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Article

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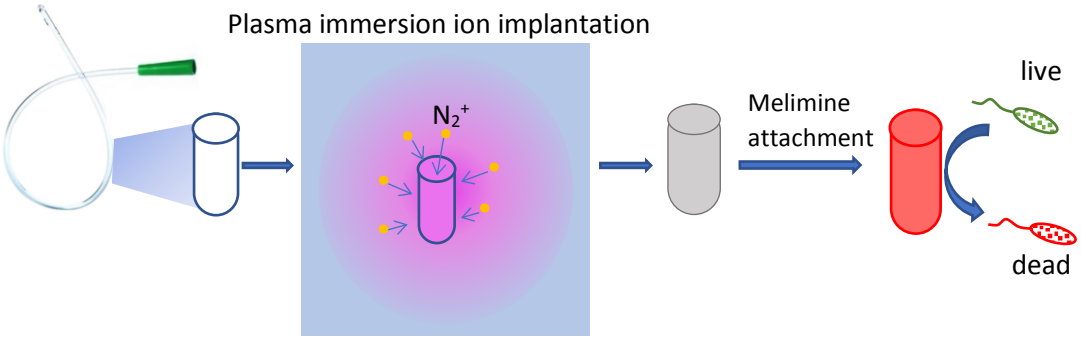
Plasma immersion ion implantation
N_2^+
Melimine
attachment
live
dead

A single step plasma process for covalent binding of antimicrobial peptides on catheters to suppress bacterial adhesion

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Abstract

Catheter-associated biofilms are responsible for a large fraction of hospital acquired infections. Antimicrobial surface coating on catheters providing prevention at source is extensively studied to reduce bacterial adhesion. Antimicrobial peptides such as melimine and Mel4, covalently linked to surfaces have shown excellent potential in animal and human studies to suppress infection without toxicity. Covalent binding of the peptides on catheter surfaces improves efficacy but so far has been implemented using multi-step wet chemical coupling that will impede widespread adoption. Here we demonstrate plasma immersion ion implantation (PIII) as a single step treatment that covalently couples antimicrobial peptides to polyvinyl chloride (PVC). Strong antimicrobial activity was demonstrated by higher than 3 log kill of *S. aureus.* A variant of the process was demonstrated as an antimicrobial treatment for chemically inert glass surfaces. Covalent coupling was rigorously tested by stringent SDS washing. We further demonstrated that the plasma treatment can effectively functionalize both internal and external surfaces of catheter tubing, reducing 99% of bacterial adhesion. The process is feasible as a patient-safe treatment for treating various types of catheters and is suitable for commercial mass production. In a logical extension of the work, the process could be adapted to bone replacement scaffolds of all types including metallic, polymeric and ceramic.

Keywords: antimicrobial peptide, melimine, plasma immersion ion implantation, plasma coating, catheters

1. Introduction

Catheter-associated urinary tract infections account for a large fraction of hospital acquired infections worldwide, with some estimates reaching as high as 80% [1]. Catheters are a common cause of bloodstream infections [2] and the infection rate is 1.8/1,000 catheter days for the general population [3] but increases to 3 - 9/1,000 catheter days when used with new-borns and oncology patients [4, 5]. Microbially-colonised central venous catheters are associated with 17.6 times greater risk of thrombosis [6]. Mortality rates from catheter-related bloodstream infection are 6-11% [7]. Patients having long-term catheterization with Foley-type urinary catheters are especially at risk of infection and can suffer from fever, bacteremia, acute or pyelonephritis, obstruction, urinary tract stones [8].

Catheter-associated infections usually begin as single species bacterial biofilms [9]. Biofilms are notoriously difficult to eradicate after they have formed. In some cases, biofilm infections can develop quickly, within 24 hours, and may progress to become multiple species biofilms, which are even more resistant to treatment especially if they contain antibiotic-resistant strains [9]. Therefore, internal and external surface treatment of catheters to block biofilm formation would significantly and positively influence clinical practice and improve patient experience in hospital [10-12].

Urinary catheters treated with antimicrobial agents are now available and some have been used in clinical settings. The antimicrobial functionality of these catheters is usually provided by impregnation of the polymer materials of the catheter with either silver or a combination of antibiotics (such as rifampin/minocycline). Silver and rifampin/minocycline impregnated surfaces have shown some antimicrobial activity, but catheters treated with these agents still fail to control urinary tract infection [10-14]. Antimicrobial peptides (AMPs) are an attractive option for a more effective inhibition of biofilm formation as they are likely to be more resilient than conventional antibiotics against the development of bacterial resistance [15]. An additional advantage is that they may be less toxic than silver to normal tissues [16]. The mode of action of AMPs is either disruption of the membrane or penetration of the membrane to initiate internal modification of cell functions [17]. The use of AMPs on titanium surfaces has been reported[18, 19] and their use on urinary catheters has been proposed in several reviews [20,

[21] along with the challenges preventing their widespread adoption. The dominant challenges that remain are the difficulty of immobilizing the AMPs to form a well-attached surface layer on the catheter, the sensitivity of AMPs to pH and a high cost.

We have developed an AMP called melimine that is effective against bacteria *in vitro* and *in vivo* [22-27]. The minimum inhibitory concentration of melimine against *S. aureus* strains ranges from 16.5 to 33.01μM [28]. Melimine and Mel4 (a shorter daughter peptide) bound to flat or nearly flat polymer surfaces using linking by chemical activation methods have been shown to prevent adhesion and biofilm formation by a broad spectrum of Gram-positive and Gram-negative bacteria and fungi. These include *Streptococcus pneumoniae, Elizabethkingia meningoseptica, Stenotrophomonas maltophilia, Escherichia coli* and *Delftia acidovorans* as well as multi-drug resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* and even the usually AMP-resistant *Serratia marcescens* and *Burkholderia cepacia*, the fungus *Candida albicans* [29] and the protozoan *Acanthamoeba* [22, 27]. Many of these microbial types are commonly found associated with urinary catheter infections [30]. Melimine is both anti-adhesive, i.e. it reduces the number of bacteria adherent to a surface, and antibacterial, i.e. it kills bacteria already adherent to a surface. Using LIVE/DEAD staining (BacLight Bacterial Viability Kit. Molecular Probes, Inc), the total number of cells of either *P. aeruginosa* or *S. aureus* are reduced when melimine is covalently bound to glass, and the number of dead cells is increased (i.e. the number stained with propidium iodide) [22]. Melimine and Mel4 coatings have superior antimicrobial activity compared to other cationic peptides such as cathelicidin LL-37, and do not loose activity in the high salt concentration of tears as does LL-37 [31]. In terms of safety, melimine and Mel4-coated contact lenses can be safely worn by rabbits [26, 32] and humans [26]. These safety and efficacy data indicate that coating catheters with melimine or Mel4 should provide a safe, non-toxic and efficacious antimicrobial functional surface. Melimine has been shown to be heat stable during autoclaving [27] and is therefore a good choice as an antimicrobial treatment for implantable devices which require sterilisation before use.

Here we address the challenge of providing a simple and strong covalent binding of AMPs to a medical device surface. Melimine and Mel4 have been applied to a variety of materials including hydroxyethyl methacrylate, silicone, titanium, glass, polystyrene and Teflon without losing activity [22, 25, 27, 29]. However, our previous coating techniques for melimine have used either inherent characteristics of the substrata such as hydroxyl or carboxyl groups that allow crosslinking with the amine groups by 1-ethyl-3-[3-

dimethylaminopropyl] carbodiimide hydrochloride (EDC) [31], or activation of surfaces with 4-azidobenzoic acid (ABA) or 4-fluoro-3-nitrophenyl azide (FNA) in methanol followed by ultraviolet irradiation [22], or activating the surfaces with plasma activated acrylic acid followed by crosslinking with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride N-hydroxysuccinimide [33], or modification of the peptides by adding cysteine to the *N*-terminal and activating the substrata with 3-aminopropyltriethoxysilane and 4-(*N* maleimidomethyl)cyclohexane-1-carboxylic 3-sulfo-n-hydroxysuccinimide ester [23, 24]. Whilst these techniques are capable of covalently binding melimine and Mel4 to the substrate, they suffer from several drawbacks. Firstly, not all substrates have inherent hydroxyl or carboxyl groups suitable for chemical linker strategies. Secondly, the residues of chemical linkers used in the coupling process may potentially adversely impact subsequent medical applications. Thirdly, the use of methanol and ultraviolet light is not appropriate for all polymers as the methanol may affect the polymer and some substrates are opaque to ultraviolet light, reducing its effect on the internal surfaces. Previous work has used an ion assisted plasma polymerised film to bind Mel4 and Casporin to titanium surfaces [29] to demonstrate the antimicrobial affect. However, the use of the PIII process on the interior surfaces of polymer tubing for AMP attachment has not been demonstrated.

In this paper, we demonstrate a linker-free single step method of surface activation to attach antimicrobial peptides covalently to polymer and glass surfaces using free radicals generated from a plasma treatment that includes a substantial amount of ion bombardment. Radical-rich surfaces can be produced on polymers using plasma immersion ion implantation (PIII) and on non-polymeric substrates using plasma immersion ion implantation with plasma chemical vapour deposition (PIIID) to form highly adherent radical-rich coatings. Both PIII treated polymers and PIIID treated surfaces have been shown to form covalent bonds with biomolecules upon contact in solution [34-37] without the need for providing any additional chemical linkers and the bond so formed has been rigorously tested for covalency [34]. We describe the use of a PIII process to prepare the surface of polyvinyl chloride (PVC) discs and a PIIID process to create a thin coating on glass cover slips with covalent binding sites for the binding of melimine in a single-step incubation. These surfaces were subsequently exposed to bacterial cultures to show antimicrobial property against *S. aureus*. We further investigate the use of PIII treatment to activate both the outer and inner surfaces of catheter tubes to covalently bind melimine and thereby reduce bacterial attachment to the catheter tubes.

2. Materials and methods

2.1 PIII treatment of PVC film and PVC catheter tubes

Plasma immersion ion implantation and plasma immersion ion implantation with plasma chemical vapour deposition were conducted in our custom-made plasma treatment system. A schematic of the apparatus can be found in our previous work [38] and in a patent [39].

PIII treatment is used for activating carbon-containing polymers to provide them with binding sites. The treatment generates ion bombardment onto the polymer surfaces to produce dangling bonds (radicals) which are active in biomolecule attachment by covalent bond formation. PVC film of 0.2 mm thickness was purchased from Goodfellow (England). Although this product is described as unplasticized, FTIR analysis (see Figure S2 in Supporting information) shows that there are several peaks that are not derived from the polyvinyl chloride structure (Figure S1 in Supporting information), leading us to believe that there are some additives in this polymer. For the PIII treatment and the subsequent analysis, PVC films were cut into 1 cm diameter discs and positioned at the bottom of a 100 ml conical flask (6 discs/flask). A copper electrode was attached outside the bottom of the flask and connected to a RUP6 pulse generator (GBS Electronik GmbH, Dresden, Germany). Nitrogen gas was introduced in the flask and regulated at 300 mTorr. A dielectric barrier discharge was created inside the flask by the application of the pulses of -7 kV to the copper electrode. The pulses were applied at a frequency of 1000 Hz with a pulse length of 40 μs for a duration of 10 minutes. The electric field accelerates positively charged nitrogen ions towards the PVC surfaces positioned inside the copper electrode area. The surface modification of PVC after the ion bombardment has been investigated in the literature [40]. PVC discs were treated on both sides for subsequent melimine attachment and bacterial attachment assay.

Commercial PVC catheters (6 mm diameter, Paralogic Pty Ltd, Sydney, Australia) were used for the PIII treatment, melimine attachment and assessment by bacterial adhesion assay. The FTIR spectrum of the inner and outer surfaces of this catheter are similar to each other but differ from the spectrum recorded from PVC film purchased from Goodfellow (see Figure 2S in Supplementary information). The inference we draw is that the PVC used for the catheter manufacture has additional additives (most likely plasticizers) not present in the PVC film. For the PIII treatment, catheter tubes were cut into 5 cm long sections and positioned on a PTFE template inside a glass treatment tube (40 mm diameter, 25 cm long) to keep them with their

axes parallel to the axis of the treatment tube. A copper electrode (5 cm long) was fitted to the outside of the glass treatment tube wall where the catheter tubes are located (see Figure 1) and connected to the RUP6 pulse generator. Nitrogen was regulated at 500 mTorr during the plasma treatment. Pulses of -10 kV, 500 Hz and 40 μs pulse length were applied to the copper electrode for a duration of 30 minutes. Samples were stored at room temperature for further experiments.

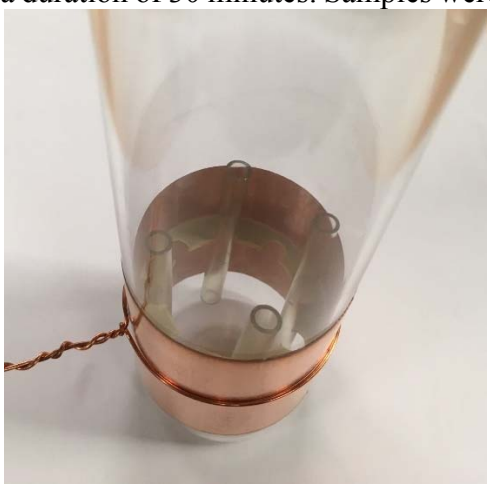


Figure 1: Catheter tubes positioned inside a glass tube with a copper electrode outside the tube for PIII treatment.

2.2 Plasma coating of glass cover slips

PIIID process was used to prepare surfaces for covalent binding when the substrate is a non-polymeric material such as glass or metal. For PIIID, a mixture of acetylene and nitrogen precursor gases with equal volumes was introduced to the flask and regulated at a pressure of 250 mTorr. Glass coverslips (13 mm in diameter) were positioned inside the copper electrode area, similar to the set-up for PIII treatment of PVC discs. Negative pulses with 40 μs pulse length and amplitude 10 kV were applied to the copper electrode at a frequency of 1000 Hz for 2 minutes to obtain a coating of approximately 40 nm thickness. Glass coverslips were coated on both sides ready for melimine attachment and bacterial assay.

2.3 Contact angle measurement

Contact angles were measured on PVC films and glass coverslips before and after the plasma treatment using a drop shape analysis system DS10 (Kruss GmbH, Germany). Two liquid probes (water and glycerol) were used with five drops on each sample for contact angle measurement and surface energy calculations using the Owens-Wendt-Rabel-Kaelble method.

2.4 Attenuated total reflectance-Fourier transform infrared (ATR-FTIR)

A Bruker Lumos FTIR microscope equipped with a micro-ATR crystal and integrated pressure control was used to analyze the infrared absorbance of the inside and outside surfaces of the catheter tube after the PIII treatment. Sixty-four scans were collected on each sample with a resolution of 4 cm^{-1}. Spectra were normalized with the intensity of C=O peak at 1724 cm^{-1} for comparison. This peak has been chosen as a monitor of the PIII treatment as it has been shown to correlate with PIII treatment time for both plasticized and unplasticized PVC [40].

2.5 Immobilization with melimine on flat samples and catheter tubes

Flat samples (untreated and PIII treated PVC discs; as-received and PIIID coated glass cover slips) were incubated with a melimine solution (2 mg/ml in phosphate buffer saline (PBS)) for 6 hours. After the incubation, they were washed with PBS four times and kept in PBS until the antibacterial assay. The same incubation was used to immobilize melimine on untreated and PIII treated tubes.

2.6 Covalency test of melimine binding on untreated and plasma treated surfaces

For covalent binding testing of melimine attached to the flat surfaces and the inner surfaces of the catheter tubes, two sets of samples were incubated with melimine as described in 2.5. After the incubation, they were washed with milli-Q water four times to remove unbound molecules and then dried in a desiccator. One set of samples was kept for X-ray photoelectron spectroscopy (XPS) analysis. The other set was further treated with 2% sodium dodecyl sulphate (SDS) for an hour with temperature regulated by a water bath (glass cover slips were treated at 70ºC, PVC discs were treated at 55ºC and catheter tubes were treated at 70ºC). The combination of SDS detergent and elevated temperature is a stringent wash that disrupts the physical absorption of molecules on a surface. After SDS treatment, samples were washed four times with milli-Q water to remove the detergent and unbound molecules before being dried in a desiccator for XPS analysis. One set of samples were incubated in PBS without melimine and used as control samples for XPS.

An XPS analysis system (Thermo Fisher Scienitific K-Alpha+) equipped with a monochromated Al Kα X-ray source was used to determine the composition of elements present on the sample surfaces with and without SDS washing. An increase in the relative concentration of nitrogen on the surface is an indication of melimine attachment. Survey scans and detailed scans of major elements (carbon, oxygen, nitrogen and chloride (on PVC discs) or silicon (on glass)) were taken on each sample for comparison. Element peak areas were

divided by element specific sensitivity factors and converted to atomic percent for each element. The results quoted are the average of two measurements on each sample.

2.7 Bacterial adhesion assay on flat samples

Antibacterial activity of melimine coated surfaces (i.e. the ability of the surfaces to kill adherent bacteria) was determined against *S. aureus* strain 38. Bacteria were grown in tryptic soy broth (TSB; Oxoid, Basingstoke, UK) for 12 h. The cells were then washed with PBS and diluted into the same buffer containing 1/10 TSB to OD_{660nm} 0.1 (1×10^8 colony forming units (CFU/ml) confirmed upon retrospective plate counts on Tryptic Soy Agar (TSA, Oxoid)). The bacterial cell suspensions were then serially diluted to 1.0×10^6 CFU/ml as a final concentration used in the adhesion assay. Melimine coated PVC and glass, together with uncoated controls and controls coated with bovine serum albumin (BSA, Sigma Aldrich), were incubated with 1 ml of bacterial suspension in 24-well plate and incubated at 37ºC for 18-24 h with shaking at 120 rpm. After incubation, the samples were mixed rapidly on a vortex mixer in a tube containing a magnetic stirring bar for two minutes in 2 ml of PBS, as previously described[31]. The resulting suspensions were serially diluted in PBS and plated on TSA. Following incubation for 18-24 h at 37 ºC , the number of live adherent bacteria were enumerated and expressed as CFU/mm^2 [41].

2.8 Evaluating S. aureus attachment on catheter tubes

Bacteria were prepared following the same procedure as described in 2.7 and diluted to 1.0×10^6 CFU/ml. Untreated and PIII treated tubes were incubated with the bacteria by filling the inside of the tubes with the bacteria suspension. Parafilm was used to block both ends of the tube to contain the suspension for 6 hours. After the incubation, the inner surfaces of the tubes were washed with PBS buffer to remove unbound bacteria.

The adherent bacteria on the tube wall were evaluated using scanning electron microscopy (SEM). To prepare the samples for SEM imaging, bacteria on the tubes were fixed with 2.5% glutaraldehyde for an hour by submerging the whole tubes in glutaraldehyde solution. After the fixation, the tubes were soaked serially in ethanol (50% ethanol for 1.5 hour, 70% ethanol for 1.5 hour, 85% ethanol for 1 hour, 95% ethanol for 1 hour) to reduce water concentration and finally the tubes were kept in 100% ethanol before dehydration step using a critical point dryer (Leica EM CPD 300). All samples were coated with a thin gold coating (15 nm) to avoid surface charge accumulation during SEM analysis. Nine images were collected on each sample

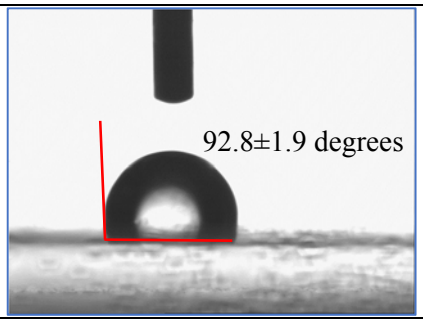
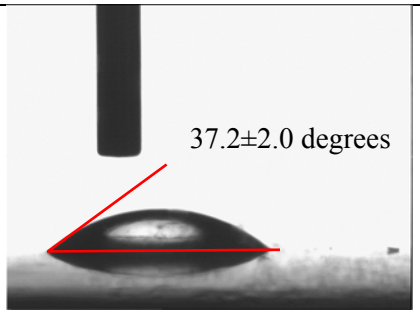
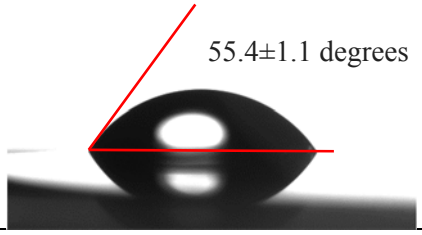
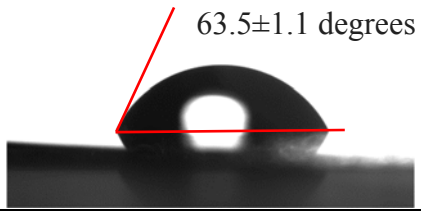
at different locations using a Zeiss Ultra Plus SEM equipped with a secondary electron detector at 5 kV. The fraction of area covered by bacteria on each image was quantified using Image J software by adjusting the contrast threshold to optimally distinguish the bright bacteria from the darker background. Results are the average of the coverage fraction on nine images with an estimated error of 10% arising from the threshold adjustment procedure in Image J.

3. Results

3.1 Effect of plasma treatment on PVC flat surfaces and glass cover slips

The PIII treatment on flat surfaces of PVC was accompanied by a visible darkening of the treated surface and changes in the water contact angle. As shown in Table 1, the water contact angle decreased substantially from 93 to 37 degree with increases in both the polar and dispersive components.

Table 1: Water contact angles and surface energy measured on PVC and glass cover slips before and after PIII and PIIID plasma treatments respectively.

	Untreated PVC	PIII treated PVC
Water contact angles		
Total surface energy	16.5±0.8 mN/m	58.68±3.85 mN/m
Polar component	2.98±0.3 mN/m	19.28±2.03 mN/m
Dispersive component	13.52±0.5 mN/m	39.40±1.81 mN/m
	As-received glass cover slips	PIIID coated glass cover slips
Water contact angles		
Total surface energy	47.37±0.87 mN/m	54.55±0.25 mN/m
Polar component	25.19±0.53 mN/m	45.90±0.17 mN/m
Dispersive component	22.18±0.34 mN/m	8.65±0.09 mN/m

PIIID coating on glass coverslips exhibited a brown appearance as a result of the formation of a polymer-like thin film that was subjected to ion bombardment during its growth. The water contact angle on as-received glass coverslips was 55 degree which increased to 63 degree after PIIID treatment, with a concurrent small increase in total surface energy (Table 1). The net increase in surface energy resulted from an increase in the polar component which was offset by a decrease in the dispersive component. These changes were attributed to the appearance of radicals in the surface caused by the ion bombardment during the PIIID process and the formation of polar groups after the reaction of radicals with the environment [38].

3.2 *Proof of covalency of melimine attachment on plasma treated surfaces*

The level of nitrogen as detected by XPS was used as the key indicator of the presence of melimine on the surface. A coverage of the surface by melimine increased the nitrogen atomic fraction by approximately 4% (Table 2). Figure 2A shows that untreated PVC immobilizes melimine, but a large fraction was removed by SDS, while the PIII treatment shows a large SDS-resistant component, which is assumed covalently bound.

Table 2: Comparison of atomic percent of elements detected by XPS analysis on untreated PVC (PVC), PIII treated PVC (PIII), after immobilisation of melimine (Mel) on these surfaces before SDS wash (PVC+Mel and PIII+Mel) and after SDS wash (PVC+Mel+SDS and PIII+Mel+SDS).

	PVC	PVC+Mel	PVC+Mel+SDS	PIII	PIII+Mel	PIII+Mel+SDS
C(%)	75.2 ± 0.0	73.1 ± 0.5	72.6 ± 0.2	73.2 ± 1.5	68.2 ± 0.3	70.7 ± 0.1
Cl(%)	15.8 ± 0.1	13.0 ± 0.6	16.8 ± 0.1	4.3 ± 0.2	4.2 ± 0.1	3.6 ± 0.1
O(%)	8.6 ± 0.2	9.9 ± 0.1	7.9 ± 0.2	14.5 ± 1.5	14.8 ± 0.1	13.7 ± 0.1
N(%)	0.4 ± 0.1	4.0 ± 0.0	2.7 ± 0.1	8.1 ± 0.2	12.8 ± 0.1	12.1 ± 0.1

Table 3: Comparison of atomic percent of elements detected by XPS on glass and glass with plasma coating (PIIID) after immobilisation of melimine before SDS wash (Glass+Mel and PIIID+Mel) and after SDS wash (Glass+Mel+SDS and PIIID+Mel+SDS). Trace amounts of Ti, Sc and K were detected on the glass cover slips but are not shown.

	Glass	Glass + Mel	Glass + Mel + SDS	PIIID	PIIID+ Mel	PIIID+ Mel + SDS
C(%)	53.6 ± 1.7	46.4 ± 0.2	61.5 ± 0.6	43.3 ± 0.1	38.4 ± 0.2	38.8 ± 0.2
Si(%)	25.8 ± 0.4	21.7 ± 0.1	28.1 ± 0.4	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1

O(%)	19.5 ± 1.3	25.2 ± 0.0	8.8 ± 1.0	44.6 ± 0.2	45.5 ± 0.0	47.2 ± 0.2
N(%)	1.2 ± 0.7	6.7 ± 0.1	1.6 ± 0.1	11.9 ± 0.2	15.8 ± 0.3	13.9 ± 0.1

Survey XPS scans on as-received glass coverslips show C, O and Si as the major elements present along with the minor constituents Ti, Sc and K. Only C, O and N are detected after plasma coating, with Si as minority constituent (Table 3), indicating that the coating provides an essentially complete coverage of the glass surface. After incubation with melimine, an increase in nitrogen was detected on both glass and PIIID treated glass. After SDS treatment, the majority of melimine on the untreated glass surface was removed, while only half of the nitrogen was removed from the PIIID treated surface (Figure 2B). This indicates melimine molecules are physically adsorbed on the untreated glass surface while a substantial fraction is covalently bound to the PIIID treated surface. The same level of silicon was detected on the plasma coated glass after SDS treatment at 70°C shows the stability of the coating on glass.

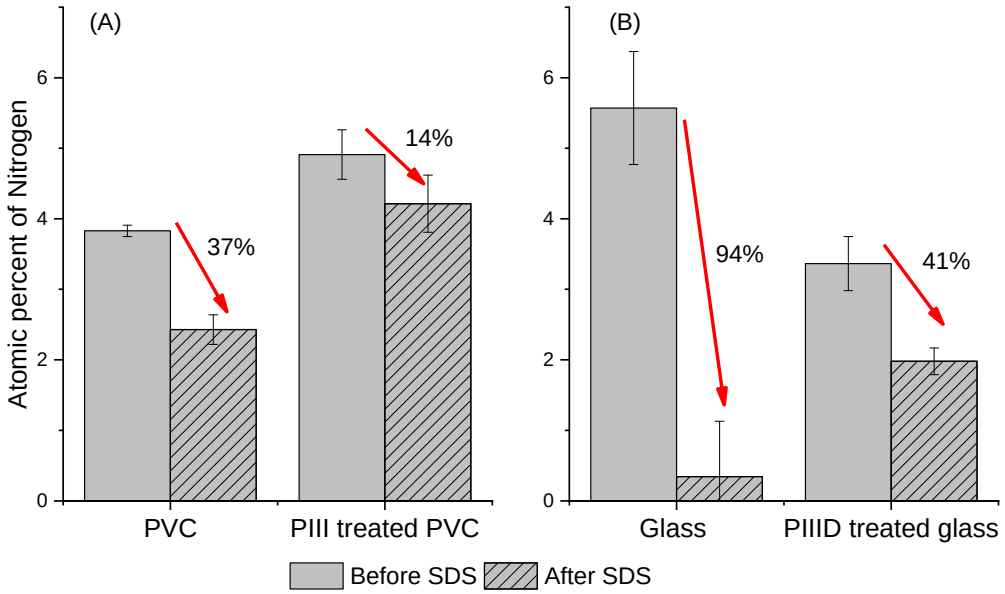


Figure 2: Comparison of the atomic percent of melimine-associated nitrogen before and after SDS wash. (A) On PVC and PIII treated PVC surfaces. (B) On glass and PIIID treated glass. The percentage losses of nitrogen after SDS wash are denoted by the arrows in the graph.

3.3 Antimicrobial effect of covalently bound melimine on Staphylococcus aureus

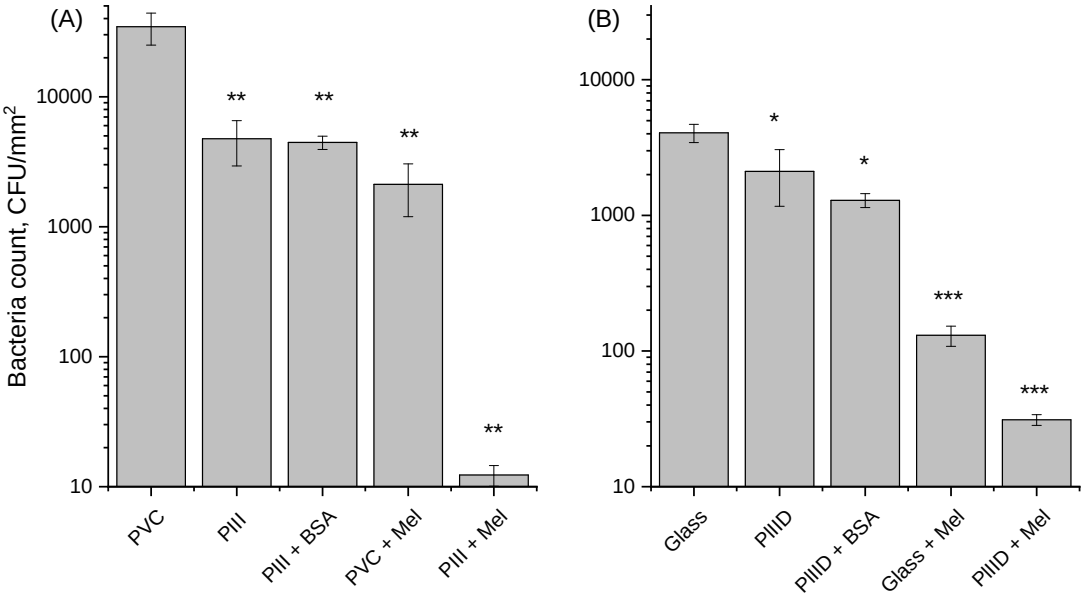


Figure 3: Bacterial count of *S. aureus* with and without melimine for: (A) flat surfaces of PVC and PIII treated PVC (PIII) and (B) Glass and PIIID treated glass (PIIID). BSA was immobilized on the PIII treated PVC and PIIID treated glass as a negative control for the antimicrobial effect of melimine. Groups significantly different from the untreated control group (PVC or Glass) are shown by a *t*-test with * $p<0.05$, ** $p<0.01$ or *** $p<0.001$.

Figure 3 compares the bacterial count of *S. aureus* on PVC and glass coverslips with and without plasma treatments and with and without melimine attachment. The untreated glass and untreated PVC surfaces show inhibition of bacterial adhesion due to the physical adsorption of melimine on those surfaces. These results are in agreement with the nitrogen level detected in XPS analysis. The activated surfaces of PIIID treated glass and PIII treated PVC without melimine showed a slight reduction in bacterial adhesion compared to untreated surfaces. Compared to the plasma activated surfaces immobilized with BSA, the plasma activated surfaces immobilized with melimine showed much stronger inhibition of *S. aureus*. The log reduction in inhibition of the PIII treated PVC incubated with melimine compared to its control (untreated PVC) is 3.4 which is comparable to the results obtained from previous work [31] where EDC coupling was used to immobilize melimine on the surface of commercially sourced contact lens made from pHEMA. The bacterial count on PIIID treated glass coverslips is reduced 130 times ($p<0.001$) compared to 40 times and 5 times reduction obtained from ABA and FNA coupling strategies [22]. Other studies have reported the use of various AMPs tethered

to the surface of polydimethylsiloxane (PDMS), a common material for urinary catheters, via epoxy groups on allyl glycidyl ether-grafted surfaces [42], polydopamine surfaces [43] or iodoacetic acid N-hydroxysuccinimide ester-treated surfaces [44]. Whilst the bacterial assays may not be directly comparable with these last three studies, the current method of attachment produced equivalent amounts of antimicrobial activity.

3.4 PIII treatment of the inner surface of catheter tubes

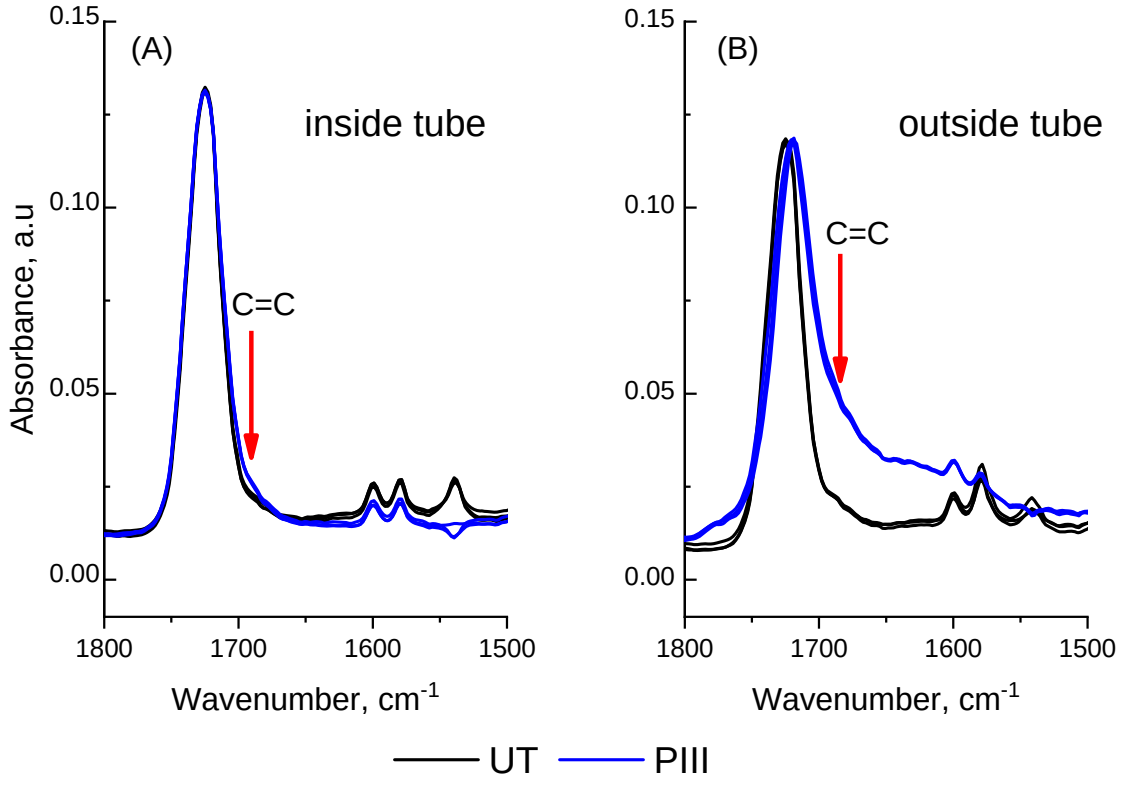


Figure 4: FTIR spectra obtained from the inside (A) and outside (B) surfaces of catheter tubes showing the changes in C=C absorbance before (UT, black lines) and after the PIII treatment (blue lines). The C=C absorbance indicated by a red arrow is associated with the ion beam modification of the polymer.

The set-up of plasma treatment on catheter tubing as shown in Figure 1 allows the whole tubes immerse in plasma resulting in ion bombardment on both surfaces of the tubes. To compare the extent of the PIII treatment on the inner and outer surfaces of the tube, both surfaces were analyzed using micro ATR-FTIR. The FTIR spectra showed that the C=C absorbance (around 1620-1680 cm^{-1}) occurred strongly on the outer surface of the tube (Figure 4A) while a moderate change was observed on the inner surface (Figure 4B). Since this

absorption is associated with ion bombardment-induced carbonization [40] the results indicate both surfaces received treatment with a somewhat heavier ion bombardment occurring on the outer surface than on the inner surface. The level of ion bombardment is proportional to the number of broken bonds (radicals) and is therefore correlated with the number of covalent binding sites [45]. Since the inner surface of the catheter is the one that is exposed to body fluids and has a higher risk of biofilm formation, we focus of the research on investigating melimine attachment on the inner surface and the subsequent bacterial adhesion. It was assumed that positive results for the inner surface imply positive results for the outer surface while not separately investigated, in view of the positive for flat surfaces.

XPS analysis on the inner surface of catheter tubes (Table 4) shows that there are changes in the atomic composition on the inner tube surface after the PIII treatment, supporting the finding from FTIR. In particular, on the PIII treated surface, oxygen increased while chlorine decreased, compared to the untreated surface. No significant level of nitrogen was detected on the PIII treated inner surface. After melimine attachment, the atomic percent of nitrogen increased on both untreated and PIII treated surfaces (Figure 5), indicating the attachment of melimine. SDS treatment at 70ºC removed 47% of nitrogen from the untreated surface while only 7% of the nitrogen was removed from the PIII treated surface. Compared to the SDS treatment on flat PVC described above, this SDS treatment on the tube surface was more stringent because of the higher temperature. The effect of SDS in removing physically adsorbed molecules is well documented and is a measure of covalency [34]. This indicates that the PIII treatment conditions used in our experiment are sufficient to create radicals for covalent attachment of melimine on the inner surface of the tube.

Table 4: Comparison of atomic percent of elements detected by XPS analysis on untreated PVC tube (PVC), PIII treated PVC tube (PIII), after immobilisation of melimine on these surfaces before SDS wash (PVC+Mel and PIII+Mel) and after SDS wash (PVC+Mel+SDS and PIII+Mel+SDS).

	PVC	PVC+Mel	PVC+Mel+SDS	PIII	PIII+Mel	PIII+Mel+SDS
C (%)	84.5 ± 0.9	79.4 ± 0.4	79.2 ± 0.1	85.9 ± 0.0	82.6 ± 0.4	82.6 ± 0.1
Cl (%)	4.6 ± 0.9	5.3 ± 0.5	8.4 ± 0.0	0.6 ± 0.1	0.9 ± 0.0	0.6 ± 0.0
O (%)	10.7 ± 0.0	13.2 ± 1.1	11.0 ± 0.1	13.4 ± 0.0	14.0 ± 0.0	14.4 ± 0.1
N (%)	0.2 ± 0.1	2.2 ± 0.2	1.3 ± 0.2	0.2 ± 0.0	2.5 ± 0.4	2.4 ± 0.0

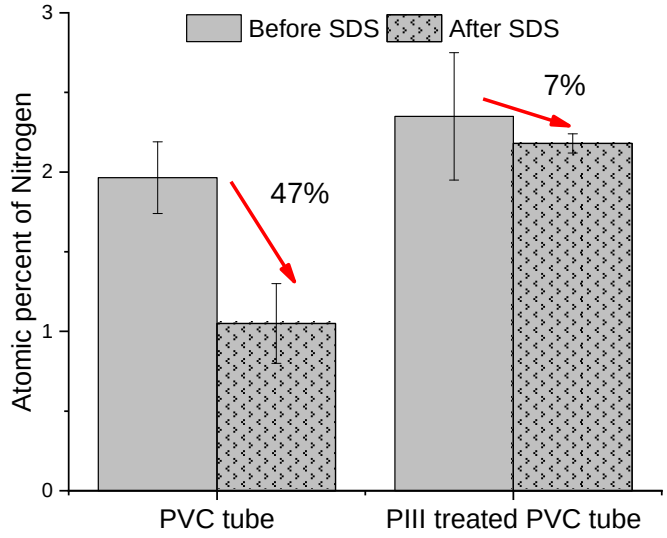


Figure 5: XPS analysis comparing atomic percent of melimine-associated nitrogen on the inner surface of catheter tubes before and after SDS washing. The percentage losses of nitrogen after SDS wash are denoted by the arrows in the graph.

To investigate the effect of melimine attachment on the catheter internal surfaces to bacterial adhesion, we incubated the catheters with *S. aureus*. SEM images taken on different locations on the inner surface of the catheters were used for qualitative and quantitative comparison. Bacteria adhered in high density and formed clusters on the inner surface of untreated catheter (Figure 6A and 6B). There was a variation of bacterial density from one location to another that required up to 9 images to be used for quantification. Compared to the untreated tubes, PIII treated tubes had lower bacterial density. Untreated tubes immobilized with melimine significantly reduces bacteria adhesion on some areas but not all (Figure 6B). On the PIII treated catheter after melimine attachment (Figure 6D), very few bacteria were found after viewing the entire surface. Compared to the untreated catheter surface, the PIII treated catheter surface with melimine covalent attachment reduces bacterial adhesion by more than 98% of the coverage fraction. The average of the bacterial coverage taken on different locations on each sample were averaged and are shown in Figure 7. The wrinkles observed on the PIII treated tube surface is due to the interaction of the modified surface with chemicals

during the fixing and dehydration stages of SEM sample preparation. We did not observe those wrinkles on PIII treated surfaces incubated with melimine only.

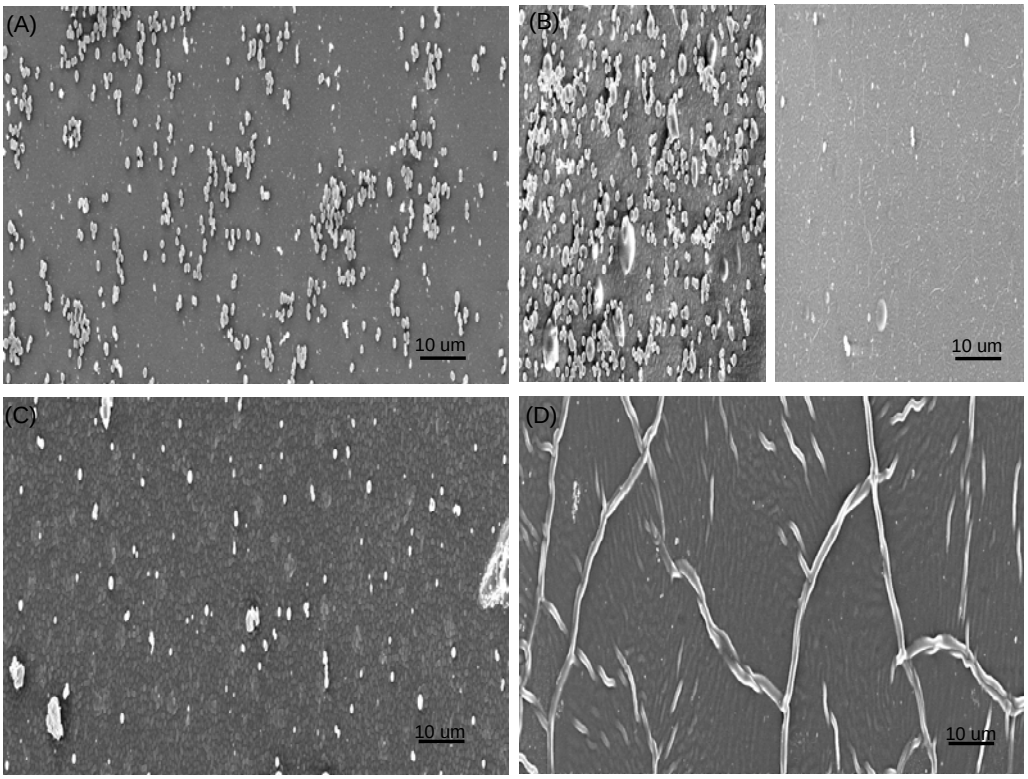


Figure 6: SEM images showing the level of bacterial adhesion of *S. aureus* on the inner surface of catheter tubes with different treatments. (A) Untreated tube, (B) Combined images of two different locations on untreated tube incubated with melimine showing a variation of bacteria adhesion on this surface, (C) PIII treated tube, (D) PIII treated tube incubated with melimine. All images were taken with the same magnification and have the same scale bar as shown in (A).

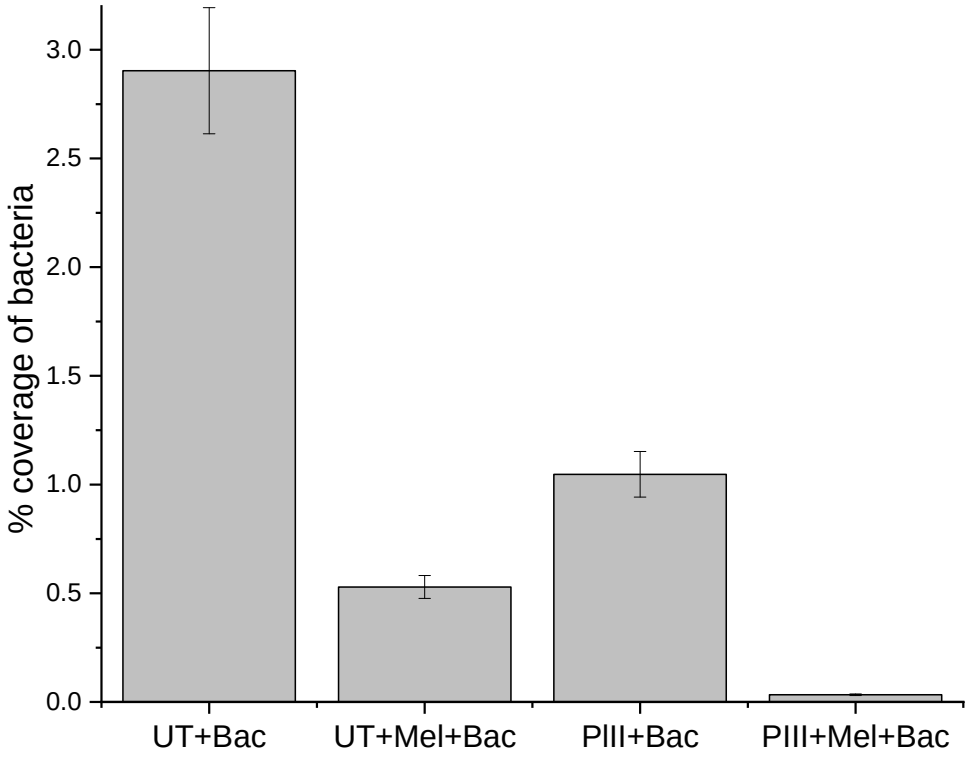


Figure 7: Comparison of *S.aureus* bacterial adhesion (Bac) on untreated and PIII treated inner surface of catheters before and after melimine attachment (Mel), based on bacterial coverage areal density on SEM images.

4. Discussion

The PIII process uses energetic nitrogen ions accelerated under a high bias to transfer their energy to polymer chains from collisions to create dangling bonds which appear not only on the surface but also in the subsurface regions of the material [46]. This process has been used to modify various carbon backbone polymers for biomolecule attachment [47]. For non-polymeric surfaces such as glass, stainless steel and titanium, a thin layer of plasma deposition from a carbon containing precursor gas can be deposited on the surface and provide radicals for biomolecule binding. The PIIID process combines the plasma deposition and ion implantation by mixing acetylene and nitrogen to enhance the radical density. This study demonstrates that the PIII technique modifies the surface of the respective materials and provides active sites for the covalent attachment of melamine while retaining their strong antimicrobial activity. Most significantly, we have shown that the PIII method can be used to treat the internal surfaces of small diameter tubing. The amount of melimine bound via PIII or PIIID surfaces can be compared to our previous strategies of binding melimine to glass

using 4-azidobenzoic acid (ABA) or 4-fluoro-3-nitrophenyl azide (FNA), and to poly-hydroxyethylmethacrylate (pHEMA) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). After controlling for %N on untreated surfaces, the %N on flat PVC activated with PIII was approximately 4.7%, on glass surfaces activated by PIIID was 3.9% and on PVC catheter tubes was 2.3%. This compares favourably with 1.7% N on ABA-treated glass, 2.6% N on FNA-treated glass [22] and 1.5% N on EDC activated pHEMA [31]. The activity of covalently bound melimine is related to the concentration of melimine on the surface [22, 23, 25]. Thus, the greater %N on PIII or PIIID-activated surfaces (which is directly related to amount of melimine) indicates that these methods for covalently binding melimine are likely to result in improved antimicrobial activity.

The availability of a convenient apparatus such as the one described here for applying PIII treatment to tubing will facilitate the translation of the process to clinical applications. The advantages of the one-step dry plasma process for providing covalent binding sites on both internal and external surfaces of tubing are many compared to the more intensive, multistep wet chemistry methods for covalent linkage that may prove cumbersome. In a straightforward extension of the work using a 15 cm electrode, we were able to demonstrate the formation of plasma throughout the 8 fr-40cm PVC catheter, confirming PIII treatment of the entire internal surface is feasible. There are many other potential applications to implantable devices that employ tubes for conveying fluids, such as implantable ventricular assistance devices, drains for removing excess or unwanted fluids from the body and blood contacting devices such as dialysis equipment and heart-lung replacement devices. In a logical extension of the work, given that we have demonstrated a treatment for chemically inert surface of glass, it will be applicable to the antimicrobial treatment of materials with internal cavities such as ceramics and metals, that are widely used for forming bone replacement implants.

5. Conclusion

This work has shown that the PIII process is capable of functionalization of polymer surface to create active sites for covalently immobilization of melimine. The covalently attached melimine-coated surfaces show stronger antibacterial activity against *S. aureus* compared to the physisorbed melimine on untreated surfaces. We have demonstrated that the PIII treatment effectively modifies the internal as well as the external surfaces of polymer tubing and is

therefore suitable as a process for treating catheters. The inner surfaces of treated tubes, though receiving less ion bombardment compared to the external surfaces, still showed covalent bonds with melimine and significantly reduced bacterial adhesion. The PIII and PIIID treatments could be readily extended to the treatment of other implantable devices with internal surfaces such as porous bone scaffolds. The combination of the antimicrobial property of melimine with a simple immobilization technique are the strong advantages of our approach. The demonstration of effective treatment for the covalent linking of antimicrobial peptides to glass opens up the possibility of treating non-polymer surfaces, including metallic and all types of non-metallic bone replacement implants.

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Supporting Information

Additional infrared spectra comparing the plasticized PVC used in our experiments to unplasticized PVC spectrum from literature.

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