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The effect of urinary Foley catheter substrate material on the antimicrobial potential of calixerene based molecules

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Running title: Material modulation of biofilms

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Abstract

Aims: This study was to investigate the antimicrobial activity of a modified calixarene polymer bound to a silicone substrate in the presence of pathogens associated with catheter infections, *Escherichia coli* and *Proteus mirabilis*.

Methods and Results: The molecule and its constituent parts, were studied bound and unbound to silicone substrates to ascertain growth effects. Minimum inhibitory and bactericidal concentrations were determined against *E.coli* and *Pr.mirabilis*. Biofilm growth was studied by immersing silicone discs seeded with either *Pr.mirabilis* or *E.coli* in artificial urine. Biofilms were assessed at 3,7 and 10 days. The coated material reduced bacterial cell density compared to the uncoated samples. Direct and indirect toxicity tests were conducted with a fibroblast cell line (3T3), coated and non-coated silicone samples were seeded with cells ($1x10^4/cm^2$) and incubated for 72h. Hoechst Propidium Iodide staining identified delayed toxic effect from the coated and non-coated material leachate in all but the platinum cured medical grade silicone which showed no evident toxicity.

Conclusions: The calixerene polymer was determined to be the active part of the coating. Biofilm formation was dramatically reduced in the coated platinum cured medical grade silicone samples but cell viability was reduced on the clinical grade silicones regardless of coating in contrast to cells seeded on the platinum cured medical grade silicone. A delayed toxic response was evident to the extract of the coated and non-coated clinical grade samples, indicating that the toxic effect is due to the underlying substrate.

Significance and Impact of Study: This study has established that the immobilised molecule enhances the antibacterial and antifouling properties of silicone, without toxicity. It also clearly demonstrates that regardless of coating efficacy the substrate material has the capacity to disrupt its potency and change the nature of the material coating.

Keywords: biofilm, catheter, colonisation, antimicrobial, biocompatibility, substrate

1 Introduction

1.1 Catheterisation

Catheterisation is one of the most common medical procedures in hospitals today, with uses ranging from the delivery of antibiotics via central venous lines to the draining of urine from the bladder. In 2014, it was reported that 23.6% patients across 183 US hospitals had acquired a catheter-associated urinary tract infection (CAUTI) (Magill *et. al*., 2014).

Patients requiring urinary catheterisation are naturally predisposed to infections of both the urinary tract and bacteraemia, specifically relating to colonisation of the device by constituent members of the normal flora. Foley catheters are designed as short term devices to drain urine from the bladder. However, in recent years, these have been increasingly employed as long-term medical devices, remaining *in situ* for up to 28 days (Donlan *et al.*, 2001). The extended use of catheters means that they are more likely to become colonised by microbial species. Mitchell *et al*. (2016) reported that of approximately 170,000 patients admitted to hospitals, 1.73% developed a Hospital Associated Urinary Tract Infections (HAUTIs), and that HAUTIs are responsible for 17% of all Health Care Associated Infections (HCAIs).

1.2 Catheter-Associated Urinary Tract Infections (CAUTIs)

Escherichia coli and *Proteus mirabilis* are key nosocomial pathogens, annually reported to be the infective agents most commonly associated with CAUTI. In 2013, Melzer & Welch reported that from CAUTI patients, the most routinely isolated organisms were *E. coli* (43.4%), and *Pr. mirabilis*, (13.3%). Whilst being primarily an intestinal bacterium, *E. coli* strains capable of causing uropathogenic

disease are referred to as UPEC *E. coli*. In the USA, CAUTI have been estimated to account for 450,000 cases of nosocomial infections, at a cost of approximately $350m (Nowatzki *et al*., 2012). Whilst the virulence factors required for pathogenesis are not fully understood, it has been hypothesised that UPEC strains initiate incidents of disease by first adhering to the surface by expression of type 1 fimbriae, or by involvement of the O, H and K serotypes (Mobley *et al.*, 1987). Similarly, the physicochemical surface properties have been implicated as a mechanism for *P. mirabilis* cells switching (Allison and Hughes, 1991; Rauprich *et al.*, 1996) between cycles of swarming and periods of colonisation and consolidation (Rauprich *et al.*, 1996). In support of this research, denser surfaces have also been shown to prevent the swarming phenomenon leading to site specific cellular attachment (Shapiro and Trubatch, 1991; Itoh *et al.*, 1999).

Biofilms are an heterogeneous matrix of exopolymers produced by microbial cells (bacteria or fungi), where the microbial cells are adherent upon a substratum, such as an indwelling catheter. The cells utilise host-derived compounds such as carbohydrates or proteins to create a protective “shell” around their cells. This acts to impede the entry of chemicals such as antibiotics (Lynch & Robertson, 2008), thus providing a relatively safe environment for the cells to develop. In the biofilm phase, individual cells express quorum sensing molecules (Miller and Bassler, 2001). These become concentrated within the biofilm matrix, and, once a threshold population is reached, the cumulative level of quorum sensing molecules trigger a physiologic change within individual bacterial cells. This leads to an up-regulation of biofilm-associated genes, and the activation of multicellular behaviour between bacterial cells in, as opposed to individual cell behaviour. Such processes are often specifically linked to disease virulence, and can include the up-regulation of motility factors (Bragonzi *et al*., 2009), as well as those associated with resistance to neutrophil-mediated killing (Bianconi *et al*., 2011). It is the quorum-induced effect that results in the bacterium expressing toxins, and thus presenting a virulence profile relating to disease states such as urinary tract infections resulting from the colonisation of catheters.

Biofilm formation on catheters is also associated with increased resistance to antibiotics commonly prescribed to treat CAUTI. A recent study in Pakistan stated that 73.4% isolates recovered from catherterised patients were biofilm producers (Sabir *et al*., 2017). This study also revealed that *E. coli* was the most routinely isolated organism, with the highest rate of resistance amongst biofilm-producing species was determined to be 87.5% to ampicillin. In 2016, Rahimi *et al.* reported that 105 from 108 methicillin resistant *Staphylococccus aureus* strains recovered from patients demonstrated a multiply resistant phenotype, and that 82.4% demonstrated resistance to over 10 antibiotics.

Further research in Australia, in 2015, revealed that 40% of isolates associated with bloodstream infections & catheter use were identified as *E. coli*, which were note dto be Extended Spectrum Beta-Lactamase producers (Bursle *et al*., 2015). This study also revealed that catheterisation increased the risk of an associated bloodstream infection developing, and importantly, that 56% of catheterisation events were unnecessary.

1.3 Catheter Coatings

The development of novel catheter coatings with anti-fouling (Ding *et al.*2012), antibacterial (Böswald *et al.*, 1999) and hydrophilic properties (Hedlund *et al*., 2001) have been developed to modulate microbial attachment, disease pathogenesis and by decreasing microbial colonisation extend the working life of the device. To date, these developments have been limited by the long term durability of the coatings (Kaye *et al.*, 1994; Stickler 1996). In such instances, the coating serves to delay the bacterial colonisation rather than prevent it, suggesting that future advances in preventing CAUTIs should focus the development of non-degradable coatings and a greater understanding of material science.

Catheters like all indwelling biomaterials must be able to perform their intended function without eliciting undesirable side effects on the local cells and tissues. Biocompatibility is dependent on the physical and physicochemical properties of the device, whether the material is toxic, and whether the device will leach its constituents or particulate material in to the surrounding milieu. An understanding of the chemical properties of the device is crucial, as complications due to reduced biocompatibility can have serious concerns for patient health. Such complications include: allergic response to the material (Warmuth and Beltrani, 1997), encrustation due to bacterial biofilm formation (Tenke *et al*., 2004), and infection resulting from bacterial entry in to underlying tissues (Arciola *et al.*, 2004). However, more subtly, recent studies have highlighted the ability of silicone to induce a proinflammatory state in human cells (Bhaskar et al, 2015), thus further complicating the host response to the infection and subsequent biofilm formation. This research underlines the need to investigate the biological effects of materials used to construct biomedical devices from the perspective of both the patient, and the infectious organisms.

1.4 Calixarene

Calixarene's are cyclic oligomers produced by the reaction of phenols and aldehydes. They share structural similarities with crown ethers, and cydodextrins, and are widely used in the recognition of biological molecules (Stone *et al.*, 2002). Biological recognition has largely focussed on interactions with amino acids and proteins of the cell surface membrane (Arena *et al.*, 2000; de Fátima *et al.*, 2009; Gualbert *et al.*, 2009). However recently, antimicrobial properties and good mammalian cell biocompatibility has also been reported (Mourer *et al.*, 2010). These compounds have also demonstrated antimicrobial activity against fungi (Coimbra de Oliveira *et al.*, 2012), Gram positive and Gram negative bacteria (Grare *et al.*, 2007), and the HIV virus (Zeng *et al.*, 2013), suggesting that these compounds could present a novel platform for the development of new antimicrobial agents.

Whilst insoluble in water, when combined with moieties such as polyethylene, the compound becomes water soluble, and can act as a potent drug delivery scaffold and interact with biological systems, believed to be initiated through binding to the cell membrane (Saluja and Singh Sekhon, 2013). Anchoring the calixarene to a silicone substratum used to construct medical devices such as catheters could present an alternative solution to prevent or reduce the level of colonisation by bacteria and fungi associated with nosocomial infections. Preserving the antimicrobial activity of the calixarene molecule whilst allowing binding to the substratum is therefore pivotal if these avenues of research are to be successful.

1.5 Aims of the Study

The purpose of this study was to two-fold, firstly to evaluate the efficacy of a novel polyethylene glycol-bound calixarene coating against a range of human pathogens over 3, 7 and 10 days to reflect clinically relevant time points of human catheterisation. Day 3 represents the idea *invivo* usage period, whilst days 7 and 10 are extensions of this to monitor bacterial colony development. Secondly, to evaluate its biocompatibility for use in biological systems. The experiments were designed to determine the effect of the full coating and its constituent parts on a range of cells, both mammalian and bacterial. The study focused on the long term efficacy of the polymer on different silicone substrates in terms of coating stability, toxicity to mammalian cells and its ability to prevent initial attachment and long-term colonisation of *Pr. mirabilis* and *E. coli*.

2 Materials and Methods

The silicone discs used throughout these experiments were sourced from three different companies in the UK [Clinical grade silicone cured with Acetic Acid (Dow, UK), Medical grade silicone cured with cerium/barium (Goodfellows, UK) and Platinum cured medical grade silicone (GB Silicone, UK)], and measured 10mm in diameter, and less than 0.5 mm in thickness. The samples were removed from

the delivery plate and washed prior to triplicate rinsing in PBS. The material was then submerged in 70% ethanol and allowed to air dry in sterile conditions prior to UV (wavelength of 250nm) sterilisation.

2.1 Minimum Inhibitory and Cidal Concentration testing of the Calixarene coating

Minimum inhibitory and bactericidal concentrations (MICs and MBCs, respectively) were determined for each of the polymer components against human urinary tract pathogens, prior to its attachment to the substratum. The full range of compounds tested is as follows: the full coating; the coating surface anchor; the coating-PEG attachment unit; the coating-PEG attachment and surface anchor combined together; and finally, the solution (phenolic composition) used to deposit the coating on to the catheter surface (0.5% w/v). The microbial human pathogens used in this study were obtained from the culture collection of the School of Pharmacy and Biomolecular Sciences, at the University of Brighton. They are: *Escherichia coli* NCTC 10418 and *Proteus mirabilis* NCTC 11938; and the minimum inhibitory and cidal concentrations were determined using the method published by the British Society of Antimicrobial Chemotherapy (BSAC; Andrews *et al*., 2006).

2.2 Bacterial Growth on substrate silicone spiked with the deposition solution

The three types of silicone were assessed for their ability to support *in vitro* growth of *Pr. mirabilis*, being one of the principle uropathogenic organisms. A total of 18 discs were used, 6 of each silicone, three of each coated with the calixarene polymer. Growth was determined at time points 24 and 48 hours incubation at 37°C. At time zero, the test wells were spiked with the deposition solution, and the resulting effect on microbial growth determined by serial dilution and spread plating.

Silicone discs were aseptically transferred into six well plates, and 2.6 mL of TSB was added to each well to submerge the disc, and 300 µL of deposition solution was added to each well to adjust the concentration to 0.02%, as this was stated to be the concentration used to coat silicone samples by

the manufacturer. Finally, 100 µL of an overnight culture (approximately $1x10^7$ CFU) was used to inoculate the samples, the plates were sealed and incubated.

2.3 Growth of biofilms on Calixarene coated and uncoated silicone substrates

Calixarene coated and non-coated silicone discs were aseptically transferred to 6-well plates and sterilized as described above. Each disc was subsequently immersed into 5 mL of 1/10th strength TSB and seeded with an overnight culture of either *Pr. mirabilis* or *E. coli*, respectively , and left for one hour at room temperature. The discs were washed in PBS, incubated and enumerated as previously described, except using artificial urine as the growth medium (see below for the formulation of the artificial urine medium). Biofilms for *E. coli* and *Pr. mirabilis* were enumerated at days three, seven and ten, and replicate discs were fixed for SEM at each time point, using the methodology outlined below.

Artificial urine (AU) was adapted from the methodology published by Stickler *et al*. (1999). The final constitution of the stock media was as follows, for 100mL total volume: 0.236g sodium disulphate, 0.065g magnesium chloride, 0.46g trisodium citrate, 0.002g sodium oxalate, 0.28g potassium dihydrogen orthophosphate, 0.16g potassium chloride, 0.1g ammonium chloride, 0.5g gelatin, and 0.1g tryptone soy broth. All media was purchased from Fisher Scientific (UK), and reverse osmosis water was used to constitute the medium, which was subsequently autoclaved at 121°C for 15 minutes. A stock solution of urea and calcium chloride was also constituted; comprising 25g of urea and 0.65g of calcium chloride in 400 mL of reverse osmosis water was also added. This was filter sterilized using a 0.2µm syringe filter (Sartorius) in a Class 2 Microbiology hood. Next, 92 mL of the stock medium was added to 8 mL of the urea/calcium chloride solution to make the working solution of artificial urine medium.

2.4 Scanning Electron Microscopy

Samples were fixed using 2.5% glutaraldehyde (manually prepared) in cacodylate buffer (pH 7.4) for one hour to remove the 'natural' water. The samples were rinsed in cacodylate buffer, and then dehydrated in serially increasing concentrations of ethanol (25%, 50%, 75% and two washes in 97%). Each wash lasted for thirty minutes, and was conducted at room temperature and atmospheric pressure. Samples were examined using a Zeiss EVO SEM (Oxford Instruments, UK).

2.5 Direct contact tests with fibroblast cell line

Sterilized calixarene coated samples (n=6), non-coated silicone samples (n=6) and tissue culture plastic (TCP) controls (n=6) were pre-conditioned with DMEM+10% FCS prior to seeding with mouse 3T3 fibroblasts at $1x10^4$ cells per cm^2. The cells were incubated for 72h, with time points at 24, 48 and 72h. At each time point, the samples were stained with hoechst propidium iodide (HPI) to identify live, dead and apoptotic/necrotic cells. Cells stained blue are alive and viable, red cells indicate DNA damage akin to cell death and apoptotic cells appear as bright blue spheres indicative of membrane clumping. Six random fields of view were imaged using fluorescent microscopy (MAKE MODEL) and total cell number enumerated to assess cellular adhesion, whilst live cell counts were used to study cell viability and proliferation.

2.6 Direct contact of unbound polymer with fibroblast cell line

The polymer loading solution was diluted down by factors of 10 to a 0 concentration. 3T3 fibroblasts were seeded $1x10^4$ per cm^2 onto TCP (n=6) and allowed to adhere for 30 minutes in 1ml DMEM+10%FCS, the adhesion medium was removed and replaced with DMEM+10%FCS containing the unbound polymer (0.2, 0.02, 0.002, 0.0002, 0.00002% and 0) the cells were incubated for 72 hours at 37°C.

2.7 Indirect contact testing with fibroblast cell line

3T3 fibroblasts were seeded $1x10^4$ per cm^2 onto pre-conditioned TCP plates (n=6). The cells were allowed to adhere in pre conditioning medium (DEME+10% FCS) prior to its replacement with 900ul fresh DMEM+10% FCA spiked with 100ul extract medium. The plate was then placed in the incubator (37°C) for 72 hours with time points at 24 and 48hrs. At each time point the cells were removed and stained using HPI (as described in section 2.5).

2.7.1 Extract medium

Extracts were derived to stimulate and/or exaggerate the clinical conditions to determine any potential hazard, they are not designed to alter or damage the test surface or chemical structure of the surface. In this study culture medium with serum (DMEM+10%FCS) was the extraction vehicle; this was chosen to allow the extraction of both polar and non-polar substances. The extraction was performed in sterile conditions using aseptic technique in accordance with ISO 10993-12, for a period of 24h at 37°C. Briefly, the sterilized coated (n=6) and non-coated (n=6) discs were placed in a sterile TCP 24 well plate, each samples was covered in 1ml DMEM+10%FCS and placed in the incubator for 24h at 37°C, standard culture media (DMEM+10% FCS) was used as a control. Once completed the supernatant was removed into separate, labelled Eppendorfs and placed in the fridge ready for use in the indirect assay. The substrate material was then discarded.

2.8 Material mapping

The clinical grade silicone samples were prepared by washing and sterilizing, prior to being coated in platinum. The platinum and cerium/barium cured medical grade samples were studied in their as received state by being mounted directly onto the SEM stubs and coated in platinum. They were investigated by backscatter imaging using the Aztec EDS system, Zeiss EVO SEM (Oxford Instruments, UK), the samples were studied using point and elemental mapping modes.

2.9 Statistical Analysis

Microbiological results were interpreted using Analysis of Variance Statistics, using Minitab® 16 (USA). Two-Way tests were employed, using a 0.05 cut-off point. Data for calixerene-coated samples were compared to analogous growth samples using un-coated silicone samples, at each specific time point stated. Biofilm percentage changes between time points were determined by using the previous time point as a reference, and nominally determining that to be 100% of the biofilm size to which subsequent growth was measured against.

3 Results

3.1 MIC and MBC analysis

The MIC and MBC values for each of the components of the coating, and the full coating are shown in Tables 1 & 2. These values represent results for planktonic cultures, *in vitro*.

The MIC and MBC values for the deposition solution against each of the test organisms in planktonic culture was determined to be 0.2% $^{w}/_{v}$, *in vitro*.

3.2 Biofilm response to Calixerene challenge

The *Pr. mirabilis* and *E. coli* biofilms increased in size on both the coated & uncoated samples (Figure 1). However, whilst the *E. coli* biofilms increased in a fairly straight increase on both coated & uncoated samples, (figure 1b), the *Pr. mirabilis* biofilms increased significantly between days 3 and 7, only to decrease by day ten on coated samples, whilst an increase between all time points was observed for uncoated samples (Figure 1a). Statistical analysis (T-test and ANOVA; Excel® and Minitab®), the results indicate no statistically significant difference for either species over the three time periods (*E. coli* on coated & uncoated substrate: p = 0.14; *Pr. mirabilis* on coated & uncoated

substrate: p = 0.28; *E. coli* c.f. *Pr. mirabilis* on coated substrate: p = 0.17). However, analysis reveals a significant difference between the growth profiles of the two closely related test bacteria on the uncoated substrate during the same test conditions (*E. coli* c.f. *Pr. mirabilis* on uncoated substrate: p = 0.02). This indicates that different species respond differently to substrates, and that catheter models for microbial colonisation should be treated cautiously when extrapolating to suggest the response of a chemical coating in relation to other infectious microorganisms.

Scanning electron microscopy (SEM) revealed that *E. coli* biofilms (Figure 2) developed much more slowly than those for *Pr. mirabilis* (Figure 3). The formation of crystals were also identified on uncoated silicone surface colonised by the Pr*. mirabilis* (micrographs not shown).

3.3 Fibroblast analysis toxicity

The direct contact assay showed reduced viability of fibroblast cells seeded on the coated clinical grade silicone (Dow, UK) and uncoated clinical grade silicone (Dow, UK) where the cells were observed to stain positive for apoptosis (red) and appeared to be clustered together (data not shown). At 24 hours, cells seeded on the medical grade (silicone (S130343, 650-236-68, Goodfellows, UK) were viable and well dispersed across the surface of the sample. Similarly, cells seeded on to the platinum cured medical grade silicone showed no positive apoptosis staining in either the coated or non-coated materials. Similar findings were noted in the direct contact assay at 48 hours. Interestingly, the uncoated platinum cured sample at 48hour incubation looked to encourage cellular aggregation unlike the more randomly distributed cells observed on the control and coated substrates (Figure 4). The incubation of the cells with various dilutions of the unbound polymer was investigated to further understand if the direct contact toxicity was a result of the polymer coating or the underlying substrate material. The images in Figure 5 highlight viable (blue) cells grown in the

presence of up to a concentration of 0.002% dilution of the initial 2% $^{v}/_{v}$ loading solution. The cells are comparable in size, number and distribution to those on the control sample.

Indirect contact testing at 24 hours showed all of the fibroblast cells in each of the extract media were viable with no sign of apoptosis. The same findings were apparent at the 48 hour time point, indicating no release of toxic leachates from the test samples. In contrast, the 72 hour HPI stain highlighted a delayed toxic (red stain) response in cells exposed to the extract medium from the clinical grade calixarene coated and non-coated samples (Figure 6). Conversely the extract from the platinum cured medical grade silicone (GB Silicone, UK) showed no evidence of apoptotic staining was observed on either the uncoated or coated samples at any of the time points studied including those incubated for 72 hours (Figure 7). This was confirmed by cell counts performed at 24 and 48 hours, respectively (Figure 8).

3.4 Material Analysis

The bulk properties of silicone were identified as silicone, oxygen and carbon, point analysis identified particulate contaminants up to 25um in diameter as nickel, chromium, iron and tin. The particulate material is more evident on the clinical grade material regardless of calixarene coating.

4.0 Discussion

This study highlights the pivotal impact of the substrate material on mammalian and bacterial cell survival. The MIC and MBC highlight that *E. coli* and *Pr. mirabilis* present different tolerance profiles for each of the individual test compounds. The addition of the anchor didn't alter the response of either pathogen; in fact the result was the same as that in contact with the free calixarene. This implies any subsequent change in inhibitory effect can be attributed to the substrate material. The dramatic increase in *Pr. mirabilis* biofilm formation over the experimental duration on the non-coated silicone when compared to the lack of increase in biofilm size in the calixarene coated silicone supports this data. Indeed, studies by Shapiro and Trubatch, 1991; Itoh et al., 1999 also

demonstrated that biofilm nature can be greatly affected by their ability to adhere onto the biomaterial surface.

However, caution must be applied when comparing the growth response of one organism to another. The data presented in this paper clearly indicates that, despite *E. coli* and *Pr. mirabilis* being closely related bacterial species, the two will develop differently on silicone catheter substrates. Models of infectious diseases must be evaluated in relation to the known or most likely infectious organisms associated with the diseases being investigated. This model would need adjusting for studies on Candidiasis, for example.

Scanning electron micrographs revealed the development of distinct multi-layered biofilms for both *E. coli* and *Pr. mirabilis* on the uncoated medical grade, platinum-cured silicone. Similarly to the MIC and MBC findings, marked differences were noted between the two species, the *E. coli* biofilms developed much more slowly when compared to *Pr .mirabilis*, it is theorised that this is as a result of the swimming and swarming phenotypes exhibited by *Pr. mirabilis*, and the positive effects that this has on bacterial growth for this species. The *Pr. mirabili*s cultured on the non-coated silicone substrates showed an increased formation of numerous crystal on the substrate surface, the crystals were as assumed to be struvite crystals. The formation of ammonia *in vivo* by bacterial species including *Pr. mirabilis* is well documented. The bacteria produce a mettaloeznyme capable of hydrolysing urea in to ammonia, resulting in an increased pH of the local host environment (Mobley *et al*., 1995). The ammonia has binds to divalent cations naturally occurring in the urine, to begin crystal formation. It has been suggested that increased ammonia production can cause damage to the uroepithelial layer of the host, leading to the release of cellular nutrients, which might cause increased bacterial growth (Johnson *et al*., 1993). In this situation, the formation of crystals and toxic damage to the human cell layers would no doubt cause great discomfort to a catheterised patient. The reduction in *E. coli* and *Pr. mirabilis* biofilm formation, and crystal formation for urease negative species such as *Pr. mirabilis*, that is afforded by calixerene coatings might prove advantageous to the

development of future coating models. However, the exact nature of the interactions between such compounds as ammonia & struvite with calixerene needs to be fully determined, for both bound and unbound calixerene.

The clinical grade silicone showed an increased cell apoptosis when incubated with the mammalian fibroblast cell line this was attributed to the underlying substrate toxicity as a result of the use of acetic acid in the curing process. As found by Okabe *et al*, the use of acetic acid in mammalian cell culture induces cell death and apoptosis. As a result any effect of the polymer calixarene polymer coating on this substrate was masked. The evidence of substrate based toxicity was further confirmed by the lack of apoptosis identified in either of the medical grade silicones, both of which promoted viable adhesion with normal cell morphology. Indeed the platinum cured substrate was shown to promote cell adhesion with evidence of cellular aggregation, the addition of the calixarene coating reduced the nature of the adhesion with fewer individual cells noted, supporting the antifouling nature of the polymer as described by Saluja and Singh Sekhon, 2013.

Systemic toxicity was investigated to ensure no leachate or peeling of the material could induce a further host response. The medical grade samples showed no toxic leachate over the tested time period however this was not the case for the clinical sample which showed an increase in cell apoptosis at 72 hours, this was identified as a response to the release of acetic acid curing agent into the media which underwent a colour change due to its phenol content from red/pink to orange/ yellow.

The clinical grade silicone (Dow, UK) uncoated and coated with the calixarene polymer had bulk properties of silicone, oxygen and carbon, in each case particulate contaminants were , investigated using point analysis and identified as nickel, chromium, iron and tin possibly artefacts of the manufacturing process. Nanoparticulate material is evident the clinical grade silicone regardless of

the presence or absence of the calixarene coating, suggesting the contaminants are embedded in the material bulk itself.

These imperfections are likely to be as a result of the manufacturing process, and it is unlikely that they would cause the toxicity identified during the direct contact experiments. Similar contaminants were observed when mapping the medical grade silicone (Goodfellows, UK), which also recorded the presence of surface debris, comprised of metallic elements such as zirconium and titanium. The platinum cured medical grade silicone (GB Silicone, UK) showed fewer areas of nano-particulate contamination and where found they were in the main part natural salts and metal such as calcium and bismuth. Individual particles containing copper, zirconium and titanium were found but not as widespread as with the earlier materials, again all are commonly found in industrial manufacturing settings.

The use of material mapping and image analyses provided crucial data which identify potential reasons why material chemistry needs to be precise in order to have a positive effect on biomedical device function, and consequently on patient suffering. It is essential to fully investigate the toxicity of potential biomaterials by simple, inexpensive testing prior to their inclusion in test matrices. In this study, the antimicrobial effect of the calixarene coating was negated by the presence of toxic particulate matter on the surface of the biomaterials. This study has produced valuable conclusions, which can be summarised as follows.

Whilst combining individual monomer units of the calixarene polymer did not decrease the effectiveness of the combined calixarene polymer, in terms of minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC), nor did the addition of the anchor molecules which facilitate adhesion to the silicone, *in vitro*. The unbound polymer appears to have species modality, whereby the MIC and MBC are similar for *Pr. mirabilis,* but the MIC is lower than the MBC for *E. coli*, which is more sensitive to lower concentrations of the unbound calixarene molecule. Mammalian cells exhibited a toxic response only at the two highest concentrations of the loading

solution, below which no adverse observations were noted up to 72 hours, but the polymer is non-toxic to mammalian cells when grafted on to a non-toxic substrate (i.e. platinum cured silicon) over 24, 48 and 72 hours in both direct and indirect testing.

The initial silicone substrates tested were toxic due to the presence of compounds remaining from the curing/manufacturing process, resulting in a level of cellular adaptation occurring in the first 72 hours of incubation with the non-platinum cured coated and uncoated silicone extract medium. The similarity in the two samples supports the theory that it is the substrate material which is leaching the toxic compound, as any toxicity from the coating would not be observed in the uncoated sample. Although positive, it is essential to keep in mind that the substrate toxicity may be masking any coating toxicity. These results support the data observed in the direct contact study and prompt the conclusion that the substrate material in the earlier investigations was probably the main causal agent for the observed toxicity. Indeed, this data taken in conjunction with the data recorded in the unbound polymer study further supports the non-toxic nature of the calixarene polymer. Cerium was also identified leading us to theorize that is was this easily oxidisable metal which was used in the curing of this silicone. Barium was also identified an alkaline metal often used to scavenge air in a vacuum system; again it is proposed that this is another curing artefact.

This paper attempts to highlight some of the issues relating to material chemistry, and the intended application of a substrate for use as a biomedical device. The results from this study show that the grade of silicone affects its toxicity to both eukaryotic & prokaryotic cells, and that this should be used as a marker to influence the selection of materials in device design. Further, the potential for adverse effects on the host tissues must be demonstrated to be negligible before a product can be promoted for use. The potential for calixerene coating to reduce bacterial biofilm formation has been demonstrated. But, this must be acknowledged to be dependent on material selection prior to use.

In April 2015, the UK NHS (National Health Service) estimated that the £1bn was spent on treating HCAIs; with £56m of this money associated with post-discharge care (Mantle, 2015). It is understood that 172% of all HCAIs in the UK are linked to UTIs (Loveday *et al*., 2014). In the USA, in 2013, it was estimated that approximately $45bn was spent on direct hospital costs for CAUTIs, with approximately 100,000 deaths annually linked to this type of infection (Kennedy *et al*., 2013). These statistics underline the pivotal importance of assigning a correct therapeutic regime for each patient, and the need to understand the interaction between microbial cell and substrate at the cellular level. Further research is needed to understand how novel materials interact with human cells to ameliorate suffering and reduce morbidity times, but also to reduce overall healthcare costs to the provider.

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Conflict of Interest

The authors of the paper declare there are no known competing interests associated with this paper or the data contained within.

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Figures list

Figure 1. The percentage change in biofilm density over a ten day period with Calixarene coated and non-coated substrates, a) Pr. Mirabilis, b) E. coli.

Figure 2. Scanning electron micrographs revealing the development of E. coli biofilms on coated and uncoated silicone sample after incubation in artificial urine at 37 oC. (a) coated silicone at day 3; (b) uncoated silicone at day 3; (c) coated silicone at day 10; (d) uncoated silicone at day 10.

Figure 3. Scanning electron micrographs revealing the development of Pr. mirabilis biofilms on coated and uncoated silicone sample after incubation in artificial urine at 37 oC. (a) coated silicone at day 3; (b) uncoated silicone at day 3 with crystal formation highlighted; (c) coated silicone at day 10; (d) uncoated silicone at day 10.

Figure 4. HPI staining of mouse 3T3 fibroblasts cells seeded onto the Calixarene coated and non-coated platinum cured medical grade substrate and a tissue culture plastic control at 24 and 48 hours (x20 magnification)(size bar 50um).

Figure 5. HPI staining of a range of percentage dilutions (0.2%, 0.02%, 0.002%, 0.0002% and 0.00002%) of the initial 2% v/v loading solution of the unbound polymer in contact with mouse fibroblasts for 72 hours (x20 magnification)(size bar 50um).

Figure 6. HPI staining of fibroblast cells incubated with the extract of the calixarene coated clinical grade and non-coated clinical grade substrate and a tissue culture plastic control at 24, 48 and 72 hours (x20 magnification)(size bar 50um).

Figure 7. HPI staining of fibroblast cells incubated with the extract of the calixarene coated platinum cured medical grade substrate, non-coated platinum cured medical grade substrate, and a tissue culture plastic control at 72 hours (x20 magnification)(size bar 50um).

Figure 8. Mouse fibroblast cell number at 24 and 48 hours on the calixarene coated platinum cured medical grade substrate, non-coated platinum cured medical grade substrate and a tissue culture plastic control at 24 and 48 hours.

Table 1. MIC values for each of the test compounds against each of the test organisms, *in vitro*.

Test Compound	**MIC ($^{w}/_{v}$)**	
	Escherichia coli	***Proteus mirabilis***
Coating-PEG attachment and surface anchor	0.0625	0.0625
Coating-PEG attachment	0.03125	0.03125
Surface anchor	0.03125	0.25
Full coating	0.03125	0.25

Table 2. MBC values for each of the test compounds against each of the test organisms, *in vitro*.

Test Compound	**MBC ($^{w}/_{v}$)**	
	Escherichia coli	***Proteus mirabilis***
Coating-PEG attachment and surface anchor	0.125	0.125
Coating-PEG attachment	0.0625	0.125
Surface anchor	0.0625	0.25
Full coating	0.0625	0.25