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Antibacterial activity studies of plasma polymerised cineole films

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Costs associated with bacterial infections in medical devices exceed $US 30 billion each year in the United States alone due to devices revisions and patient treatment. Likewise, in 2012-2013, 126 surgical bacterial infections cost a single Australian state over $AUD 5 million. In the search for coatings that can prevent bacterial attachment and reduce medical and human costs, a number of studies have explored the application of antibacterial and anti-fungal essential oils. Traditionally the antibacterial properties of tea tree oils have been linked to their major component terpinen-4-ol, with little focus on the second component, 1,8-cineole (Co). In this study we explore the antibacterial behaviour of solutions of cineol and demonstrate its ability to significantly reduce *Escherichia coli* viability in solution. However, one of the challenges with essential oils is their limited reactivity and solubility, creating a significant limitation for translating these antibacterial oils into coatings for medical devices. Previous studies have shown that plasma polymerised thin films can be produced from 1,8-cineole (ppCo), though it is unknown if the antibacterial activity can be retained. Herein, we report the behaviour of ppCo films when exposed to different solvents, and the interaction of these coatings films with two bacteria (*Escherichia coli* and *Staphylococcus aureus)* commonly related to the failure of medical devices. While a reduction on bacterial attachment was observed onto the ppCo film and the control hydrophobic surface, only the ppCo coatings resisted biofilm formation after 5 days of incubation with *Escherichia coli*. Additionally, ppCo films showed to be non-adherent and non-cytotoxic to mammalian fibroblast. The combination of these two findings suggests that while the ppCo films retained part of the antimicrobial activity of the cineole oil, any leachables that may be released from the coating are also not cytotoxic or cell disruptive to mammalian cells. These coatings present a promising approach toward creating biocompatible antimicrobial coatings from Australian essential oils.

Introduction

Problems associated with biofilm formation cost billions of dollars every year. In 2006 the cost of hospital acquired infections (HAI) in Victoria (Australia) exceeded $AUS 40 million/year.[1] In 2012-2013, 126 surgical infections cost over $AUD 5 million dollars [2] and, in 2009, HAI cost exceeds $US 30 billion annually in the United State alone.[3] On ship hulls, it has been estimated that a biofilm of just a few hundred microns yields an average 20 % increase in fuel consumption due to increased drag.[4] In the medical industry, bacterial infection results in the failure of biomedical devices and is the cause of numerous conditions requiring medical intervention (e.g. dental plaque and wound infection), affecting the shelf-life of diagnostics and sensors.[4, 5] Techniques to minimise biofilm formation are thus required urgently.

Biofilm formation begins with the attachment of bacteria to a surface,[5] and is influenced by both bacterial and surface factors. Bacterial strain, morphology, membrane free energy and cell wall chemistry play an important role,[6-8] but are difficult to manipulate or control. Accordingly, a more promising strategy involves engineering surfaces to prevent bacterial attachment. To date, many approaches have been trialled including materials that prevent protein adsorption,[9] materials with specific topographical features[10] and the surface immobilisation of antimicrobial and antibacterial molecules including furanones and antimicrobial peptides.[11]

The antibacterial properties of essential oil solutions including tea tree have been established against a number of wild type and multidrug-resistant organisms.[12] However, the use of essential oil or its components for engineering coatings have not been widely explored due to limitations linked to the

oil properties. One of the challenges of creating coatings from these oils is their solubility and limited routes for surface immobilisation. Plasma polymerisation overcomes these challenges, providing the possibility of fabricating coatings from low volatility monomers.[13, 14] Plasma polymerisation is a dry single-step process that allows the fabrication of conformal thin films with controlled thickness and different functionalities. Generally, plasma polymerised thin films show strong adhesion to a wide range of surfaces.

Traditionally, the antibacterial properties of tea tree oils have been linked to their major component; terpinen-4-ol and thin plasma polymer films have been fabricated and an attempt to examine the antibacterial activity was made.[14, 15] While plasma polymer films formed from the minor component 1,8-cineole have been fabricated previously,[13] neither the stability or antibacterial activity of the oil or films has been explored. In this study, the activity of 1,8-cineole against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus (S. aureus)* in solution was evaluated based on the kill-rate approach. Coatings derived from 1,8-cineole were then fabricated by plasma polymerisation and characterised for stability in aqueous solution, surface wettability by contact angle, elemental composition by X-ray photoelectron spectroscopy (XPS), surface morphology by atomic force microscopy (AFM), and antibacterial and biofilm forming properties by exposure to *E. coli* and *S. aureus* inoculated media. Hydrocarbon-rich 1,7-octadiene plasma polymer films (ppOct) were used as a hydrophobic negative control and clean glass slides were using as a hydrophilic positive control. As the coatings are intended for use in biomaterials and tissue contact applications, the eukaryotic cell cytotoxicity of the ppCo films was also examined by direct cell contact viability assays.

Materials and methods

Materials

Silicon wafers (orientation <100>; thickness 600-650 μm; single side polished, resistivity 14.0-22.0 Ohm cm) were purchased from Micro Materials & Research Consumables. Microscope slides were purchased from Sail Brand (Jiangsu, China). Reagents were obtained from commercial vendors and used without further purification: Isopropyl alcohol (IPA, 99.8 % purity) from Chem-Supply Pty Ltd (Adelaide, Australia), glycerol (99.5 % purity, MW 92.09, ρ=1.256-1.261) from BDH (Poole, UK), 1,8-cineole (99.5% purity, MW 154.249, ρ= 0.9225) from FGB Natural Products (Melbourne, Australia), 1,7-Octadiene (98.50 % purity, MW 110.20, ρ= 0.74) from Acros Organics (New Jersey, USA), sodium chloride (99.8 % purity, MW 58.44) from Riedel-de Haen (Seelze, Germany), di-sodium hydrogen phosphate (99 % purity, MW 141.96) from Chem-supply (Adelaide, Australia), potassium chloride (99.5 % purity, MW 74.55) from Sigma Aldrich (St Louise, USA) and potassium phosphate monobasic (KH2PO4, 99 % purity, MW 136.09) from Astral Scientific (Gymea, Australia). LIVE/DEAD® BacLight™ Bacterial Viability (3μM propidium iodide (PI) and 0.5μM of SYTO 9 green fluorescent nucleic acid stain) were purchased from Invitrogen (Grand Island, USA), and agar media and broth media from Oxoid Ltd (Hampshire, UK). Milli-Q grade water (18 mΩ Millipore, Australia) was used in all the experiments. Clinical isolates *Escherichia coli* (*E. coli*, 25922 ATCC) and *Staphylococcus aureus* (*S. aureus*, 25923 ATCC), and murine fibroblasts L929 (ATCC CCL-1), were purchased from American Type Culture Collection (ATCC, USA). Dulbecco's phosphate buffered saline (DPBS), MEM + GlutaMAXTM-I, non-essential amino acids (NEAA), and Antimycotic-Antibiotic (Anti-Anti) were obtained from GIBCO (Carlsbad, USA). Fetal bovine serum (FBS) was obtained from SAFC Biosciences (St Louise, USA). Calcein-AM and DilC12(3) were obtained from BD Biosciences(Franklin Lakes, USA). Tryple Express was obtained from Invitrogen (Grand Island, USA). Plasticware used for mammalian culture were obtained from Nunc (Rocherster, USA).

Plasma Polymerisation

Plasma polymerisation was carried out in a 20 L, 35 cm total length, custom-built stainless steel T-shaped reactor with stainless steel end plates sealed with Viton O-rings detailed previously.[16] Briefly, plasma was ignited within the reactor using a 13.56 MHz generator (Coaxial Power Systems Ltd., UK) coupled to an aluminium internal disc electrode (170 mm) with a matching network via an impedance matching network. Flow rate of the monomer was controlled using a needle valve and the plasma unit pressure was monitored using a Pirani gauge. Prior to plasma polymerisation, the monomer was degassed using at least three freeze-pump-thaw cycles. Silicon wafer and glass slide substrates were cleaned of organic contamination by sonication in IPA for 5 min at room temperature, and then blown dry with high purity nitrogen gas immediately before plasma deposition. Substrates were placed in the reactor and the vessel was evacuated down to base pressure (1×10^{-3} mbar). Monomer vapour was then introduced to the chamber and an operating pressure of 2×10^{-2} mbar was maintained at the defined monomer flow rates, as determined using the technique outlined by Griesser and Gengenbach.[17] After deposition, monomer flow was maintained for another 5 min to quench any radicals on the surface. The monomer valve was then closed and the plasma unit evacuated to remove any residual monomer from the system. Plasma deposition conditions were 20 W deposition power, 2 sccm monomer flow rate, and 20 minutes deposition time for each monomer.

Surface Characterisation

Static contact angles were measured on each of the samples using a contact angle goniometer (Ramé-Hart, Inc., Mountain Lakes, NJ, USA). A ~2μL droplet of Milli-Q water was lowered manually onto the sample using a needle, and the contact angle was manually measured on the right side of the droplet. The measurements were repeated in three different positions on each sample, and the samples were prepared in

triplicate (n=9). To evaluate the coating stability, samples were placed in beakers and immersed in 100 mL of either 150 mM PBS, broth media or Milli-Q water and allowed to soak at room temperature for ~16 ± 0.5 hours, after which time they were rinsed in Milli-Q water and dried under a stream of nitrogen gas. These samples were analysed by XPS and AFM.

X-ray photoelectron spectroscopy (XPS) was performed using an AXIS-HSi spectrometer (Kratos Analytical Inc., Manchester, UK) with a monochromated Al K_α source at a power of 144 W (12 kV × 12 mA), a hemispherical analyser operating in the fixed analyser transmission mode and the standard aperture (analysis area: 0.3 mm × 0.7 mm). The total pressure in the main vacuum chamber during analysis was less than 10^{-8} mbar. Survey spectra were acquired at a pass energy of 160 eV. To obtain more detailed information about chemical structure, high resolution spectra were recorded from individual peaks at 40 eV pass energy. Data processing was performed using CasaXPS processing software version 2.3.16 (Casa Software Ltd., Teignmouth, UK). All elements present were identified from survey spectra. The atomic concentrations of the detected elements were calculated using integral peak intensities and the sensitivity factors supplied by the manufacturer. Binding energies were referenced to the aliphatic hydrocarbon peak at 285.0 eV. High resolution C1s spectra were fitted with Gaussian-broadened Lorentzian functions (30GL). Peaks were restricted to full width half maximum (FWHM) between 1.2 - 1.6 eV. Five distinct binding energy shifts were used in curve fitting, namely C1: hydrocarbon component (C-C, C-H) at 285 eV, C2: secondary shift associated with carboxylic groups (C*COO) at 285.6 eV, C3: ether and hydroxyl component (C-O), and C-N at 286.5 eV, C4: carbonyl and amide contributions (C=O, O-C-O, N-C=O) at 288 eV, and C5: carboxylic component (CC*OO) at 289 eV.

The XPS overlayer algorithm was used to approximate the amount of protein adsorbed onto surfaces. The overlayer algorithm relates the atomic concentration of nitrogen measured on the surface, the N_∞ nitrogen in a protein (estimated ~ 14 %), the mean free path of nitrogen photoelectrons (λ = 3.18 nm for Al K_α radiation), total thickness of protein film (d), and the angle of the sample relative to the detector (θ).[18]

The thickness of the films was measured by AFM imaging over a well-defined step created by partially masking the Si wafers before plasma polymerisation (10 % w/v polystyrene in toluene, dried in air).[19] An Asylum Research MFP-3D AFM (Santa Barbara, CA, USA) was used to image the films using tapping mode with ultra-sharp silicon nitride tips (NSC15 non-contact silicon cantilevers, MikroMasch, Spain). The tips used in this study had a typical force constant of 40 N/m and a resonant frequency of 320 kHz. Tips were ozone cleaned for 20 minutes prior to use to remove organic contaminant using a UV-ozone Procleaner (BioForce Nanosciences, Inc.). Typical scan settings included the use of an applied piezo deflection voltage of 0.6 – 0.8 V and a scan rate of 0.45 – 0.8 Hz. All images were processed by a 1st order flattening algorithm and line scans were generated using Igor Pro and Gwyddion 2.30 software. Section analysis across the step indicated the film thickness. A minimum of nine data points were obtained from three different samples to calculate the average thickness of the films. Igor Pro and Gwyddion 2.30 software were also used to establish the roughness of surface of the films. All roughness parameters were calculated over an area of 25 μm^2.

Microbiological studies

Lyophilized *Escherichia coli* (*E. coli*, 25922 ATCC) and *Staphylococcus aureus* (*S. aureus*, 25923 ATCC) were reconstituted in broth media and grown overnight at 37°C and 100 rpm. The optical density at 600 nm (OD_{600}) of the overnight inoculum was measured to be 0.875 for *E. coli* (equivalent to 3 x 10^8 CFU/mL) and 1.307 for *S. aureus* (1.34 x 10^9 CFU/mL). The overnight inoculum was diluted 1:1 using a glycerol solution (30 % v/v) and stored at -80 °C until required. The relationship between the OD_{600} and the CFU/mL of each bacterium in liquid culture was established by preparing serial 10-fold dilutions to reach 1:10^7. A 100 µL aliquot of 10^{-5}, 10^{-6} and 10^{-7} dilutions was plated onto agar media in triplicate, and incubated overnight at 37 °C; the CFU from the different plates was then correlated to the measured OD_{600}.

Prior to each experiment, three tubes of the relevant bacterium were taken from -80°C storage and placed in the 4°C fridge to thaw. The tubes were centrifuged at 37°C, 1420 rpm for 20 minutes to obtain a pellet, which was resuspended in warm broth media and grow overnight. The bacteria cultures were diluted into warm fresh broth media until an $OD_{600\ nm}$ of approximately 0.1 was obtained and incubated at 37°C and 100 rpm until the bacteria were in logarithmic growth phases ($OD_{600\ nm}$ ~ 0.3).

The kill-rate studies with the essential oil were performed using a variation on the National Committee for Clinical Laboratory Standards guidelines.[20] After the triplicate cultures reached a logarithmic growth phase, 1,8-cineole was added to give final concentrations of 0.05, 0.25, 0.35 and 0.5 % v/v, and the samples were gently mixed by inversion of the tubes several times. All samples were then cultured at 37°C and 100 rpm with the $OD_{600\ nm}$ measured at intervals over 24 hours. The OD_{600nm} of each time point was correlated to CFU/mL. The estimated fraction of bacteria surviving in the 1,8-cineole was calculated by dividing the number of CFU/mL after treatment by the number of CFU/mL in the control sample (i.e. bacterial culture without 1,8-cineole). The % of bacteria survival was calculated relative to the control sample considered as 100 % survival.

Bacterial attachment

To establish the antibacterial properties of the plasma polymer thin films, the different substrates (ppCo, ppOct, and glass) were placed in individual wells of a 6-well tissue culture plate, covered with 4 mL/well of media inoculated with bacteria in the logarithmic growth phase. Samples were cultured at 37°C for 18 hours. Substrates were then triple rinsed with sterilised NaCl (0.85 % v/v). To establish cell viability, washed samples were incubated with PI and SYTO 9 solution. Samples were

incubated in the dark at room temperature for 30 minutes. Surfaces were gently rinsed with copious amounts of Milli-Q water then stored in clean 6-wells plates until further analysis.

As a control, the above procedure was repeated in the three different substrates but for the broth media being absent the bacterial inoculum.

To study the biofilm forming properties of the systems, samples were incubated as for the 18 hours study and after this time, the inoculated media was removed and replaced with 4 mL of warm broth media (no bacteria). Broth media was replaced in this way every 24 hours for 5 days. On the fifth day, the substrates were then rinsed with NaCl, stained for viability, and then rinsed and stored as described above. As negative controls, ppCo films, ppOct films and glass were immersed in broth media over 5 days.

Confocal laser scanning microscopy (CSLM)

Bacterial viability on the plasma polymer films and glass was imaged using a 100x oil immersion lens on an inverted confocal laser scanning microscope (FV1000 Olympus with IX81). An excitation wavelength of 488 nm for SYTO 9 (500 nm emission observed), and 543 nm for PI (617 nm emission observed) were used. For assessment of bacterial attachment, the number of bacteria was estimated from at least three randomly recorded images, from which five representative 20 μm by 20 μm areas were selected for cell counting. Image analysis was done using ImageJ software, where the sharpen function was applied to all images prior to manual counting. For assessment of biofilm formation, at least three representative recorded images were used. Image analysis was done using ImageJ software, where the sharpen function was applied before transformation to 8-bit, then the brightness and the contrast were adjusted to improve the distinction between covered and non-covered areas, and the threshold adjusted until the area covered with biofilm was highlighted and calculated.

Mammalian cell direct contact assay

An L929 mouse fibroblast monolayer in a T75 flask was rinsed twice with DPBS and then all cells were stained with the membrane dye DilC12(3) by addition of 15 mL of a 10 μg/mL solution in media (MEM + GlutaMAX^{TM}-I, supplemented with 1% v/v NEAA, 1% v/v Anti-Anti). The flask was incubated at 37^{o}C in a 5 % CO_2/air atmosphere for 60 min after which excess DilC12 (3) was removed by rinsing the cell monolayer with DPBS. Meanwhile, samples were sterilized by immersion in 2X Anti-Anti solution for at least 60 min. Tissue culture polystyrene wells, treated similarly, were used as controls. L929 mouse fibroblasts were trypsinized with 3 mL Tryple Express, rinsed twice by centrifugation (240 g for 5 min in 30 mL media), and re-suspended at 75000 cells/mL. ppCo samples were placed in the wells of a 12-well plate and then an aliquot of cells added (1.2 mL, 25000 cells/cm^2 of well area) and incubated overnight at 37^{o}C in a 5 % CO_2/air atmosphere. Non-adherent cells, present on both the ppCo coated wafer and on the surrounding tissue culture polystyrene, were removed by rinsing the samples in fresh media. Any remaining cells were further stained for viability with 2 μM Calcein-AM in DPBS for at least 20 minutes. Samples were removed from the wells and inverted prior to imaging with a fluorescent microscope (Nikon Eclipse TE2000-U). DilC12(3) was excited at 510-560 nm with >590 nm emission observed, and Calcein excited at 465-495 nm with 515-550 nm emission observed.

Results and Discussion

Characterisation of plasma polymerised thin films

The static contact angle measured for ppCo film (77 ± 2 degrees) was lower than reported literature values.[13] A lower contact angle suggests that more oxygen was incorporated in the thin film or the surface was less polar than the coating previously reported. However, that observation is difficult to confirm as no XPS data is available from the previous studies. The differences between the previously reported coatings and the films presented in this paper are likely due to differences in the plasma reactors employed, including geometry, electrode type and electrode location which would induce differences in the chemical and physical properties of the resultant film. The contact angles for the hydrocarbon rich ppOct film (90 ± 1 degrees), and for the glass slides (20 ± 4 degrees), correlate well with literature values.[7, 21]

XPS analysis of the ppCo and ppOct films revealed coatings rich in carbon with an O/C ratio of 0.10 and 0.09, respectively (Table 2); the elemental composition for ppCo compared well with the theoretical O/C ratio of the monomer while the composition of ppOct was in agreement with previous studies.[16, 22] While the 1,7-octadiene monomer does not contain oxygen, the creation of radicals within the ppOct film is known to lead to the rapid oxidation upon exposure to the atmosphere.[16, 22] When films were exposed to water or PBS, a small increase in oxygen content was observed. For ppCo, the O/C ratio increased to 0.11 and 0.12 when exposed to water and PBS respectively, while for ppOct, the O/C ratio increased to 0.10 when exposed to water and PBS (see Table 3). Thus these minor changes in O content are likely associated with ageing of the films.[22] After incubating the films in broth media, XPS analysis indicated the introduction of nitrogen (6.1 ± 0.2 % for ppCo and 5.7 ± 0.1 % for ppOct), and a significant increase in the oxygen content indicative of protein adsorption from the media. In all samples examined, no Si associated with the underlying Si wafer substrate was detected indicating that all films were >10nm thick.

Analysis of high resolution C1s spectra of the ppCo and ppOct stored in air, water and indicated minor changes in the composition of samples after storage (Table 3). For samples exposed to broth media, additional contributions were identified and can be associated with the introduction of amide, amine and carboxyl species associated with protein adsorption (Figure 1). Overall, the XPS results indicate that both ppCo and ppOct films are stable with minimal changes in the elemental composition and no evidence of delamination of the films.

Table 2: XPS elemental analysis of ppCo and ppOct deposited on Si wafer (n≥3). Listed are the mean values (± standard deviation) based on at least 3 analyses performed on each sample.

	% C	% O	% N	O/C	N/C
ppCo					
Monomer (theoretical)	91	9	-	0.10	-
As deposited stored in air	90.6 ± 0.1	9.4 ± 0.1	-	0.10	-
16 h immersed- water	90.2 ± 0.2	9.8 ± 0.2	-	0.11	-
16 h immersed- PBS 150	89.4 ± 0.5	10.6 ± 0.5	-	0.12	-
16 h immersed- broth media	80.1 ± 0.4	13.8 ± 0.2	6.1 ± 0.2	0.17	0.08
ppOct					
Monomer (theoretical)	100	-	-	-	-
As deposited stored in air	91.5 ± 0.1	8.5 ± 0.1	-	0.09	-
16 h immersed- water	90.9 ± 0.2	9.1 ± 0.2	-	0.10	-
16 h immersed- PBS 150	90.9 ± 0.2	9.1 ± 0.2	-	0.10	-
16 h immersed- broth media	82.5 ± 0.2	11.8 ± 0.1	5.7 ± 0.1	0.14	0.07

Table 3: Component fitting of high resolution C 1s spectra of ppOct and ppCo before and after exposure to water, PBS and broth media as determined by XPS (n≥4). Listed are the mean values (± standard deviation) based on at least 4 analyses performed on each sample.

	C1	C2	C3	C4	C5
ppCo					
Monomer (theoretical)	80	-	20	-	-
As deposited stored in air	84 ± 0.3	-	13.8 ± 0.1	2.3 ± 0.2	-
16 h immersed- water	83.9 ± 0.1	-	13.6 ± 0.1	2.5 ± 0.1	-
16 h immersed- PBS 150	82.3 ± 0.4	-	14.8 ± 0.4	2.9 ± 0.2	-
16 h immersed- broth media	72.1 ± 0.6	2.4 ± 0.1	17.5 ± 0.5	5.5 ± 0.1	2.4 ± 0.1
ppOct					
Monomer (theoretical)	100	-	-	-	-
As deposited stored in air	87.3 ± 1.0	-	10.2 ± 0.7	2.6 ± 0.3	-
16 h immersed- water	86.7 ± 0.3	-	10.6 ± 0.3	2.7 ± 0.1	-
16 h immersed- PBS 150	86.4 ± 0.4	-	10.7 ± 0.3	2.9 ± 0.1	-
16 h immersed- broth media	77.0 ± 1.1	3.3 ± 0.2	12.6 ± 1.2	3.8 ± 0.3	3.3 ± 0.2

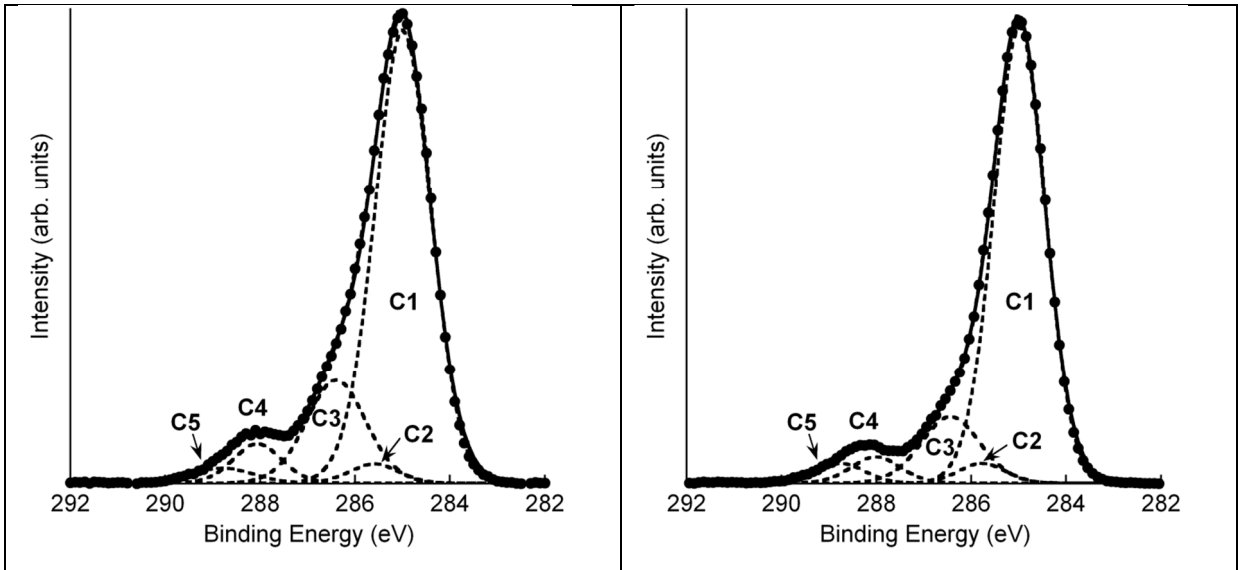

Figure 1: Curve fitting example of C1s spectra for a) ppCO and b) ppOct immersed in broth media 16 h, determined by XPS. Circle markers represent experimental data while overlaying solid line represents spectrum fit obtained from components.

Table 4: Thickness measurements (n≥9) and roughness parameters (n≥6) of ppCo and ppOct as deposited, and after exposure to water and phosphate buffered saline (PBS, 150 mM) with an incubation time of 16 hours. Listed are the mean values (± standard deviation) based on the specified analyses performed on each sample.

	Thickness (nm)	R_a (nm)	R_q (nm)	Rmax (nm)
ppCo				
As deposited stored in air	56.7 ± 2.8	< 1	< 1	9 ± 4
16 h immersed- water	57.5 ± 1.2	-	-	-
16 h immersed- PBS 150	54.1 ± 2.9	-	-	-
ppOct				
As deposited stored in air	75.4 ± 2.1	< 1	< 1	11 ± 6
16 h immersed- water	74.4 ± 2.5	-	-	-
16 h immersed- PBS 150	73.8 ± 3.8	-	-	-

Using the XPS overlayer algorithm, the amount of protein adsorbed onto ppCo was estimated to be ~ 220 ng/cm^2 and on ppOct ~ 199 ng/cm^2. The presence of a low concentration of protein on the hydrophobic substrates was expected as hydrophobic materials are known to adsorb significantly lower amounts of protein than hydrophilic materials.[23]

To complement the XPS study, the thickness of the ppOct and ppCo films was assessed by AFM step height measurements (Table 4). As deposited, ppCo and ppOct were 57nm and 75nm thick, respectively, and no statistical significant changes in the thickness of the film were observed after immersion in water or PBS. ppCo had a roughness average (R_a) and root means square roughness (R_q) of ~1 nm, and a maximum height (R_{max}) of 11 ± 6 nm (see Table 4); R_a, R_q and R_{max} for samples immersed in water and PBS are within an error. Similar values for R_a, R_q and R_{max} were obtained for ppOct, indicating that the films were conformal and did not swell on exposure to the solutions.

Kill-rate of 1,8-cineole against Staphylococcus aureus and Escherichia coli

Measurements of kill rate for a variety of concentrations of the 1,8-cineole monomer were performed for both *S. aureus* and *E. coli*. Figure 2 illustrates the relative viable count of *S. aureus* incubated with 1,8-cineole. At the lowest concentration of 0.05 % v/v there was no statistically significant change in the number of viable bacteria when compared with control cultures (Figure 2a). At higher concentrations, a significant decrease in viable cells was observed after 5 hours when 1,8-cineole was applied at 0.5 % v/v. After 16 hours, the growth of the cells recovered and reached a 100 % of relative viable count at 24 hours. The growth recovery observed was shown to be independent of the 1,8-cineole concentration.

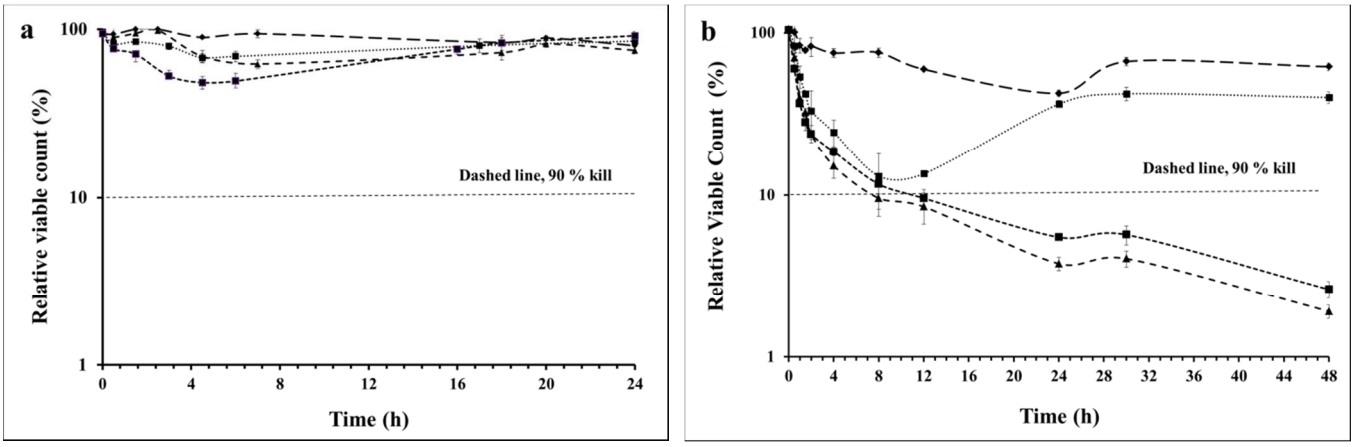

Figure 2: Relative viable count (%) of a) *Staphylococcus aureus* and b) *Escherichia coli* after exposure to 1,8-cineole determined by time-kill studies. Circles represents 0.05 % v/v, squares 0.2 %v/v, triangles 0.35 % v/v, and crosses 0.5 % v/v 1,8-cineole in broth media. Repeat experiments gave similar results for both bacteria with no significant difference between duplicate samples (data validated by t-test), and the data represent the average of the measurements (± standard deviation). Smoothed lines are added to assist comprehension, not as true interpolations.

The recovery of bacteria after exposure to essential oils has been observed previously. Examining ceftazadime-resistant *Pseudomonas aeruginosa* exposed to cloned tea tree oil, May *et al*.[12] observed an initial decrease in viable cell count in the first two hours, followed by a recovery over the next 4 hours.

Figure 2b shows the influence of the same concentrations of 1,8-cineole on *E. coli* growth rates. At the lowest concentration, there was no significant change in the number of viable cells compared with the controls. At 0.20 % v/v, the viability dropped with almost 90 % of the bacteria killed after 8 hours of incubation. Cells recovered their standard growth patterns after 48 hours. At the higher concentrations of 0.35 % v/v and 0.5 % v/v, cell viability dropped to 10 % after 8 hours, and this reduction in cell viability was increased to 99 % at 48 hours. There were no substantial differences in the reduction of viability of bacteria between 0.35 % v/v and 0.5 % v/v of 1,8-cineole indicating that 0.35 % v/v is the minimal concentration of 1,8-cineole required. Gustafson *et al.* attributed the effect of tea tree oils on *E. coli* to the stimulation of autolysis in exponential and stationary phases.[24] However, this study did not link this effect to the minor component of tea tree oils, 1,8-cineole. It is difficult to isolate the factors that influence in the bacterial recovery without a more in depth study in the mode of action of 1,8-cineole.

Bacterial attachment and growth, and biofilm formation on glass, ppCo and ppOct.

The antibacterial activity of ppCo films was evaluated against *S. aureus* and *E. coli*. The behaviour of ppCo film was compared to a ppOct (hydrophobic control) and clean glass surfaces (hydrophilic control).

Two-dimensional CSLM images of *S. aureus* attached to ppOct and ppCo films after 18 hours of culture are shown in Figure 4a and 4b. A significant reduction in bacterial attachment was observed for both films when compared to the hydrophilic controls with $1.3 \times 10^7 \pm 1.1 \times 10^6$ bacteria / cm^2 on ppCo (63% reduction compared to glass control, $p < 0.01$) and $1.2 \times 10^7 \pm 1.3 \times 10^6$ bacteria / cm^2 on ppOct (64% reduction compared to glass control, $p < 0.01$) (Figure 3). While a significant reduction of bacteria was observed on ppCo when compared to the hydrophilic control, no significant variation was observed in the total number of bacteria attached to ppCo when compared to the ppOct (Figure 4). A relatively small number of hydrophilic bacteria were expected to attach to ppOct, as hydrophobic hydrocarbon-rich materials are known to limit bacterial attachment and protein adsorption,[25], and this was observed. However, a comparable decrease in the total bacteria / cm^2 on ppCo was surprising given ppCo is more hydrophilic. *S. aureus* is relatively hydrophilic at pH between 5-11.[26, 27] Unsurprisingly, a relatively larger number of cells attached to the hydrophilic glass slide ($3.44 \times 10^7 \pm 1.55 \times 10^7$ bacteria / cm^2; Figure 3), in agreement with results reported elsewhere for attachment on hydrophilic surfaces.[27] The colonisation data retrieved from one sample to the next was highly variable, as can be seen by the standard deviation for the glass sample.

In terms of the colonisation morphology, grape-like clusters were observed on all three surfaces. For the glass slide, some areas had a high density of attached cells, which indicates that 18 hours was sufficient for an initial biofilm to form that resisted the rinsing process. A green background can be observed in several images for the plasma polymer thin films and is related to auto-fluorescence indicating the presence of unsaturated carbon bonds within the film.[28, 29]

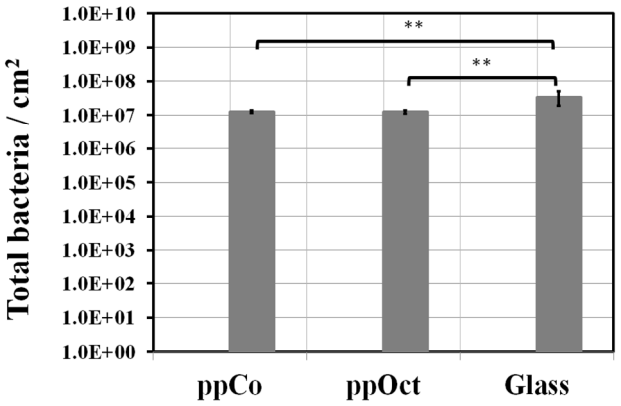

Figure 3: Staphylococcus aureus attached to ppCo films, ppOct films and glass. Samples were immersed in bacterial culture for 18 hours. The number of bacteria attached is shown as total bacteria / cm^2 (± standard deviation, ** $p < 0.01$).

E. coli attachment and growth on ppCo, ppOct, and glass slides were also evaluated (Figure 5). A considerable reduction in the number of cells quantified on ppCo surfaces was observed, with a decrease of 98 % compared with the glass surfaces ($1.5 \times 10^5 \pm 7.1 \times 10^4$ bacteria / cm^2, Figure 6). A reduction in the number of cells attached to ppOct was also observed ($9.2 \times 10^5 \pm 8.7 \times 10^5$ bacteria / cm^2, Figure 6) which is an 85 % decrease in attachment when compared to glass. As illustrated by the variation in the cell counts, bacterial attachment on ppOct was heterogeneous (see Figure). An average of $6.3 \times 10^6 \pm 2.2 \times 10^6$ bacteria / cm^2 was detected on the hydrophilic surfaces (see Figure 6). As for *S. aureus*, a lower number of bacteria were expected on the hydrophobic substrates and this was observed.[25] A significantly smaller number of *E. coli* (10^6) attached compared with *S. aureus* (10^7). *E. coli* is more negatively charged than *S. aureus*, therefore charge interactions likely reduced the *E. coli* attachment.[10] In the case of ppCo and ppOct, chain-like colonisation was not evident, but was observed on the glass samples (Figure 5 a) and b) compared to Figure c)). For glass, several areas of the surface showed traces of biofilm remaining after the rinsing.

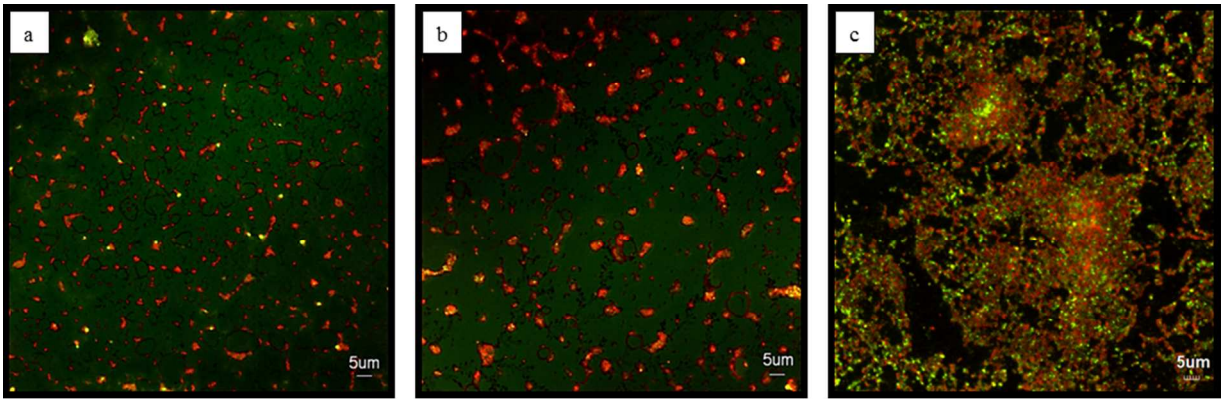

Figure 4: CSLM images of *Staphylococcus aureus* attached to a) ppCo, b) ppOct and c) glass slides. Samples were immersed in bacterial culture for 18 hours. Green represents live cells and red represents dead cells. Images have been corrected in brightness and contrast. Representative images from the three samples examined.

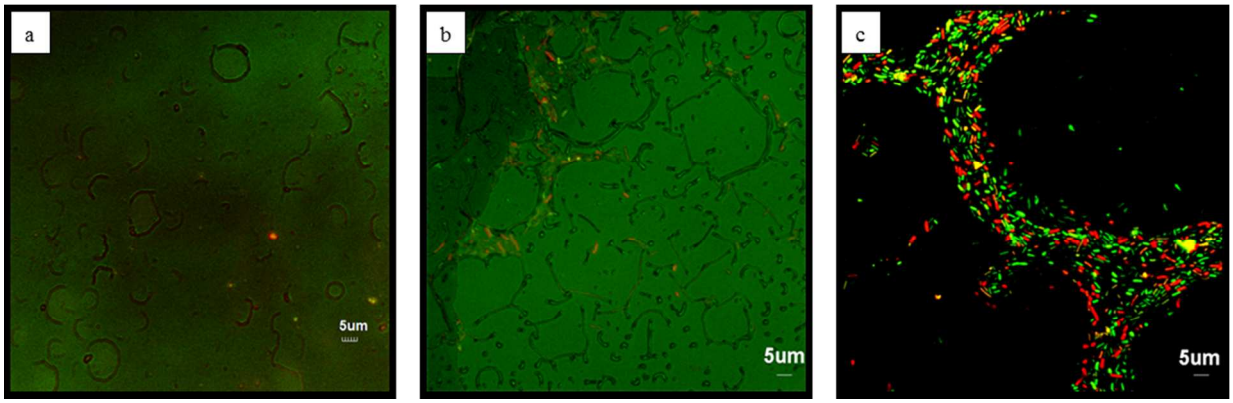

Figure 5: CSLM images of *Escherichia coli* attached to a) ppCo, b) ppOct and c) glass slides after 18 hours incubation. Green represent alive cells, red represent dead cells. Images have been corrected in brightness and contrast. Representative images from the three samples examined.

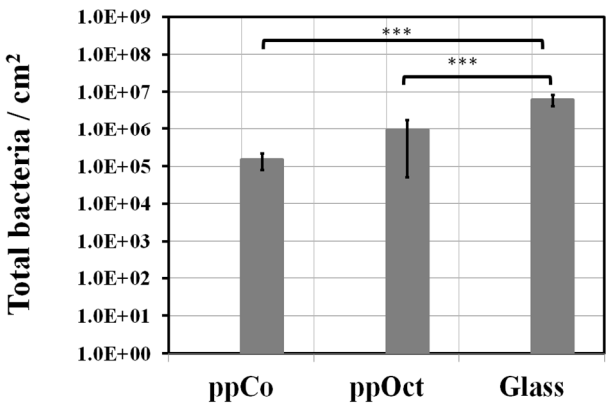

Figure 6: *Escherichia coli* attached to ppCo films, ppOct films and glass slides. Samples were immersed in bacterial culture for 18 hours. The number of bacteria attached is shown as total bacteria / cm^2 (± standard deviation, *** $p < 0.001$).

The results of the bacteria attachment studies on ppCo and ppOct indicated that the surfaces appeared to prevent significant bacterial attachment. However, other studies have shown that a low initial attachment does not necessarily result in reduction of biofilm formation.[7] To assess the ability of the ppCo in preventing bacteria colonisation over time, samples were immersed for five days with *E. coli* (Figure 8).

Analysis of the biofilm formation on ppCo films showed an isolated sample with less than 1 % of the surface was covered with biofilm while the remaining samples were free from bacteria (Figure 7). In the area covered with biofilm, an even ratio of viable and unviable was found. On ppOct, a significant reduction in biofilm formation was noted when compared to the glass sample (see Figure 7), and several areas showed that the bacteria within the biofilm appeared to have low viability. As expected, biofilm formed on clean glass slides after five days of exposure, with approximately 23 ± 8 % surface coverage. Likewise, viable and unviable cells were found in even ratio.

Similar colonisation patterns were found in all hydrophilic samples indicating a more homogeneous colonisation on the surfaces once the biofilm is formed. The formation of a mature biofilm that remain after the rinsing process is as expected after 5 days of culture. These results demonstrated that by extending the assay time, significant difference in the behaviour of ppCo and ppOct exist ($p < 0.1$, Figure 7). The reduced bacterial attachment of the ppCo was maintained for at least five days, which prevented the formation of biofilm. Thus, the reduction of the bacterial attachment onto ppCo is likely related to the antibacterial properties of the monomer in addition to the moderate hydrophobicity of the resultant film. Further studies are required to identify the exact mechanism for the prevention of bacterial attachment observed on the ppCo thin films.

To ensure that the antibacterial behaviour was not due to essential oil or other agents leaching from the ppCo films, a 100 µl aliquot of the *E. coli* after exposure to ppCo for 18 hours was plated on agar and immersed overnight at 37°C, 100 r.p.m. The same procedure was performed with *E. coli* exposed to clean glass slides and ppOct. No significant changes were observed on the growth pattern of the *E. coli* in any of the cases. These observations confirmed that there was no substantial leaching from the ppCo films into the media.

Although the mechanism by which the inhibition of biofilm formation occurs remains unknown, the data suggests that to some extent the original structure of the 1,8-cineole is retained within the thin film. The 1,-8cineole structure is formed by a cyclic ether and a monoterpenoid, and previous studies have reported that the presence of ether functional group on a surface can inhibit biofilm formation.[30] In addition, monoterpenoids have been used as a biocidal agents.[31] In addition, Mishra *et. al.* [32] proved that the original structure of a selected molecule can be retained after plasma processes and reported a specific examples using maleic anhydride. Further, Vasilev *et. al.* [11] showed that biocidal agents such as furanones can be immobilized onto different surfaces and still retain their antimicrobial activity. To confirm this theory, further studies are required to corroborate that the original structure of the 1,8-cineole has been retained and responsible for the antimicrobial activity shown by the ppCo films.

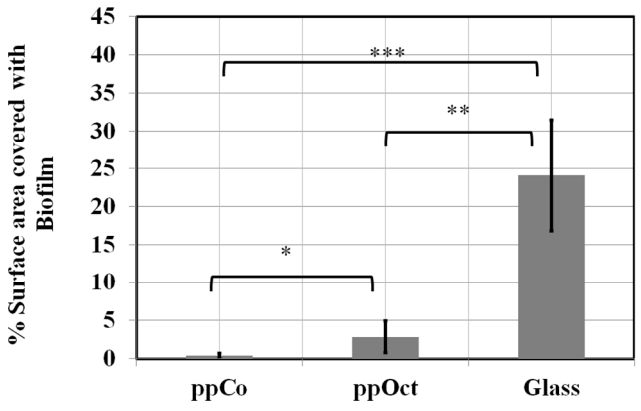

Figure 7: Surface area covered by *Escherichia coli* biofilm formed on ppCo, ppOct, and glass. Samples were immersed in bacterial culture for 5 days (± standard deviation, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.1$).

Mammalian cell viability

Biomedical devices that can mitigate or eliminate the issues associated with bacterial fouling are of importance for successful medical application. However, for many applications, for example *in vivo* use, a complicating factor is that a device must simultaneously display (i) toxic or inhibitory properties towards bacterial cells and (ii) non-toxic or favourable properties towards mammalian cells. As a preliminary study, we cultured murine fibroblast cells on/in the

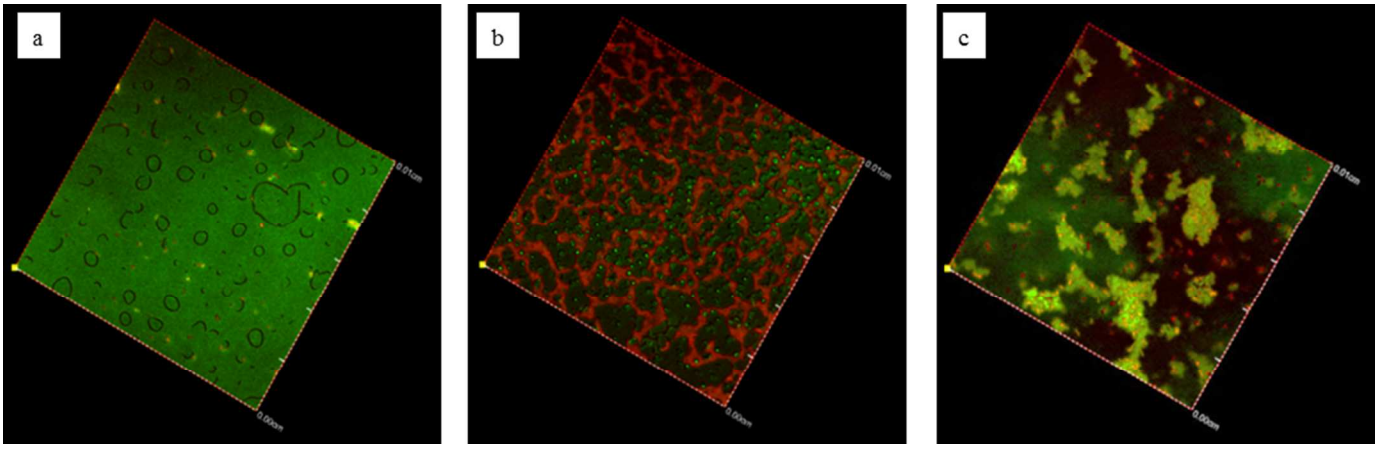

Figure 8: Two-dimensional confocal laser scanning microscope (CSLM) images of *Escherichia coli* biofilm formed on a) ppCo, b) ppOct and c) glass. Samples were immersed in bacterial culture for 5 days – live/dead staining. Representative images from the three samples examined.

presence of ppCo coated Si wafers placed in the walls of a tissue culture plate. At 23 hours total culture, no cells could be found adhered to the ppCo (data not shown). Further, all cells attached to the surrounding TCPS appeared to have spread morphology comparable to that of control wells. Finally, there was no significant evidence of cell death, as indicated by retention of membrane integrity, and retention of esterase activity, shown in Figure 9.

These preliminary results are very promising. It is not surprising that the cells did not attach to this hydrophobic surface as it is well established that the extracellular matrix (ECM) proteins required for cell adhesion tend to adsorb in low quantities and/or denature on hydrophobic surfaces.[33, 34] The absence of observed cytotoxicity suggests that direct contact with the ppCo coating does not cause cell death. Further, the morphology and viability of cells adherent to the surrounding TCPS suggests that any leachables that may be released from the ppCo coating, while not being antibacterial, are also importantly not cytotoxic or cell disruptive to mammalian cells.

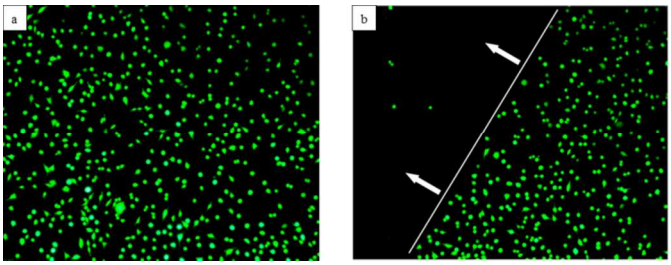

Figure 9: Fluorescent microscopy images of viable cells at 23 hours, as indicated by Calcein generation and retention within the cellular membrane, on a) a TCPS control surface, and b) a TCPS surface immediately adjacent the location of a ppCo coated Si wafer (now removed, but indicated by the white line and arrows).

Conclusions

A plasma polymerisation route was used to engineer thin films from 1,8-cineole. The moderate hydrophobicity of the ppCo was confirmed. XPS data revealed that ppCo are chemically stable when immersed in water and PBS. The results suggested that there is a greater amount of hydrocarbon compared with the theoretical composition. No thinning of the films was detected when samples were immersed in water and PBS. AFM analysis of the ppCo films indicated that the films were smooth. The antibacterial activity of 1,8-cineole in solution was tested against *E. coli* and *S. aureus*. 50 % of *S. aureus* cells were killed after 5 hours in the presence of 0.5 % w/v of 1,8-cineole. However, the cells recovered over time. 1,8-cineole was more effective against *E. coli*, where 90 % of the cells were killed after 8 hours by concentrations above 0.35 % w/v. A larger exposure of the *E. coli* to 1,8-cineole resulted in 99 % of bacteria killed after 48 hours.

Retention of the antibacterial activity of the 1,8-cineole when used to form a plasma polymer thin film (ppCo) was demonstrated. A reduction of 64 % of *S. aureus* was observed compared to glass slides. A similar decrease was obtained for ppOct films. A larger reduction on the attachment of *E. coli* was observed with 98 % less cells attached to ppCo when compared to glass. When the experiment was extended to 5 days to examine biofilm formation with *E. coli*, a statically significant difference was observed between the ppCo and ppOct films. On ppCo less than 1 % of the surface was covered with biofilm. For intended biomedical application, preliminary studies suggest that ppCo is non-cytotoxic.

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Notes and references

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