An antimicrobial impregnated urinary catheter that reduces mineral encrustation and prevents colonisation by multi-drug resistant organisms for up to 12 weeks

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Abstract

Two major complications of indwelling urinary catheterisation include infection and mineral encrustation of the catheter. Our antimicrobial urinary catheter (AUC) impregnated with rifampicin, triclosan, and sparfloxacin has demonstrated long-term protective activity against major uropathogens. This study aimed to firstly assess the ability of the AUC to resist mineral encrustation in the presence and absence of bacteria. Secondly, it aimed to investigate the AUC's anti-biofilm activity against multi-drug resistant organisms. There was no difference in surface roughness between AUC and control segments. In a static and a perfusion model, phosphate deposition was significantly reduced on AUCs challenged with *P. mirabilis*. Furthermore, none of the AUCs blocked during the 28 day test period, unlike controls. The AUC prevented colonisation by methicillin-resistant *Staphylococcus aureus*, methicillin-resistant *Staphylococcus epidermidis*, extended-spectrum beta-lactamase producing *E. coli*, and carbapenemase-producing *E. coli* for 12 consecutive weekly challenges. All three drugs impregnated into the catheter continued to exert protective activity throughout 12 weeks of constant perfusion. The drugs appear to migrate into the crystalline biofilm to continually protect against bacteria not it direct contact with the catheter surface. In conclusion, the AUC reduces mineral encrustation and may increase time to blockage in the presence of *P. mirabilis*, and does not predispose to mineral deposition under other conditions. It also offers 12 weeks of protection against multi-drug resistant bacteria.

Keywords: Urinary catheters; anti-infective agents; catheter obstruction; catheter-related infection; catheters, indwelling; microscopy, atomic force; struvite

1 Introduction:

Indwelling urinary catheters rapidly become colonised by bacteria with almost 100% of catheters colonised after 28 days[1]. It is important to distinguish between bacterial colonization of the catheter and catheter-associated urinary tract infection (CAUTI). Bacteria

in the urine without symptoms, asymptomatic bacteriuria, is not usually an indication for antibiotic treatment, but users of indwelling urethral urinary catheters are at risk of CAUTI which involves invasion of the bladder by bacteria and presence of symptoms such as bladder pain, fever, and frequency of passing urine. Patients who depend, often for life, on long term catheters (in place for 28 days or greater), are at an increased risk of repeated infections, repeated courses of antibiotics and their associated side effects, and catheter blockage caused by mineral encrustation. These problems have been compounded by the increasing role of resistant bacteria. CAUTI and its treatment have profound effects on quality of life, mental health, and associated costs.

All urinary catheter types are susceptible to mineral encrustation, a complication resulting from crystal deposits on the catheter surface[1]. Some bacteria produce urease, an enzyme which hydrolyses urea from the urine into ammonia and carbon dioxide. The production of ammonia results in an alkaline urinary pH, causing precipitation of urinary minerals such as calcium, magnesium, and phosphate and their deposition on the catheter surfaces[2]. Crystal deposits can occlude the catheter lumen or drainage eyelets causing reflux of urine to the kidneys and/or bypass of urine around the outside of the catheter[2, 3]. Some catheter users experience blockages as frequently as every seven to 10 days[4].

Urease-producing uropathogens include *Proteus* spp., *Morganella morganii*, *Staphylococcus aureus,* and *Staphylococcus saprophyticus*[2]. *P. mirabilis* is most frequently cited as the cause of encrustation and catheter blockage due to the ability of its urease to hydrolyse urea six-25 times faster than ureases from other species[5]. *P. mirabilis* forms a crystalline biofilm in which the biofilm bacteria grow amongst ammonium magnesium phosphate (struvite) and calcium phosphate crystals[6]. The polysaccharide matrix formed by *P. mirabilis* biofilm initiaties crystallisation by binding calcium and magnesium ions and is thus able to maintain crystallisation and encrustation of the catheter[7].

The clinical, personal, and financial burden of CAUTI and subsequent blockages is vast and yet no commercial technology exists to prevent CAUTI in patients who require long-term indwelling urinary catheters (more than 28 days) to manage their bladder. Costs and

morbidity are further increased if CAUTI is caused by a multi-drug resistant organism[8]. A multi-centre randomised controlled trial of a commercially available silver-alloy catheter and a previously available nitrofurazone-coated catheter for use under 28 days did not significantly reduce clinical episodes of CAUTI[9]. A commercially available 0.3% triclosan balloon inflation fluid intended to reduce mineral encrustation and blockage due to the activity of triclosan against *Proteus mirabilis*[10], has recently become available, but this technology is suitable only for short-term use. There are no anti-infective or anti-encrustation catheters for long-term use.

An antimicrobial urinary catheter (AUC) with protective activity for the lifetime of a long-term urinary catheter (up to 12 weeks) has been developed in an effort to provide long-term protection [11]. The drug release profile and drug distribution in silicone urinary catheters impregnated with rifampicin, triclosan, and sparfloxacin have been previously characterised [11, 12]. Importantly, these initial assessments demonstrated that antimicrobial impregnation of silicone urinary catheters did not adversely affect the mechanical properties of the catheter nor inflation and deflation of the balloon. Time-of-flight secondary ion mass spectrometry studies indicated that the drug molecules are impregnated throughout the silicone material including the intraluminal surface, extraluminal surface, and balloon. The AUC has demonstrated between seven and 12 weeks of protection against bacterial colonisation in a clinically predictive in vitro flow model [11]. The AUC aims to avoid the development of resistance by using antimicrobials locally and not systemically, and by optimising its design in line with the Dual Drug Principle. This states that the use of two antibiotics of two different classes prevents emergence of resistance, as the likelihood of bacteria developing two simultaneous mutations is greatly reduced[13].

This study further aims to understand firstly if the antimicrobial impregnation process affects the surface roughness of the catheter, which if increased may predispose the AUC to increased crystallisation and encrustation. Surface roughness was quantified by atomic force microscopy, and as a consequence of surface changes, phosphate deposition on the catheter surface was measured in the absence and presence of urease-producing bacteria.

The test bacteria included *P. mirabilis* as it represents a robust crystalline biofilm former with an efficient urease enzyme. *S. saprophyticus* also produces a urease enzyme but one that is less efficient than that of *P. mirabilis*, and served as an example of an uropathogen with moderate urease activity. *E. coli* is the most common causative organism of CAUTI but does not produce urease and was the urease-negative control. Secondly, this study investigated the efficacy of the AUC against multi-drug resistant (MDR) gram-positive and gram-negative organisms including methicillin-resistant *S. aureus* (MRSA) and carbapenemase-producing *E. coli,* which are resistant to the newer antimicrobials developed for use against such bacteria.

2 Materials and Methods

2.1 Antimicrobial impregnation of silicone urinary catheters

The method of antimicrobial impregnation of silicone materials has been previously described [14, 15]. Briefly, 1.0% w/v triclosan (Irgasan, BASF Ludwigshafen, Germany), 0.2% w/v rifampicin (Sigma), and 1.0% w/v sparfloxacin (Sigma-Aldrich) were dissolved in chloroform (Analytical reagent grade, Fisher Scientific, Loughborough, United Kingdom). Silicone catheters (16 Ch BARDIA® AQUAFIL®, Bard Medical), with any plastic components removed, were immersed in the antimicrobial-chloroform solution for one hour, after which they were removed and the chloroform was evaporated under constant air flow for at least 12 hours. The catheters were rinsed in absolute ethanol (Fisher Scientific) to remove any surface aggregates. Catheters or 1.0 cm longitudinally cut segments were sterilized by autoclaving at 121°C for 15 minutes.

2.2 Isolation and characterization of uropathogens for encrustation studies

F2627 *S. saprophyticus*, F2647 *E. coli*, and F2629 *P. mirabilis* were isolated from indwelling urinary catheters and ureteral stents collected at Nottingham University Hospitals

NHS Trust, Nottingham, United Kingdom. General microbiological identification, including urease production, was performed and identification was confirmed by API (BioMerieux, Marcy l'Etoile, France). Antimicrobial susceptibility testing was carried out according to EUCAST guidelines[16].

2.3 Isolation and characterisation of MDR uropathogens for efficacy studies

Bacteria of interest were isolated from clinically relevant samples, particularly from the lumens of urinary catheters and ureteral stents collected at Nottingham University Hospitals NHS Trust, Nottingham UK. A New Delhi metallo-beta-lactamase (NDM-1) producing *E. coli* isolated from a mid-stream urine specimen was donated by NHS Dumfries and Galloway. General microbiological identification was performed and identities were verified by API (Biomérieux).

Antimicrobial susceptibility testing was carried out according to the EUCAST guidelines[16, 17]. Methicillin resistance by staphylococci was determined by disc diffusion using a cefoxitin disc (30 µg) on Mueller-Hinton agar (MHA) (Oxoid). *E. coli* were screened for extended-spectrum beta-lactmase (ESBL) production by disc diffusion using cefpodoxime (10 µg), ceftazidime (10 µg), ceftriaxone (5 µg) discs (Oxoid Ltd., Basingstoke, United Kingdom) Confirmatory testing was carried out using the gradient test method using both cefotaxime/cefotaxime + clavulanic acid and ceftazidime/ceftazidime + clavulanic acid strips (Etest, Biomérieux) on MHA. Carbapenemase production was detected initially by disc diffusion to meropenem (10 µg). Specific metallo-beta-lactamase (MBL) activity was detected using the Total Metallo-beta-lactamase Confirm Kit: MBLs (Rosco Diagnostica, Taastrup, Denmark) and the procedure and interpretation of results were carried out according to the manufacturer's instructions. The precise MBL was verified by the Antimicrobial Resistance and Healthcare Associated Infections Reference Unit (London, United Kingdom)[18].

The bacterial test panel included F4001 methicillin-resistant *S. epidermidis* (MRSE), F4142 methicillin-resistant *S. aureus* (MRSA), F3991 methicillin-susceptible *S. aureus* (MSSA), F3802 NDM-1 producing *E. coli*, and F3986 ESBL- producing *E. coli.*

2.4 Bacterial attachment assay

A bacterial attachment assay was used to verify the ability of the test bacteria to attach and remain attached to silicone that would be used in the subsequent models[19, 20]. Bacterial attachment to 1.0 cm longitudinally cut silicone catheter segments was quantified by detaching bacteria into surrounding 1.0 mL PBS by unheated sonication for five mins at 50 kHz (Precision Ultrasonic Cleaner DP201-00, Ultrwave Ltd., Cardiff, UK). Previous validation in our laboratory showed that sonication for five minutes achieved the best balance of detaching bacteria without killing bacteria[21]. Further details can be found in Supplementary Methods 1.

2.5 Encrustation studies

2.5.1 Atomic force microscopy

Artificial urine (AU) was prepared according to the formula by Griffith et al[22] and adjusted to a pH of 6.1 (urinary pH ranges from 5.52-6.97 in adults [23]) before being filter sterilised.1.0 cm longitudinally cut segments in tripicate were added to the AU and incubated at 37°C with rocking for either one hour, one week, or two weeks. Sterile, fresh AUCs and silicone segments without soaking were included as controls. A chloroform-only control was also used, as its swelling action on silicone could have altered the surface in the absence of antimicrobial molecules.

For imaging by AFM, a small section of the 1.0 cm longitudinally cut catheter segment was cut away and placed on a microscope glass slide with the lumen side facing up. The slide was pre-coated with Araldite glue and dried. Three separate samples from each experimental group were imaged using a Bruker ICON FastSCan Bio microscope in

Peakforce in air mode using Tap150A tips with an 8nm radius. Image preparation and surface roughness analysis were carried out using Nanoscope Analysis v1.5 software (Bruker Corporation, Massachusetts, USA).

2.5.2 Investigation of mineral encrustation prevention – static model

Phosphate precipitation was measured by a colourimetric assay based on the method by Mahadevaiah et al[24]. Disodium hydrogen phosphate in increasing concentrations was added to 0.5 mL of 5.528 $x10^{-3}$ M ammonium molybdate (Hopkin and Williams, Essex, UK) and 3.0 mL 0.25 N sulphuric acid (Fisher Scientific) to create the calibration plot. 1.0 mL of 2.0833$x10^{-3}$ M sodium sulphide (Scientific Laboratory Supplies, Nottingham UK) was added and the solution was incubated at room temperature (20-25°C) for 20 minutes. Absorbance was measured against milliQ water by spectrophotometer (Jenway UV/Vis, Staffordshire, UK) at 715 nm.

F2629 *P. mirabilis*, F2627 *S. saprophyticus*, and F2647 *E. coli* were subcultured on blood agar overnight at 37°C. A loopful of the fresh culture in 20.0 mL tryptone soya broth (TSB, Oxoid) was incubated for four hours at 37°C with shaking at 200 rpm (Orbital Incubator 1500, Stuart, Staffordshire UK). The bacterial suspension was adjusted to A_{490} 0.6 – 0.7 and 5.0 µL was added to 50.0 mL filter-sterilised AU (adjusted to pH of 6.1) in triplicate. An additional 50.0 mL filter-sterilised AU adjusted to a pH of 6.1, 7.1, or 8.3 using 1 M sodium hydroxide (Fisher Scientific) was also prepared.

1.0 mL of the bacterial inoculated AU or the pH-adjusted AU was added to 1.5 mL microcentrifuge tubes in triplicate. At 0, 4, 24, 48 and 72 hours the phosphate precipitate was harvested by centrifugation (3000 rpm for five minutes) (Centaur 2 Centrifuge, MSE, Sussex, UK), and resuspended in 1.0 mL sterile milliQ water. The 1.0 mL suspension was added to the reagents described previously and spectophotometrically measured. The assay was repeated in which 1.0 mL of bacterial inoculated AU or pH-adjusted AU was added to 1.0 cm longitudinally cut AUC segments or silicone segments and incubated at 37°C with

shaking at 200 rpm for 0, 4, 24, 48, or 72 hours. For each time point the phosphate attached to three catheter segments was harvested by sonication in 1.0 mL sterile milliQ water for five minutes. The sonicate was transferred to the reagents described previously and measured by spectrophotometer.

2.5.3 Investigation of mineral encrustation prevention – In vitro flow model

A clinically predictive in vitro flow model has been previously described in the literature [20] [19] [25]. AU was perfused through silicone catheters and AUCs to determine the time to blockage and phosphate deposition on the catheter lumens in the presence and absence of *P. mirabilis.* Briefly, the AUCs in triplicate (with balloon and drainage ports removed) with an all-silicone control were aseptically inserted into a multi-chamber water jacket. The water jacket was maintained at 37°C by a heating circuit. AU was pumped through the catheters at a rate of 30 mL/hour [26] using a peristaltic pump (Watson-Marlow Ltd., Falmouth, UK) which regulated flow (Figure 1). The waste AU was collected in a 30 L waste collection container. Control and experimental catheters were challenged weekly with an early log phase culture of 10^5 CFU/mL inoculum. AU was perfused through the catheters for one hour to prime the tubing and form a conditioning film. 2.0mL of the inoculum was inserted into the catheter tubing and was retained in the catheter tubing for one hour to allow bacterial attachment by clamping (straight-jaw surgical clamps) tubing 2 and tubing 4 in close proximity to the catheter tubing. Flow was then restarted. This was carried out weekly for each challenge.

Bacterial growth was monitored for each control and AUC after inoculation and then daily by disconnecting the outlet tubing and collecting the effluent into a sterile bijou bottle (Sterilin). The collected effluent and its dilutions were spread onto cysteine-lactose-electrolyte-deficient agar (Oxoid) for *P. mirabilis* and onto blood agar for the other isolates. The plates were incubated overnight and colonies were enumerated.

The in vitro challenge model ran for 28 days or until blockage of the catheter, which was determined by leaking proximal to the test catheter and subsequent examination by microscope of the catheter lumen for occlusion. One 1.0 cm long segment of the catheter tubing was cut away from an area within the water jacket using a sterile scalpel, placed in 2.0 mL of cold acetone, and refrigerated for scanning electron microscopy (SEM). Another was cut away and placed in a clean, dry glass bottle for x-ray photoelectron spectroscopy (XPS).

The remaining section of the catheter tubing was dried at 40°C for 24 hours. After drying, the catheter tubing was filled with approximately 1.0 mL of milliQ water and the ends were clamped. The clamped tubing was sonicated for five minutes at 50 kHz. The sonicate was drained into a sterile bijou bottle and was added to the colourimetric reagents described above and measured spectrophotometrically at 715 nm. The minimum level of detection was 2.2 ppm.

2.5.4 Scanning electron microscopy

Fixed 1.0 cm catheter segments were cut longitudinally using a sterile scalpel to expose the inner lumen. The lumen samples were dehydrated by tetramethylsilane (Sigma-Aldrich) [27] and fixed onto specimen stubs (12.5 mm diameter, Agar Scientific, Stansted, UK). The samples were gold sputter-coated for 300 seconds and imaged using a Jeol JSM-6060 scanning electron microscope (JEOL Ltd., Tokyo, Japan). The accelerating voltage was 30 kV and working distance was 10.0mm at x65 magnification.

2.6 Efficacy studies

2.6.1 In vitro flow model

The same in vitro flow model as described previously was employed to determine the ability of the AUC to resist bacterial colonisation. 20% aqueous TSB was used as the perfusion medium instead of AU as it has similar peptide and amino acid content and a

similar pH (6.9 at 24.4°C) at this concentration but is more convenient to produce in significant quantities. AUCs in triplicate and a control catheter were challenged by each microorganism weekly as described previously. The in vitro flow model ran until the end of 12 weeks or until the AUCs became colonised, which was determined by counting colonies in the effluent from each catheter daily. Success in the in vitro flow model, eradication of the attached microorganisms, was determined as CFU/mL = 0 of the test organism in the effluent at the end of the weekly challenge period.

2.6.2 High-performance liquid chromatography

AUCs were removed from the in vitro flow challenge model at failure or at the end of 12 weeks and kept at -20°C until drug extraction. Freshly impregnated unperfused catheters were also processed. Drug was extracted from three 1.0 cm long catheter segments in chloroform. The drug extraction process was repeated three times for each segment to ensure complete drug extraction and the extracts in chloroform per sample were pooled together. The chloroform was evaporated off and drug residues were stored at -20°C until reconstitution with methanol. HPLC analysis was performed by an Agilent 1100 HPLC machine with a variable wavelength UV detector (Agilent Technologies, Berkshire, UK) (Supplementary Method 2). Standards of all three drugs were prepared in methanol. All experiments were carried out in triplicate and the calibration curves demonstrated good linearity with R^2 values of >0.990 for all curves.

2.6.3 XPS Analysis

The AUCs and control segments perfused with AU and challenged with *P. mirabilis* were further assessed for drug content at the exposed surface. Catheter segments exposed to *P. mirabilis* were chosen due to the robust nature of the *P. mirabilis* crystalline biofilm which may form a thick mineral layer on the catheter surface. If there are minerals, a proteinaceous conditioning film, or biofilm on the catheter surface it is important to

understand if the antimicrobials can migrate through the surface deposits to exert their antmicrobial effect. Catheters were stored in clean glass vials until processing by a Kratos AXIS ULTRA instrument with a monochromated Al kα X-ray source (1486.6eV). The 1.0 cm catheter segments were cut longitudinally using a clean, sterile scalpel. Samples were mounted lumen side-up on a Kratos sample bar with double-sided tape (Sellotape). The sample bar was inserted into the airlock and the pressure was pumped to approximately 3 x 10^{-7} Torr overnight. It was transferred to the analysis chamber where the pressure remained at 5 x 10^{-9} Torr or less. Two areas of approximately 700 x 300 µm from the catheter lumens were analysed with low resolution wide scan at pass energy 80 eV and high resolution spectra at pass energy 20 eV on relevant energy ranges for the elements detected. The data were collected using Kratos VISIONII software and processed with CASAXPS software (version 2.3.17) with Kratos relative sensitivity factors.

2.7 Statistical analysis

Graphing and statistical analysis were carried out using GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla California, USA). Data analysed using unpaired multiple comparisons t-tests were corrected for using the Holm-Sidak method and $p<0.05$ was considered significant.

3 Results

3.1 Bacterial attachment screening assay

The three encrustation strains and five efficacy test bacteria were able to attach and maintain attachment to silicone urinary catheters over 72 hours (data not shown).

3.2 Encrustation studies

3.2.1 Atomic force microscopy

The surface of a silicone, non-soaked catheter appeared to be composed of nano-sized peaks and spikes (see Figure 2a for examples of images generated by AFM analysis of silicone controls and AUC segments). The spiky surface is apparent after soaking at all three time points and remains consistent. Of interest, the appearance of the AUC segments without soaking compared to the silicone catheters has a different appearance despite mean surface roughness values as represented by the root mean square roughness (R_q) values of 34.77 ± 5.95 and 56.6 ±14.09 respectively, which were not significantly different across groups according to one-way ANOVA of p=0.0806 (Figure 2b). Despite having non - significantly different R_q values to the silicone segments, the surface of the non-soaked AUC segments appears to have less numerous sharp spikes and instead fewer spikes, but wider, flatter areas. The same can be said of the silicone and AUC segments soaked for one hour in which there is no significant difference between group means, but the topography of the AUC 1 hr soaked segments is composed of more plateaus and fewer spikes compared to the silicone 1 hr soaked segments.

An important limitation of this method is that of the 1.0 cm longitudinally cut catheter segments, only a 2.0 μm x 2.0 μm area is measured and quantified so it is possible that chance would allow for rougher or smoother areas to be sampled, which may be responsible for the large standard deviation for the AUC – 1 wk group. This outlying group also does not correspond to the mean values of AUC – 1 hr and AUC – 2 wks, which were not significantly different from the Control (No soaking) mean, and in fact it would be expected that those segments soaked for two weeks would have a greater surface roughness than those soaked for one week. The R_q values for the all-silicone segments soaked for one week (Plain – 1 wk) were consistent with the control and all-silicone segments soaked for one hour and soaked for two weeks.

3.2.2 Static model of encrustation

The mean of the optical density values was interpolated from the calibration plot to give the amount of phosphate precipitated from the AU adjusted to a pH of 6.1, 7.1, or 8.3. Figure 3a shows that the method has the sensitivity to detect differences in phosphate precipitation under different conditions. At 48 hours 1347.0 ± 76.33 mg/L, 697.1± 78.81 mg/L and 359.1± 201.9 mg/L of phosphate were precipitated from AU adjusted to pH of 8.3, 7.1, and 6.1 respectively. Phosphate precipitation in the presence of the three bacteria appeared to be influenced by the activity of the urease enzyme and its ability to moderate the urinary pH as expected (Figure 3b).

Significantly less phosphate was attached to the AUC segments incubated with *P. mirabilis* at 48 hours (p=0.004, unpaired t-test), 72 hours (p=0.007), and 96 hours (p=0.011) compared to the control catheter segments (Figure 4). Phosphate attached to the control and AUCs was not significantly different from those catheter segments incubated in AU with *S. saprophyticus*, *E. coli* or AU adjusted to the three different pH values. This suggests that the antimicrobial impregnation process reduces mineral deposition on the catheter surface in the presence of *P. mirabilis*.

3.2.3 In vitro flow model of encrustation

All control catheters inoculated with *P. mirabilis* blocked before the end of the 28 days test period. The average time until blockage was 21.7 days (range 16-26 days). None of the AUCs blocked during this time period. The corresponding AUC was removed from the model for analysis at the time of control catheter blockage. The amount of phosphate attached to the lumens of the AUCs perfused with AU inoculated with *P. mirabilis* was significantly less (p=0.0197, paired t-test) than the phosphate attached to the control catheters. These results correspond to the findings of the static model, in that the AUC is able to reduce phosphate attachment in the presence of *P. mirabilis*.

None of the AUCs or silicone controls perfused with AU only (without *P. mirabilis*) blocked within the 28 day test period. In the absence of bacteria, there is no significant difference (p=0.599, paired t-test) between the amount of phosphate attached to the AUC and silicone catheters after being exposed to flow conditions for 28 days which corresponds to the results of the static model of encrustation as there was no significant difference in phosphate deposition between the AUC and silicone control catheter segments in the absence of bacteria.

3.2.4 Scanning electron microscopy of catheters in the flow model

SEM images showed fewer deposits on AUC lumens compared to the matched silicone catheter lumens when inoculated with *P. mirabilis*. For example, the AUCs removed at 23 and 16 days when the silicone controls blocked, were virtually free of mineral deposits. The catheters removed at 26 days had more mineralisation on the catheter surface compared to the others removed earlier. However, the AUC had a single layer of widely spaced minerals, whereas the silicone matched control had much larger three-dimensional clusters of minerals (Figure 5). While SEM is not quantitative, it does provide visual indications of the reduction of mineral deposition of the AUC surfaces when inoculated with *P. mirabilis*.

3.3 Efficacy studies

3.3.1 In vitro flow challenge model

The AUC prevented colonisation by MSSA, MRSE, ESBL *E. coli* and NDM-1 *E. coli* (Figure 6a) for 12 weeks and prevented colonisation by MRSA for 10-12 weeks (Figure 6b). In the case of MRSA, at 11 weeks one catheter failed to kill 100% of bacteria during the challenge period, another failed at 12 weeks, and the third catheter successfully prevented colonisation for the entire 12-week experimental period. The control catheters were successfully colonised by the bacterial isolates each week indicating that the bacterial isolates were capable of attachment and colonisation throughout the test period.

3.3.2 HPLC

HPLC Drug content analysis of catheter segments before and after constant perfusion and bacterial challenge for 12-13 weeks showed that sparfloxacin and triclosan were readily detectable in the catheter segments. Rifampicin was detectable after perfusion in the majority of catheters, but the amount was often below the limit of quantification. A summary of the drug content of AUCs after 12-13 weeks of perfusion and bacterial challenge can be found in Table 1. The majority of each drug was eluted over 12-13 weeks so that some detectable drug remained, but the majority had been eluted. This data combined with that from the in vitro flow model suggests that the remaining amount is sufficient to offer protection, but that the antimicrobials are eluted at a rate such that they are exhausted at the end of catheter lifetime.

Table 1: Proportional drug content (w/w) and total drug content (mg) with interquartile range (IQR) of silicone catheters impregnated with rifampicin, sparfloxacin, and triclosan and after constant perfusion and weekly bacterial challenge for 12-13 weeks.

	Drug content at impregnation		**Drug content after 12-13 weeks perfusion**		**Drug eluted over period of perfusion**	
	Median proportional drug content (w/w) (IQR)	Total drug content (mg) (IQR)	Median proportional drug content (w/w) (IQR)	Total drug content (mg) (IQR)	Median proportional drug eluted	Median drug eluted (mg)
Rifampicin	0.080% (0.013%)	8.81 (5.08)	0.0046%* (0.0029%)	0.51* (0.33)	94.09%*	8.29*
Sparfloxacin	0.70% (0.16%)	90.83 (34.88)	0.20% (0.10%)	22.41 (11.21)	75.33%	68.42
Triclosan	1.08% (0.14%)	121.99 (40.9)	0.021% (0.023%)	2.33 (2.55)	98.09%	119.66

* indicates rifampicin samples below the limit of detection and quantification were not included in the calculation

3.3.3 XPS analysis

XPS is a surface-sensitive technique, detecting the uppermost 10 nm of the sample, therefore it is uniquely sensitive to thin film formation on a silicone surface. XPS is also sensitive to the light elements (C, N, and O) and exhibits chemical shifting of the detected peaks dependent on the chemical environment of these light atoms. From Table 2 the

principal detected elements are O, C, and Si, which are the detectable elements of silicone[28]. It appears that some of the surface analysed has a film thickness of less than 10 nm or that the biofilm/mineral encrustations are patchy and bare areas of silicone are detectable. As there was 50.0% and greater C in all samples, the extra carbon is a likely result of deposition of cellular and organic material. The O and Si were slightly less than 25% consistently and this is probably due to cells and mineral deposits that prevented some of the catheter surfaces from being detected. Phosphorous (P) and calcium (Ca) are markers of mineral encrustation and Ca is significantly reduced ($p=0.0281$, post-hoc (one-way ANOVA) multiple comparisons with Dunnett's correction) on the AUCs exposed to *P. mirabilis* compared to control catheters exposed to *P. mirabilis.* Although there was no statistically significant difference in P between groups ($p=0.1165$, one-way ANOVA), no P was detected on AUCs exposed to *P. mirabilis*. This reinforces the results of the other assays that the AUC reduces mineral encrustation.

Table 2: X-ray photoelectron spectroscopy. Mean and standard deviation of atomic % estimated from the photoelectron peak intensities composition of silicone (Plain) and antimicrobial-impregnated urinary catheter catheter (AUC) segments perfused with artificial urine alone (AU) or artificial urine inoculated with *Proteus mirabilis* (Proteus) for 28 days. C: Carbon, O: oxygen, Si: Silicon, Cl: chlorine, N: nitrogen, F: fluorine, P: phosphorous, Ca: calcium

	C 1s	**O 1s**	**Si 2p**	**Cl 2p**	**F 1s**	**N 1s**	**P 2p**	**Ca 2p**
AUC Proteus	56.4 ± 3.3	21.7 ± 2.3	17.2 ± 1.4	0.4 ± 0.3	1.1 ± 0.3	2.9 ± 0.2	0	0.05 ± 0.08
Plain Proteus	53.3 ± 1.3	24.1 ± 0.4	16.3 ± 6.7	0.7 ± 0.6	0	3.8 ± 3.7	0.6 ± 0.6	0.5 ± 0.3
AUC AU	53.5 ± 1.5	23.7 ± 1.9	19.2 ± 1.7	0.2 ± 0.2	1.00 ± 0.5	2.0 ± 0.6	0.1 ± 0.2	0.1 ± 0.08
Plain AU	52.3 ± 2.1	24.7 ± 0.6	21.5 ± 2.3	0.03 ± 0.06	0	1.0 ± 0.7	0.02 ± 0.03	0.1 ± 0.06

Fluorine (F), an elemental component of sparfloxacin, was detected only in the AUC segments supporting the view that the antimicrobials at the surface were detected by XPS. There is no F in the AU formula. The amount of N detected varied between 1.0-3.8 atomic % (Table 2). There are three possible contributions to the N 1s peak, namely; antibiotics (rifampicin and sparfloxacin), urea in the form of NH_2, and proteins. The dosing of the antibiotics into the silicones is relatively low so a very small N 1s contribution from the

antimicrobials is expected even for an unexposed catheter. The catheter spectra discussed herein are all for materials exposed to AU and, therefore, are likely to have some coverage of biofilm or encrustation, and most of the N 1s signal is likely to come from that. In all four samples, the N1s peak consists of a low binding energy at approximately 400 eV consistent with ureic acid and a higher binding energy at approximately 402 eV, which can be attributed to several possible forms of polymeric nitrogen[29]. The peak positions observed exclude the possibility of nitride and nitrate forms, which would be at lower and higher binding energies, respectively, than the observed peaks. It is difficult to accurately interpret the origin of the nitrogen species, however, as stated, they are consistent with the expected urea and proteins from cell growth on the catheter surface[30]. The amount of N can be taken as a rough guide to the severity of growth and should correlate with other assessment methods.

4 Discussion:

In this study, silicone urinary catheters impregnated with 0.080% w/w rifampicin, 0.704% w/w sparfloxacin, and 1.084% w/w triclosan were investigated for their surface roughness properties and propensity for mineral encrustation and ability to resist colonisation by MDR organisms. These studies determined that surface roughness was not affected by the antimicrobial impregnation process or by depletion of the antimicrobial molecules over time. When exposed to bacteria with increasing urease activity and artificial urine of three increasingly alkaline concentrations mineral encrustation (as measured by phosphate) did not significantly differ between silicone control and antimicrobial impregnated catheters. There was significantly less mineral deposition on the antimicrobial impregnated catheters inoculated with *P. mirabilis* compared to the control inoculated with *P. mirabilis* 24-72 hours after exposure to the bacterial-AU inoculum. Of interest, there was increased phosphate attachment universally to the AUCs in the presence and absence of bacteria at 0 and 4 hours compared to the controls. Before soaking and at 0 hours, the AUCs are considerably more hydrophilic (water contact angle of 79.95° ± 3.88°) than silicone controls (water contact angle of 90.68° ± 0.85) [12]. Hydophilic surfaces have a greater surface

energy which may serve to attract phosphate, however, within 24 hours phosphate attachment is reduced to less or no different from the controls. The anti-encrustation properties of the AUC in the presence of *P. mirabilis* were investigated in a flow model which demonstrated that the AUC prevented blockage and significantly reduced mineral encrustation. This was further examined by SEM.

The AUCs and controls were challenged weekly for 12-13 weeks with MDR *S. aureus* and *E. coli* in the in vitro flow challenge model and prevented colonisation by ESBL *E. coli*, NDM-1 *E. coli*, MSSA, and MRSE for 12-13 weeks and prevented colonisation by MRSA for 10-12 weeks. The mechanism of colonisation prevention was investigated by XPS and HPLC. XPS demonstrated the presence of fluorine atoms in conditioning films on AUCs and not on the control catheters suggesting sparfloxacin as the source. Nitrogen was present on the surfaces of all experimental and control catheters, but the shape of the peaks from AUCs was different to the control catheters suggesting rifampicin and sparfloxacin as a source of nitrogen contributing to the peak shape. HPLC demonstrated the presence of the antimicrobials in the AUCs after 12-13 weeks of perfusion in amounts that were greatly reduced compared to the amount initially impregnated. This is important to the design of the catheter as it demonstrates a balance of having enough drug molecules to protect the catheter, but also that is has allowed diffusion through the silicone to replace molecules that have been rinsed away by flow.

4.1 Encrustation studies

AFM studies demonstrated that neither antimicrobial impregnation nor soaking to deplete surface molecules resulted in significant differences in surface roughness values. This may be clinically relevant to mineral encrustation as an increase in surface roughness or depressions may increase the number of nuclei available to initiate the crystallisation process[31, 32]. This hypothesis is supported by Santin et al., in which they perfused hydrogel-coated ureteral stents with concentrated urine and commented that the hydrogel-

coating was degraded by the urine and that this '…[favoured] the formation of irregularities on the surface which may represent preferential sites for the massive deposition of organic matter for the nucleation of crystals'[33].

Therefore, the mineral encrustation/crystallisation potential of the AUC was investigated in a clinically predictive flow model using artificial urine, which is a consistent, reproducible medium unlike urine from donors, replicates the protein content of urine[34] and produces a conditioning film that replicates in vivo urological conditioning films[11, 35]. AU has also been shown to produce crystals similar to those found in human urine[36]. A spectrophotocolourimetric method was employed to quantify phosphate deposition on the catheter surfaces as phosphate is a key component of struvite crystals ($NH_4MgPO_4 \cdot 6H_20$), which are often the byproduct of urease - producing bacteria, amorphous phosphate crystals which are formed in alkaline urine, and of calcium phosphate, which is a key component of urinary stones[37]. The extent of mineral encrustation has been previously detected using atomic absorption spectroscopy[3, 38, 39]. However, a key disadvantage is that phosphate interferes with the detection of calcium[40], another common component of urinary crystals, and phosphate is difficult to detect as the wavelength required to excite phosphorous electrons is within the ultraviolet spectrum[41]. Therefore, atomic absorption spectroscopy typically measures magnesium and calcium, and calcium detection may be reduced in the presence of phosphate. A spectrophotocolourimetric method was advantageous as it is relatively simple and inexpensive so many samples can be analysed, and phosphate could be quantified.

The method relies on the formation of phosphomolybdate $(PO_4Mo_{12})^{3-}$, which creates a blue colour, the intensity of which can be measured[24] and correlated to phosphate concentration according to Beer-Lambert's law[42]. The method was validated for the purposes of these experiments by measuring phosphate precipitation in AU when in the presence of bacteria and pH-adjusted AU. The minimum limit of detection was 2.2 ppm, which although not as sensitive as atomic absorption spectroscopy, was able to detect significant differences in mineralisation, which are likely to be clinically relevant.

4.2 Efficacy studies

Previous Time-of-Flight Secondary Ion Mass Spectrometry studies of the AUC demonstrated the presence of the three antimicrobials distributed evenly throughout the silicone and that they are able to diffuse through it [11]. This appears to be consistent with the results found here in that antimicrobials are likely able to diffuse from the catheter into the conditioning film and they are able to replace molecules at the surface that are rinsed away by flow over 12 weeks, suggesting that the conditioning film becomes *de facto* the secondary antimicrobial surface. If the antimicrobials were unable to diffuse through the silicone to replace lost drug molecules, a consistent killing effect of the catheter over 12 weeks would not be observed. The catheters containing *P. mirabilis* were chosen for the XPS studies as *P. mirabilis* forms a robust crystalline biofilm which would increase the thickness of the surface deposits. This was considered a 'worst case scenario' of mineral deposition on the surface and would be a conservative indicator of the ability of the antimicrobial drug molecules to migrate through the surface accretions.

Success in the in vitro flow model was conservatively determined as bacterial eradication, but in vivo a significant reduction might be considered a success in certain clinical circumstances. 20% TSB was chosen as the perfusion medium as the constituent peptide content at this concentration was equivalent to that of urine and the medium is readily available in the large quantities needed. Furthermore, TSB does not readily form crystals on the catheter surfaces, which was important so that the AUCs did not block during the 12 week period of constant medium perfusion, as this assay was measuring bacterial attachment and not propensity for mineral encrustation. These studies contribute to the confidence in the ability to prevent colonisation as previous studies have tested other ESBL *E. coli* and MRSA strains, which also demonstrated 12 weeks of protection under flow conditions [11].

4.3 In vivo studies

All antimicrobials impregnated into the catheter have been used extensively clinically and therefore their safety profile is well-established. However, the in vivo safety of our antimicrobial-impregnation technology has been verified elsewhere. For example, segments of a continuous ambulatory peritoneal dialysis catheter impregnated using the same method but containing rifampicin, triclosan, and trimethoprim were implanted intraperitoneally in mice. At surgery there were only minimal inflammatory changes seen, attributable to the surgical implantation, and there were no differences in inflammatory reactions at 7 and 31 days between the control and antimicrobial-impregnated segments[20]. A hydrocephalus shunt and external ventricular drain impregnated with rifampicin and clindamycin by this method are in use clinically in 47 countries with no reports of adverse events [43, 44]. Importantly, a pilot tolerability and patient acceptability human clinical trial of the AUC reported here has recently been completed in 30 patients with results showing that the AUC is safe and acceptable to patients with no evidence of toxic or inflammatory reaction[45].

5 Conclusions

The impregnation process of silicone urinary catheters with three antimicrobials does not increase the surface roughness of the catheter surface. Studies of phosphate deposition on the catheter surfaces in an initial static model and then a perfusion model demonstrated that when inoculated with *P. mirabilis* phosphate deposition was significantly reduced on the AUCs. When exposed to other conditions such as incubation with AU adjusted to three pH values and when inoculated with *S. saprophyticus* and *E. coli*, there were no differences in phosphate deposition on the AUC catheters and segments. The AUC may increase the time to blockage in clinical use by reduction of mineral deposition caused by *P. mirabilis*.

To the authors' knowledge, this is the first antimicrobial catheter technology with 12 weeks of protective activity against carbapenemase – producing (NDM-1) *E. coli*. Investigations into its mechanism of action show that the antimicrobials are eluted over the

12 week period of use and that they are able to migrate into bacterial and non-bacterial conditioning films that form on the catheter surface to prevent bacterial colonisation. These results suggest that the AUC may prevent CAUTI including by MDR organisms and reduce mineral encrustation in long-term urinary catheter use. This would reduce antibiotic prescribing and associated side effects and avoid development of resistance [20], as well as the need for early catheter changes due to blocked catheters which have a significant impact on a patient's quality of life. In vivo studies have shown that the formulation used in the catheter is safe for human use.

Supplementary Material:

The following material is supplied as supporting information:

- Supplementary Method 1: Bacterial attachment assay method
- Supplementary Method 2: High-performance liquid chromatography of antimicrobial urinary catheter segments method
- Supplementary Table 1: Maintenance tryptone soya broth concentrations per test isolate used in the bacterial attachment assay

Acknowledgements:

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Data Availability:

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

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Figure Legends:

Figure 1: Diagram of the set-up of the in vitro flow model. Tubing is labelled numerically and apparatus are labelled alphabetically. Components include A. artificial urine or tryptone soya broth reservoir, B. peristaltic pump, C: multichannel water jacket, D: waste collection container. 1: reservoir tubing, 2: pump tubing, 3: urinary catheter (silicone or antimicrobial), 4: Outlet tubing

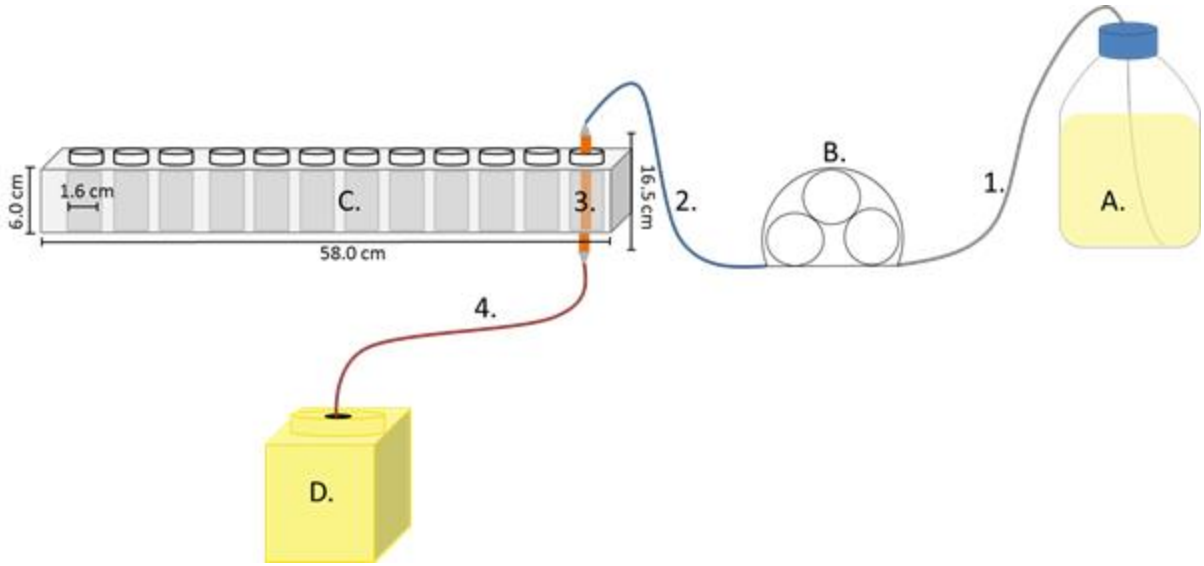


Figure 2. Atomic force microscopy results of silicone (plain) and antimicrobial-impregnated urinary catheter (AUC) 1.0 cm longitudinally cut segments soaked in artificial urine (AU) for 1 hour, 1 week, or 2 weeks with controls. a) AFM 3D height sensor images representative of each group. For a better view of topographic details, the vertical (Z) and lateral (X-Y) dimensions are not proportionally plotted. b) R_q values (nm) in triplicate with standard deviation.

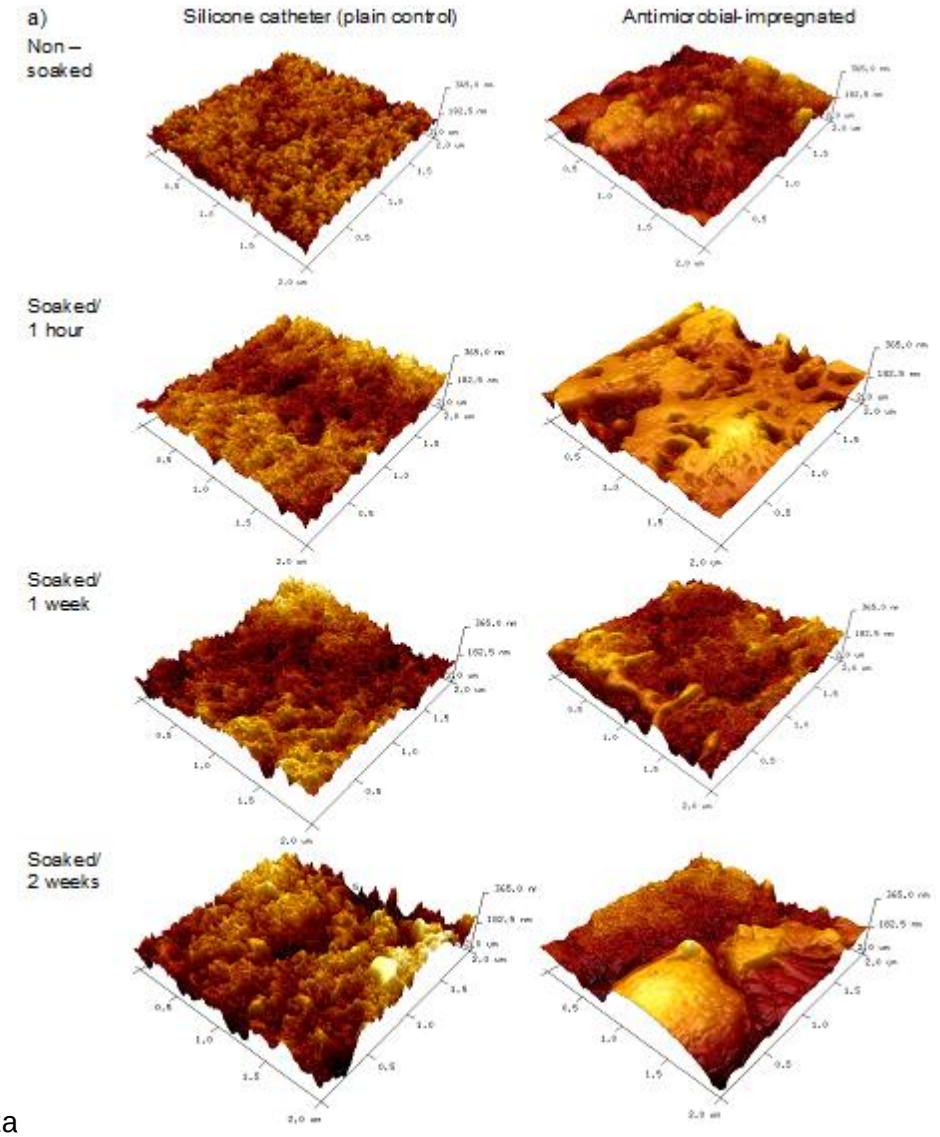


Fig 2a

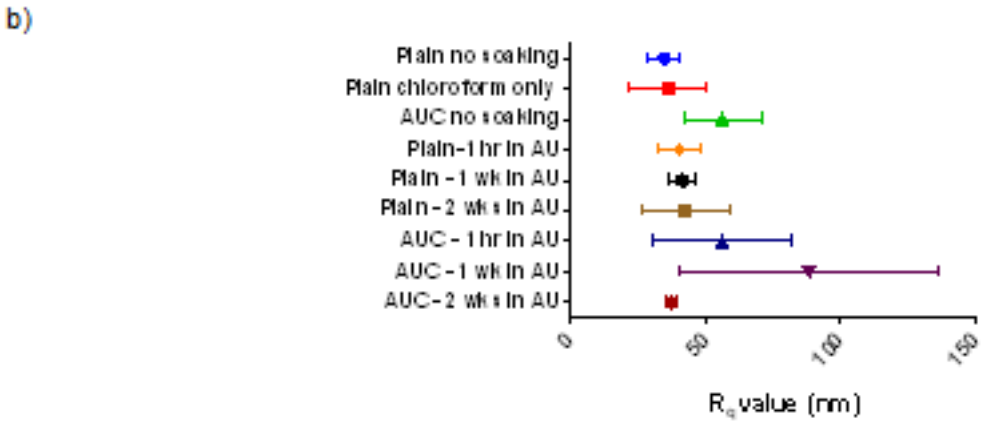


Fig 2b

Figure 3: Mean phosphate (complete line) (mg/L) and standard deviation in AU a) adjusted to varying pH values or b) inoculated with bacteria overlaid with pH of the AU (dotted line)

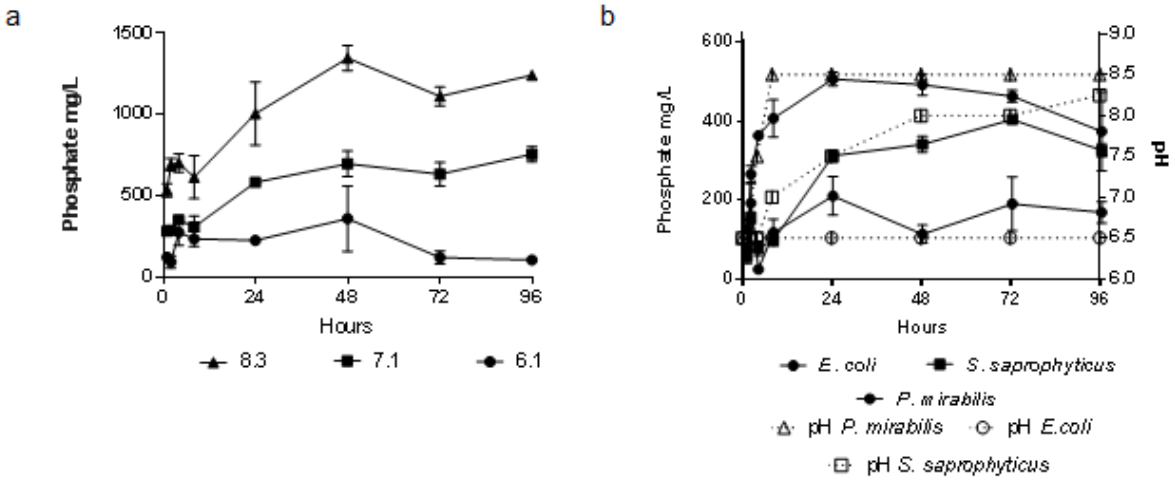


Figure 4: Phosphate (mg/L) attached to silicone and antimicrobial impregnated catheter (AUC) segments incubated statically with a) artificial urine (AU) inoculated bacteria or b) pH-adjusted artificial urine (AU).

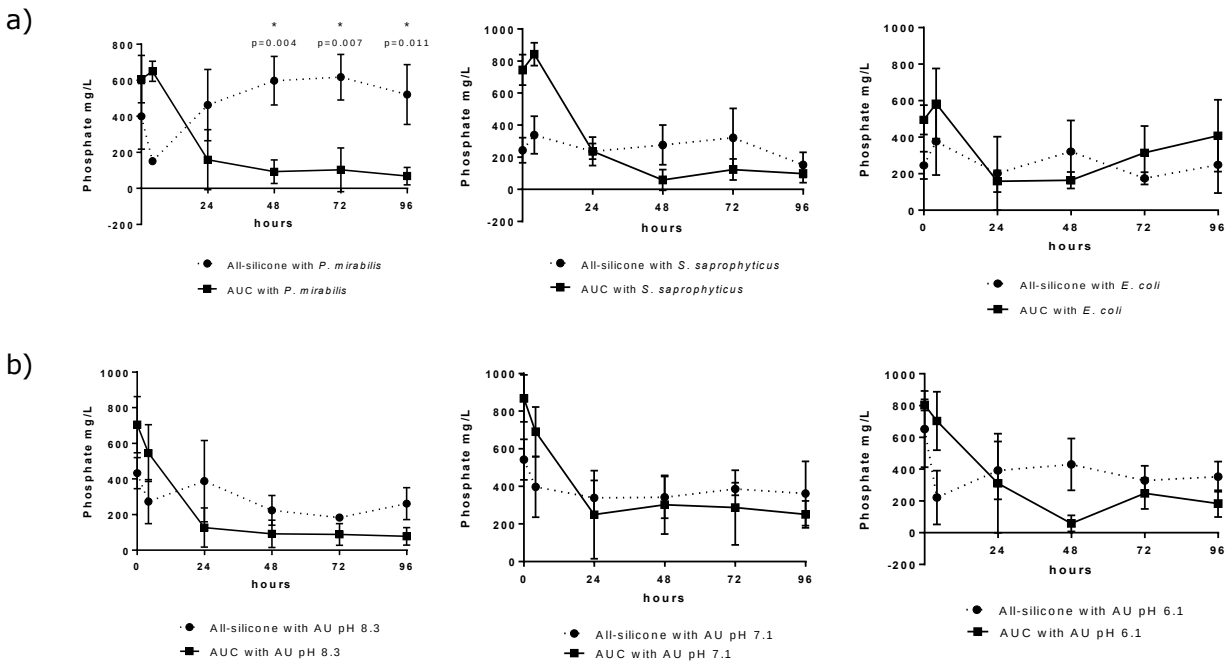


Figure 5: Scanning electron microscopy images of the lumens of a) silicone and b) antimicrobial impregnated catheters perfused with artificial urine and inoculated with *P. mirabilis* visualised at x65 magnification. Silicone catheters and AUCs are matched and imaged at the day of blockage of control.

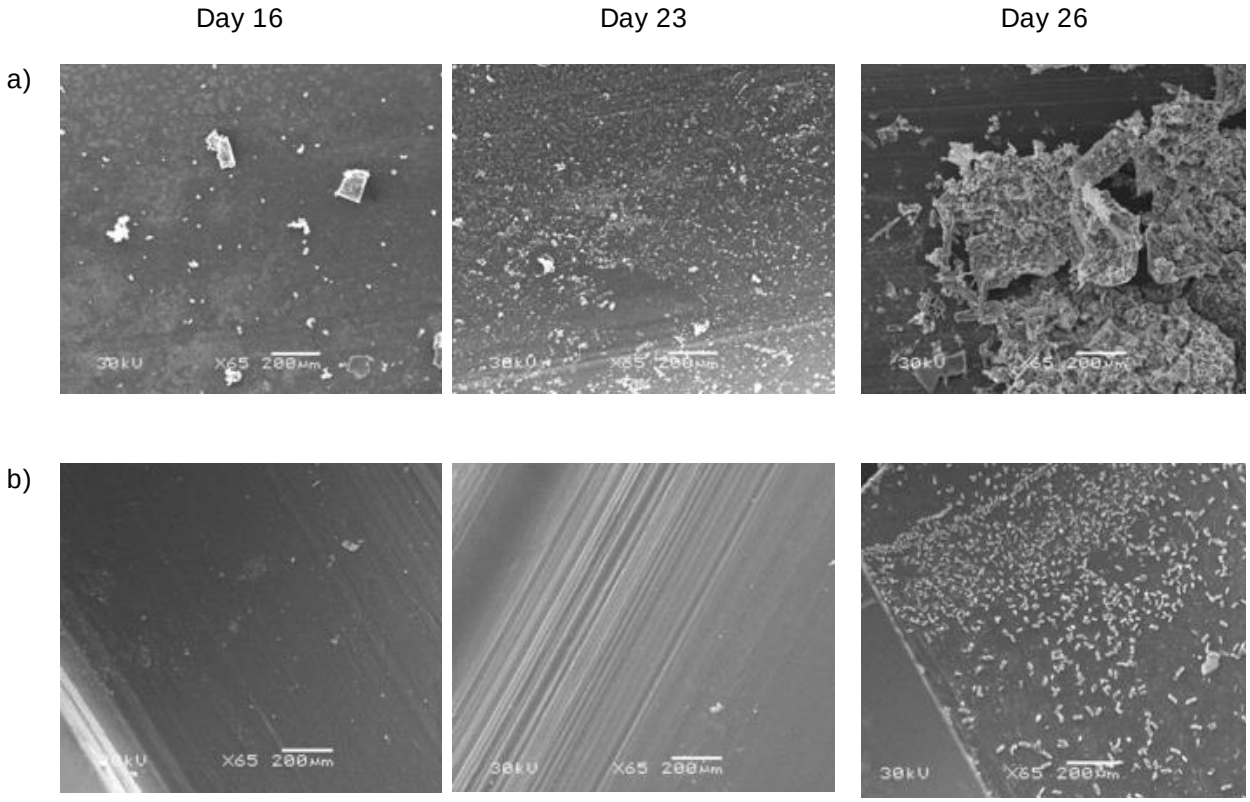


Figure 6: Examples of colonisation of silicone controls and antimicrobial urinary catheters (AUCs) by a) NDM-1 producing *E. coli* (data displayed as mean CFU/mL with SD), b.) MRSA (data display *point of failure of one AUC, +point of failure of a second AUC, Syringe symbol indicates point of bacterial challenge of catheters. c) antimicrobial impregnated catheter segment after 12 weeks of bacterial inoculation and perfusion and d) silicone catheter segment one week after bacterial inoculation and perfusion.

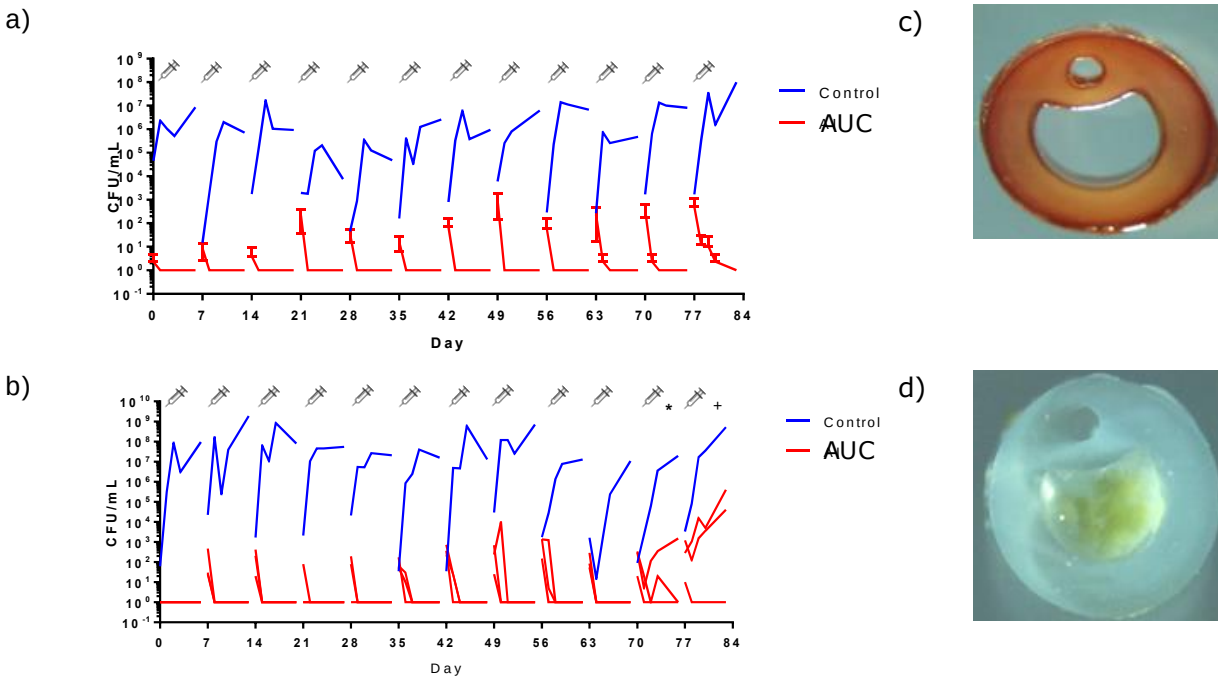


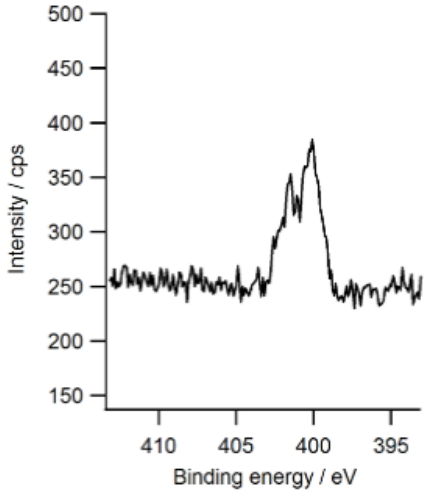
Figure 7: N 1s peaks generated by x-ray photoelectron spectroscopy. AU: artificial urine; Proteus: *Proteus mirabilis*

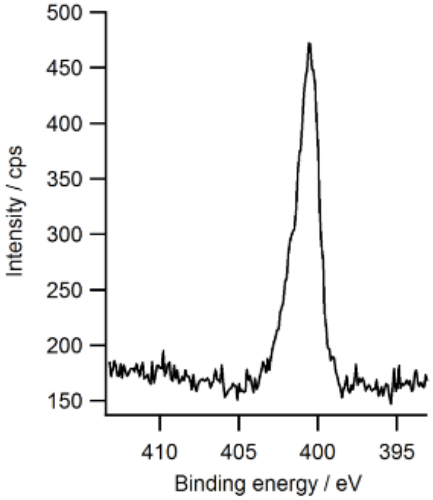
Graphic

Antimicrobial urinary catheter (AUC)

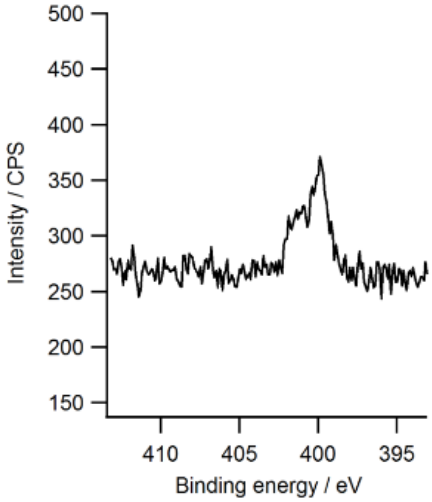
All-silicone control (Plain)

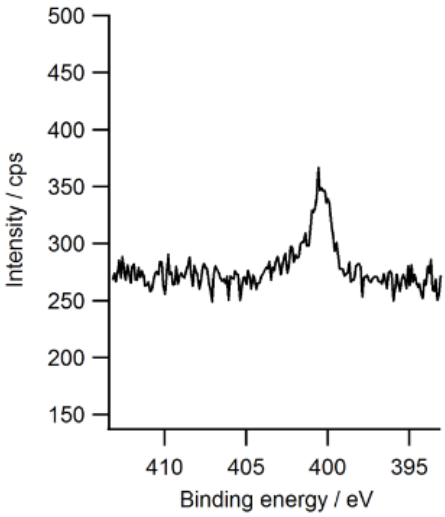
Perfused with AU and Proteus





Perfused with AU only





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Supplementary material

An antimicrobial impregnated urinary catheter that reduces mineral encrustation and prevents colonisation by multi-drug resistant organisms, for up to 12 weeks

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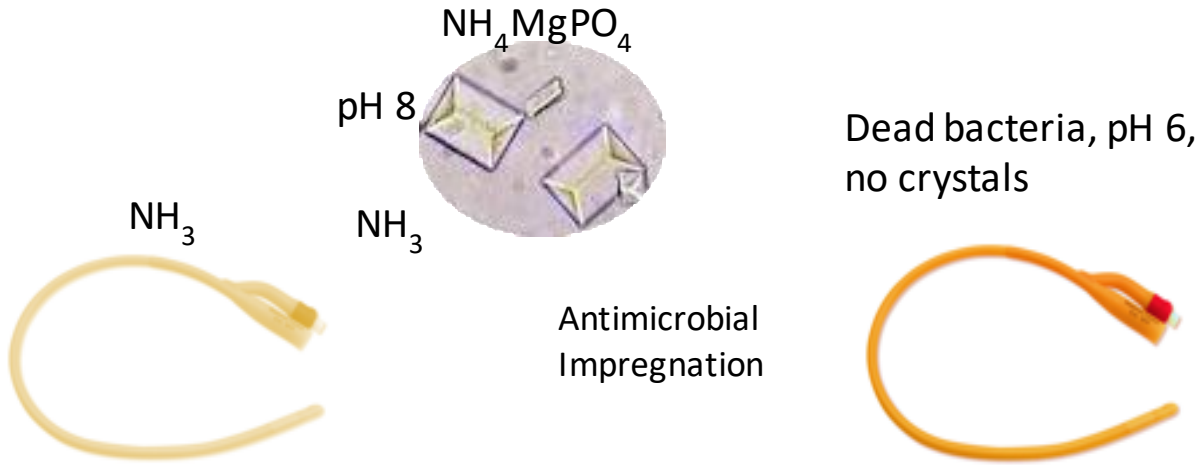
Supplementary Methods

1

Graphical Abstract:



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