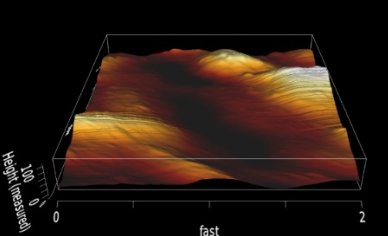
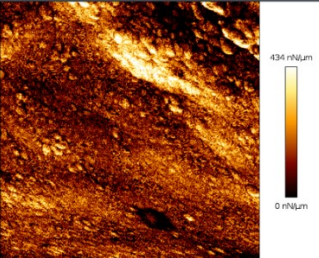
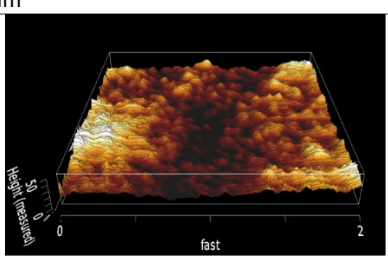
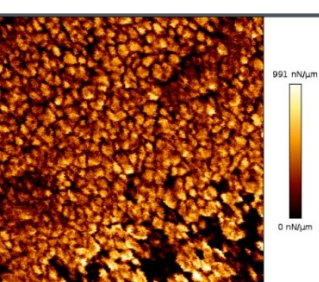
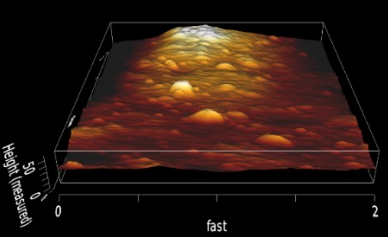
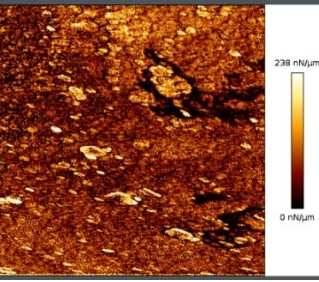
Per 100 mL	3D Roughness	2D Stiffness
0.1g LMW chitosan 0.2g ZnO	 R_a 2.6 nm R_q 3.5 nm	
0.1g MMW chitosan 0.2g ZnO	 R_a 2.5 nm R_q 3.5 nm	
1.5g LMW chitosan 0.2g ZnO	 R_a 24.6 nm R_q 29.6 nm	
1.5g MMW chitosan 0.2g ZnO	 R_a 16.9 nm R_q 22.1 nm	
1.5g LMW chitosan 0.2g ZnO NaOH-HW	 R_a 5.2 nm R_q 6.7 nm	
1.5g MMW chitosan 0.2g ZnO NaOH-HW	 R_a 5.6 nm R_q 7.9 nm	

Figure 6.6 AFM images of some of the chitosan-ZnO nanocomposite films prepared in Part 1, 3D roughness and 2D stiffness has been provided for the same image.

AFM images of a selection of samples were obtained in this study, see **Figure 6.7**. Surface topographical uniformity is lost, and an aggregated surface film can be observed in the LMW samples, with the ZnO nanoparticles being spread randomly under these conditions. This behaviour is also mirrored in the MMW PVP films.

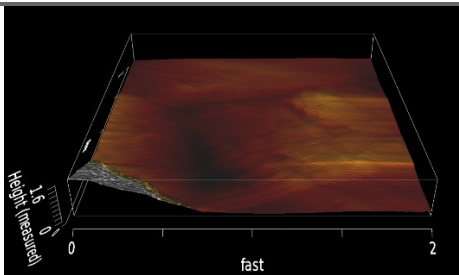
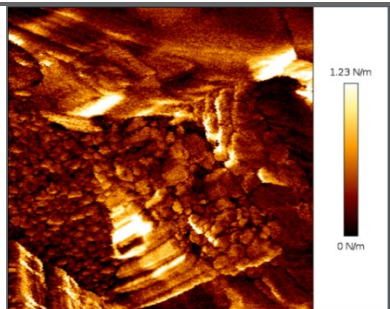
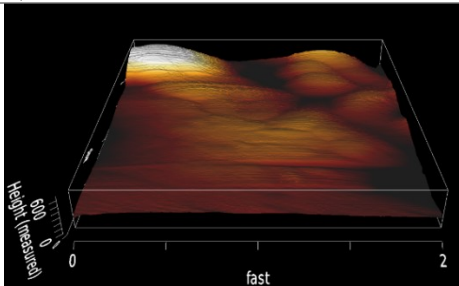
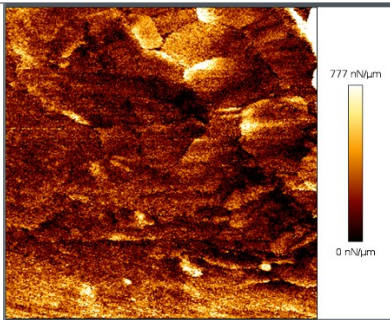
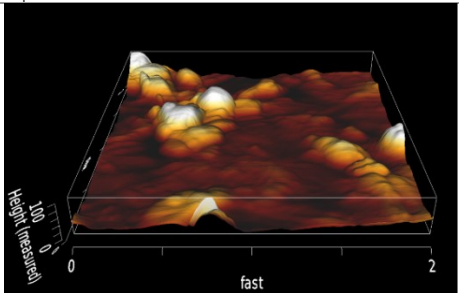
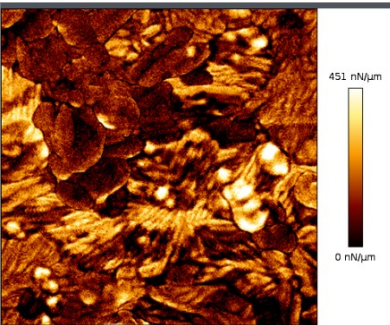
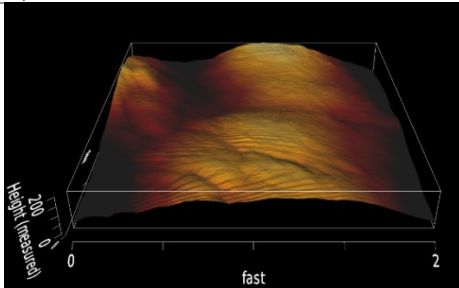
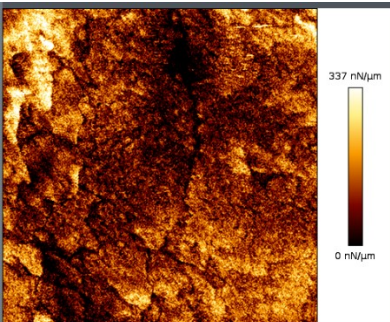
Per 100 mL	3D Roughness	2D Stiffness
0.5g LMW chitosan (10% acetic acid)	 R_a 92.2 nm R_q 157.4 nm	 1.23 N/m 0 N/m
0.5g LMW chitosan 1.5 g ZnO (10% acetic acid)	 R_a 60.0 nm R_q 90.6 nm	 777 nN/μm 0 nN/μm
MMW PVP 5% (10% acetic acid)	 R_a 10.4 nm R_q 14.9 nm	 451 nN/μm 0 nN/μm
MMW PVP 5% 1.5g ZnO (10% acetic acid)	 R_a 32.6 nm R_q 38.4 nm	 337 nN/μm 0 nN/μm

Figure 6.7 AFM images of chitosan-ZnO, chitosan, PVP, PVP-ZnO composite films prepared on UVO-SA oxidised HDPE surfaces using dip-coating approach. Images have 526 x 526 pixel resolution at 2 x 2 μm scale, N=1.

6.2.3 Antimicrobial Performance Against *E. coli* Early-Stage Biofilm

The efficacy of these materials against the early-stage biofilm of *E. coli* has been assessed using the viable plate counting assay (protocol found in Chapter 3). The results show that there was no significant loss or change in colonies grown from using these nanocomposite coatings compared to the untreated HDPE and oxidised UVO-SA material. Also, no conclusive antimicrobial effect is observed from these coatings even though great care has been implemented to evaluate different experimental conditions and formulations, **Table 6.3**. The highest loss in *E. coli* viability was observed for the more higher concentrations of LMW chitosan (1.75 g) and in the presence of 0.75 g ZnO, with a 2.9 log reduction observed, photos of the agar plates are provided in **Figure 6.8**. Such a trend was not observed for the MMW chitosan-ZnO samples prepared, and this material can be concluded as not efficacious. This material had the highest concentration of ZnO and chitosan possible with this formulation (1 % acetic acid), and modulation to higher concentrations by using 10 % acetic acid did not afford heightened antimicrobial effects.

Alternatively, slight reductions in *E. coli* viability was observed for LMW and MMW 1.5 g chitosan formulations (containing 0.2 g ZnO/100 mL), formulations 5 and 6 in **Table 6.3 (A)** giving log 5.7 and 4.3 respectively. The NaOH-HW performed has shown to remove the slight antimicrobial property present in these surfaces, formulations 7 and 8 in **Table 6.3 (A)**.

A	Polymer (x/100 mL solvent)	ZnO (g/100 mL solvent)	Solvent (100mL)	NaOH- HW Yes/No	<i>E. coli</i> CFU/mL (Log₁₀)
1	LMW 0.1 g chitosan	0.2	1% acetic acid	No	6.4 +/- 0.1 {p=2x10 ⁻³ }
2	MMW 0.1 g chitosan	0.2	1% acetic acid	No	6.3 +/- 0.1 {p=2x10 ⁻² }
3	LMW 0.1 g chitosan	0.2	1% acetic acid	Yes	6.4 +/- 0.2 {p=9x10 ⁻³ }
4	MMW 0.1 g chitosan	0.2	1% acetic acid	Yes	6.2 +/- 0.1 {p=8x10 ⁻² }
5	LMW 1.5 g chitosan	0.2	1% acetic acid	No	5.7 +/- 0.1 {p=0.01}
6	MMW 1.5 g chitosan	0.2	1% acetic acid	No	4.3 +/- 0.3 {p=4x10 ⁻⁷ }
7	LMW 1.5 g chitosan	0.2	1% acetic acid	Yes	6.3 +/- 0.6 {p=0.3}
8	MMW 1.5 g chitosan	0.2	1% acetic acid	Yes	6.0 +/- 0.1 {p=0.6}
9	LMW 1.75 g chitosan	0	1% acetic acid	No	6.6 +/- 0.1 {p=2x10 ⁻⁵ }
10	MMW 1.75 g chitosan	0	1% acetic acid	No	6.3 +/- 0.1 {p=0.02}
11	LMW 1.75 g chitosan	0.75	1% acetic acid	No	3.2 +/- 0.5 {p=2x10 ⁻⁶ }
12	MMW 1.75 g chitosan	0.75	1% acetic acid	No	5.9 +/- 0.3 {p=0.6}
13	LMW 0.5 g chitosan	0	10% acetic acid	No	6.1 +/- 0.1 {p=0.4}
14	LMW 0.5 g chitosan	1.5	10% acetic acid	No	5.8 +/- 0.2 {p=0.1}
15	LMW 1 g chitosan	0	10% acetic acid	No	6.5 +/- 0.1 {p=3x10 ⁻⁴ }
16	LMW 1 g chitosan	1.5	10% acetic acid	No	6.1 +/- 0.1 {p=0.4}
17	MMW 5%v PVP	0	10% acetic acid	No	6.0 +/- 0.5 {p=0.8}
18	MMW 5%v PVP	1.5	10% acetic acid	No	6.0 +/- 0.2 {p=0.9}

B	Controls	<i>E. coli</i> CFU/mL (Log₁₀)
1	HDPE UVO-SA oxidised	6.0 +/- 0.4
2	HDPE control (untreated)	6.1 +/- 0.3

Table 6.3 Viable plate counting assay of early-stage biofilm with *E. coli* using HDPE oxidised samples coated with MMW and LMW chitosan-ZnO thin-films, PVP films in table **A** and relevant controls provided in table **B**. T-tests were performed to compare results to UVO oxidised HDPE control, {p=value}.

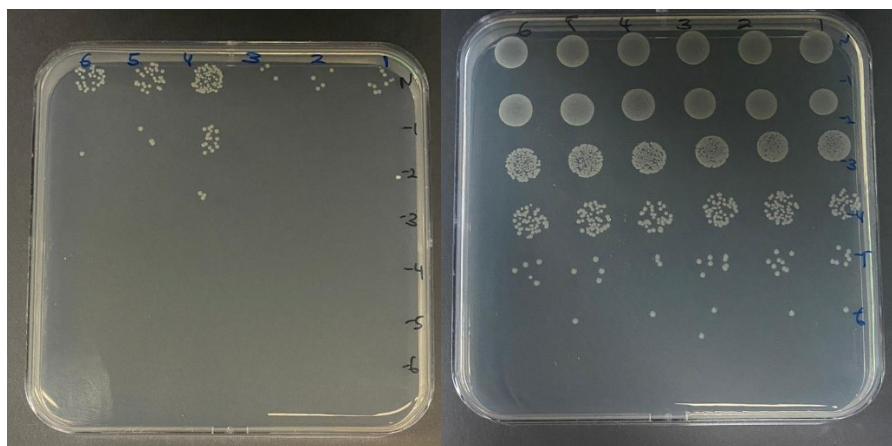


Figure 6.8 Photos of *E. coli* agar plates exposed to serial dilution from N (neat) to -6; left: HDPE oxidised with LMW chitosan 1.75 g/0.75 g ZnO; right: HDPE untreated control surface.

The variability of the chitosan film preparation to achieve an antimicrobial effect has also been observed by others. Fernandez-Saiz *et al.* performed a detailed study on HMW chitosan and found that a ‘just-formed chitosan’ film can show a 7-log reduction in *S. aureus*, whereas an NaOH neutralised film, a 48-h old-film, and a chitosan solution containing the same amount of high MW chitosan gave no reduction of *S. aureus* colonies. This ultimately revealed that in order for a biocidal result to be seen for HMW chitosan in their system, a gelled and viscous acidic solution form of the film is needed, where the amine groups are protonated/ionised [455]. Other film variables which were thought to impact on antimicrobial performance included the quality of chitosan which varies depending on its source, and can cause disparities in their crystallinity, physiochemical characteristic and ash contents [456]. These observations are important as the physical properties of chitosan in aqueous solution depends on the DDA and the acetyl group distribution in the polymer chains, with uneven acetyl group distribution leading to lower solubility and encouraging aggregate formation [457]. Deacetylation is also important for influencing the MW (MW decreases with decreasing deacetylation), tensile strength, biodegradability, crystallinity (greater acetylation causes greater crystallinity), hydrophilicity, chemical reactivity and bio-response [458].

The failure of forming an effective significant antimicrobial performance of these materials against *E. coli*, even after extensive modulation of various parameters in this study, suggests that intrinsically there may be a loss of active antimicrobial agent across all samples. Chitosan antimicrobial activity is largely agreed to be attributed to the primary amine functionalities present in the structure of chitosan [188]. The ninhydrin assay is a common method used to validate the presence of free amine groups in chitosan as this has shown to correlate well with the antimicrobial performance attained by chitosan containing materials [459,460]. Ninhydrin can react with primary amine groups to form a purple solution, 2,2-dihydroxyindane-1,3-dione (also known as Ruhemann's purple), which has a maximum absorption at 570 nm ($\epsilon = 22000$). A selection of reference samples were examined using this assay and the absorption at 570 nm was evaluated, see **Table 6.4**.

The selection of samples was based on antimicrobial results, see **Table 6.3**. The higher concentration of chitosan exhibited a slight reduction in *E. coli* viability (1.5 g MMW/LMW chitosan – 0.2g ZnO samples). The higher ZnO concentration showed with increased LMW chitosan concentration showed the most significant antimicrobial performance (1.75g LMW chitosan-0.75g ZnO). The results presented showed that the most antimicrobial surface found and prepared was the 1.5 g LMW chitosan-1.75g ZnO sample, which revealed the presence of free amine moieties but not uniformly across the sample. All other samples remained yellow and did not show a colour change to purple. Full spectral data can be obtained in the Appendix under section Chapter 6[D] for all samples.

Samples	Absorption at 570 nm
Untreated control	0.013 +/- 0.004
UVO-SA oxidised	0.011 +/- 0.006
MMW 1.5 g chitosan- 0.2 g ZnO	0.017 +/- 0.006
LMW 1.5 g chitosan- 0.2 g ZnO	0.020 +/- 0.011
LMW 1.75 g chitosan-0.75 g ZnO	0.096 +/- 0.047
MMW 1.75 g chitosan-0.75 g ZnO	0.028 +/- 0.008

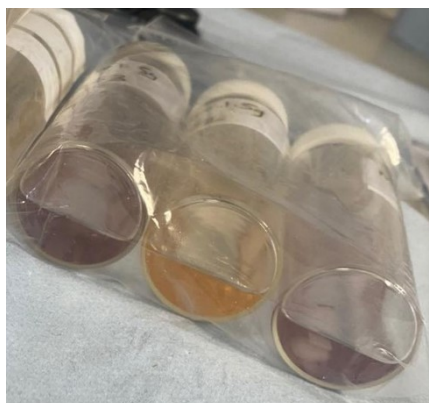


Table 6.4 *Ninhydrin assay results on chitosan-ZnO nanocomposite films formed on UVO-SA oxidised HDPE materials, (+/- SD). A photo of 1.75g LMW chitosan – 1.75g ZnO film has been provided below, with n=3 where it can be observed that 2/3 samples has turned purple.*

The DDA is a key parameter that governs the properties and behaviour of chitosan and is defined as the proportion of *N*-acetyl-glucosamine units present in the polymer. The degree of acetylation (DA) is usually between 13-40% because it is difficult to fully deacetylate chitosan [461]. FTIR is a quick technique that is commonly used for the evaluation of DDA. DDA values can change depending on the (i) type and level of impurities, (ii) source, (iii) polymer morphology, and (iv) deacetylation conditions of chitin. Evaluation methods for the DA of chitosan using FTIR by researchers vary, depending on the calculation they have employed, and each contain their own limitation and advantages. For example, errors made from samples containing high values of DA, variations in sample drying, and peak resolution (i.e. many peaks appearing in the region of the reference peak)[462]. The chitosan ordered (MMW/LMW) both are said to have a DDA between 75-85%; ATR-FTIR was used to evaluate and confirm the DDA, and to determine if this was a limiting factor in this project. **Equation 6.1** has been used to calculate the % DDA [463].

$$\% DDA = \left(\frac{A_{1320}}{A_{1420}} - 0.3822 \right) \times \frac{1}{0.03133} \quad \text{Equation 6.1}$$

The MMW/LMW chitosan starting materials used in this study was vacuum dried under a desiccant (silica) overnight and evaluated under ATR-FTIR (Perkin Elmer). The results found that for the LMW chitosan powder a % DDA of 76.4 $\pm$ 0.2 % was obtained, and for MMW chitosan powder a % DDA of 86.7 $\pm$ 3.1 was obtained using three separate spectra, values used can be found in the Appendix Chapter 6[E]. These results suggest that during the preparation of the films, the free amine groups have become unavailable for antimicrobial activity, and in turn explains the generally poor antimicrobial performance of these materials.

6.3 Conclusion

The most effective formulation reported here achieved a 2.9 log reduction was a LMW chitosan nanocomposite coating containing 1.75 g chitosan and 0.75 g ZnO nanoparticles in 1 % acetic acid solvent (100 mL), dip-coated at pH 6 to reduce *E. coli* early-stage biofilms, compared to the HDPE untreated control. This formulation contained maximum saturation of chitosan and ZnO at 1% acetic acid. Attempts to improve the anti-biofilm and antimicrobial attributes of this material by increasing the concentration of chitosan and ZnO in the nanocomposite film formulation using a more acidic solvent system (10 % acetic acid, 100 mL) did not prove efficacious. The anti-biofilm performance seen in the 1.75g LMW chitosan-0.75g ZnO formulation was later attributed to the presence free amino groups which was confirmed by the ninhydrin test for primary amines. This work supports the hypothesis mentioned by many researchers that the antimicrobial property of chitosan is due to the presence of free amine groups in the film.

ZnO nanoparticles contained within the films found independently to show that a tendential increase in its concentration in the formulation, offered a gradual (small) reduction in the *E. coli* early-stage biofilm viability when LMW chitosan was used with 1% acetic acid solvent system.

The lack of success from the efforts to prepare and optimise different chitosan formulations to produce a highly antimicrobial surface on HDPE, suggests that the

antimicrobial active agent (-NH₂) groups are highly sensitive to the solution conditions in the preparation of a chitosan composite films.

CHAPTER 7

Concluding Discussion

7.1 Summary

This research project has evaluated the modification of a range of polyolefinic industrial materials, with HDPE being used as the model substrate primarily under investigation. A thorough evaluation of surface polymer oxidation has been performed which provided a detailed understanding of sulfuric-chromic acid oxidation for polyolefinic materials and the chemical basis for its antimicrobial performance, as well as its limitations which will govern its application scope. Sulfuric-chromic acid oxidation of HDPE and PP proved efficacious against Gram-negative organisms of *E. coli* and *P. aeruginosa* when examined, with both reduced adhesion and reduced viability observed in confocal laser scanning microscopy experiments, and viable cell enumeration studies respectively. The use of fluorescent dye DiBAC₄(3) revealed a significant loss in membrane potential upon exposure to sulfuric-chromic acid oxidised surfaces. Furthermore, a reduction in adhesion was observed with increasing sulfuric-acid oxidation time, which was shown to be stable under standard room storage conditions even after 3 months, when tested with HDPE substrate. This sulfuric-chromic acid oxidative treatment was also essential in maximising and optimising the antimicrobial performance of SnO₂ thin-film coated polymers, as it helped provide both an anti-adhesive and bactericidal surface as demonstrated by the Syto™9/PI Viability Kit. In addition, the sulfuric-chromic acid pre-treatment has helped to reduce SnO₂ leaching when these coatings were rinsed. Furthermore, surface oxidation was a powerful tool to investigate the performance of chitosan-ZnO coatings because it provided a strong foundation for adhesion of the nanocomposite film onto the HDPE substratum; as without this surface treatment, the coated film would easily peel away from the polymer.

Overall sulfuric-chromic acid oxidation of HDPE showed a 5.2-6 log reduction in the early-stage biofilm of *P. aeruginosa*, and a 3-log reduction when PP was used. This difference was attributed to the higher level of labile Cr for HDPE found from surface characterisation. No antimicrobial performance with the viable cell enumeration was

observed using a Gram-positive organism which was related to the presence of the thicker peptidoglycan layer that make up its cell wall. There was no influence of surface topographical changes caused from oxidation on antimicrobial response. Furthermore, comparison of alternate chemical oxidation approaches (UVO and hot sulfuric-acid treatment) revealed that only sulfuric-chromic acid oxidation resulted in significant reduction in colony counts, which pertained to its ability to produce labile-chromium containing species on the surface, whereas the other oxidising treatments did not show any loss in colony counts compared to the untreated HDPE surface. However, evaluation of microbial attachment of these alternate oxidising treatments were not performed in this project. Furthermore, detailed comparison of Cr-oxyhydroxide coatings with sulfuric-chromic acid treatment approach on HDPE further confirmed that the extensive loss of labile Cr species is essential in producing an antimicrobial result.

The excellent performance of sulfuric-chromic acid HDPE in reducing colony formation in Gram-negative organisms was not demonstrated by any of the other antimicrobial polymers prepared in this research project. Recyclability experiments of the oxidised HDPE samples in an aqueous environment showed an extensive loss of the antimicrobial specie which was not based on the presence of the biofilm on the surface, but from the excessive washing and cleaning that produced a loss of labile Cr species. Furthermore, this behaviour also explains why mature biofilm when exposed to a 24 hr hydrated environment, did not prove efficacious.

Alternatively, evaluation of SnO₂ thin-films using CLSM-based assays revealed the maintained stability and performance of the SnO₂ coating in both ethanol and water environments for extended time periods, as well as in saline, with maintained PI dye uptake observed. However, with the viable enumeration assay, the oxidised HDPE with Film A/B showed reduced viability growth only for 10 and 30 min oxidised samples, whereas the HDPE samples without pre-treatment that had been coated with Film A/B showed the largest reduction (4-log reduction) in colony counts compared to the HDPE untreated control. Furthermore, the use of this technology was efficacious but not substantial, with PP and PC polymers that were not pre-treated (oxidised), based on confocal microscopy studies. However, SnO₂ thin-films were unable to show any loss in

viability against the early-stage biofilms of *S. aureus* and *E. coli* when investigated, as well as for mature 24 hydrated biofilm of *P. aeruginosa*.

A thorough formulation evaluation of chitosan-ZnO coatings with optimised antimicrobial performance was conducted. The most effective formulation that achieved a 3.4-log reduction with *E. coli* was a LMW chitosan nanocomposite coating containing 1.75 g chitosan and 0.75 g ZnO nanoparticle in 1 % acetic acid solvent (100 mL), dip-coated at pH 6. Furthermore, the anti-biofilm performance seen in the 1.75g LMW chitosan-0.75g ZnO formulation was later attributed to the presence free amino groups which was confirmed by the ninhydrin test for primary amines. It was found that ZnO nanoparticles within the films in the presence of 1% acetic acid formulation resulted in a gradual reduction in *E. coli* viability with an increase in its concentration in the formulation. It was found that efforts to increase the concentration of chitosan and ZnO by using lower pH solvent system via 10% acetic acid, proved ineffective in offering an antimicrobial performance as a coating.

7.2 Future Work

Future work for this project could venture into many avenues as there are different areas that can be further investigated to confirm findings found.

This project has shown that oxidisation using sulfuric-chromic acid oxidisation on HDPE reduces the early-attachment of bacteria as well as the culturability and viability of recovered cells of Gram-negative bacteria. Examination of the effects of other oxidisation methods (such as UV/ozone and sulfuric acid treatment) did not show a similar reduction in culturability. The attachment behaviour of these alternate (UV/ozone and sulfuric acid treatment) methods were not investigated in this research project. However, previous research has confirmed that oxidisation using similar alternate methods on different polymers in literature (UV/ozone, plasma treatment...) has shown to correlate to reduced early-stage bacterial attachment, therefore further research into validating the early-stage attachment of bacteria against a range of varied polymer surface oxidisation approaches across a range of polymer materials in a cross-comparative large high-through put study would bring insight into not only the early-stage attachment behaviour of bacteria but also the mechanism of the antiadhesive property that has been observed with oxidised polymers in the past. For example, which oxidised functional group is primarily contributing to reduced attachment of bacteria and whether it pertains to the proposed water barrier theory, which mentions that a strong hydration layer on the polymer surface serves as an energetic barrier to prevent adhesion.

This project has revealed the efficacy of labile chromium surface species in helping to produce an AMP, however due to the very small residual amounts of this species available the approach is limited when sulfuric-chromic acid oxidisation is employed as here the chromium is acting as a catalyst to aid the oxidisation of the polymer surface. As a result, in order to produce a more antimicrobial surface strategy, one approach can look into further investigating synthesis routes that aid the grafting or incorporation of labile chromium with a slow release mechanism, in order to achieve a more effective AMP technology.

Another important area of future work can look into the antimicrobial efficacy of the AMP promising approaches presented in this thesis against real-world environmental assays in order to find a more precise application scope for these technologies. For instance, the antimicrobial performance of sulfuric-acid oxidised HDPE/PP, and SnO₂ coated polymers can be investigated in an assay that evaluates biofilm development in air under dry conditions, which mirrors situations where microbial surface contamination from hand-skin touching occurs on contact points or from sneezing onto a surface which later dries down. This is important as mature biofilm studies performed under a 24-h hydrated environment in the presence of nutrient rich media did not show any antimicrobial result for these materials. However, such an environment is more stressed and favours microbial growth and biofilm proliferation due to the nutrients and water available as these AMP approaches do not contain a lot of antimicrobial active agents to effectively combat such a system. As a result, environmental experiments under dry conditions can help shed light on the uses and limitations of these AMP approaches for use in a hospital environment, in public spaces, as product packaging, and its efficacy as well as stability.

The formulation of chitosan-ZnO nanocomposite coatings as an effective AMP proved difficult in this research project, therefore another avenue of future work could investigate improving the stability of the formulation to ensure that the free amine groups of chitosan are available and surface exposed, in order for a more antimicrobial coating to be synthesised as the free amine was confirmed to be the active specie contributing to loss in microbial viability against early-stage biofilms of *E. coli*.

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Chapter 2 Appendix

(A) Fowke's Theory Example Calculation

Example calculation from a contact angle experiment, $\theta_{\text{DIM}}: 52.92^\circ$ and $\theta_{\text{Water}}: 95.8^\circ$,

$$W_{SL} = 2 \left(\sqrt{\gamma_{LV}^D \gamma_{SV}^D} + \sqrt{\gamma_{LV}^P \gamma_{SV}^P} \right) = \gamma_{LV} (\cos \theta + 1)$$

Where $\gamma_{LV}^D = 50.8$, $\gamma_{LV}^P = 0$, and $\gamma_{LV} = 50.8$ for DIM

Where $\gamma_{LV}^D = 51.0$, $\gamma_{LV}^P = 21.8$, and $\gamma_{LV} = 72.8$ for water

Given that $\gamma_{LV}^P = 0$ for DIM, the γ_{SV}^D can be determined as the equation becomes,

$$2 \sqrt{\gamma_{LV}^D \gamma_{SV}^D} = \gamma_{LV} (\cos \theta + 1)$$

Rearranging to make γ_{SV}^D the subject,

$$\left(\frac{\gamma_{LV} \times (\cos \theta + 1)}{2} \right)^2 / \gamma_{LV}^D = \gamma_{SV}^D$$

$$\left(\frac{50.8 \times (\cos (52.92) + 1)}{2} \right)^2 / 50.8 = \gamma_{SV}^D$$

$$32.6 = \gamma_{SV}^D$$

Now γ_{SV}^D can be determined from the water contact angle using the calculated γ_{SV}^D value, see below.

$$W_{SL} = 72.8 (\cos 95.8 + 1) = 65.4$$

$$65.4 = 2 \left(\sqrt{\gamma_{LV}^D \gamma_{SV}^D} + \sqrt{\gamma_{LV}^P \gamma_{SV}^P} \right)$$

$$32.7 = \sqrt{(51)(32.6)} + \sqrt{(21.8)\gamma_{SV}^P}$$

$$3.0 = \gamma_{SV}^P$$

To calculate γ_{SV} is the sum of γ_{SV}^P and γ_{SV}^D determined,

$$\gamma_{SV} = \gamma_{SV}^P + \gamma_{SV}^D$$

$$\gamma_{SV} = 32.6 + 3.0$$

$$\gamma_{SV} = 35.6$$

Chapter 3 Appendix

(A) General Excel formulas used

Average

The AVERAGE function in Excel has been utilised. This is described as the arithmetic mean (**Equation A.1**), which is calculated by adding a group of numbers and dividing by the count of those numbers:

$$AVERAGE = \frac{[item\ 1] + [item\ 2] + [item\ 3] \dots}{Total\ number\ of\ items} \quad \text{Equation A.1}$$

Standard Deviation

The SD(P) excel formula has been used as a measure of standard deviation (SD) in this thesis, which is based on the entire population given as arguments (ignores values and texts) and is a measure of how widely the values are dispersed from the average value in the data set, **Equation A.2**:

$$SD(P) = \sqrt{\frac{\sum (x - \bar{x})^2}{n}} \quad \text{Equation A.2}$$

where x is each value from the population, $\bar{x}$ is the AVERAGE, and n is the sample size.

Standard Error

Standard error (SE) measures the accuracy with which a sample distribution represents a population by using the SD determined. The SE has been calculated using the below equation, **Equation A.3**. The Excel SQRT function syntax has been used in the denominator to obtain the SE:

$$SE = \frac{SD(P)}{\sqrt{n}}$$

Equation A.3

where SD(P) is the SD, and n is the sample size.

(B) ICPMS standard calibration for testing chromium

Calibration was performed using a 23-elemental component reference mixture (Aristar, Lot. 386065) was used for the standard. Samples contained 2% nitric acid for ionisation.

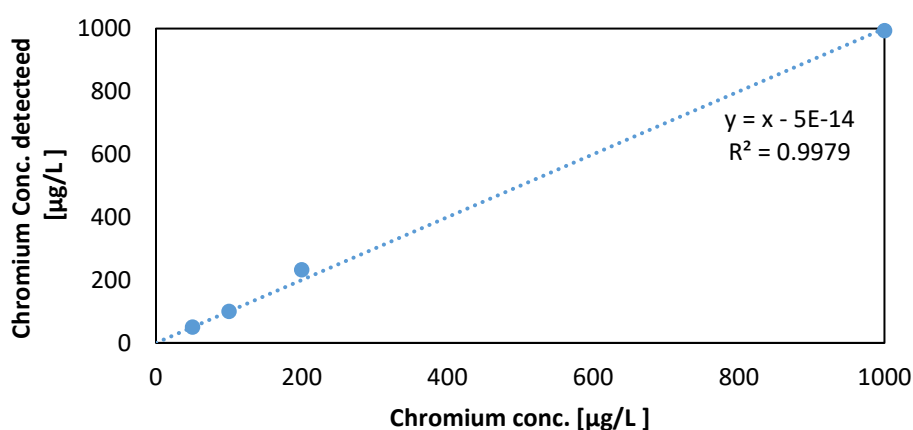


Figure A.1 ICPMS standard calibration for testing elemental chromium. The instrument set-up was carried out by Kaho Cheung, analytical technician at Imperial College London, department of chemical engineering.

(C) ICP-MS standard calibration for testing tin

Calibration was performed using standards prepared with $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ starting reagent from serial dilution with DI water to the required calibration concentrations of 1, 10, 100, 500 and 1000 ppb. Samples contained 2% nitric acid for ionisation.

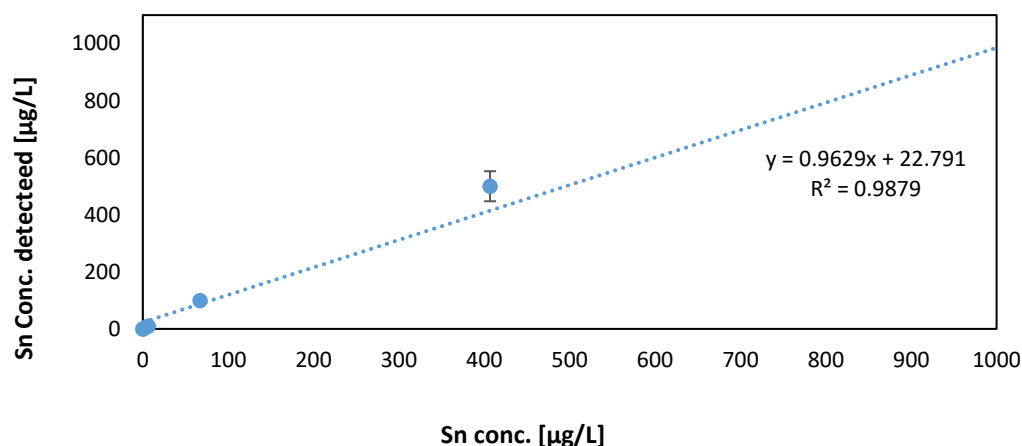


Figure A.2 ICP-MS calibration standards used to determine the concentration of Sn in rinsed washings from SnO₂ thin-film coatings on HDPE and sulfuric-chromic acid oxidised HDPE. The standards were pre-prepared, and the instrument set-up was carried out by Kaho Cheung, analytical technician at Imperial College London, department of chemical engineering.

(D) Optimisation and selection of CLSM Leica systems settings (gain, threshold, gating and optical z-slice)

Samples were prepared as outlined in Section 3.4.5 (Viability Assay for Early-stage Biofilm Viability Imaging). Four samples of HDPE (unmodified) were used in this experiment, whereby two samples were exposed to *P. aeruginosa* early-stage biofilm, and two samples were kept as controls that were exposed to the stains in the absence of *P. aeruginosa*. To find the most optimal threshold (TH), the TH was modulated from 60, 70, 80, 90, 100, 110 and 120, at varying gains settings from 10 to 200 to yield the signal-to-noise ratio (S:N). TH values of 90 and 110 show the best S:N ratio for Syto™9, and TH values of 80 and 90 shows the best S:N ratio for PI under these conditions for *P. aeruginosa* tested. Overall TH 90 provides a good signal to noise ratio for both Syto™9 and PI separate channels. It is important for the PI S:N to be optimal as these values are generally low in signal for the controls and some samples and as a result can be affected heavily by error.

TH: 60	Gain	Syto™9 (S:N)	PI (S:N)		TH: 70	Gain	Syto™9 (S:N)	PI (S:N)
	10	0.0	0.5			10	0.00	0.50
	50	1844.5	0.0			50	0.00	0.00
	100	700.5	10.7			100	700.49	10.65
	150	119.9	2.4			150	119.85	2.37
	200	15.7	1.2			200	15.72	1.23
TH: 80	Gain	Syto™9 (S:N)	PI (S:N)		TH: 90	Gain	Syto™9 (S:N)	PI (S:N)
	10	0.0	0.5			10	0.0	0.00
	50	0.0	0.0			50	0.00	0.00
	100	700.5	127.8			100	2025.9	127.8
	150	121.1	2.3			150	643.5	9.8
	200	122.8	2.3			200	124.0	2.3
TH: 100	Gain	Syto™9 (S:N)	PI (S:N)		TH: 110	Gain	Syto™9 (S:N)	PI (S:N)
	10	0	0.5			10	0.0	0.0
	50	0	0			50	0.0	0.0
	100	4355.0	89.0			100	4355.0	89.0
	150	643.5	9.8			150	643.5	9.8
	200	124.0	2.3			200	124.0	2.3
TH: 120	Gain	Syto™9 (S:N)	PI (S:N)					
	10	0.5	0.5					
	50	0	0					
	100	962.4	0					
	150	3072.2	122.7					
	200	810.9	10.8					

Table A.1 Modulation of gain from 10 to 200 at TH (threshold) 60 to 120. The gating was kept constant at 3 ns delay. The best value for signal-to-noise (S:N) ratio in each instance has been highlighted in grey.

At 100 gain and threshold 90, the gating was modulated from 0 ns delay to 4 ns delay to determine the best setting. We found that 2 ns delay showed the best result overall, therefore this was used as the setting moving forward and kept constant.

Gating delay (ns)	Syto™9 (S:N)	PI (S:N)
0	1244.6	42.2
1	1381.7	50.8
2	1350.8	81.9
3	837.2	48.8
4	1032.7	65.2

Table A.2 Modulating gating to determine best setting for gating using the Leica CLSM. The threshold was kept at 90 and the gain at 100 for both Syto™9 and PI channels.

CLSM optical z-slice utilised for imaging can be modulated, larger optical slices cause the pinhole to be widened and in turn reducing its resolution and capturing unwanted noise. Therefore, the chosen optical slice was a compromise between visual clarity and sampling most of the early-stage biofilm to be analysed which was decided as 10 µm optical slice at 25x magnification using a hydrated/wet lens. Obtaining biofilm stacks was disregarded as it is time consuming and would cause great variability in sampling due to time delays caused by the stack measurement itself and the large volume of samples and repeats that were needed to be evaluated, as well as the restricted access available to the CLSM. Analysis of this chosen setting (10 µm) was performed for HDPE untreated exposed to *P. aeruginosa* early-stage biofilm at 1 µm increment stacks which showed that 10 µm roughly encompassed 83% of the biofilm.

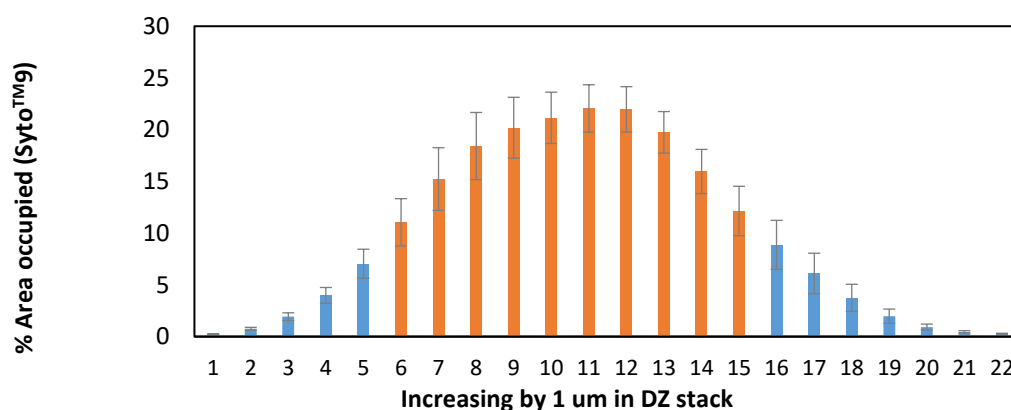


Figure A.3 Stacks of 1 μm increment was obtained from 0 to 200 for early-stage *P. aeruginosa* biofilm. Orange bars represents the 10 μm that would be captured, and the blue represents the region excluded, TH 90, gating 2 ns, gain 100.

(E) Calibration for Viability Invitrogen kit using CLSM with *P. aeruginosa*, *E. coli* and *S. aureus* organisms

Calibration studies have been performed to validate that the stains used in the viability assay does show a difference when known concentrations of live and dead bacteria are prepared and stained. Initially it was found that simply placing a solution mixed with a set amount of live and dead cells on a reference inert surface did not show representative changes in ratios of Syto™9/PI because the dead bacteria did not stick well to a 'non-active' surface compared to the live bacteria. For example, if you were to place a solution of 90% dead bacteria on a surface, and therefore it would appear as though the surface had a lot of living surface bound bacterial cells as the dead bacteria did not adhere.

To overcome this issue, suction filtration was used to draw down all the bacteria in solution onto a filter paper, the inner filter lining was soaked in media (100% tryptic soy broth) solution prior to prevent bacteria from dying from dehydration during filtration, see photos below.

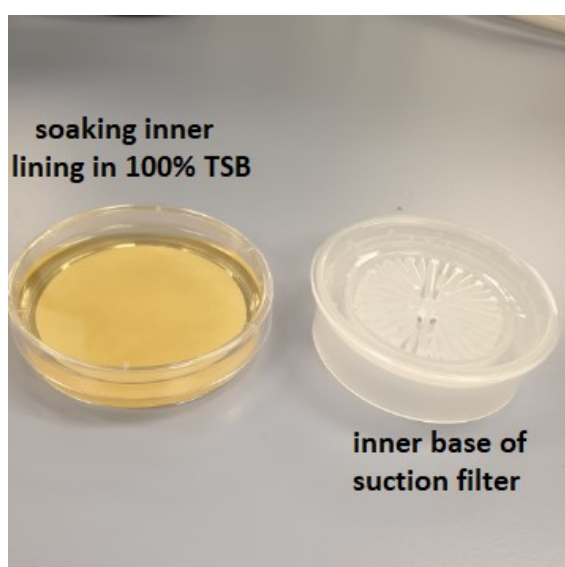


Figure A.4 Photos taken to show how the filter system was set up for calibration experiments for the CLSM viability assay.

The method was adapted from the usage manual of the probes by suppliers (Invitrogen) for the LIVE/DEAD[®] BacLight Kit L7012. Sample bacteria (30 μ L) was extracted from a cell bank and placed in 100% tryptic soy broth (TSB) (30 mL) overnight at 32.5[°] C as advised. After 18-24 hr, the inoculant was split into two centrifuge vials and centrifuged for 15 min at 3500 min^{-1} . The supernatant was removed, saline (20 mL) was added to one and 70% isopropyl alcohol (IPA) (20 mL) was added to the other; the IPA treated batch formed the 'dead' batch of bacterial cells. These were incubated for 1 hour at 32.5[°] C with constant shaking. Both vials were centrifuged again (3500 min^{-1}), the supernatant was removed and saline (15 mL) was added to both. A UV-Vis spectrophotometer was used to prepare 0.2 optical density (OD) at 600 nm wavelength for each sample, found to correspond to 4.9×10^7 CFU/mL after 15 hr of growth on agar plates. Different concentrations of mixtures of live and dead bacteria were prepared from 0 to 100% viability (1 mL total volume). Syto[™]9 (1.5 μ L) and PI (1.5 μ L) was added to each mixture and left for 5 min prior analysis was to be performed in the dark. To this solution, saline was added (10 mL) and the solution was filtered in a sterile suction filter (0.45 μ m pore) with inner sponge lining soaked in 100% TSB for 3-4 hr prior use as *P. aeruginosa* viability reduces significantly when dehydrated without this step. After the sample was filtered, the filter was imaged using the CLSM to image bound cells at the surface of the filter. Image-J (FIJI) and Comstat 2.1 was used for the conversion to ometif and analysis of threshold and percentage surface area coverage of the cells in the

images captured with optical section set to 10 µm in thickness, 1024 x 1024 resolution with a 25x magnification lens.

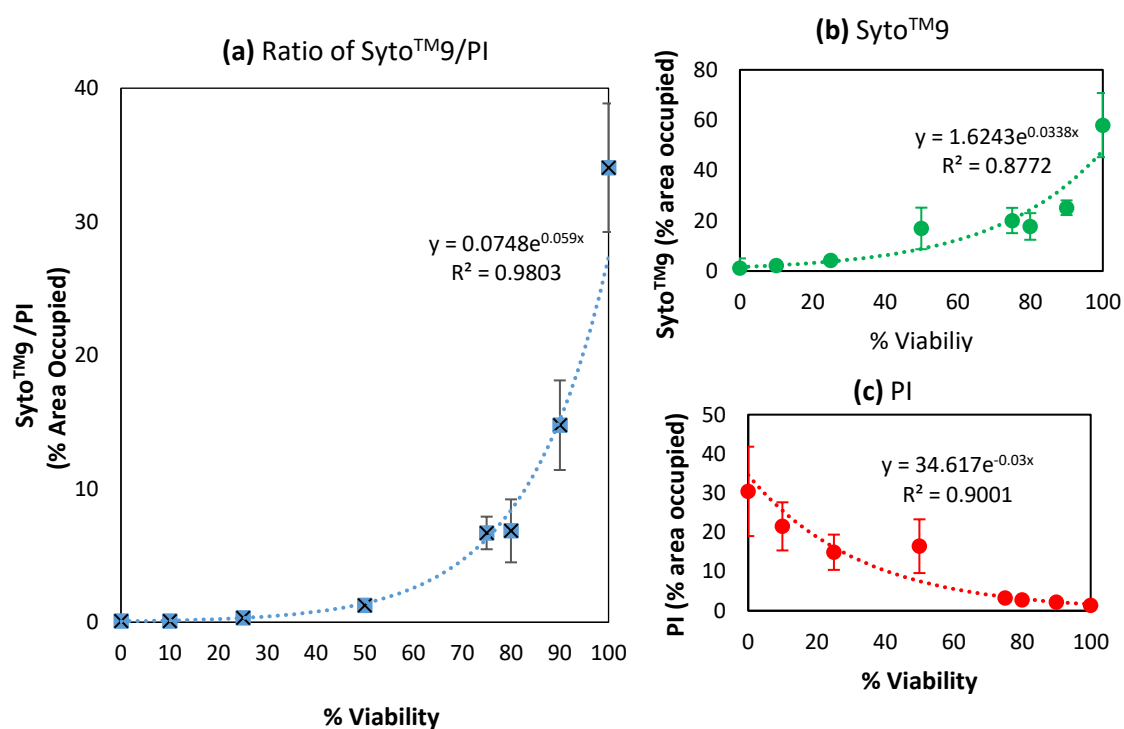


Figure A.5 Calibration Curve for *P. aeruginosa* performed using suction filtration and imaged CLSM, where (a) provides the ratio of Syto™9/PI, (b) Syto™9 channel only, (c) PI channel only. $N=5$ (number of biological repeats on separate occasions), $\pm$ SE, data analysed using Image J with the Comstat 2.1 plugin, 1024 x 1024 pixel resolution with a 25x Magnification.

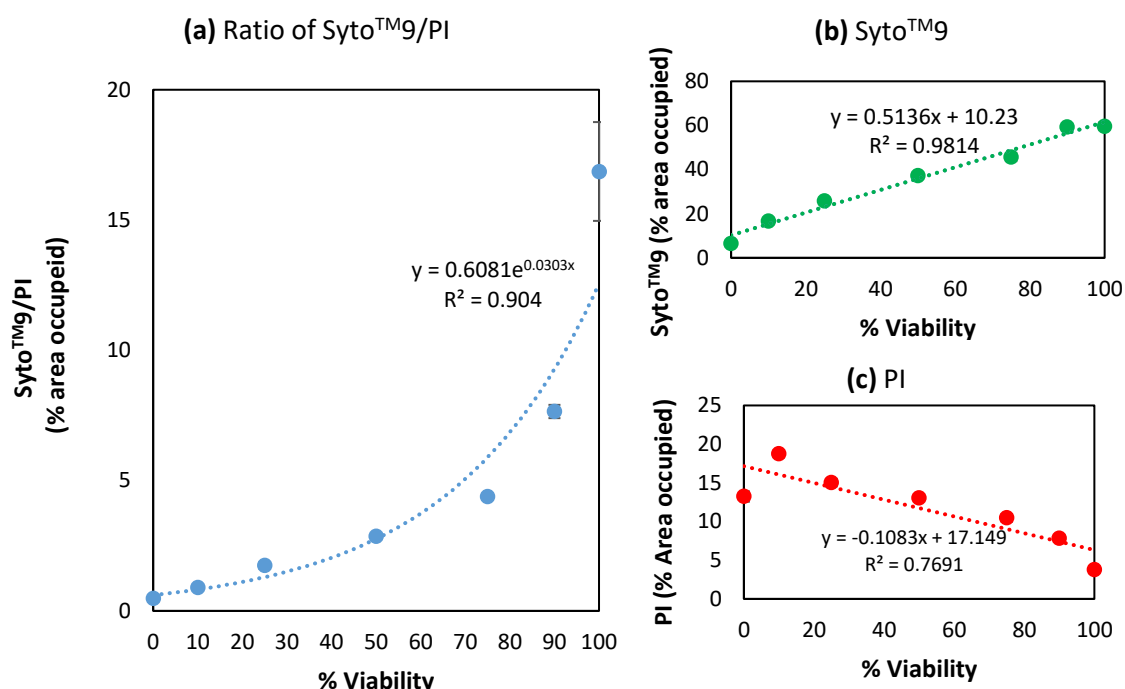


Figure A.6 Calibration Curve for *S. aureus* (ATCC 6538) performed using suction filtration and imaged CLSM, where (a) provides the ratio of Syto™9/PI, (b) Syto™9 channel only, (b) PI channel only. N=5 (number of images taken), +/- SE, data analysed using Image J with the Comstat 2.1 plugin, 1024 x 1024 pixel resolution with a 25x Magnification.

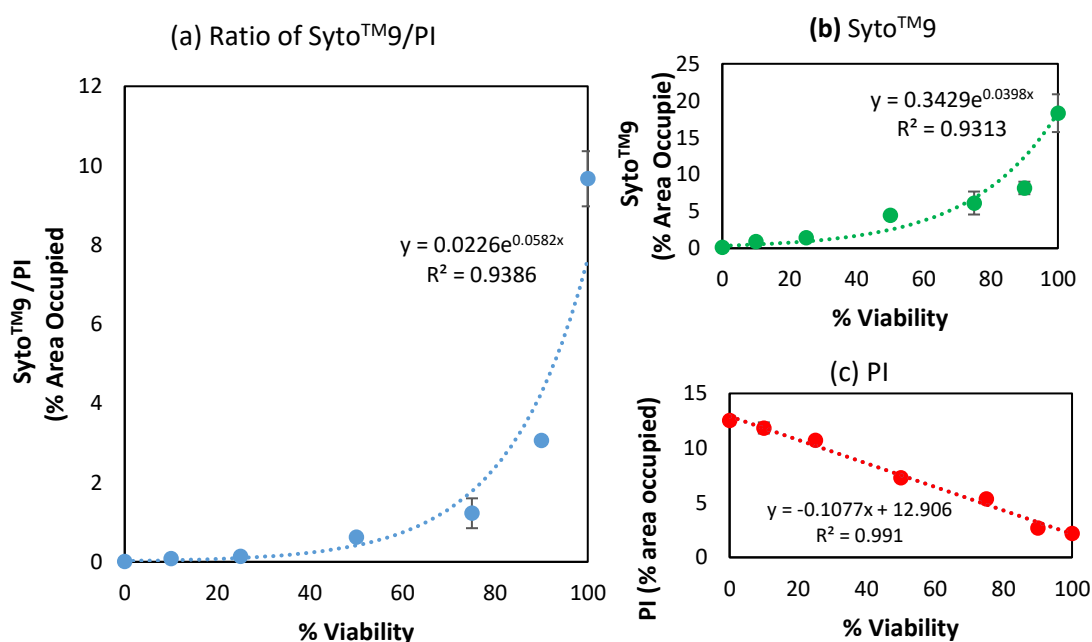


Figure A.7 Calibration Curve for *E. coli* (ATCC 10536) performed using suction filtration and imaged CLSM, where (a) provides the ratio of SytoTM9/PI, (b) SytoTM9 channel only, (b) PI channel only. *N*=2 (number of biological repeats), +/- SE, data analysed using Image J with the Comstat 2.1 plugin, 1024 x 1024 pixel resolution with a 25x magnification.

(F) Evaluation of methods to remove biofilm from a sample surface

(i) Sonication of surface with biofilms in the presence of surfactants

Selected reference samples were used to assess the effectiveness of sonication to remove the early-stage biofilm of *P. aeruginosa* via CLSM imaging. Pre-sonication procedure follows the IAA, which experimental method has been given in Chapter 3 under sub-section 3.3.4. For the post-sonication procedure, *P. aeruginosa* was cultured overnight in 100% TSB, the solution was diluted to 0.2 OD (600 nm, 4.9×10^7 CFU/mL) with 100% TSB. 200 μ L of the suspension was pipetted onto a surface and left static for 30 mins. The surface was then rinsed in 3 beakers of water (100 mL, 4.5cm²/s) to remove planktonic cells. The samples were placed face-down in MLBTL (modified letheen broth with tween and lecithin) surfactant solution. The MLBTL solution containing the samples were sonicated for 10 mins at RT in aims of removing the early-stage *P. aeruginosa* biofilm. The samples were extracted and placed on a flat bench-top, 200 μ L of SytoTM9 (5 mM) probe solution in 1/1000 was pipetted onto the biofilm and left for 5 mins in the dark. The probe solution was expunged using a tissue (avoiding surface contact) and replaced with water (200 μ L) for hydrated dip-lens CLSM imaging of the biofilm. Image-J (FIJI) and Comstat 2.1 software were used for the conversion to ome-tiff format and analysis of threshold and percentage surface area coverage of the cells in the images captured with optical imaging section set to 10 μ m in thickness, with a 1024 x 1024 pixel resolution using a 25x magnification liquid lens.

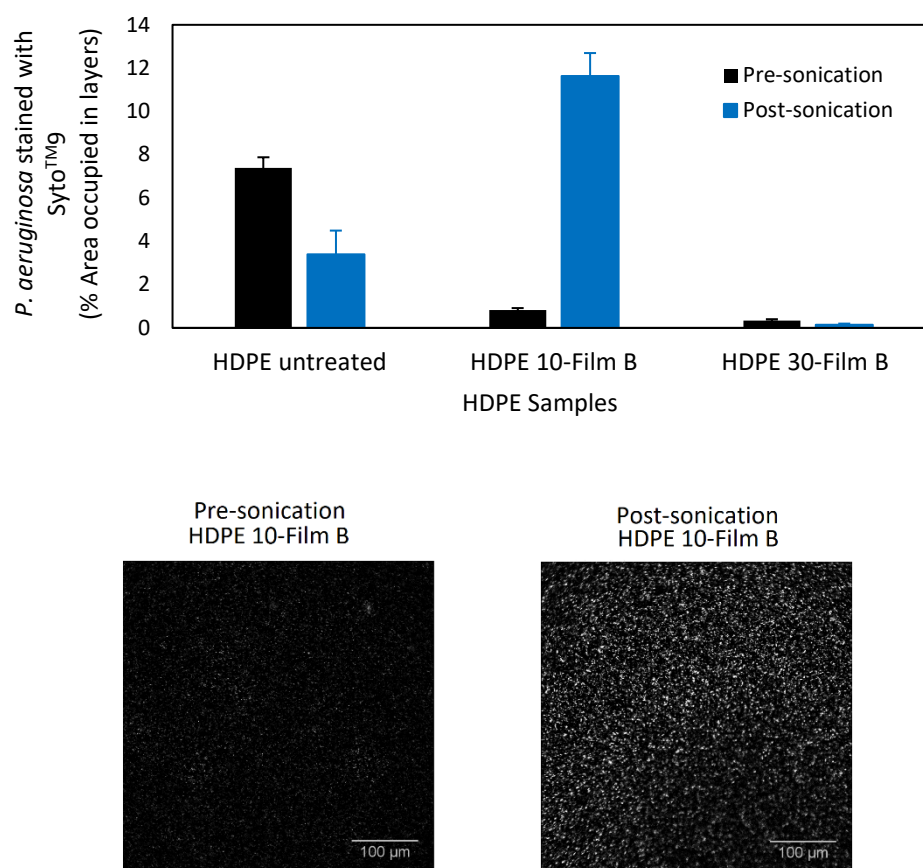


Figure A.8 CLSM sonication assay using SytoTM9 with *P. aeruginosa*, the samples were imaged pre- and post- sonication to determine the effectiveness of this approach in removing the biofilm. Example images of HDPE 10 mins sulfuric-chromic acid oxidised with Film B coating has been provided beneath, scale bar showing 100 μm.

(ii) Agitation of surface with biofilms

Stainless-steel holders were bespoke manufactured to hold the HDPE coupons within a 24-well plate as HDPE floats. These were manufactured by the Imperial workshop, in the Chemical Engineering department at Imperial College London. This set-up was utilised for the purpose of ensuring the HDPE surfaces are hydrated and suspended in the centre of each well for maximised exposure for biofilm maturation to form an early-stage biofilm, in a suspension of media. Images of the holders can be found below.

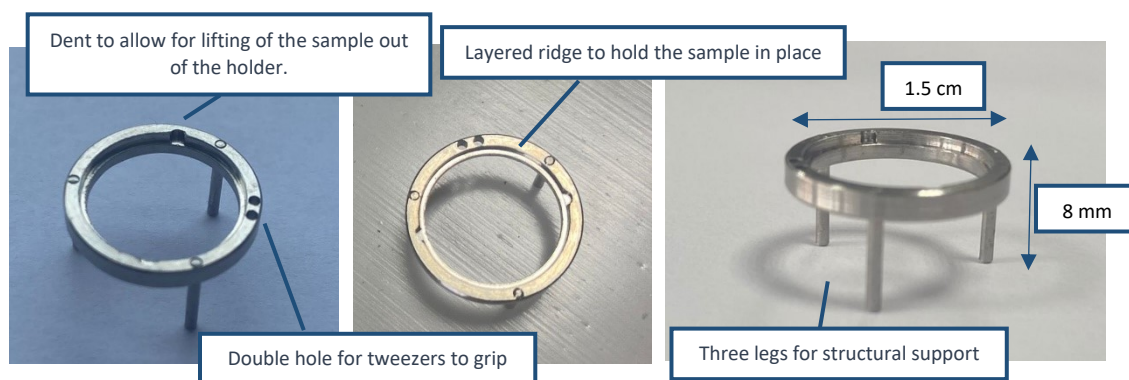


Figure A.9 Sample holders for OD-plate reader experiment for evaluation of early-stage biofilm viability.

Selected reference samples were used to assess the effectiveness of sonication to remove the early-stage biofilm of *E. coli* via CLSM imaging. *E. coli* (Mg 1655 with Plasmid Pet33Bt) was cultured overnight in LB containing 0.1% kanamycin, the solution was diluted to 0.05 OD with LB containing 0.1% kanamycin. 1 mL of this solution is added to a 24-well plate, the ring holders containing the sample are placed inside the well using sterile tweezers, and a further 1 mL of this solution is added. The plate was placed in a static incubator for 30 mins at 37 °C for early-stage biofilm maturation. The samples were taken out and placed into a fresh 24-well plate containing 1 mL of PBS, after a further 1 mL of PBS was added on top (total volume 2 of PBS 2 mL). 1 mL was suctioned from the well using a pipette and a further 1 mL of PBS added, to rinse the planktonic cells. The samples were removed from the holders and placed into a new 24-well plate (500 μ L with 0.1% kanamycin), this was placed in a shaker incubator for 2 h under agitation at 37 °C. The resultant solutions were collected and placed in a fresh 24-well plate, and recorded on an OD-plate reader for 12 h.

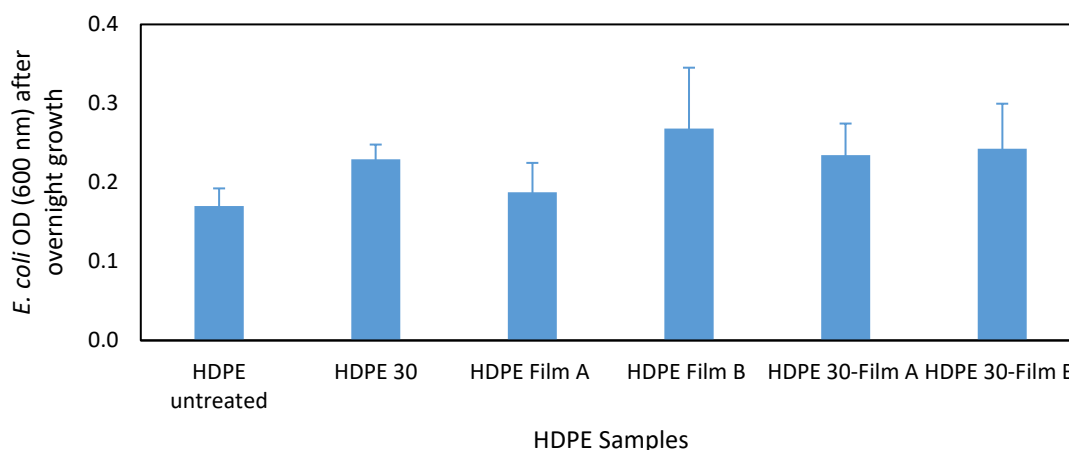


Figure A.10 Results of HDPE modified samples from the use of agitation within an OD plate reader, a shaker incubator was used to dislodge the early-stage biofilm of *E. coli*. Results are an average of two biological repeats conducted on separate days, blank corrected, with 3 samples each, +/- SE.

(iii) Glass beads of surface with biofilms

Selected reference samples were used to assess the effectiveness of sonication to remove the early-stage biofilm of *E. coli* via CLSM imaging. *E. coli* (Mg 1655 with Plasmid Pet33Bt) was cultured overnight in LB containing 0.1% kanamycin, the solution was diluted to 0.05 OD with LB containing 0.1% kanamycin. 1 mL of this solution is added to a 24-well plate, the ring holders containing the sample are placed inside the well using sterile tweezers, and a further 1 mL of this solution is added. The plate was placed in a static incubator for 30 min at 37 °C for early-stage biofilm maturation. The samples were taken out and placed into a fresh 24-well plate containing 1 mL of PBS, after a further 1 mL of PBS was added on top (total volume 2 of PBS 2 mL). 1 mL was suctioned from the well using a pipette and a further 1 mL of PBS added, to rinse the planktonic cells. The samples were removed from the holders and placed separately into scintillation vials, containing fresh LB media and 0.1% kanamycin (500 µL). 3 beads/6 beads glass beads (5 mm diameter) were added to each scintillation vial. This was vortexed at high speed for 20 sec. The resultant solution was pipetted into a fresh 24-well plate and recorded on an OD-plate reader for 12 h.

In addition to this, a set of samples included were not PBS rinsed to determine the efficacy of PBS washing in dislodging in removing the planktonic cells.

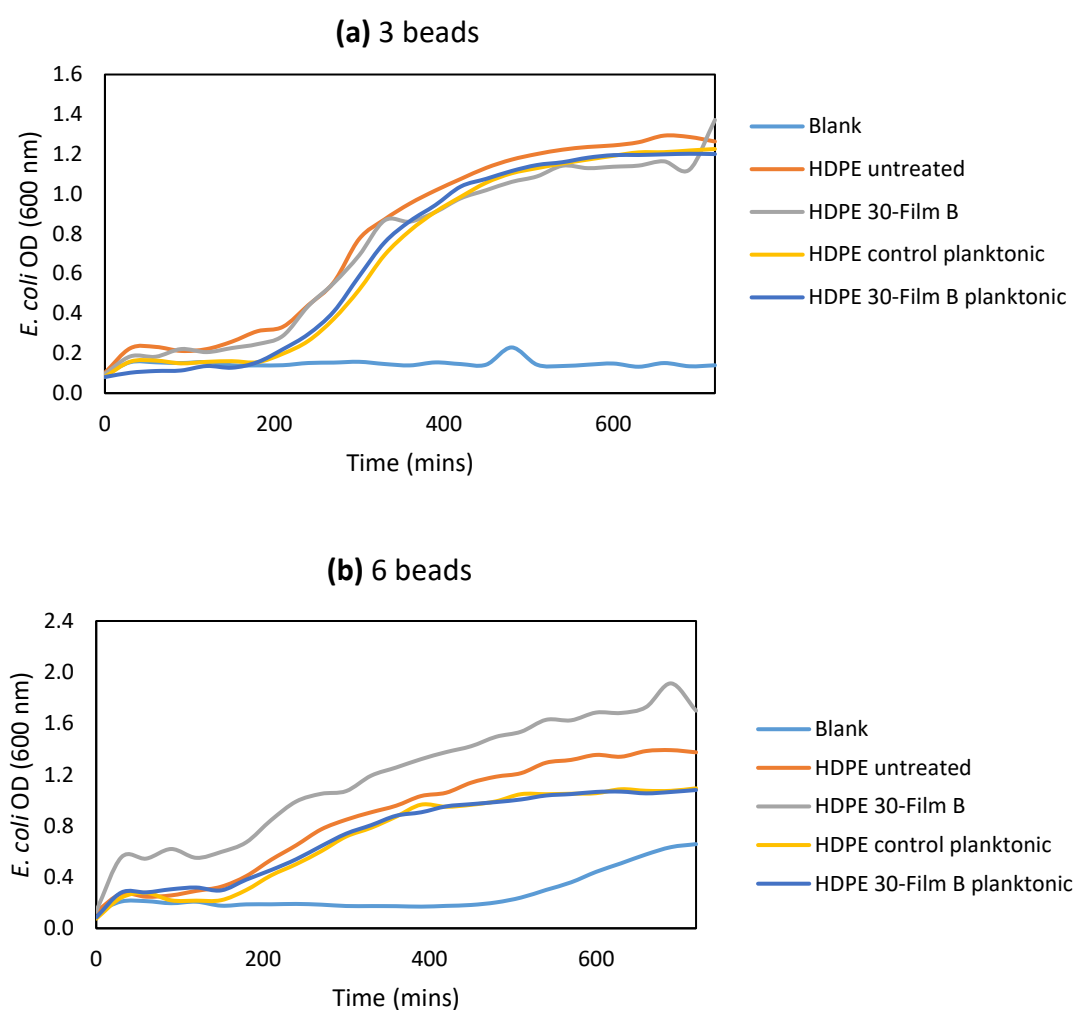


Figure A.11 The OD-plate reader results from the use of glass beads to remove the early-stage biofilm. Planktonic samples refer to samples that do not under-go the PBS washing steps, where in (a) 3 beads used, (b) 6 beads used. Raw data provided, results are an average of 3 samples each at each time interval.

(iv) Scraping of surface with biofilms

Selected reference samples were used to assess the effectiveness of sonication to remove the early-stage biofilm of *P. aeruginosa* via CLSM imaging. *P. aeruginosa* (NCTC 12924) was cultured overnight in 100% TSB, the solution was diluted to 0.2 OD (600 nm, 4.9×10^7 CFU/mL) with 100% TSB. 200 μ L of the suspension was pipetted onto a surface

and left static for 30 mins. The surface was then rinsed in 3 beakers of water (100 mL, 4.5cm²/s) to remove planktonic cells. 200 µL of Syto-9TM (5 mM) probe solution in 1/1000 was pipetted onto the biofilm and left for 5 mins in the dark. The probe solution was expunged using a tissue (avoiding surface contact) and replaced with water (200 µL) for hydrated dip-lens CLSM imaging of the biofilm. Image-J (FIJI) and Comstat 2.1 software were used for the conversion to ome-tiff format and analysis of threshold and percentage surface area coverage of the cells in the images captured with optical imaging section set to 10 µm in thickness, with a 1024 x 1024 pixel resolution using a 25x magnification liquid lens. 1 mL of PBS was placed on the surface, a cell-scraper was used to lift the biofilm off and re-suspend it into the PBS solution, the PBS solution was extract, this was repeated once more in the aim of removing the early-stage biofilm from the surface. The samples were imaged again, using 200 µL of water and a wet-lens to obtain the post-scraping images.

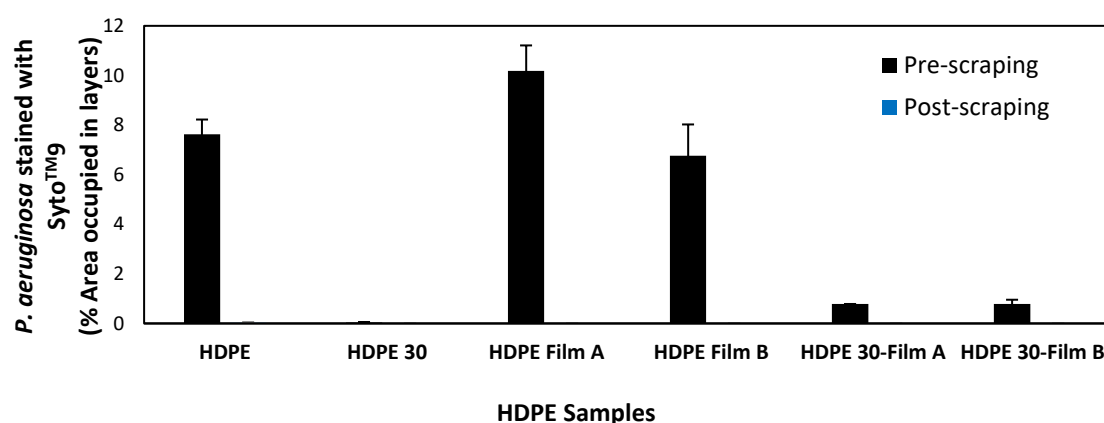


Figure A.12 CLSM imaging used to evaluate the scraping method of the early-stage biofilm using SytoTM9 with *P. aeruginosa*, the samples were imaged pre- and post- scraping.

Chapter 4 Appendix

(A) Water advancing contact angle measurements after rinsing, for HDPE sulfuric-chromic acid oxidised at 0, 2, 10 and 30 min

Test samples were vertically dipped into three containers of deionized water (30 mL) sequentially by hand, this rinsing was monitored at 3 secs per rinse and left to air dry before being measured for water advancing contact angle measurements. The data was collected using a Krüss Drop Shape Analyzer (DSA25) with the Advance Software with an automated liquid dispenser operating at 2.67 $\mu\text{L/s}$. An initial droplet of 5 μL was used, and this was increased by a series of 1 μL aliquots to record the advancing contact angle. The advancing contact angles reported were an average of the right- and left-hand side of the contact angles using the tangent setting from the software and consisted of a minimum of 3 different droplets ($N \geq 3$), $\pm$ SE.

Sulfuric-chromic acid submersion time (min)	Original (°)	Further Rinsed (°)
0	91.7 $\pm$ 1.4	90.3 $\pm$ 1.3
2	51.7 $\pm$ 2.8	50.9 $\pm$ 2.7
10	46.4 $\pm$ 1.5	45.8 $\pm$ 1.3
30	46.0 $\pm$ 1.2	46.7 $\pm$ 1.5

Table A.3 Water advancing contact angle of HDPE surfaces upon sequential rinsing for sulfuric-chromic acid oxidised fresh samples.

(B) XPS C1s core level scan of 20, 45 and 60 minute treated sulfuric-chromic acid HDPE samples

XPS was performed using high-resolution X-ray photoelectron spectroscopy (XPS), with the Thermo Scientific K-Alpha⁺ instrument at 2×10^{-9} mbar base pressure. This instrument used a monochromated micro-focused Al K α X-ray source ($h\nu = 1486.6$ eV) and a 180° double-focusing hemispherical analyser accompanied by a 2D detector. The X-ray source operated at 6 mA emission current and 12 kV anode bias thus obtaining a

spot size of 400 μm^2 . An electron flood gun was used to minimize the sample charging that occurs when exposing an insulated sample to an x-ray beam. Charge neutralization was deemed to have been achieved by monitoring the energy of the C 1s signal of adventitious carbon present. All XPS spectra were recorded using the Advantage Data System software as was the quantification analysis.

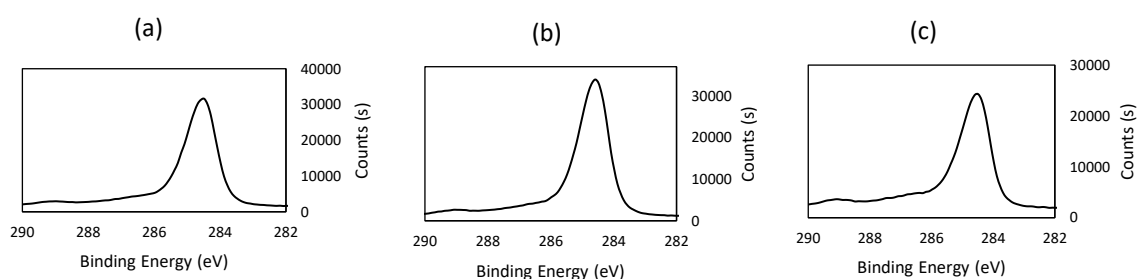


Figure A.13 XPS core level C 1s scan of HDPE oxidised with sulfuric-chromic acid solution at 70 °C with (a) 20 min, (b) 45 min and (c) 60 min submersion time periods.

(C) Agar disk diffusion results with *P. aeruginosa* and *E. coli*

Agar disk diffusion was performed using *P. aeruginosa* (PA01) and *E. coli* (Mg1655) on HDPE untreated and HDPE 30 min oxidised with sulfuric-chromic acid treatment.

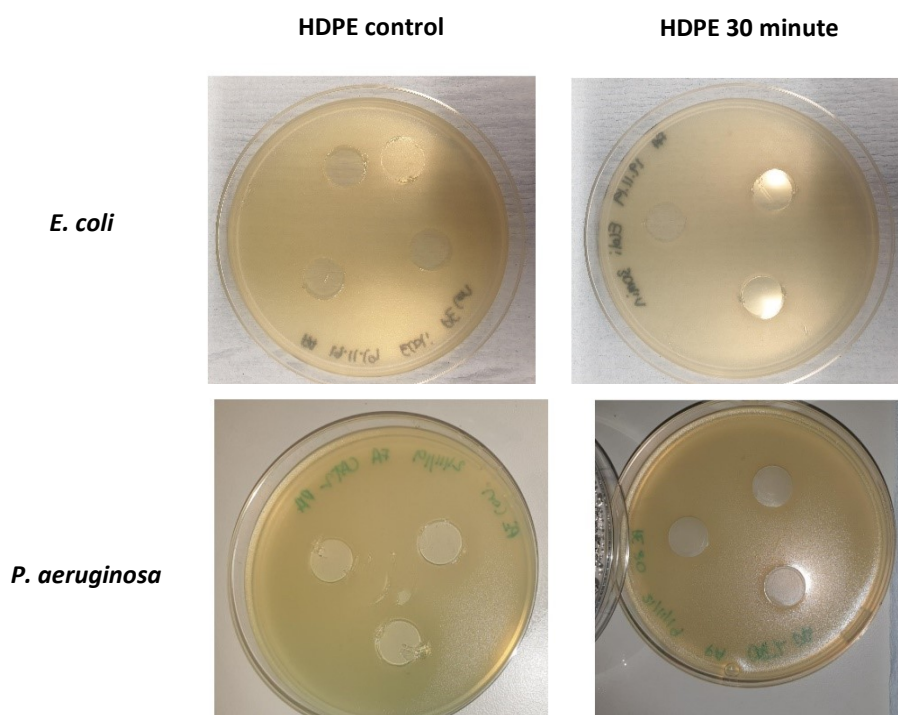


Figure A.14 Agar disk diffusion assay to determine leaching of any antimicrobial reagents from the surface against *E. coli* (Mg1655) and *P. aeruginosa* (PA01).

(D) Gating and thresholds used for FC using DiBAC₄(3) with *P. aeruginosa* and dye optimisation

Forward scatter (FSC) and side scatter (SSC) are signals generated from the scattering or refraction of the laser light by the cell or particle being measured during the experiment. To minimise the influence of noise (bubbles/debris/electronic noise) as the bacteria are very small and can be found at the lower end of the detection limits (1-2 µm in size), a gate (R2) was prepared based on controls in the FSC and SSC plot. These controls can be seen below.

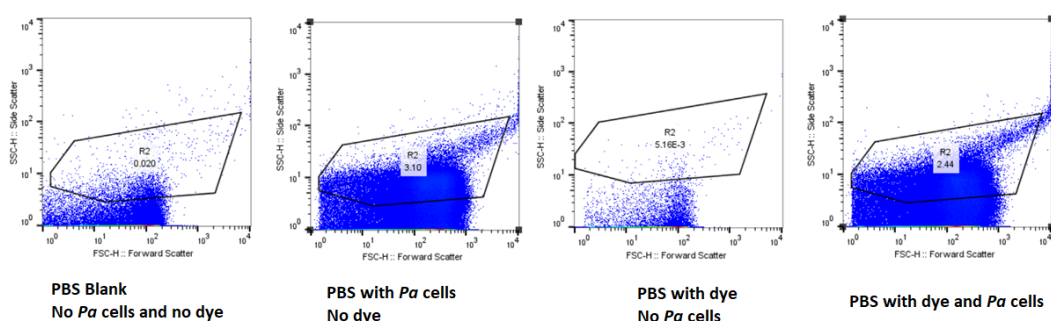


Figure A.15 Gating of forward and FCS and SSC plots named R2, with DiBAC₄(3) as the dye.

This gating was then used during dye calibration studies to determine the concentration of dye for the experiment. Calibration/titration was important to achieve optimal signal to noise, for example to achieve good separation between close signals. The calibration studies were conducted at high and low concentrations of *Pseudomonas aeruginosa* (at high and low optical densities) as the number of recovered cells from biofilm studies can be varied. Increasing DiBAC₄(3) showed that 0.625 µg/L provided the best staining of the cells without largely shifting the control peak (peak for unstained cells) significantly, and provided significant separation between peaks.

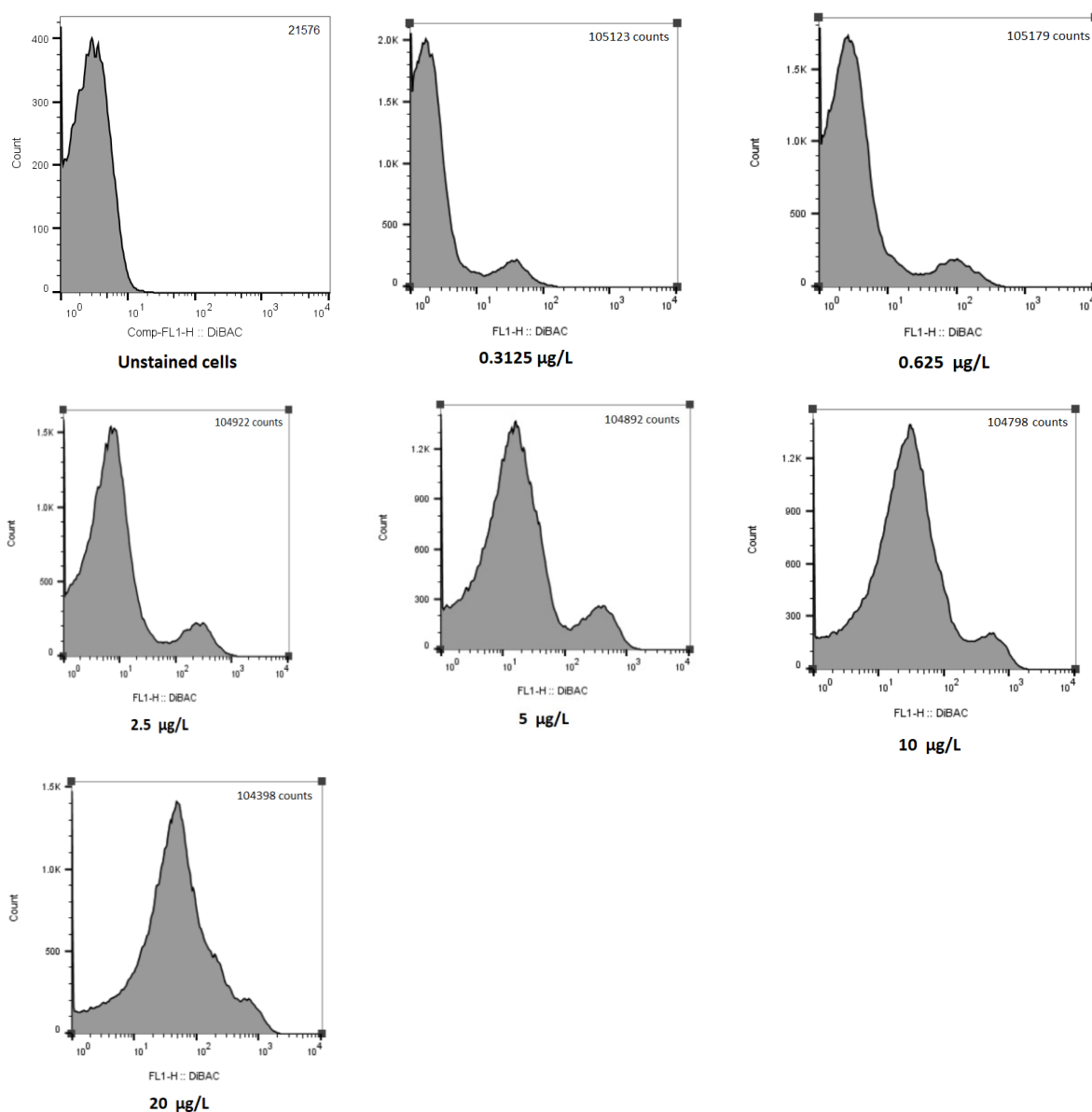


Figure A.16 Histogram plots of DiBAC4(3) stained *P. aeruginosa* with increasing dye amount for high concentrations of bacterial cells (*P. aeruginosa* OD 1 at 600 nm in PBS buffer solution). The concentration of dye used is provided underneath each histography graph.

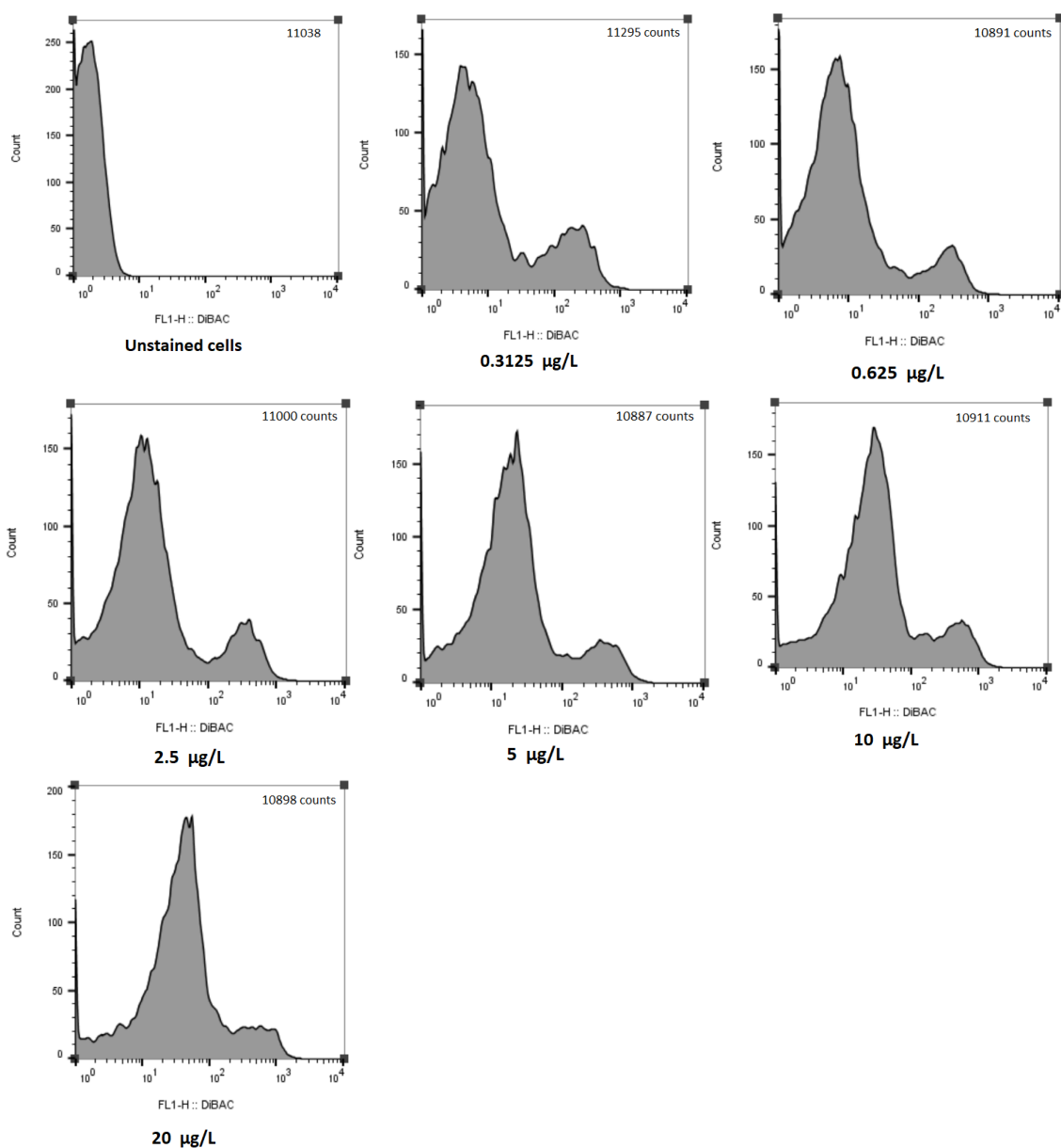


Figure A.17 Histogram plots of DiBAC₄(3) stained *P. aeruginosa* with increasing dye amount for low concentrations of bacterial cells (*P. aeruginosa* OD 0.05 at 600 nm in PBS buffer solution). The concentration of dye used is provided underneath each histography graph.

(E) Time-progression of sulfuric-acid and UV/ozone using ATR-FTIR

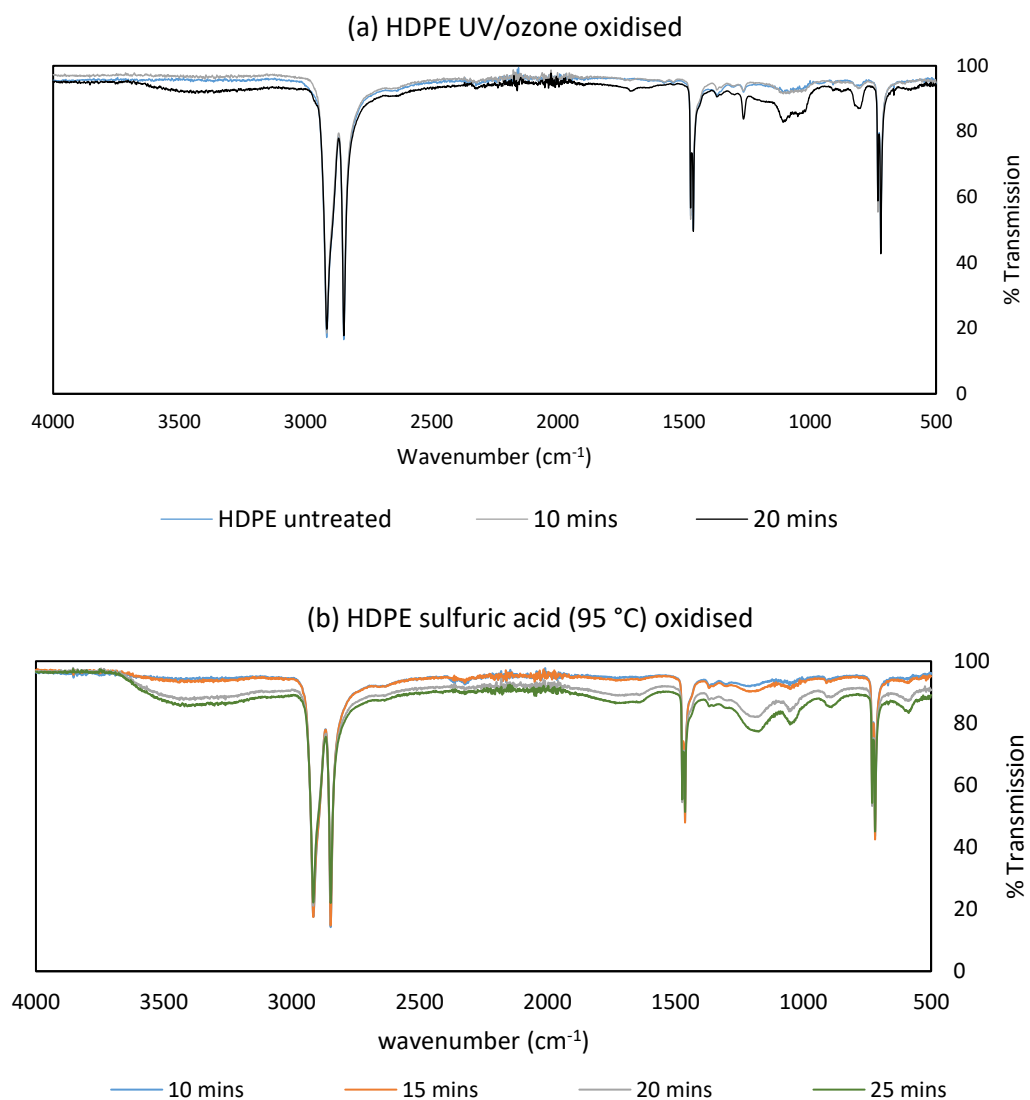
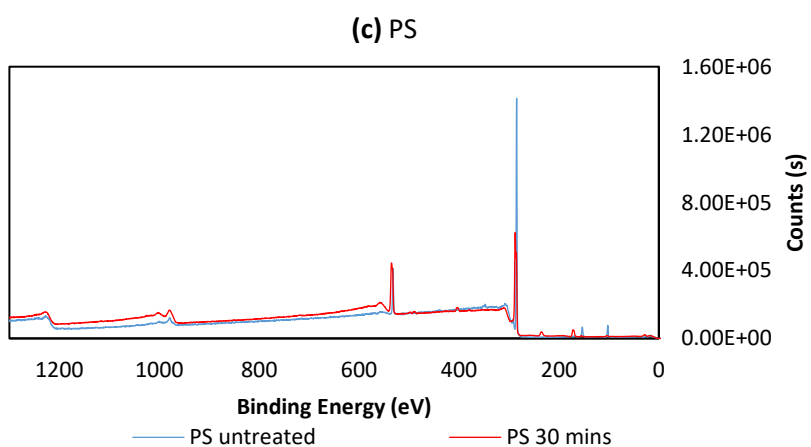
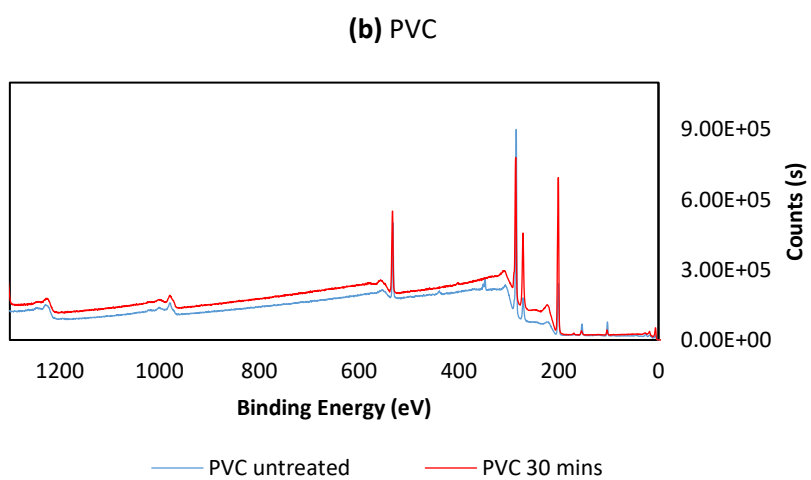
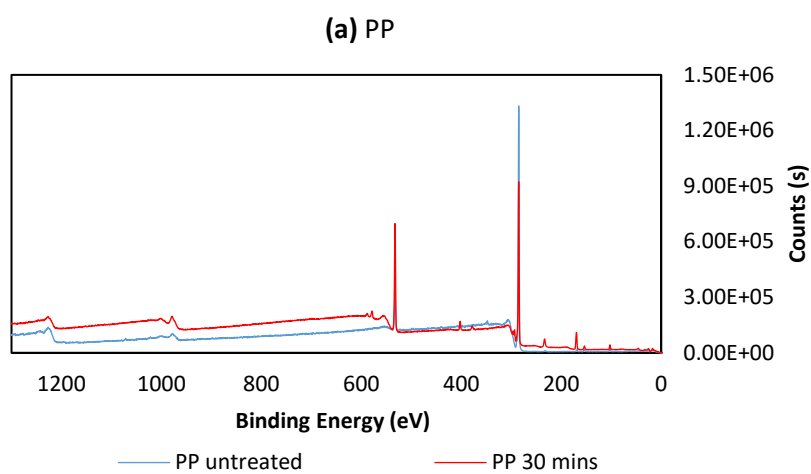


Figure A.18 ATR-FTIR of (a) HDPE UV/ozone oxidised and (b) HDPE sulfuric acid oxidised at different exposure periods.

(F) XPS survey scans of sulfuric-chromic acid oxidised PP, PC, PVC and PS



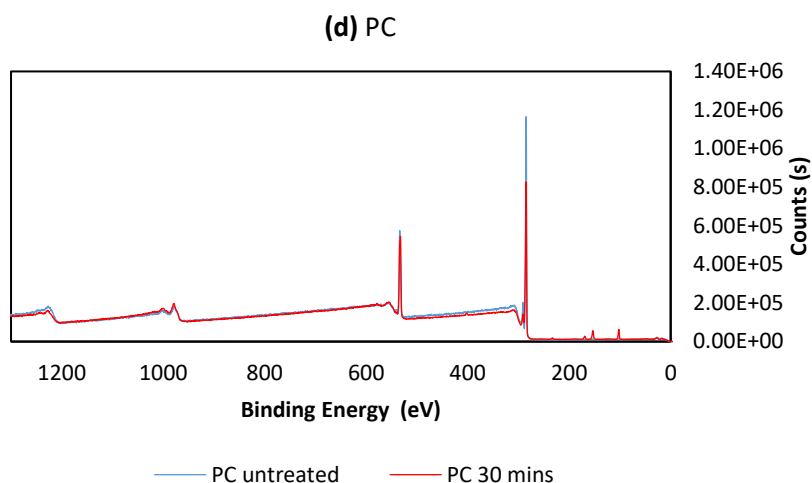
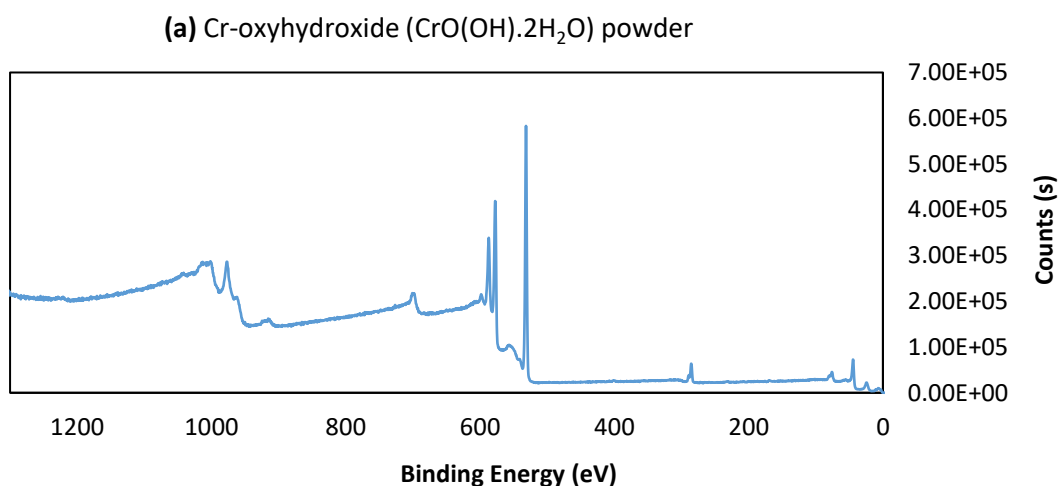


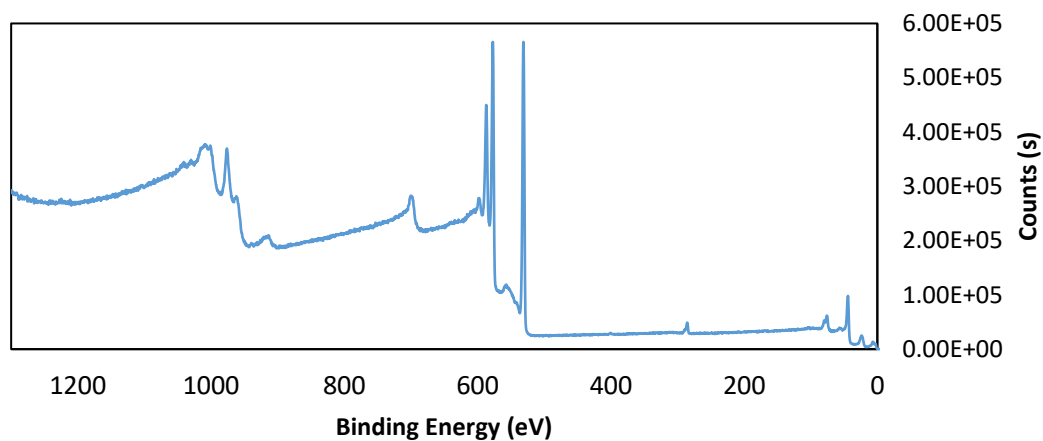
Figure A.19 XPS survey scans of polyolefins untreated and oxidised with sulfuric-chromic acid for 30 mins, for samples (a) PP, (b) PVC, (c) PS and (d) PC.

(G) XPS survey scan of Cr-oxyhydroxide samples

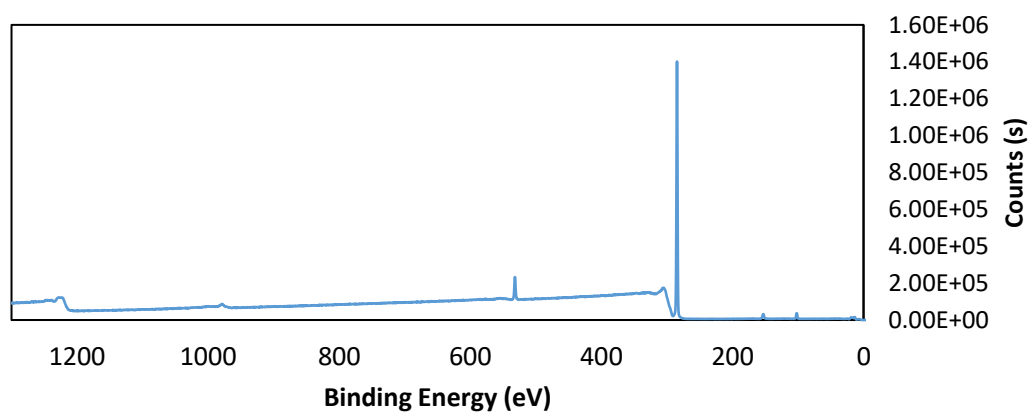
XPS was performed as outlined in Chapter 3, Cr-oxyhydroxide powders were stuck to carbon tape to be measured.



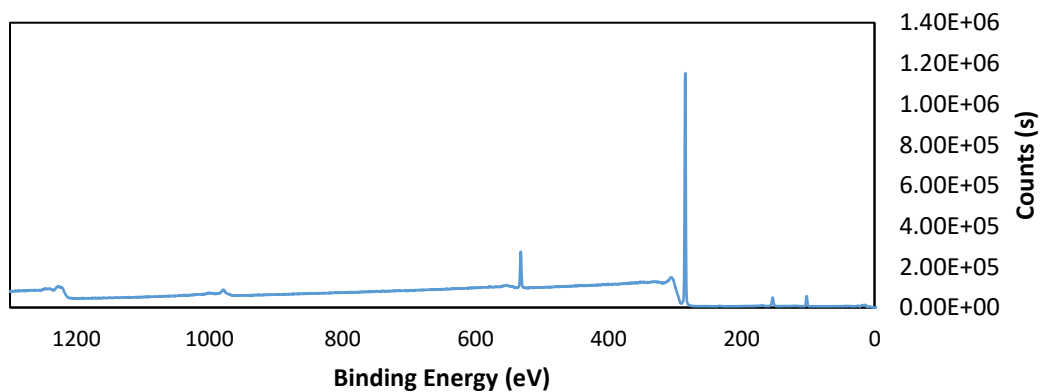
(b) Cr-oxyhydroxide ($\text{CrO}(\text{OH}) \cdot 2\text{H}_2\text{O}$) powder HT



(c) HDPE with Cr-oxyhydroxide ($\text{CrO}(\text{OH}) \cdot 2\text{H}_2\text{O}$)



(d) HDPE with Cr-oxyhydroxide ($\text{CrO}(\text{OH}) \cdot 2\text{H}_2\text{O}$) HT



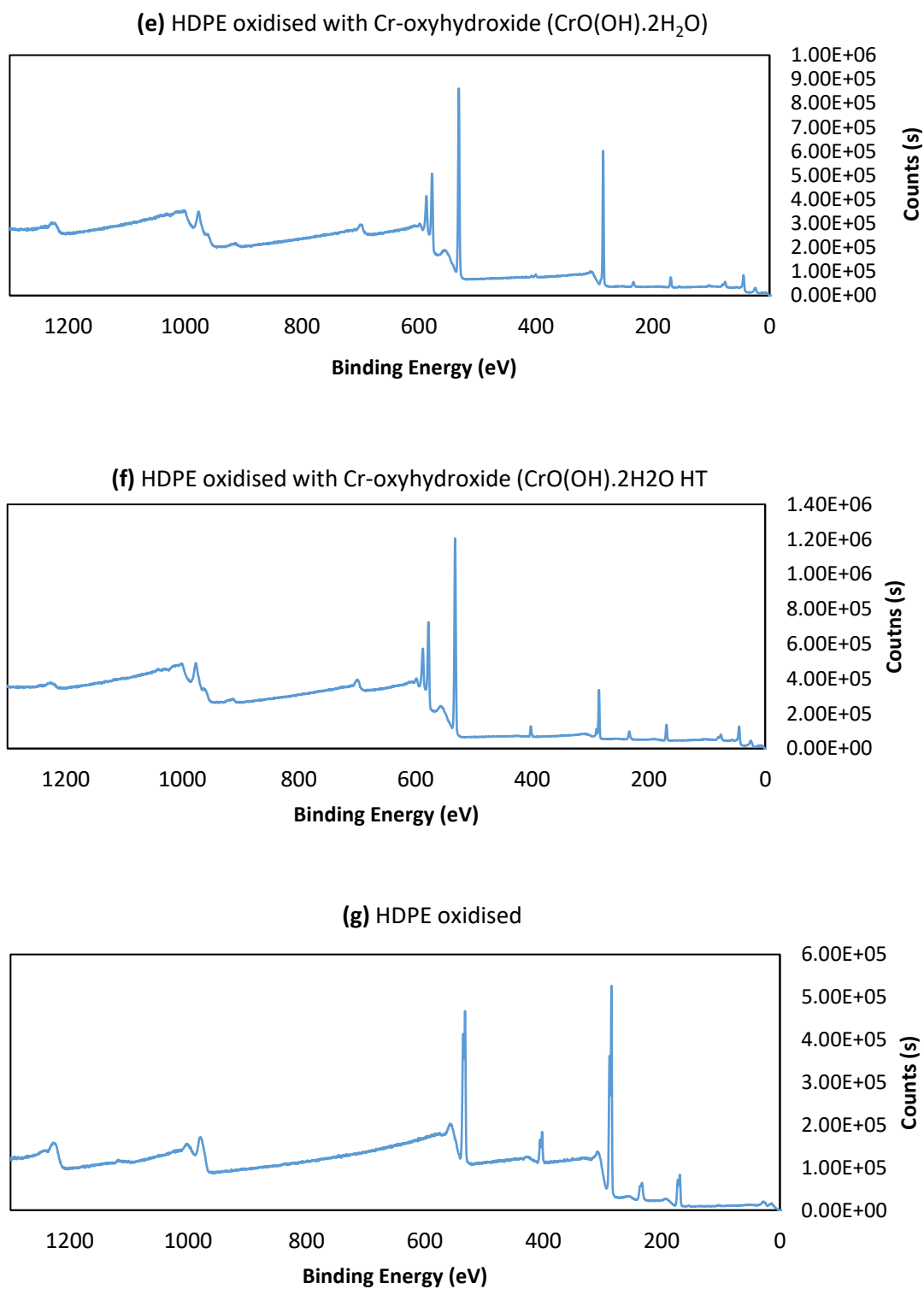


Figure A.20 XPS full survey scans of Cr-oxyhydroxide samples (a-f) and control (g). HDPE oxidised refers to HDPE that has been pre-treated with UV/ozone and sulfuric acid heated oxidation treatment.

Chapter 5 Appendix

(A) Refractometry Results of Tin-hydrosol A and Tin-hydrosol B

A Ceti Convex bench-top refractometer was employed, measured at RT (22 °C).

Tin-hydrosol A	Tin-hydrosol B
1.3334	1.3330

Table A.4 Refractive index measured, results are an average of 5 repeats, +/- 0.0002 for both (standard deviation).

(B) UV/vis spectroscopy results of tin-hydrosol A & tin-hydrosol B

Instrument absorbance reliability is limited to no more than 2, therefore the samples were diluted from the original concentration prepared to obtain reliable spectra. The results show little to no absorption at 830 nm for tin-hydrosol A and therefore this was designated the lowest possible absorbance value permitted by the instrument (0.001), and little absorption for tin-hydrosol B of ca. 0.1.

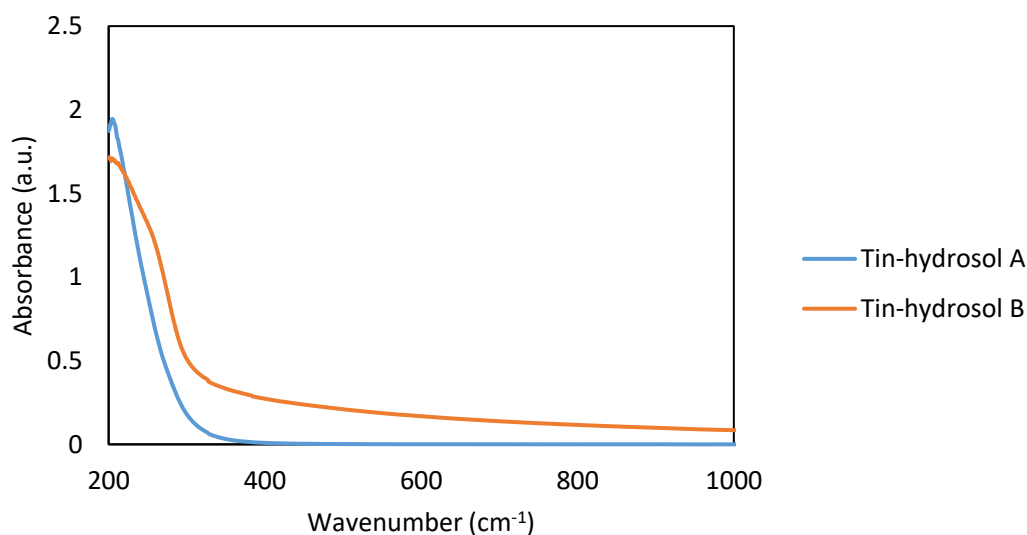


Figure A.21 For tin-hydrosol A the solution had to be diluted 1/50 and for tin-hydrosol B the solution diluted 1/20 to obtain absorbance values less than 2.

(C) DLS Malvern μ V Zetasizer parameters

The following information has been largely sourced from the instrument manual for the Malvern μ V Zetasizer. The detected light by randomly scattered particles gives a fluctuating intensity signal. The intensity will change over time as particles continue to diffuse in solution. The speed of intensity fluctuations relates to the particles diffusion rate. Therefore, smaller particles give rise to more rapid intensity fluctuation as they diffuse more quickly. Random fluctuations are interpreted in terms of the autocorrelation function which can be used to determine the diffusion coefficient (D), this is achieved by comparing the intensity signal at short intervals and comparing to see if the signals are weakly or strongly correlated and thus the correlation reduces with time to give a decay in relation to the first reported intensity signal. This decay is interpreted to give D. The Stokes Einstein equation is then used to obtain the radius (R) of your particle using the D obtained. See the below equations.

Equation Name	Equation	Key
Correlation function for a monodisperse sample	$g(\tau) = B + \beta e^{-2\Gamma\tau}$	$g(\tau)$, correlation function B, baseline of the correlation function β , correlation function amplitude at zero delay Γ , decay rate τ , Time delay (t+ δ t)
Decay rate	$\Gamma = Dq^2, D = \frac{\Gamma}{q^2}$	D, Stokes Einstein's Diffusion coefficient q, Scattering vector
Scattering vector	$q = \frac{4\pi n}{\lambda} \sin \frac{\theta}{2}$	θ , Scattering angle n, refractive index λ , incident laser wavelength
Stokes Einstein's Equation	$D = \frac{kT}{6\pi\eta R}$	k, Boltzmann's distribution, η , Solvent viscosity T, Temperature (kelvin) R, Radius

Table A.5 Equations utilised in the determination of particle size using DLS.

The polydispersity index (PDI) is determined from the fit of a polynomial to the log of the correlation function. The provides a cumulant analysis to give data on sample heterogeneity. The coefficients are determined using solvent viscosity and instrumental constants that have been unspecified. The coefficient of the squared

term c is the PDI. The value of b is the second order cumulant and is the z-average diffusion coefficient.

$$\ln [g(\tau)] = a + b\tau + c\tau^2 + d\tau^3 + e\tau^4 + \dots$$

$$PDI = \frac{2c}{b^2}$$

To elucidate a definition for the number and volume distributions from DLS a presumed scenario has been provided as follows; a sample of solution containing equal quantities (1:1) of 5 and 50 nm particles will show a number distribution as equal peaks at 5 nm and 50 nm diameters, whereas a volume distribution will give 1000 times larger peak for the 50 nm than the 5 nm. This is because the volume distribution provides the volume of space occupied by a particle at a particular size, which for the 50 nm sized particles gives an area that is 1,000,000 times larger (1:1,000,000 ratio). See below for a graphical illustration. The volume and number distribution are calculated from the intensity distributions that can also be obtained from DLS.

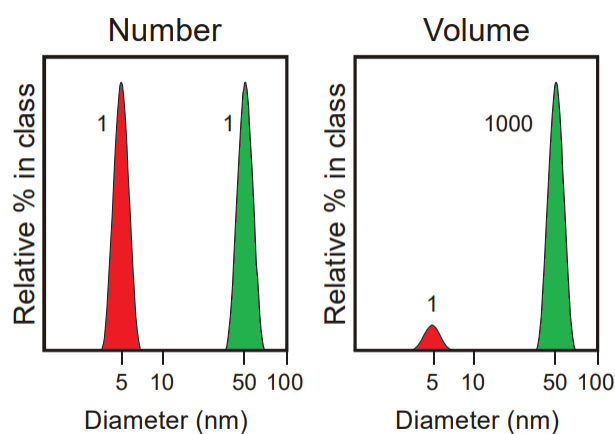


Figure A.22 Hypothesised DLS data of a 1:1 sample containing 5 nm and 50 nm particles, obtained from the instrument manual.

(D) DLS graphs for Tin-hydrosol A

- Batch 1 (Number distribution)

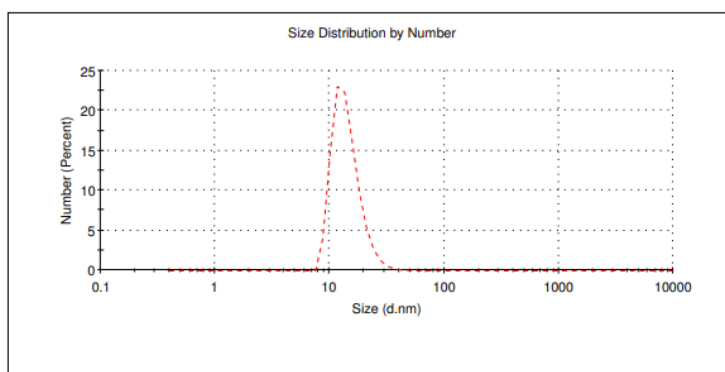
System

Temperature (°C): 19.9 Duration Used (s): 60
Count Rate (kcps): 290.6 Measurement Position (mm): 3.00
Cell Description: Disposable low volume c... Attenuator: 0

Results

	Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm): 32.34	Peak 1: 14.27	100.0	4.369
Pdl: 0.257	Peak 2: 0.000	0.0	0.000
Intercept: 0.879	Peak 3: 0.000	0.0	0.000

Result quality **Good**



- Batch 1 (Volume distribution)

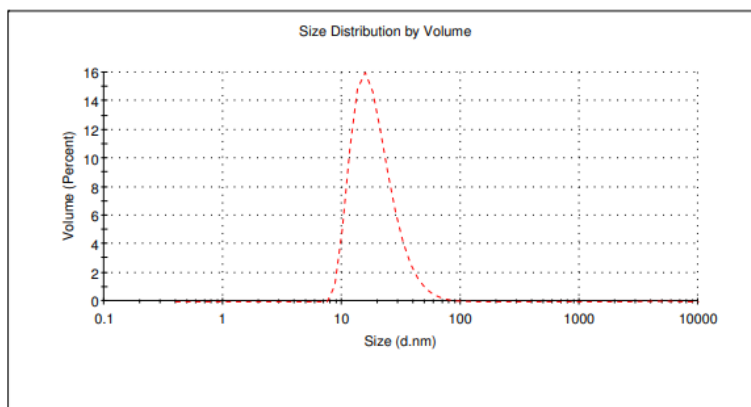
System

Temperature (°C): 19.9 Duration Used (s): 60
Count Rate (kcps): 290.6 Measurement Position (mm): 3.00
Cell Description: Disposable low volume cuv... Attenuator: 0

Results

	Size (d.nm):	% Volume:	St Dev (d.nm):
Z-Average (d.nm): 32.34	Peak 1: 19.77	99.9	9.400
Pdl: 0.257	Peak 2: 4656	0.1	995.5
Intercept: 0.879	Peak 3: 0.000	0.0	0.000

Result quality **Good**



- Batch 2 (Number distribution)

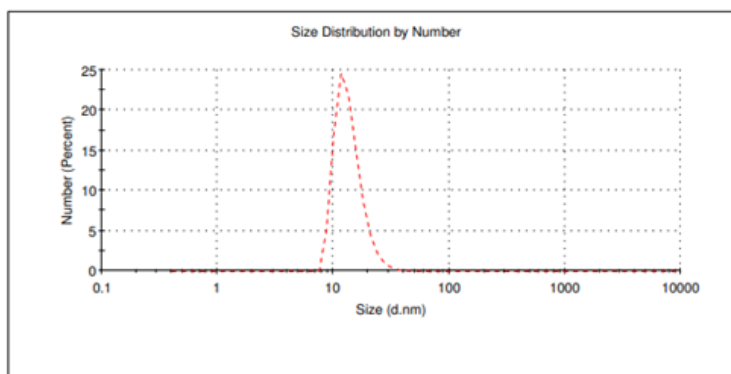
System

Temperature (°C): 20.1 Duration Used (s): 60
 Count Rate (kcps): 294.2 Measurement Position (mm): 3.00
 Cell Description: Disposable low volume c... Attenuator: 0

Results

	Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm): 32.51	Peak 1: 13.80	100.0	4.092
Pdl: 0.328	Peak 2: 0.000	0.0	0.000
Intercept: 0.880	Peak 3: 0.000	0.0	0.000

Result quality Good



- Batch 2 (Volume distribution)

System

Temperature (°C): 20.1 Duration Used (s): 60
 Count Rate (kcps): 294.2 Measurement Position (mm): 3.00
 Cell Description: Disposable low volume cur... Attenuator: 0

Results

	Size (d.nm):	% Volume:	St Dev (d.nm):
Z-Average (d.nm): 32.51	Peak 1: 18.81	99.9	9.072
Pdl: 0.328	Peak 2: 3532	0.1	1440
Intercept: 0.880	Peak 3: 0.000	0.0	0.000

Result quality Good

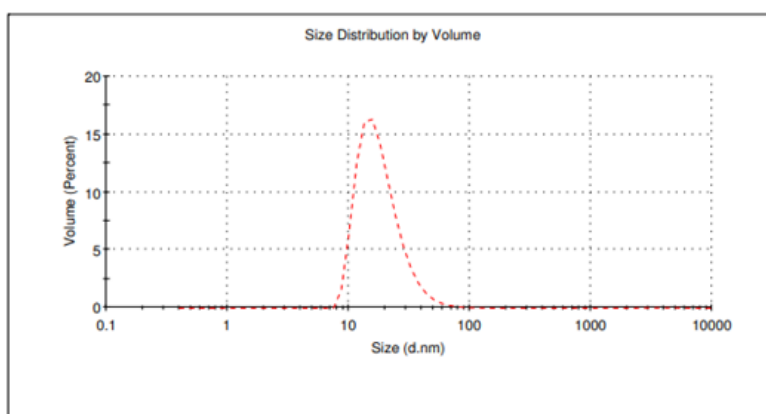


Figure A.23 DLS results (Zetasizer μ V instrument, Malvern Panalytical) for tin-hydrosol A showing the number and volume distribution results for two samples prepared separately, Batch 1 and Batch 2.

(E) DLS Graphs for Tin-hydrosol B

- Batch 1 (Number distribution)

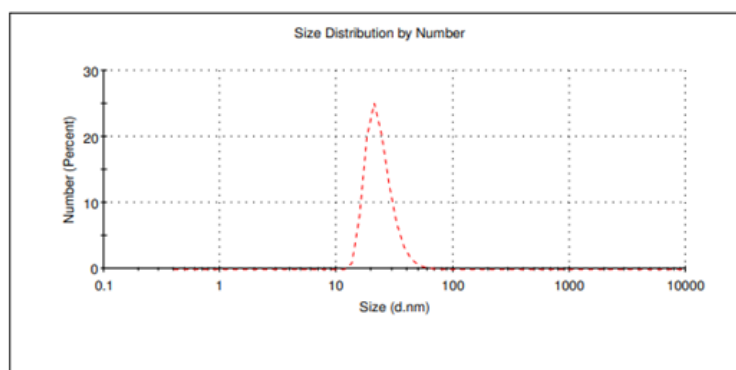
System

Temperature (°C): 20.1 Duration Used (s): 60
Count Rate (kcps): 315.4 Measurement Position (mm): 3.00
Cell Description: Disposable low volume c... Attenuator: 0

Results

	Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm): 50.39	Peak 1: 23.66	100.0	6.860
Pdl: 0.264	Peak 2: 0.000	0.0	0.000
Intercept: 0.863	Peak 3: 0.000	0.0	0.000

Result quality **Good**



- Batch 1 (Volume distribution)

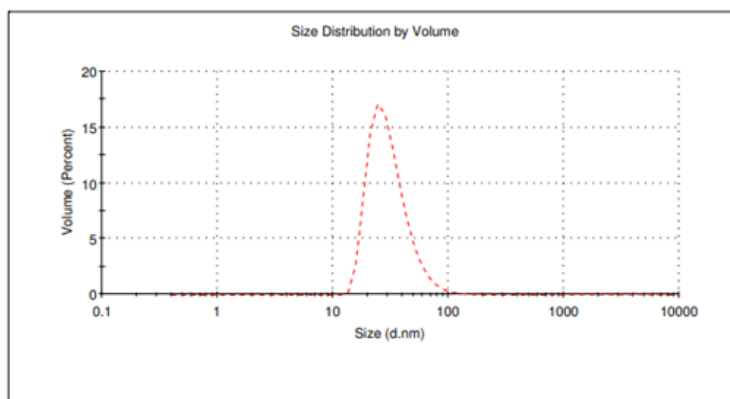
System

Temperature (°C): 20.1 Duration Used (s): 60
Count Rate (kcps): 315.4 Measurement Position (mm): 3.00
Cell Description: Disposable low volume cuv... Attenuator: 0

Results

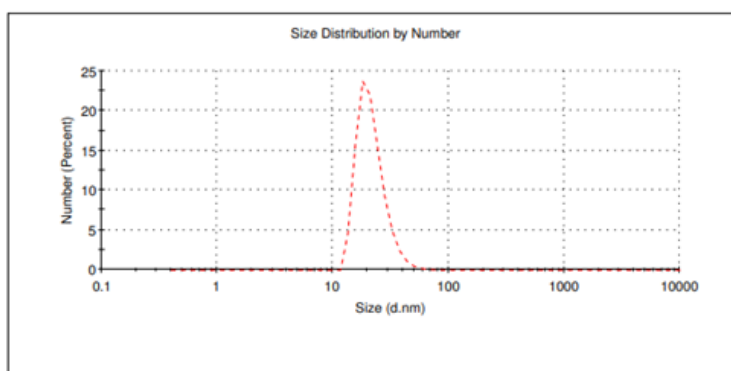
	Size (d.nm):	% Volume:	St Dev (d.nm):
Z-Average (d.nm): 50.39	Peak 1: 31.57	99.4	14.39
Pdl: 0.264	Peak 2: 1533	0.3	519.7
Intercept: 0.863	Peak 3: 3799	0.3	1147

Result quality **Good**



- Batch 2 (Number distribution)

System				
Temperature (°C):	19.9	Duration Used (s):	60	
Count Rate (kcps):	280.0	Measurement Position (mm):	3.00	
Cell Description:	Disposable low volume c...	Attenuator:	0	
Results				
		Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm): 50.36	Peak 1:	21.86	100.0	6.729
Pdl: 0.252	Peak 2:	0.000	0.0	0.000
Intercept: 0.862	Peak 3:	0.000	0.0	0.000
Result quality Good				



- Batch 2 (Volume distribution)

System				
Temperature (°C):	19.9	Duration Used (s):	60	
Count Rate (kcps):	280.0	Measurement Position (mm):	3.00	
Cell Description:	Disposable low volume cuv...	Attenuator:	0	
Results				
		Size (d.nm):	% Volume:	St Dev (d.nm):
Z-Average (d.nm): 50.36	Peak 1:	30.59	99.3	14.96
Pdl: 0.252	Peak 2:	4513	0.7	1083
Intercept: 0.862	Peak 3:	0.000	0.0	0.000
Result quality Good				

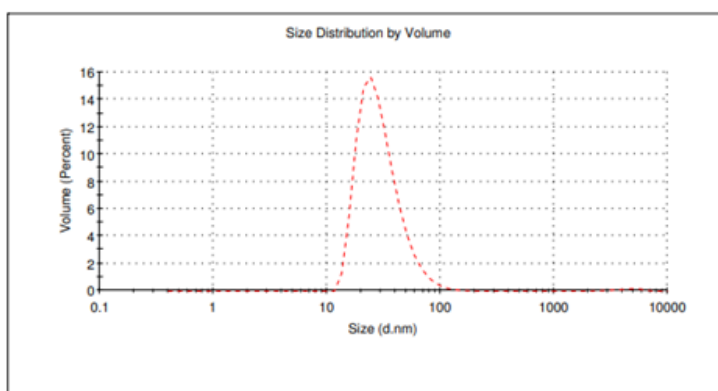


Figure A.24 DLS results (Zetasizer μ V instrument, Malvern Panalytical) for tin-hydrosol B showing the number and volume distribution results for two samples prepared separately, Batch 1 and Batch 2.

(F) AFM determination of SnO₂ nanoparticle size using cross-section measurements with JPK processing software

The height shows the most accurate representation of size, and in this instance, we see 10 nm size observed for this particle being measured, from a HDPE surface with Film B. AFM settings can be found in Chapter 3.

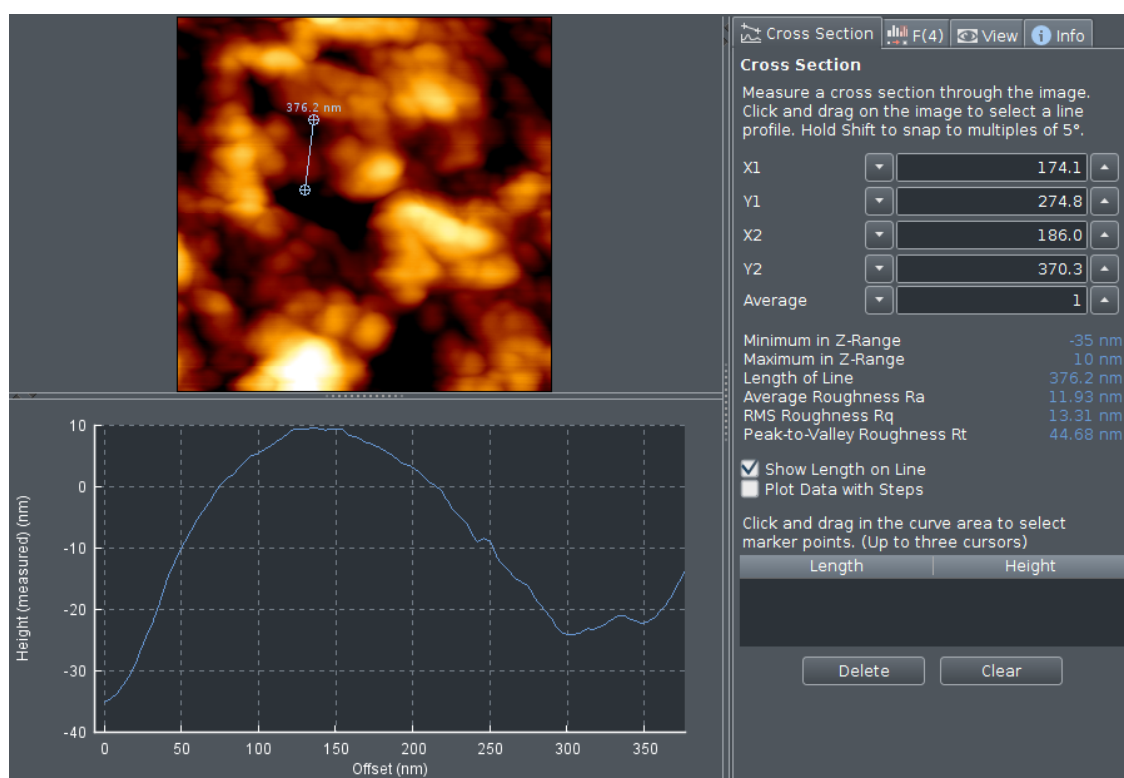
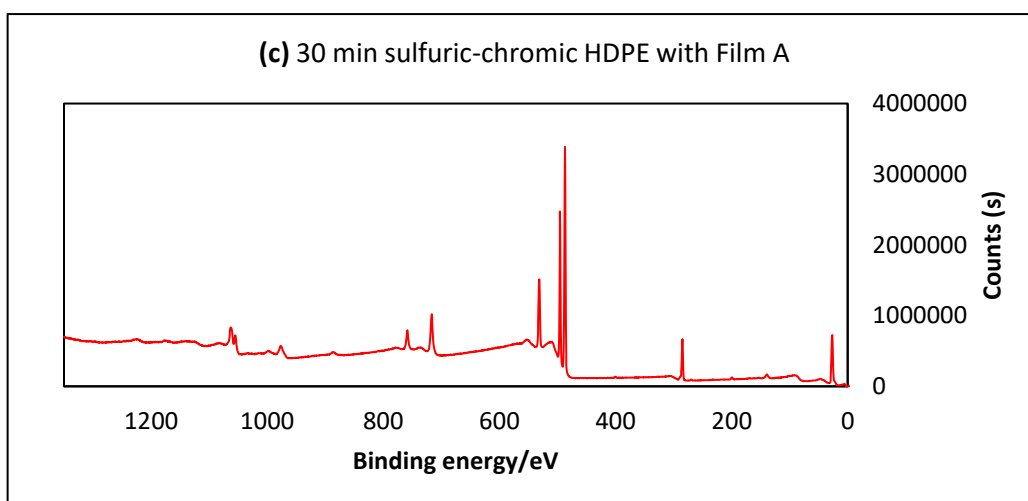
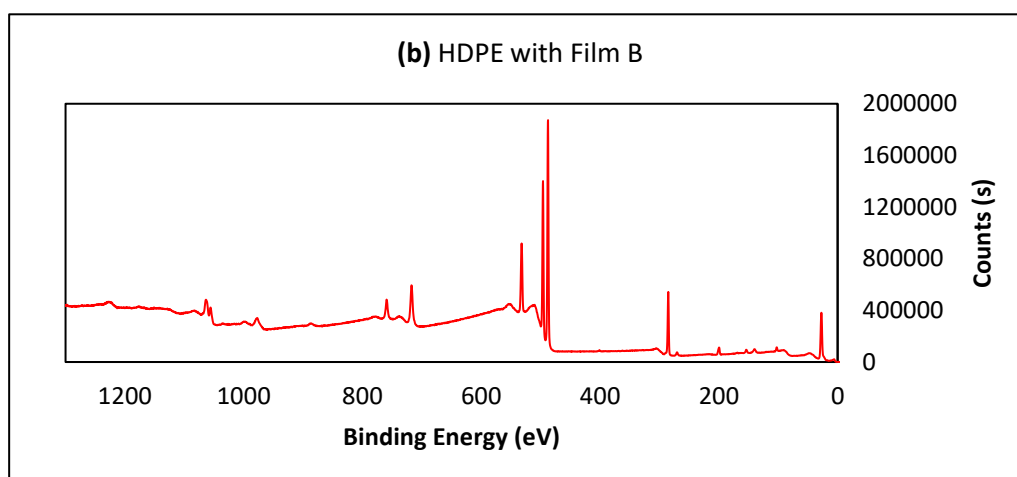
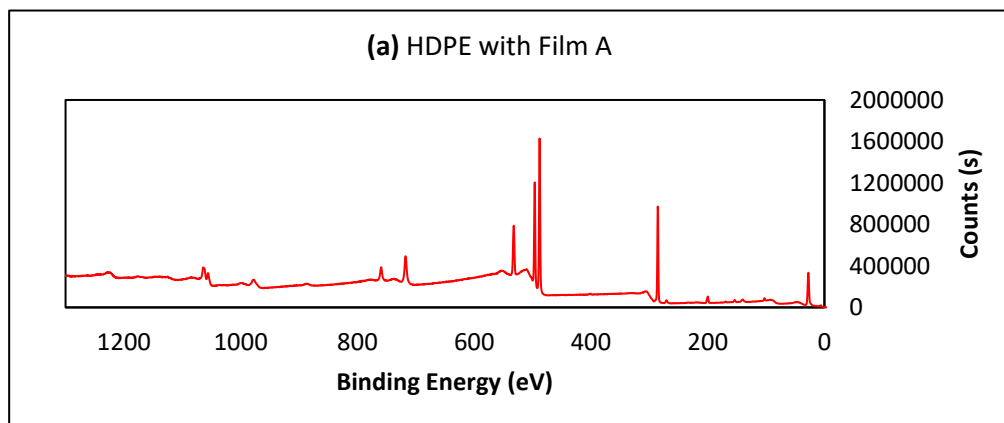


Figure A.25 AFM analysis of particle size based on cross-sectional height evaluation to determine the diameter from utilising z-scale.

(G) XPS survey scan for Film A and Film B on HDPE and HDPE 30 minutes sulfuric-chromic acid oxidised



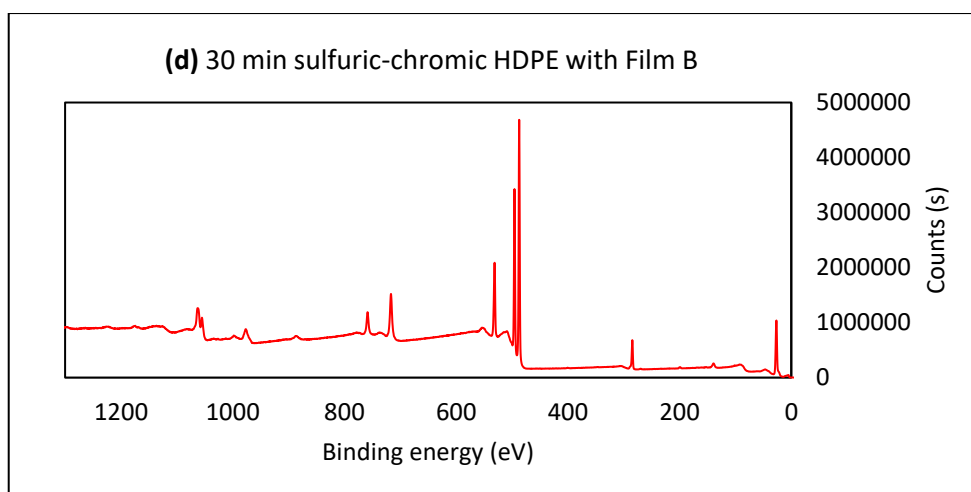


Figure A.26 XPS survey scan of SnO_2 coated HDPE samples, where (a) Film A, (b) Film B, (c) 30 min oxidised Film A and (d) 30 min oxidised Film B.

(H) T20 saline delay CLSM viability assay with *P. aeruginosa* with SnO_2 coated HDPE samples

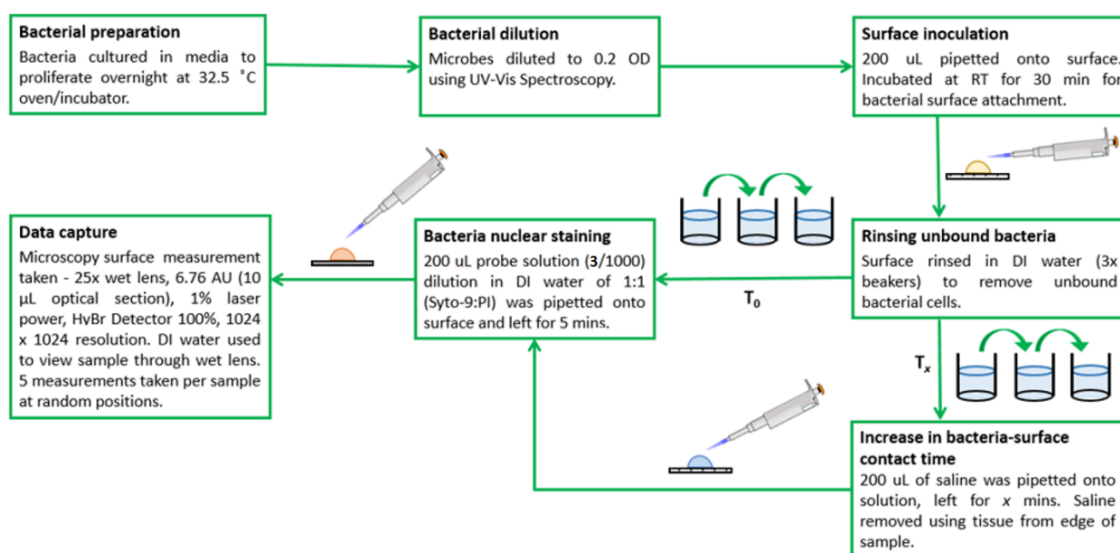


Figure A.27 Step-by-step flow chart illustrating the surface viability assay using the LIVE/DEAD BacLight kit for early-stage biofilm viability evaluation, with T_x as 20 min delay experiment with exposure to sterile saline.

Chapter 6 Appendix

(A) Evaluation of ZnO nanoparticle dissolving in 1% acetic acid

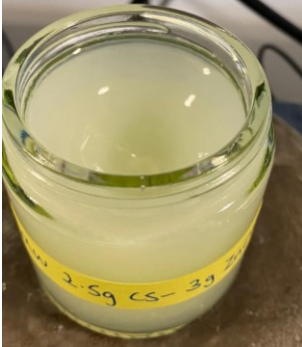
Concentration	Photograph	Observation
0.5 g ZnO		clear solution, dissolved
0.75 g		clear solution, dissolved
1 g		partially dissolved
3 g		partially dissolved

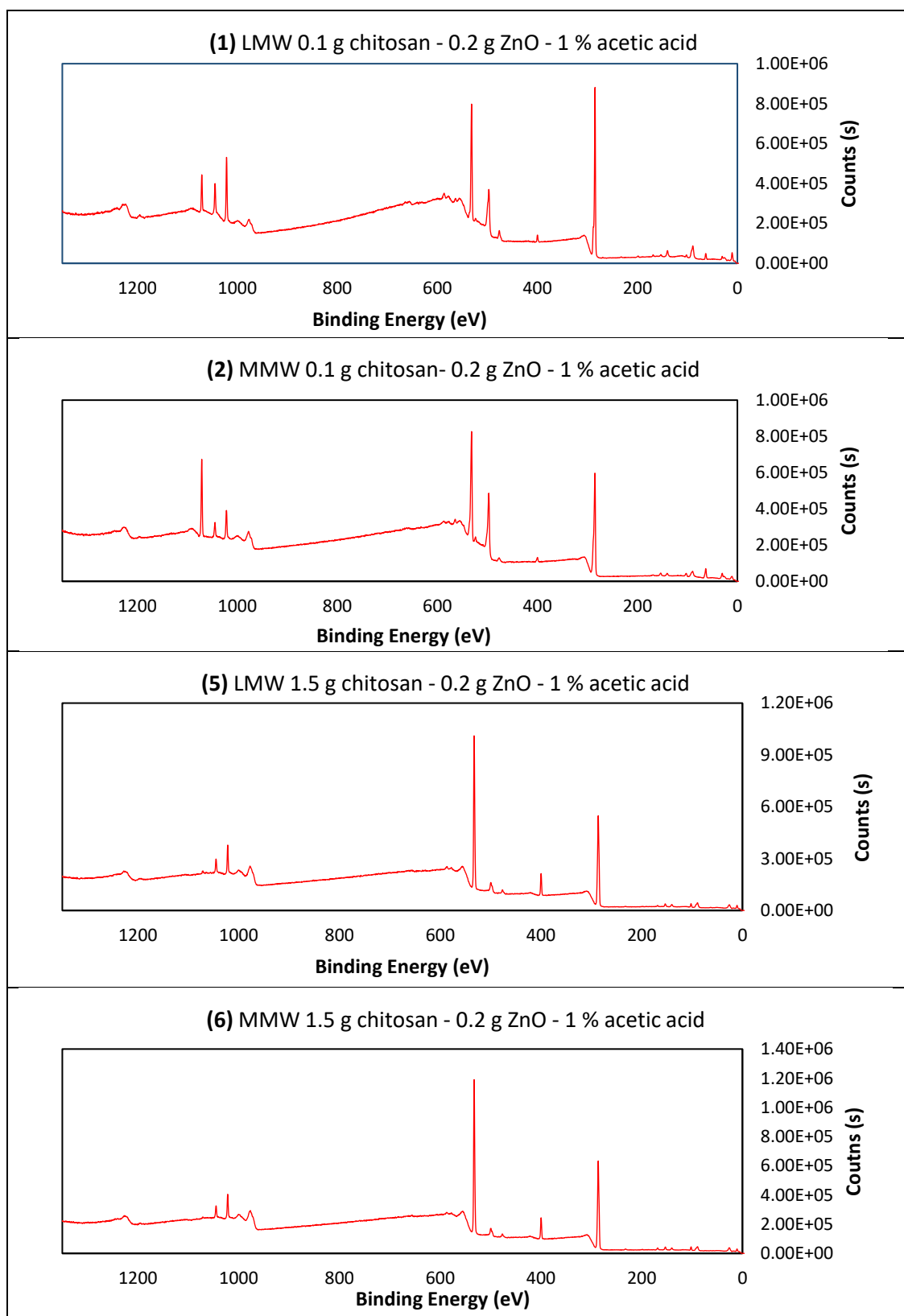
Table A.6 Solubility evaluation of commercial ZnO in 1% acetic acid, 100 mL total volume.

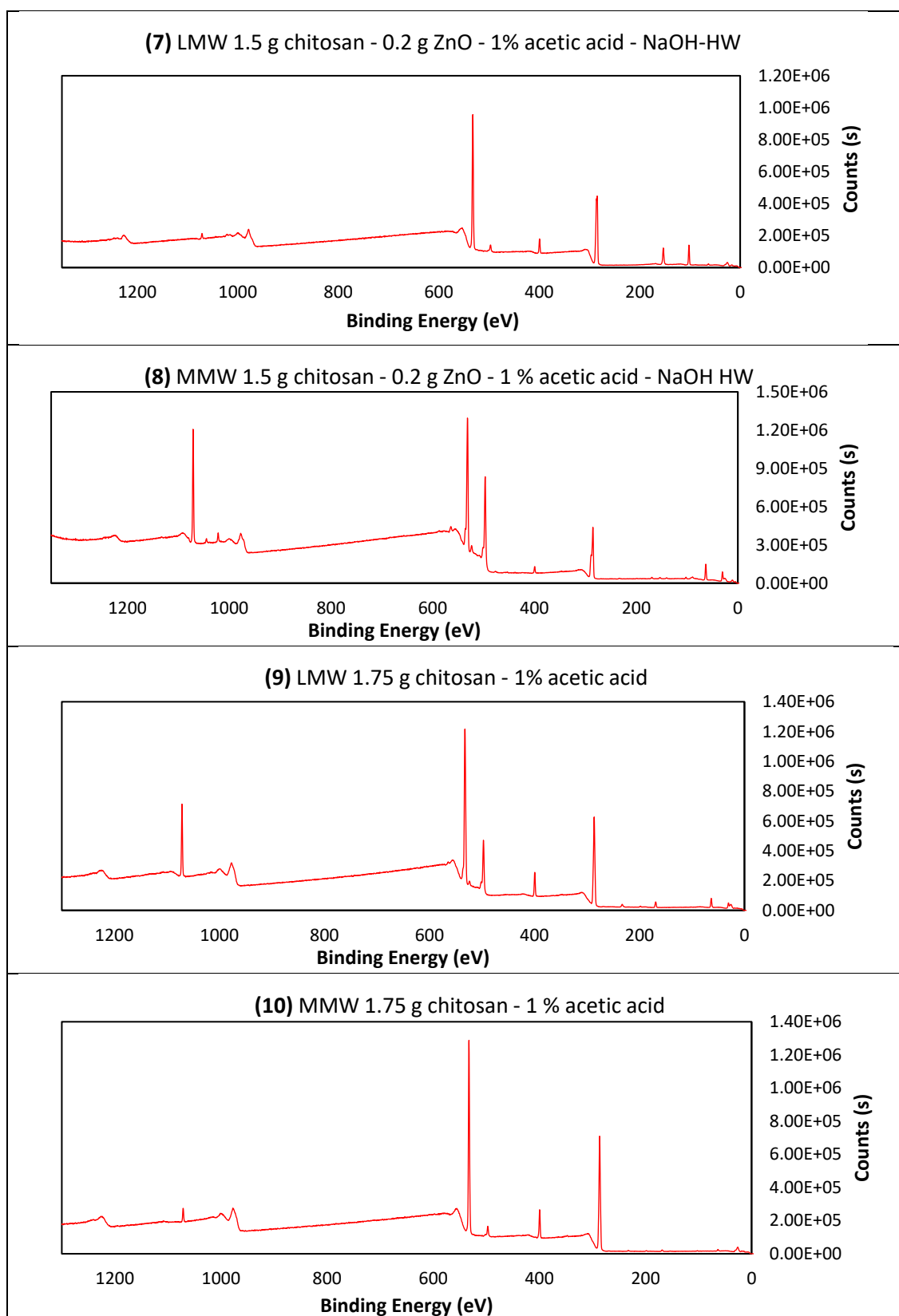
(B) Contact angle and surface energy results of chitosan-ZnO films

	Polymer (x/100 mL solvent)	ZnO (g/100 mL solvent)	Solvent (100mL)	NaOH- HW Yes/No	Water contact angle (°)	DIM contact angle (°)	γ_{sv} (mJ/m ²)
1	LMW 0.1 g chitosan-	0.2	1% acetic acid	No	13.0 +/- 1	53.1 +/- 3.0	77.0 +/- 0.5
2	MMW 0.1 g chitosan	0.2	1% acetic acid	No	45.8 +/- 4.0	37.9 +/- 1.1	52.7 +/- 2.8
3	LMW 0.1 g chitosan	0.2	1% acetic acid	Yes	60.1 +/- 5.1	43.1 +/- 3.3	45.4 +/- 2.9
4	MMW 0.1 g chitosan	0.2	1% acetic acid	Yes	41.4 +/- 3.9	29.3 +/- 2.1	56.4 +/- 2.6
5	LMW 1.5 g chitosan	0.2	1% acetic acid	No	79.8 +/- 9.6	45.1 +/- 0.8	36.9 +/- 5.0
6	MMW 1.5 g chitosan	0.2	1% acetic acid	No	79.7 +/- 18.7	26.5 +/- 3.0	46.9 +/- 14.2
7	LMW 1.5 g chitosan	0.2	1% acetic acid	Yes	39.2 +/- 2.1	22.8 +/-	58.2 +/- 0.6
8	MMW 1.5 g chitosan	0.2	1% acetic acid	Yes	61.1 +/- 9.7	32.9 +/- 1.8	45.3 +/- 4.9
9	LMW 1.75 g chitosan	0	1% acetic acid	No	12.5 +/- 1.5	52.9 +/- 1.8	77.1 +/- 1.4
10	MMW 1.75 g chitosan	0	1% acetic acid	No	26.8 +/- 0.5	56.1 +/- 1.3	70.1 +/- 1.0
11	LMW 1.75 g chitosan	0.75	1% acetic acid	No	91.0 +/- 3.1	52.3 +/- 0.7	35.6 +/- 1.7
12	MMW 1.75 g chitosan	0.75	1% acetic acid	No	80.4 +/- 3.5	53.0 +/- 1.0	32.3 +/- 1.1
13	LMW 0.5 g chitosan	0	10% acetic acid	No	34.5 +/- 2.6	48.3 +/- 3.0	61.7 +/- 2.7
14	LMW 0.5 g chitosan	1.5	10% acetic acid	No	60.7 +/- 2.6	54.2 +/- 1.1	40.7 +/- 2.0
15	LMW 1 g chitosan	0	10% acetic acid	No	37.4 +/- 0.8	50.4 +/- 0.9	59.7 +/- 0.5
16	LMW 1 g chitosan	1.5	10% acetic acid	No	51.5 +/- 1.9	55.4 +/- 0.7	48.1 +/- 1.8
17	MMW 5%v PVP	0	10% acetic acid	No	12.2 +/- 1.1	51.1 +/- 0.7	76.5 +/- 0.7
18	MMW 5%v PVP	1.5	10% acetic acid	No	43.8 +/- 2.7	38.0 +/- 1.2	54.1 +/- 2.0

Table A.7 Water and DIM advancing contact angle results, and the corresponding surface free energy, calculated using Fowke's Theory.

(C) XPS Survey Scans of CS-ZnO Samples





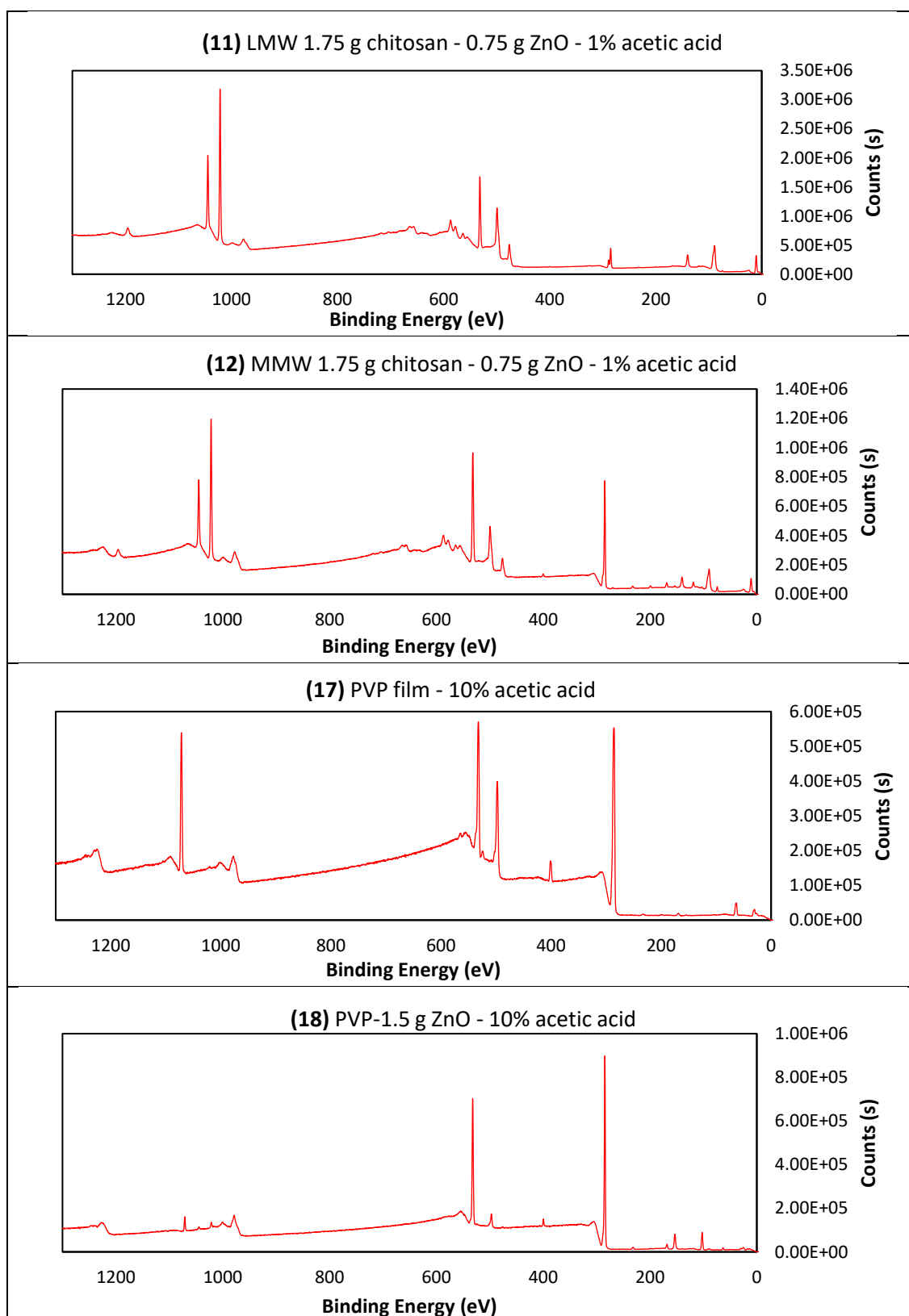
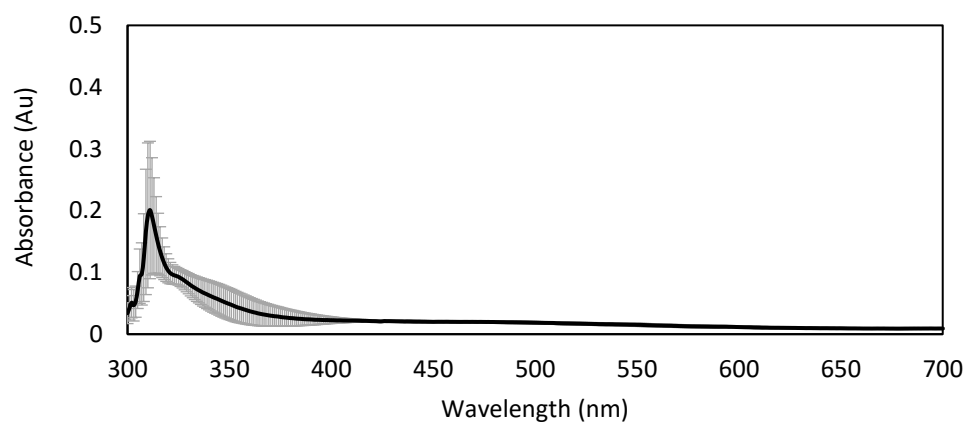


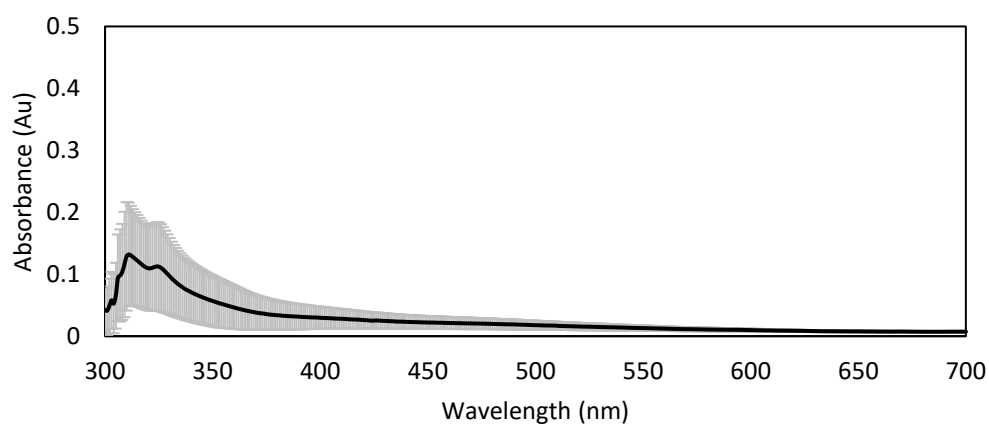
Figure A.28 XPS survey scan of CS-ZnO coatings outlined in Table 6.2 in Chapter 6.

(D) UV-Vis full spectra from ninhydrin assay

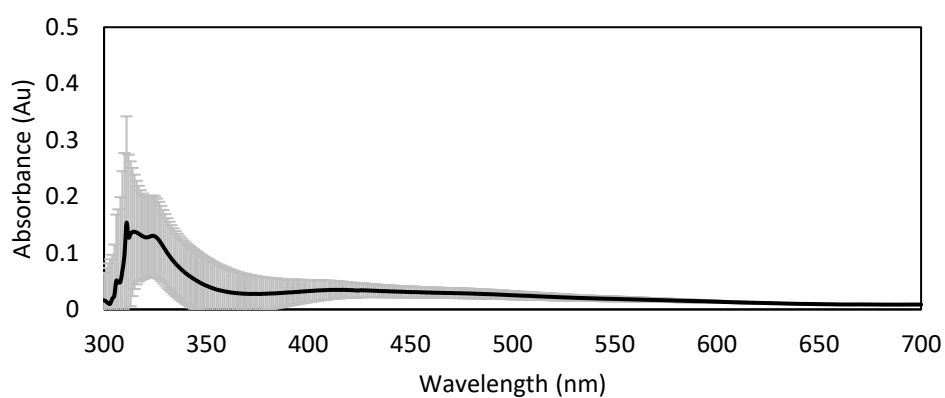
(a) HDPE control



(b) HDPE UVO-SA oxidised



(c) MMW 1.5 g CS - 0.2 g ZnO



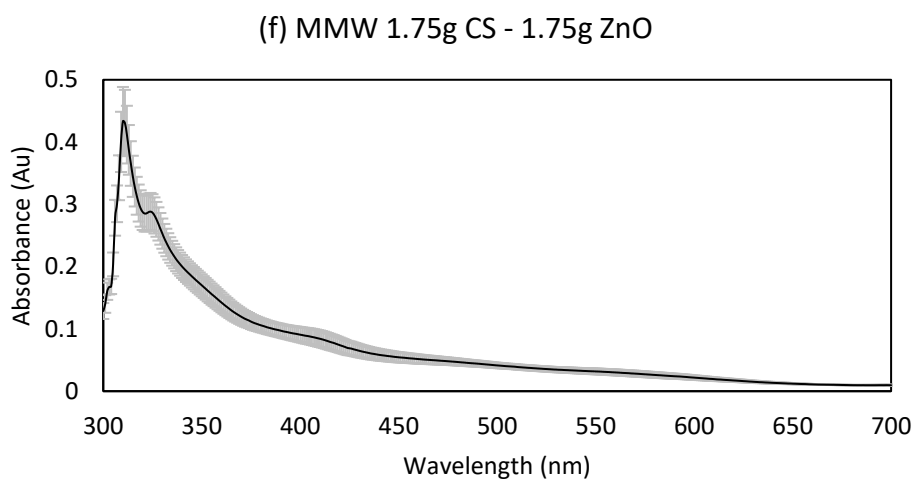
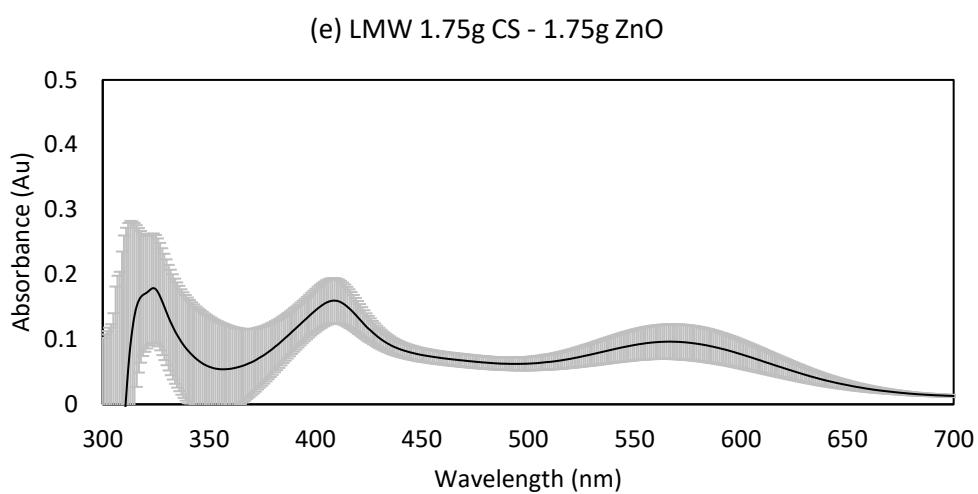
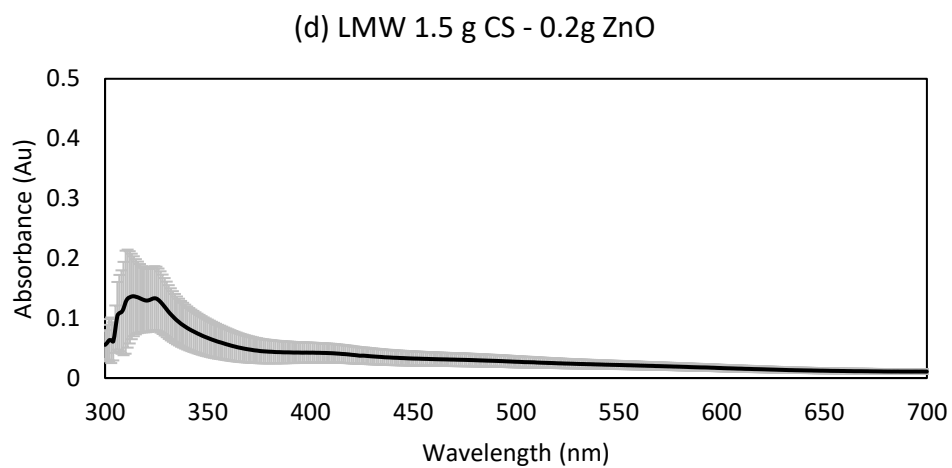


Figure A.29 UV-vis spectrophotometer results between 300-700 nm as an average of three separate samples, error bars show +/- SE, samples examined were (a) control HDPE sample, (b)

HDPE UVO-SA oxidised, (c) MMW 1.5 g CS – 0.2 g ZnO, (d) LMW 1.5 g CS – 0.2 g ZnO, (e) LMW 1.75g CS– 1.75g ZnO, and (f) MMW 1.75g CS – 1.75 ZnO.

(E) ATR-FTIR values used for the determination of % DDA of LMW/MMW chitosan powders

	LMW chitosan powder A_{1320}/A_{1420}	MMW chitosan powder A_{1320}/A_{1420}
Run 1	0.1074/0.0954	0.0979/0.1456
Run 2	0.0832/0.0738	0.0485/0.0536
Run 3	0.0927/0.0831	0.0659/0.0806

Table A.8 Absorbance values obtained from ATR-FTIR for three separate spectra obtained of the LMW and MMW chitosan powders that have been commercially.