<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Surface characteristics and antimicrobial properties of modified catheter surfaces by polypyrogallol and metal ions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Balne, Praveen Kumar; Harini, Sriram; Dhand, Chetna; Dwivedi, Neeraj; Chalasani, Madhavi Latha Somaraju; Verma, Navin Kumar; Barathi, Veluchamy Amutha; Beuerman, Roger; Agrawal, Rupesh; Lakshminarayanan, Rajamani</td>
</tr>
<tr>
<td><strong>Date</strong></td>
<td>2018</td>
</tr>
<tr>
<td><strong>URL</strong></td>
<td><a href="http://hdl.handle.net/10220/45081">http://hdl.handle.net/10220/45081</a></td>
</tr>
<tr>
<td><strong>Rights</strong></td>
<td>© 2018 Published by Elsevier B.V. This paper was published in Materials Science and Engineering: C and is made available as an electronic reprint (preprint) with permission of Elsevier B.V. The published version is available at: [<a href="http://dx.doi.org/10.1016/j.msec.2018.04.095">http://dx.doi.org/10.1016/j.msec.2018.04.095</a>]. One print or electronic copy may be made for personal use only. Systematic or multiple reproduction, distribution to multiple locations via electronic or other means, duplication of any material in this paper for a fee or for commercial purposes, or modification of the content of the paper is prohibited and is subject to penalties under law.</td>
</tr>
</tbody>
</table>
Surface characteristics and antimicrobial properties of modified catheter surfaces by polypyrrogallol and metal ions

Authors:
Praveen Kumar Balne\textsuperscript{1}, Sriram Harini\textsuperscript{2}, Chetna Dhand\textsuperscript{2,3}, Neeraj Dwivedi\textsuperscript{4}, Madhavi Latha Somaraju Chalasani\textsuperscript{5}, Navin Kumar Verma\textsuperscript{5}, Veluchamy Amutha Barathi\textsuperscript{1,3,6}, Roger Beuerman\textsuperscript{2,3}, Rupesh Agrawal\textsuperscript{*7}, Rajamani Lakshminarayanan\textsuperscript{*2,3}.

Affiliations:
\textsuperscript{1}Translational Pre-Clinical Model Platform, Singapore Eye Research Institute, The Academia, 20 College Road, Discovery Tower, Singapore - 169856.
\textsuperscript{2}Anti-Infectives Research Group, Singapore Eye Research Institute, The Academia, 20 College Road, Discovery Tower, Singapore - 169856.
\textsuperscript{3}Ophthalmology and Visual Sciences Academic Clinical Program, Duke-NUS Graduate Medical School, Singapore 169857.
\textsuperscript{4}Department of Electrical and Computer Engineering, National University of Singapore, 3 Engineering Drive 3, Singapore 117583.
\textsuperscript{5}Lee Kong Chian School of Medicine, Nanyang Technological University, Experimental Medicine Building, Singapore 636921.
\textsuperscript{6}Department of Ophthalmology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 119077.
\textsuperscript{7}National healthcare Group Eye Institute, Tan Tock Seng Hospital, Singapore - 308433.
\textsuperscript{*}These authors contributed equally

*Corresponding authors:
Dr. Rajamani Lakshminarayanan, Anti-Infectives Research Group, Singapore Eye Research Institute, Level 6, The Academia, 20 College Road, Discovery Tower, Singapore - 169856.
E-mail: lakshminarayanan.rajamani@seri.com.sg
Telephone: +65-65767276

Dr. Rupesh Agrawal, National healthcare Group Eye Institute, Tan Tock Seng Hospital, Singapore – 308433.
E-mail: rupesh_agrawal@ttsh.com.sg
Telephone: +65-90613202
Abstract:

Catheter associated infections (CAIs) are the major cause of nosocomial infections leading to increased morbidity, mortality rates and economical loss. Though the antibiotic coated surface modified catheters are reported to be effective in preventing CAIs, presence of sub-lethal concentrations of antibiotics in long term instilled catheters poses a risk of development and spread of drug resistant microbial strains. Herein, we have developed an antibiotic-free alternative strategy to coat catheter surfaces using pyrogallol (PG) and metal ions (Ag+/Mg2+). Surface characteristics, antimicrobial and anti-biofilm properties with hemocompatibility of the coated catheters were studied. Structural characteristics of coated catheters were similar to the uncoated catheters with improved wettability. All the coated catheters with PG and different PG/metal ion combinations exhibited broad spectrum antibacterial activity. Catheters coated with PG/metal ions combination showed effective antibiofilm properties against MRSA strains. None of the coated catheters showed any significant hemolysis for rabbit erythrocytes. In addition, polypyrogallol (pPG) coating attenuated the hemolytic properties of silver without altering the antimicrobial properties. The inherent antimicrobial properties of the coating agent along with antimicrobial metal ions broaden the application landscape which includes coating of other medical devices, clean room construction and development of antimicrobial surfaces. The chemical formulation can also be used to design antiseptic solutions to prevent healthcare associated infections.

Keywords: Catheter coating; Pyrogallol; Metal ions; Antimicrobial; Broad-spectrum; Non-hemolytic.
1. INTRODUCTION

Catheter associated infections (CAIs) such as urinary tract, blood-stream and respiratory tract infections are the most common hospital acquired infections (HAI) contributing to higher morbidity and mortality rates [1-4]. In a recent report from International nosocomial infection control consortium (INICC), data collected from 703 intensive care units (ICUs) in Europe, Latin America, Southeast Asia, Eastern Mediterranean and Western Pacific countries showed that the rate of intravascular catheter associated bloodstream and urinary tract infections was 4.1 and 5.07 per 1,000 central line or catheter days, respectively [5]. The rate of these infections were high compared to United States ICUs (0.8 and 1.7 per 1,000 central line or catheter days respectively) [6]. Pathogenic bacteria such as Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii and Enterococcus faecalis are some of the common bacterial strains isolated from the infected patients [5, 7].

The evolution of resistance to antibiotics/antimicrobials is the major problem associated with CAIs. Bacterial contaminations to the catheters originates from the patient’s skin commensal flora, healthcare personnel’s exogenous flora and contaminated devices or fluids [8]. The adhesion of bacteria to the catheter surfaces is the key step which leads to the formation of biofilms and responsible for the eventual antimicrobial resistance [9]. Coating of catheters with antifouling and antimicrobial compounds which inhibits microbial adhesion is an effective approach to prevent microbial contamination. There are several such compounds used to coat the catheter surfaces to prevent the CAIs with varying success rates [9-11].

Catheter surfaces coated with various antiseptic agents (such as silver nitrate, chlorhexidine, triclosan and antimicrobial peptides) or broad-spectrum antibiotics such as ciprofloxacin, norfloxacin, ofloxacin, sparflloxacin, nitrofurazone, nitrofurantoin, gentamicin, tigecycline,
rifampin and minocycline showed efficient in vitro and in vivo antimicrobial activity against several pathogenic bacteria [12-20]. The catheter surfaces coated with antibiotics showed better antimicrobial activity than antiseptics coated catheters [13, 18]. However infections caused by intrinsic drug resistant strains and development of drug resistance in bacteria when exposed to sub-lethal concentrations of antibiotics limit the utility of antibiotic coated catheters, thus highlighting the need for development of alternative antimicrobial coating strategies to prevent the CAIs [21].

Naturally occurring polyphenolic compounds containing catechol groups undergo oxidative polymerization under wide variety of conditions forming uniform material independent coatings on various substrates [22-24]. Recent studies further established that some of the polyphenol compounds display potent antimicrobial and anti-biofilm activities in both monomeric and polymeric forms [25, 26]. In addition, polyphenols such as caffeic acid or nordihydroguaiaretic acid in combination with other antibiotics synergistically enhanced antibacterial activities of the drugs [27, 28]. Silver ions (Ag⁺) have been shown to complex with β-lactam antibiotics and the organometallic complex showed synergistic activity against multi-drug resistant strains of Gram-positive and Gram-negative bacteria [29]. A sustained release of antimicrobial silver ions was accomplished by taking advantage of the chelating properties of the phenolic groups [30-32]. While silver ions display inherent antimicrobial potential, Workentine et al. showed that a number of other metal ions also possess antibacterial and anti-biofilm properties dictated by the ions-specific physico-chemical properties [33]. Metal ions such as calcium and magnesium (Ca²⁺ and Mg²⁺) possess species specific antimicrobial activity against S. aureus strains [34]. Here we report the utility of a phenolic compound, pyrogallol (PG) alone and in combination with metal ions (Ag⁺ and Mg²⁺) to develop potent antimicrobial surface coatings for catheters and investigated their antimicrobial, anti-biofilm and hemocompatibility properties.
2. MATERIAL AND METHODS

2.1. Reagents

Pyrogallol (PG), sodium bicarbonate (NaHCO₃), sodium periodate (NaIO₄), silver nitrate (AgNO₃), magnesium chloride (MgCl₂), paraformaldehyde, glutaraldehyde and resazurin sodium salt were purchased from Sigma-Aldrich (Singapore). The microbial culture media Mueller Hilton broth (MHB), Mueller Hilton agar (MHA) and tryptic soy broth (TSB) were procured from Acumedia, Neogen Corporation, Michigan, USA. All the reagents were of analytical grade and used without any further purification.

2.2. Bacterial strains

The antimicrobial efficacy was evaluated against both the reference strains from American Type Culture Collection (ATCC) and clinical isolates. The details of the Gram positive bacterial strains used in this study were ATCC strains: 1) *Staphylococcus aureus* 29213 (SA 29213), 2) *Staphylococcus epidermis* 35984 (SE 35984), 3) *Enterococcus faecalis* 29212 (EF 29212) and a clinical strain 4) Methicillin resistant *Staphylococcus aureus* DM9808R (MRSA DM9808R). The details of the Gram negative bacteria used in this study were ATCC strains: 5) *Pseudomonas aeruginosa* 9027 (PA 9027), 6) *Escherichia coli* 10536 (EC 10536), 7) *Klebsiella pneumoniae* 10031 (KP 10031) and 8) *Acinetobacter baumanii* 19606 (AB 19606).

2.3. Antimicrobial susceptibility testing

To assess the antimicrobial activity of PG, microbial susceptibility testing was performed in cation-adjusted Mueller Hilton broth (CAMHB) using broth micro-dilution methods in accordance with the CLSI guidelines in 96 well polystyrene plates. A fresh stock solution of 10 mg/mL of PG was prepared in de-ionized water. CAMHB was used to prepare serial 2-fold dilutions of the stock solutions in 96 well plates starting at a concentration of 1000
µg/mL down to 7.8 µg/mL in half steps. The initial microbial inoculum was adjusted such that each well contained approximately 5× 10⁵ CFU/mL. The plates were incubated at 35 °C and the OD600 was measured immediately and at each hour for 24 hours using TECAN microplate reader (TECAN Infinite 200, Austria). The lowest PG concentration at which no visible growth was observed was recorded as the minimum inhibitory concentration (MIC) value. The dilutions were performed in duplicates and the average MIC was reported.

2.4. Development of PG coating with/without metal ions on catheter surface

In order to develop antifouling surfaces, a facile coating strategy was devised for adherence of PG on the surfaces of medical devices (Eg. catheters, contact lens, etc.). Suction catheters (Unomedical, Singapore) were used as model devices to optimize the coating strategy. The catheter tube was cut into pieces with the length of 1-1.3 cm, weighed and coated with PG at concentrations of 2% and 1% (w/v) in 10 mM sodium bicarbonate (NaHCO₃) buffer (pH 8.5) and 100 µM sodium periodate (NaIO₄) under constant stirring at 250 rpm for 24 h at 37 °C and then rinsed with de-ionized water and dried before further characterization. In order to improve the antimicrobial efficacy of the PG coated catheter tubes, antimicrobial metal ions including silver ions (Ag⁺) and magnesium ions (Mg²⁺) were incorporated in the form of their salts, silver nitrate (AgNO₃) and magnesium chloride (MgCl₂), respectively, into the coating solution. The salt concentration was varied from 0.05, 0.025 and 0.0125% w/v and all the components such as PG alone, PG with AgNO₃ and PG with MgCl₂ were added together in the buffer solution at the same time to minimize the steps involved in the coating process.
Table 1 summarizes the sample abbreviations and concentrations of the coating components respectively.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>PG (w/v)</th>
<th>AgNO₃ (w/v)</th>
<th>MgCl₂ (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPG_2%</td>
<td>2%</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>pPG_1%</td>
<td>1%</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>pPG_2%_Ag_0.05%</td>
<td>2%</td>
<td>0.05%</td>
<td>Nil</td>
</tr>
<tr>
<td>pPG_2%_Mg_0.05%</td>
<td>2%</td>
<td>Nil</td>
<td>0.05%</td>
</tr>
<tr>
<td>pPG_1%_Ag_0.025%</td>
<td>1%</td>
<td>0.025%</td>
<td>Nil</td>
</tr>
<tr>
<td>pPG_1%_Mg_0.025%</td>
<td>1%</td>
<td>Nil</td>
<td>0.025%</td>
</tr>
<tr>
<td>pPG_1%_Ag_0.0125%</td>
<td>1%</td>
<td>0.0125%</td>
<td>Nil</td>
</tr>
<tr>
<td>pPG_1%_Mg_0.0125%</td>
<td>1%</td>
<td>Nil</td>
<td>0.0125%</td>
</tr>
</tbody>
</table>

Table 1: Different concentrations of PG and metal ions used for coating the catheters.

2.5. Antimicrobial properties of coated catheters

2.5.1. Bacterial contact inhibition assay. The catheter tubes (coated and uncoated) were placed in sterile microcentrifuge tubes. Bacterial culture (1 mL) with an initial inoculum equivalent to $10^5$ colony forming units (CFU)/mL in 17% MHB in PBS was added to the catheters and incubated at 35 °C in a shaking incubator at 300 rpm for 24 h. After incubation, these catheter tubes were washed twice with PBS and transferred to another microcentrifuge tubes containing 10 mM phosphate buffered saline (PBS) followed by short sonication for 20 s. A 100 µL of this suspension was serially diluted and plated using MHA. The plates were incubated at 35 °C for 24 h before enumerating the colonies. The experiment was performed in triplicates and the average of the three values was reported as CFU/mL and log₁₀ reduction.

2.5.2. Biofilm inhibition assay. Overnight grown mid-log phase bacterial cultures of MRSA DM9808R and E. coli 10536 were pelleted by centrifugation at 3000 rpm for
10 minutes and the pelleted cells were washed twice with 10 mM PBS. The cell densities were adjusted to $3 \times 10^3$ CFU/mL in TSB. Each coated catheter tube was placed in a sterile microcentrifuge tube containing 1 mL of this bacterial suspension and incubated at 37 °C. Each tube was cut into 2 halves one for each resazurin assay and FESEM analysis and the biofilm formation was analysed at day 1, 3 and 7 post-inoculum.

2.5.3. Resazurin assay. At each time point, catheter tubes were taken out from the bacterial culture and washed twice with 10 mM PBS to remove the planktonic cells. Catheter tubes were placed in a sterile 96 well plate and 200 µL of 0.1 mM resazurin (in TSB) was added to each well and incubated at 37 °C for 2 h. After incubation, the samples were transferred to fresh 96 wells plate and the amount of reduced resazurin (resorufin) was determined by recording the absorbance at 560 nm. Residual amount of the oxidized resazurin was quantified by measuring the absorbance at 620 nm and the corrected $A_{560}$ values ($AR_{560}$) were calculated using the following formula:

$$AR_{560} = A_{560} - (A_{620} \times R_0)$$

Where $R_0 = AO_{560}/ AO_{620}$

$AO_{560}$ and $AO_{620}$ – absorbance of resazurin (0.1 mM in TSB) at 560 nm and 620 nm respectively.

$A_{560}$ and $A_{620}$ – absorbance of the samples at 560 nm and 620 nm respectively.

Lower the $AR_{560}$ value higher is the inhibition of bacterial growth or biofilm disruption [35].

2.5.4. FESEM to visualize bacterial adhesion and biofilm formation on catheter surface. At each time point the catheter tubes incubated with bacterial suspensions were taken out and washed with sterile 10 mM PBS to remove the planktonic cells.
The bacteria adhered to the catheter surface were fixed using a mixture of 3% paraformaldehyde and 1.5% glutaraldehyde in 10 mM PBS for 15 minutes for immediate use or stored at 4 °C until further use. Prior to the FESEM analysis the catheters were dehydrated step by step with 50%, 60%, 70%, 80%, 90% and 100% ethanol for 10 minutes each and the tubes were allowed to air dry soon after the addition of hexamethyldisilazane (Sigma-Aldrich, Singapore). The processed tubes were analyzed using FESEM technique.

2.6. Hemocompatibility assessment

The hemocompatibility of the coated catheter tubes were assessed using hemolysis assay [36]. Fresh blood was obtained from New Zealand white rabbit and the red blood cells (RBCs) were isolated by centrifugation at 3000 rpm for 10 minutes and washed twice with sterile 20 mM PBS. RBCs were diluted to a concentration of 4% with 20 mM PBS and 750 µL of this suspension was added to each microcentrifuge tube containing various coated catheters (Length = 1cm, weight ~ 100 mg). A 2% Triton-X 100 was used as positive control while 20 mM PBS buffer served as the negative control. The tubes were incubated in a shaking incubator at 37 °C at 300 rpm speed for 8 h. After incubation, the RBC suspension from each tube was centrifuged at 3000 rpm for 3 minutes. A 100 µL of the supernatant was then transferred to 96 well plates and its absorbance at 576 nm was measured using TECAN microplate reader (TECAN Infinite 200, Austria). The percentage hemolysis was calculated using the following equation:

\[
\text{% Hemolysis} = \left( \frac{\text{Abs}_{\text{catheter}} - \text{Abs}_{\text{negative}}}{\text{Abs}_{\text{positive}} - \text{Abs}_{\text{negative}}} \right) \times 100
\]

where \(\text{Abs}_{\text{catheter}}\), \(\text{Abs}_{\text{negative}}\) and \(\text{Abs}_{\text{positive}}\) correspond to the absorbance value recorded for different catheter samples, positive control (Triton X-100) and negative control (20 mM PBS), respectively, at 576 nm.
2.7. Characterization of the coated catheters

2.7.1. Surface morphology of the coated catheters. The coated catheter’s surface morphology was analyzed using FEI-QUANTA 200F field emission scanning electron microscope (FESEM) after sputter coating with Platinum (JEOL JSC-1200 fine coater, Japan) at an accelerating voltage of 5 kV. The FESEM analysis was also employed to study the bacterial attachment and biofilm formation on the surface of uncoated and coated catheters.

2.7.2. Atomic force microscopy (AFM) studies. Innova AFM (Bruker, Bruker Corporation, MA, USA) equipped with a silicon cantilever having a tip of radius $\sim$8 nm in tapping mode was used to measure the surface topology and roughness of the uncoated and coated catheters at three different locations (30 μm × 30 μm).

2.7.3. X-ray Photoelectron Spectroscopy (XPS). Kratos AXIS UltraDLD (Kratos Analytical Ltd) employing a monochromatic Al-Kα X-ray source (1486.71 eV) in ultrahigh vacuum (UHV) conditions of $\sim$10-9 Torr was used to record the in-depth analysis of various chemical states of catheters. During analysis, the high resolution spectra were deconvoluted using various Gaussian-Lorentzian components with the background subtracted in Shirley mode.

2.7.4. Surface wettability of the catheters. VCA Optima Surface Analysis system (AST products, Billerica, MA) was used to record the dynamic contact angle of water on the surface of the various coated catheters. A 1 μL of de-ionized water was placed on the surface of the flattened coated catheters and the advancing contact angle was measured at different time points for 10 minutes. The contact angle was recorded in triplicates and the average value was reported.
2.8. Statistical analysis

One-way analysis of variance (ANOVA) with Tukey’s test was used on data obtained for the log reduction comparison against a panel of 9 bacterial strains. Log reductions were considered statistically significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Antimicrobial activity of PG and polypyrrogallol (pPG)-coated catheters

To ascertain the antimicrobial properties of PG, we determined its MIC values against Gram-positive and Gram-negative bacterial strains. **Figure 1** shows the concentration-dependent growth kinetics of *S. aureus* ATCC 29213 and *E. coli* ATCC 10536 in the presence of PG. Against the *S. aureus* strains, a complete inhibition was observed at a PG concentration of 31.25 $\mu$g/ml whereas a slightly higher concentration (125 $\mu$g/ml) was required against *E. coli*. MIC values of PG against a panel of Gram-positive strains (*S. aureus*/MRSA strains and *S. epidermidis*) varied from 15.6 - 31.25 $\mu$g/mL (7 different strains), indicating potent antimicrobial activity of PG against pathogenic strains. These results indicate that the presence of additional outer membrane in *E. coli* confers greater protection than *S. aureus* against PG. Yoda et al reported similar differences in the susceptibility of *S. aureus* and *E. coli* against epigallocatechin gallate (EGCG) [37]. These authors showed that a higher affinity of EGCG for peptidoglycan, the major cell wall component of *S. aureus*, and the lack of affinity for lipopolysaccharide, the major outer membrane component of *E. coli*, was responsible for the differences in the sensitivity. Therefore, it is plausible that a higher affinity of PG for peptidoglycan could account for the higher sensitivity of *S. aureus* than *E. coli*. 
**Figure 1.** Growth kinetics of SA 29213 and EC 10536 in 2×, 1× and 0.5× MIC of PG a) OD<sub>600</sub> vs time (h) of SA 29213 and b) OD<sub>600</sub> vs time (h) of EC 10536.

Next, the suction catheters were coated in PG solution (0.1%, 1% and 2%, w/v) under alkaline conditions supplemented with NaIO<sub>4</sub> for 24 h. The color of the catheters turned brown confirming the formation of pPG coating. Radial disc diffusion results indicated a clear zone of inhibition for the pPG-coated catheters using 1% and 2% w/v of PG, whereas no growth-inhibition was observed for pristine or catheter coated with 0.1% w/v PG (Figure 2a - d). Disc diffusion assay indicated that pPG coating conferred complete protection against MRSA and suggesting a concentration-dependent antimicrobial effect. To confirm the antibacterial properties of pPG-coated catheters, we determined the amount of *S. aureus* and *E. coli* cells attached to the catheter surface after incubating in PBS-buffered MHB media that promotes bacterial adhesion and proliferation. Later, the amount of bacteria attached to the surface was quantified and expressed in terms of CFU/mL (Figure 3). A significant *S. aureus* and *E. coli* growth was observed on pristine catheters as the colony counting indicated too numerous to count. Catheters pPG_1% and pPG_2% showed >6 log<sub>10</sub> reduction in the viability of *S. aureus*. However, a concentration-dependent decrease in *E. coli* burden was observed as no bacteria could be detected on pPG_2% whereas pPG_1% displayed >4 log<sub>10</sub> decrease in bacterial viability. SEM studies further confirmed the increased abundance of bacterial cells on pristine catheters. However, barely detectable colonies could be identified in pPG_2% and the bacterial cells lost smooth surface morphology and appeared truncated,
confirming the antibacterial effect of pPG-coating under these conditions. *(Supplementary Figure S1)*. To examine this remarkable effect, we determined the antimicrobial activities of pPG-coated catheters against a range of Gram-positive and Gram-negative bacteria. Under these conditions, pristine catheter promoted bacterial adhesion, as indicated by the presence of significant amounts of bacteria (>10⁹ CFU/mL) from the bacterial enumeration assay. However, depending on the Gram-negative strains and the concentration of PG in the coating solution, pPG-coated catheters displayed substantial decrease in bacterial adhesion, indicated by nearly no detectable colonies in 8 bacterial strains tested for pPG_2% catheters *(Figure 3)*.

In a systematic study, Taguri et al., compared the antimicrobial properties of simple and complex polyphenols and determined that pyrogallol had superior antimicrobial activity than catechol [38]. Their results further suggested that complex phenols containing PG subunits displayed more potent antimicrobial properties than polyphenols with catechol or resorcinol groups. Though the mechanisms of action of polyphenols remain complex, it has been suggested that the formation of reactive oxygen species could be responsible for the bactericidal properties [39-41].
Figure 2. Antimicrobial properties of pristine and pPG-coated catheters against MRSA 700699 strains assessed by disc diffusion assay. a) Pristine; b) – d) pPG-coated catheters with 0.1%, 1% and 2% initial PG (w/v) concentration, respectively. Note the appearance of brown coloration of catheters and a clear zone of inhibition as the PG concentration was increased.
**Figure 3.** Antimicrobial properties of pristine and pPG-coated against a panel of Gram-negative and Gram-positive bacteria. The strains are indicated in the figure. The horizontal dotted lines represent the minimum detection limit of the bacteria. The data represents mean±sd.

### 3.2. Anti-biofilm properties of pPG-coated catheters

As the pPG-coated catheters prevented the bacterial attachment, we next investigated the efficacy of coated catheters to prevent biofilm formation. Pristine, pPG_1% and pPG_2% catheters were immersed in media containing bacterial inoculum that promoted the biofilm
formation. SEM and resazurin assays were used to confirm the morphology of bacterial biofilms and metabolic activity of the microbial cells. To infer if the inner and outer surfaces had similar/different bacterial bioburden, SEM of both the surfaces were imaged. The results suggested higher MRSA aggregates on the outer surface of the uncoated catheters than inner surface after 24 h (Figure 4a). The pPG coated catheters (pPG_1% and pPG_2%), however, completely abrogated the formation of MRSA biofilms as no trace of bacterial cells could be observed on both inner and outer surfaces (Figure 4a). Consistent with the SEM studies, resazurin assays confirmed weak bacterial metabolic activity on pPG coated catheters (Figure 4b). As the incubation time was increased to 72 h, higher populations of bacterial aggregates were observed on the outer surfaces than on the inner surfaces of the pristine catheters whereas no substantial adherent bacterial cells or aggregates were observed on pPG-coated catheters (Figure 5a). Resazurin assays confirmed the lack of metabolic activity on pPG-coated catheters even after 72 h incubation (Figure 5b). Taken together, these observations establish the anti-biofilm properties of the pPG coating against MRSA strains for at least 72 h. However, both pPG_1% and pPG_2% catheters lacked anti-biofilm properties against E. coli strains, as substantial adherent cells and bacterial aggregates were observed on the surface after 24 h (Supplementary Figure S2).

Interestingly, we observed substantial differences in MRSA biovolume between inner and outer surfaces of pristine catheters. However, no such difference in bioburden was observed when tested on E. coli strains. AFM studies (Supplementary Figure S3) indicated that the root mean square roughness for the inner surface was lower (31.5±4.2 nm) than outer surface (153.3±12.3 nm). It is likely that the smooth texture of the inner surfaces could be responsible for poor MRSA adhesion and biofilm formation than the outer surface. These results corroborate with previous observations that nanopatterning of surfaces could inhibit the colonization of S. aureus more significantly than E. coli [42, 43].
Ni et al reported that pyrogallol and several of its analogues can inhibit autoinducer-2 (AI-2) mediated quorum sensing in *V. harveyi* at sub-micromolar concentration, thus inhibiting the biofilm formation [44]. These authors showed that the formation of borate complex of aromatic vicinal diol and its binding to the LuxP receptor was responsible for the anti-biofilm activity. Thus, it is likely that the lack of antibiofilm properties of pPG coated catheters could be due to the formation of complex bulky polymeric structures which could not bind to the receptor sites.
Figure 4. Antibiofilm properties of pristine and pPG-coated against MRSA DM9808R strains after 24 h. a) SEM images showing the bacterial aggregates formed inner and outer surfaces of the catheters after 24 h. Note that there were no bacterial cells attached to pPG-coated catheters. Scale bar = 1 µm. b) Assessment of metabolic activity of the bacterial cells by resazurin assay. Note a substantial loss of metabolic activity on pPG-coated catheters. The data represents mean±sd from two independent triplicates.
Figure 5. Antibiofilm properties of pristine and pPG-coated against MRSA DM9808R strains after 72h. a) SEM images revealing the bacterial aggregates formed on the inner and outer surfaces of the catheters after 72 h. Scale bar = 1 μm. b) Assessment of metabolic activity of the bacterial cells by resazurin assay. Note a substantial loss of metabolic activity on pPG-coated catheters. The data represents mean±sd from two independent triplicates.
3.3. Effect of incorporating antimicrobial metal ions on antimicrobial properties of coated catheters

A characteristic feature of polyphenols containing catechol motifs is their ability to interact with metal ions in various metastable redox states [45]. Recent studies have shown that Mg\(^{2+}\) display potent antimicrobial activity against *S. aureus* both in planktonic and biofilm form [46, 47]. To broaden the antimicrobial potential of catheters, mineralized coating was developed on their surface from buffer solution containing PG with silver or magnesium salt. After optimization, we examined the antimicrobial properties catheters coated with 1% (w/v) PG and 0.0125% (w/v) AgNO\(_3\) and MgCl\(_2\) were used and followed the labelling of pPG\(_{1\%}\)Ag/Mg. Bacterial adhesion test indicated that the mineral coating did not interfere with the antimicrobial properties of pPG, in fact showed enhancement in their antimicrobial potential ([Supplementary Figure S4](#)) as discussed ahead. Mineralization of the catheters prevented MRSA biofilm formation on both inner and outer surface of the catheters ([Figure 6a](#)). Resazurin assay confirmed the lack of any metabolic activity on mineralized pPG coated catheters ([Figure 6b](#)). Interestingly, the anti-biofilm activity was retained for 7 days when compared to pPG coated catheters (which lasted for 72 h) as no microbial colonization or metabolic activity could be detected on pPG\(_{1\%}\)Ag and pPG\(_{1\%}\)Mg catheters ([Figure 7a-d](#)). In addition, pPG\(_{1\%}\)Ag catheters displayed anti-biofilm properties against *E. coli* strains for 24 h as no bacterial cells could be detected on catheters, whereas pPG\(_{1\%}\) or pPG\(_{1\%}\)Mg lacked any anti-biofilm properties ([Figure 8a-d](#)). Together, these results demonstrate that pPG coating from a solution containing antimicrobial metal ions enhanced the sterility of the surfaces.
Figure 6. Antibiofilm properties of pPG_1%_Ag and pPG_1%_Mg coated against MRSA DM9808R strains after 24 h. a) SEM images indicating absence of any microbial cells formed inside and outside of the coated catheter surfaces. Scale bar = 1 µm. b) Assessment of metabolic activity of the bacterial cells by resazurin assay. Note a substantial loss of metabolic activity on pPG-coated catheters. The data represents mean±sd from two independent triplicates.
Figure 7. Antibiofilm properties of pPG_1%_Ag and pPG_1%_Mg coated against MRSA DM9808R strains after 7 days. For clarity only outer surfaces are presented in the figure a) SEM images showing the formation of dense aggregates of MRSA cells on the surface of the uncoated catheter. b) and c) SEM images indicating the presence of few planktonic MRSA cells on pPG_1%_Ag and pPG_1%_Mg coated catheter surfaces compared to the uncoated catheter, respectively. Scale bar = 1 µm. d) Assessment of metabolic activity of the bacterial cells by resazurin assay. Note a substantial loss of metabolic activity on pPG-coated catheters after incorporation of Ag+ or Mg2+ ions. The data represents mean±sd from two independent triplicates.
Figure 8. a) SEM images displaying the antibiofilm properties of pPG_1%, b) pPG_1%_Ag and c) pPG_1%_Mg coated catheters against *E. coli* 10536 strains after 24 h. Scale bar = 1 µm. d) Assessment of metabolic activity of the bacterial cells by resazurin assay. Note that pPG_1%_Ag coated catheters prevented the formation of *E. coli* biofilms for 24 h. The data represents mean±sd from two independent triplicates.

Bacterial adhesion test confirmed that pPG-mineral coatings did not impair the antimicrobial properties as similar log₁₀ reduction was observed between pPG-coated and pPG-minerals coated catheters. The presence of pPG and mineralization prevented the MRSA biofilm formation for at least 7 days whereas pPG-coating could prevent the biofilm formation for 3 days. pPG_1%_Ag coatings on the catheters delayed the biofilm formation of Gram-negative *E. coli* strains. SEM images indicated substantial decrease in bacterial populations of *E. coli* on the inner surfaces of the catheters coated with pPG_1%_Ag in comparison to pPG_1% or
pPG_2% catheters. These results demonstrate that simultaneous silver and pPG coating would broaden the anti-biofilm properties of the catheters.

The adhesion of bacteria to the surfaces depends on several factors such as surface roughness, degree of hydrophobicity, nanopatterning and chemical structure. Each of these factors alone or in tandem could contribute to the challenges associated with designing antifouling or antimicrobial surfaces. Similar to our study, two previous studies reported the delayed adhesion of *E. coli* to the silver impregnated catheters. Johnson et al. compared commercially available silver-alloy coated catheters and nitrofurazone coated catheters for the adherence properties of 11 pathogenic microbes responsible for CAUTI. Their results suggested that nitrofurazone coated catheters showed significant reduction in bacterial adherence and biofilm formation in comparison to silver-alloy coated catheters [17]. Similarly, Desai et al., reported the lack of antimicrobial properties of silver impregnated catheters [48]. Their results suggested that silver-alloy impregnated catheters did not inhibit the *E. coli* adhesion to the catheter surface as no significant difference in the bacterial bioburden was observed in comparison to catheter without silver alloy. However, Roe et al., reported that plastic catheters coated with silver nanoparticles showed significant *in vitro* antimicrobial and antibiofilm activity against *E. coli, Enterococcus spp, S. aureus*, coagulase-negative *Staphylococci, P. aeruginosa* and *C. albicans* [49]. These authors demonstrated that the release of ionic silver for an extended period of time was responsible for potent antimicrobial and antibiofilm properties. Thus, we suggest that the strong interaction between silver and vicinal diols in pPG could prevent the release of silver ions, thus attenuating the antibiofilm properties.

### 3.4. Hemocompatibility of pPG coatings

To confirm the biocompatibility of pPG coating with or without the antimicrobial metal ions, we determined % hemolysis by exposing the rabbit erythrocytes to the catheters for 24 h. The
coated catheters did not show any statistically significant hemolysis in comparison to pristine catheters (p>0.05), indicating good biocompatibility for blood cells (Figure 9). However, catheters soaked in silver nitrate solution displayed substantial hemolytic activity, indicating pPG-coating attenuated the hemolytic activity of silver or silver ions.

Figure 9. Hemolytic activity of pristine and pPG- or pPG-metal ions coated catheters after incubation in rabbit red blood cells for 24 h. Note that none of the coated catheters display any hemolytic activity whereas catheters coated with Ag⁺ without pPG displayed substantial hemolytic activity. The data represents mean±sd from two independent triplicates.

3.5. Surface characterization of pPG coated catheters

To discern the surface properties of uncoated and coated catheters, we analysed the catheter surfaces in terms of gross morphology (SEM), surface wettability (contact angle measurements) and chemical composition (XPS). SEM images indicated no discernible differences in the gross morphologies of catheter inner surface after coating pPG or pPG containing silver/magnesium ions (Figure 10a, c, e, g). However, the outer surfaces indicated the presence of few electron dense particulate structures on the outer surfaces of pPG, pPG_1%_Ag and pPG_1%_Mg catheters (Figure 10b, d, f, h). SEM studies also showed that simultaneous pPG and mineralization did not alter the surface properties of catheters in comparison to catheters coated with pPG alone. Surface wettability or the degree of hydrophobicity was assessed by the contact angle measurements at 10 minutes after
application of the water droplet. The results indicated that contact angle ($\theta_{\text{static}}$) decreased after pPG coating (Figure 11a, b) as indicated by a decrease in $\theta_{\text{static}}$ values from $93.3\pm3.9^\circ$ to $61.6\pm4^\circ$. Incorporation of silver and magnesium along with the pPG coating did not alter $\theta_{\text{static}}$ values significantly (Figure 11c-e). These results suggest that pPG coating with or without the metal ions enhanced the wettability of the surface when compared to pristine catheter surface. Contact angle measurements together with antimicrobial and anti-biofilm assay results indicated that pPG with metal ions coating rendered the surface less hydrophobic than pristine surface, yet prevented the formation of MRSA biofilms for 72 h.

XPS analysis was performed for the pristine and pPG coated catheters containing metal ions (pPG$_1$%Ag and pPG$_1$%Mg) in order to confirm the formation of mineralized coatings on catheter surface. All samples showed the presence of carbon (C 1s peak), oxygen (O 1s peak) and nitrogen (N 1s peak), while weak Ag and Mg peaks appeared in pPG$_1$%Ag and pPG$_1$%Mg samples, respectively, confirming successful incorporation of metal ions (Figure 12a). Low concentration of metal ions (0.0125% w/v) could be responsible for the manifestation of trivial peaks for Ag and Mg. While the spectral shape of C 1s, N 1s and O 1s spectra appear to be similar for pristine and pPG$_1$%Ag samples, a dramatic change in the spectral shape was observed for pPG$_1$%Mg in terms of substantial peak broadening and splitting. The observed spectra indicated extensive changes in the bonding pattern at the catheter surface covered with the formation of pyrogallol-magnesium mineral coating.

To obtain an insight into a surface chemical bonding of pristine and modified catheters, the deconvolution of C 1s, N 1s and O 1s spectra was performed (Figure 12b). The deconvolution of C 1s spectra of all samples revealed four constituent peaks C$_1$, C$_2$, C$_3$ and C$_4$ which correspond to C-C/C-H, C-N, C-O and C=O bonding, respectively [50]. The deconvolution of N 1s spectra revealed three constituent peaks namely N$_1$, N$_2$ and N$_3$ for all samples which assign to R$_2$NH, RNH$_2$ and C=NR bonding, respectively [51]. Similarly, the
Deconvolution of O 1s spectra revealed two constituent peaks O₁ and O₂ in all samples which correspond to C=O and C-O bonding, respectively. The N 1s and O 1s spectra of pPG_1%_Mg sample revealed some additional peaks namely N₄ peak in N 1s spectrum which assigns to N-Oₓ bonding and O₃ and O₄ peaks in O 1s spectrum. The emergence of O₃ peak in pPG_1%_Mg catheter at ~ 533.5 eV can be assigned to formation of magnesium carbonate [52-54]. The origin of magnesium carbonate peak in O 1s spectrum of PG_1%_Mg may be resulting from the reaction of MgCl₂ and sodium bicarbonate salts available in the buffering medium used for coating. On the other hand, O₄ peak can be attributed to adsorbed water peak [52]. XPS results confirm the formation of mineral phase on polypygallol coating, thus conferring increased protection against MRSA adhesion and biofilm formation.

It has been well documented an average surface roughness in the range of only few nm or sub nm could facilitate bacterial adhesion although the type of biomaterial, chemical composition of the surface, surface free energy and surface hydrophobicity as well contribute to the microbial colonization and biofilm formation [55, 56]. Thus a more facile approach in making antifouling (or antimicrobial surfaces) would be active coating strategies which show on-surface antimicrobial activity to exercise contact killing of the adhering bacteria [57-59]. Xin et al’s work on polymer coating on catheters proposed that the hydrophobic component in their polymer enhances the interaction in the lipid domains of the bacterial membrane and providing more bactericidal action to the coating [36]. Hydrophilic materials and coatings provide low adhesion surfaces. In our approach, we used simple organic molecule (pyrogallol) which has inherent antimicrobial properties and readily forms adherent coatings on polymeric surfaces and established the antimicrobial properties and biocompatibility. The present study opens up the possible applications of antimicrobial coatings of pPG with metal ions. The versatility of the coating conditions may be expanded to cover both polymeric and metallic surfaces, thus conferring potent antimicrobial properties.
Figure 10. SEM images of inner and outer surfaces of pristine and pPG-coated catheters. Note that pPG coating with or without antimicrobial silver or magnesium ions (0.0125% w/v) did not alter the morphology of the surfaces. Scale bar = 1 µm.
Figure 11. Wettability of catheter surfaces determined by water contact angle measurements. Digital photos of the water droplets placed on catheter surfaces a) Pristine; b) pPG_1%; c) pPG_1%_Ag and d) pPG_1%_Mg. e) Average static contact angle ($\theta_{\text{static}}$) determined after 10 minutes. The Ag$^+$ and Mg$^{2+}$ concentrations were 0.0125% w/v. The data represents mean±sd from two independent duplicates.
Figure 12. a) High resolution C 1s, N 1s, O 1s, Ag<sup>+</sup> 3d and Mg<sup>2+</sup> 2p spectra and b) Deconvolution of C 1s, N 1s and O 1s spectra of pristine (Cath) and mineralized pPG coated catheters (Ag/Mg_Cath) showing various constituent peaks.
4. CONCLUSION

In summary, we have demonstrated a simple methodology of coating catheter surfaces with pyrogallol alone and in combination with metal ions Ag⁺ and Mg²⁺. This method of coating is easy, cost effective and develops disinfected surfaces having broad spectrum antimicrobial activity with no toxic effects on rabbit RBCs. This method of coating could be a promising alternative to antibiotic coated catheters which prevents the development of drug resistant strains and controls the spread of healthcare associated infections.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgements

This research is supported by the Singapore National Research Foundation under its Translational and Clinical Research Flagship Program (NMRC/TCR/008-SERI/2013) and administered by the Singapore Ministry of Health’s National Medical Research Council. RL thanks the funding support from National Medical Research Council's Co-operative Basic Research Grant (NMRC/CBRG/0048/2013). This research is also supported by the Singapore Ministry of Health’s National Medical Research Council under its Centre Grant Programme-Optimization of core platform Technologies for Ocular Research (INCEPTOR)-NMRC/CG/M010/2017_SERI.
References


