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Anhydrous polymer-based coating with sustainable controlled release functionality for facile, efficacious impregnation and delivery of antimicrobial peptides

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Abstract

Anhydrous polymers are actively explored as alternative materials to overcome limitations of conventional hydrogel-based antibacterial coating. However, the requirement for strong organic solvent in polymerization reactions often necessitates extra protection steps for encapsulation of target biomolecules, lowering encapsulation efficiency and increasing process complexity. This study reports a novel coating strategy that allows direct solvation and encapsulation of antimicrobial peptides (HHC36) into anhydrous polycaprolactone (PCL) polymer-based dual layer coating. A thin 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) film is layered onto the peptide-impregnated PCL as a diffusion barrier, to modulate and enhance release kinetics. The impregnated peptides are eventually released in a controlled fashion. The use of 2,2,2-trifluoroethanol (TFE), as polymerization and solvation medium, induces the impregnated peptides to adopt highly stable turned conformation, conserving peptide integrity and functionality during both encapsulation and subsequent release processes. The dual layer coating showed sustained antibacterial functionality, lasting for 14 days. *In vivo* assessment using an experimental mouse wounding model demonstrated good biocompatibility and significant antimicrobial efficacy of the coating under physiological conditions. The coating was translated onto silicone urinary catheters and showed promising antibacterial efficacy, even outperforming commercial silver-based Dover catheter. This anhydrous polymer-based platform holds immense potential as an effective antibacterial coating to prevent clinical device-associated infections. The simplicity of the coating process enhances its industrial viability.

1. Introduction

Bacterial nosocomial infection represents one of the biggest problems faced by healthcare facilities worldwide. A recent survey by the United States Center for Disease Control and Prevention reported an estimate of 722,000 cases of nosocomial infections annually. This number represents approximately 10% of all the patients admitted into acute care hospitals in the United States (Wenzel, 2007), and affects millions of patients worldwide each year. 50% of these infections are related to implantable medical devices (Guggenbichler, Assadian, Boeswald, & Kramer, 2011), with urinary catheter-associated urinary tract infection (CAUTI) accounting for the majority (19%) of such device-associated infections (Maki & Tambyah, 2001; Zorgani, Abofayed, Glia, Albarbar, & Hanish, 2015).

Clinical treatment for nosocomial infections usually involves systemic administration of antibiotics. Heavy reliance and abusive use of antibiotics has resulted in the rapid emergence of antimicrobial resistant microbes, especially in patients with indwelling medical device. For example, there has been a rise in UTI patients (many of whom had CAUTI) developing ciprofloxacin resistance from 3% in 2000 to 17.1% in 2010 (Sanchez, Master, Karlowsky, & Bordon, 2012; Zowawi et al., 2015). Confronted by these issues, an optimized delivery platform, which provides localized, site-specific delivery of antibacterial agents, may be a good alternative to the conventional systematic antibiotic treatment.

The use of controlled release coatings for localized, site-specific delivery of antibacterial agent to the infected site, has been developed and implemented on a variety of implantable medical devices. Examples include Dover^{TM} silver-coated urinary catheters and Rochester Medical Magic³ nitrofurazone catheters. Mixed results were observed for such coated medical device implants (Lee et al., 2017; Riley, Classen, Stevens, & Burke, 2017). A possible reason accounting for the inconsistency of these antibacterial coatings could be due to the inability of these coatings to sustain a continuous, significant release profile for prolonged infection control.

Confronted by the limitations of existing antibacterial coatings, the development of a superior coating to effectively target and prevent device-associated bacterial infection is urgently needed, which forms the basis of this study. This study reports the development of a novel dual layer anhydrous polymer-based coating strategy that allows direct solvation, impregnation and sustained release of a potent antimicrobial peptide (AMP) candidate, HHC36. This AMP-releasing coating platform provides an immediate, practical and cost effective solution to minimize device-associated infection incidences, directly benefitting patients, care givers and physicians.

2. Materials and Methods

2.1. Materials

Peptide HHC36 (KRWKWWRR) was synthesized by Bio Basic Inc. (Canada) using commercial Fmoc chemistry to a purity of >90%. Chemicals were purchased from Sigma Aldrich (Singapore), unless otherwise stated. Human uropathogenic *Escherichia coli* strain UTI89 (*E. coli*) and the fluorescent protein-expressing strain (*E. coli*-GFP) (Wright, Seed, & Hultgren, 2005), *Staphylococcus aureus* ATCC 25923 (*S. aureus*) and *Pseudomonas aeruginosa* PAO1 (*P. aeruginosa*) were used for respective antimicrobial and anti-biofilm studies. Dover^{TM} silver coated 100% silicone Foley catheters (Dover catheter) were purchased from Covidien Private Limited (USA).

2.2. Synthesis of HHC36-impregnated dual layer coating (PCL(P)-POPC(P))

HHC36-laden dual layer coatings were prepared in wells of 96-well plates using a simple drop-and-dry technique. 10% (w/v) poly- ϵ -caprolactone (PCL) solution was prepared by dissolving 0.5 g PCL pellet in 5.0 mL 2,2,2-trifluoroethanol (TFE). 5.0 mg HHC36 was subsequently loaded into 500 μ L 10% (w/v) PCL solution and the mixture was vortexed and allowed to stand under atmospheric condition (25°C, 1 h) to facilitate complete solvation of the peptide. 100 μ L of the HHC36-rich PCL solution was transferred into each well and dried under atmospheric

condition (25°C) for 16 h for vaporization of the TFE solvent and form the single layer HHC36-impregnated PCL film (PCL(P)). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (Avanti Polar Lipids Inc., USA) was dissolved in ethanol to obtain a final concentration of 26 mM POPC solution. 100 µL of the POPC solution was pipetted onto the PCL(P) surface, and dried in air under room temperature (25°C, 16 h). HHC36 from the basal PCL(P) diffused into the newly formed POPC layer to form the dual layer AMP-laden PCL-POPC coating (PCL(P)-POPC(P)). Thickness of PCL(P)-POPC(P) film was measured using a digimatic thickness gauge (574-401, Mitutoyo, Japan). (P) annotates impregnation of HHC36 peptide within the respective layers.

2.3. PCL(P)-POPC(P) coating surface characterization

Scanning electron microscopy (SEM)

Surface morphologies of PCL(P) and PCL(P)-POPC(P) coatings were processed and viewed under SEM (JSM6390LA, JEOL, Japan). Briefly, sample coatings were sputter-coated with a thin layer of platinum (JFC-1600, JEOL, Japan) and kept in a 40°C oven before viewing.

Energy dispersive X-ray spectroscopy (EDS) and surface elemental mapping

Surface elemental composition and mapping of platinum sputtered PCL(P)-POPC(P) coatings were studied using a field emission scanning electron microscope (FESEM) (JSM6701, JEOL, Japan) equipped with EDS (X-Maxⁿ, Oxford Instruments, UK) analysis. Analysis was carried out at beam current of 11 µAh while ion energy was maintained at 15 kV.

Attenuated total reflectance fourier transform infrared (ATR-FTIR) spectroscopy

Transmission spectrum was recorded using a FTIR spectrometer (Spectrum One, Perkin Elmer, USA) with a MIRacle ATR accessory unit (PIKE technologies, USA) attached. Transmittance was recorded between 1000 cm⁻¹ to 4000 cm⁻¹, with 64 scans conducted for each run.

2.4. HHC36 peptide release kinetics

In vitro HHC36 release profile from the PCL(P)-POPC(P) coating, in wells of 96 well plate, was determined by absorbance measurement at 280 nm using Nanodrop 2000 (Thermo Scientific, USA). PCL(P)-POPC(P) coating in each well was exposed to 100 μ L of phosphate buffered saline (PBS) solution and incubated at 37°C. After 24 h incubation, the PBS solution (100 μ L) was withdrawn and sampled for peptide release. The well was subsequently replenished with 100 μ L of fresh PBS. The process was repeated on a daily basis for 14 days. A series of HHC36 standards with concentrations ranging from 4 μ g/mL to 1000 μ g/mL was used for calibration purposes.

2.5. Released HHC36 conformation and functionality assessment

Circular dichroism (CD) spectroscopy

Far UV spectroscopy analysis of the released and native HHC36 in (i) deionized water and (ii) 50% TFE, was performed using Chirascan Circular Dichroism Spectrometer (Applied Photophysics Ltd, U.K.). Spectra were recorded between 200 nm and 260 nm at room temperature (25°C), in 1 nm steps. All measurements were carried out in 0.1 mm quartz cuvette. Blank spectra of solutions without peptides, obtained under identical conditions, were recorded as background and subtracted from the spectra generated from each peptide-containing sample.

1 dimensional Nuclear magnetic resonance (1D-NMR) spectroscopy

NMR experiments were performed using a Bruker DRX 600 MHz spectrometer (Germany), equipped with cryo-probe and pulse field gradients. NMR data processing and analysis was carried out using Bruker Topspin program. 1D proton spectra was recorded with 0.5 mM of the native and PCL(P)-POPC(P) released peptide (from 24 hours post initiation of release) in water (pH 3.0) containing 10% deuterium oxide and 4,4-dimethyl-4-silapentane-1-sulfonic acid as internal standard. Spectral width was maintained at 14 ppm and a total of 32k data points were collected for 16 rounds of scan at 25°C. The solvent signal was suppressed by

WATERGATE w5 pulse sequences with gradient using double echo method (Sklénar, Piotto, Leppik, & Saudek, 1993).

Fluorescence spectroscopy

Fluorescence emissions of released and native HHC36 in (i) deionized water and (ii) 10 μ M lipopolysaccharide (LPS), were measured with a fluorescence spectrometer (LS5, Perkin Elmer, USA) at 25°C using a quartz cuvette of 0.5 cm pathlength. Emission spectra were recorded between 300 nm and 400 nm, with excitation at 280 nm. Excitation and emission slit widths were set to 5.0 nm.

2.6. PCL(P)-POPC(P) antimicrobial properties determination

PCL(P)-POPC(P) coatings, in 96-well plates were subjected to an adapted broth microdilution antimicrobial assay (Wiegand, Hilpert, & Hancock, 2008). 100 μ L of *E. coli* culture (5×10^4 CFU) in nutrient rich *Mueller Hinton* (MH) broth were added to the PCL(P)-POPC(P)-coated wells, and incubated at 37°C for 24 h. Bacterial growth in the resulting suspension was assayed by CFU enumeration. The long term antimicrobial activity of the coating was investigated by subjecting the PCL(P)-POPC(P) coating to 14 consecutive cycles of the antimicrobial assay as described above. CFU counts of each overnight suspension were determined after every cycle of the antimicrobial assay, as an indication of bacterial growth. Non AMP-impregnated PCL-POPC coating (PCL-POPC) was used as control for the long term antimicrobial activity study.

2.7. PCL(P)-POPC(P) *in vivo* antibacterial efficacy and safety study

E. coli-GFP was cultured at 37°C in Luria-Bertani (LB) broth under static conditions. The infecting inoculum was prepared by harvesting the bacterial pellet in PBS and normalizing the suspension to $2 - 4 \times 10^6$ CFU/ml. The experimental mouse model of excisional wound infection was performed as described by Chong *et al.* (Chong *et al.*, 2017a). Briefly, male wild-type C57BL/6 mice (7-8 weeks old, 22 to 25g; InVivos, Singapore) were anesthetized with 3 % isoflurane. Hair on the back of the mice was trimmed and blade shaven after application of

Nair™ shaving cream (Church and Dwight Co, Charles Ewing Boulevard, USA). Skin surface was disinfected with 70% ethanol before wounding using a 6 mm biopsy punch (Integra Miltex, New York, USA). Wounds were inoculated with 10^4 (10 μ l) CFU *E. coli*-GFP, left to air-dry for 5 min before introducing PCL(P)-POPC(P), containing 1 mg of HHC36, and non AMP-impregnated control coatings (PCL-POPC) to cover the wound cavity. Wounds were sealed with a dressing (Tegaderm™ 3M, St Paul Minnesota, USA). Mice were euthanized at 24 h post infection by carbon dioxide inhalation and a 1 cm² area surrounding the wound site was excised and homogenized in 1 ml PBS. Bacterial burden was assessed by CFU enumeration. Mouse experiments were performed with ethical approval by the ARF-SBS/NIE Nanyang Technological University Institutional Animal Care and Use Committee under protocol ARF-SBS/NIE-A0198.

2.8. Application of PCL(P)-POPC(P) coating onto commercial silicone Foley catheter

100% silicone Foley catheter (16 Fr, Multigate, Australia) was cut into 1.0 cm (length) segments and physically coated with the PCL(P)-POC(P) coating *via* dip-coating. Briefly, the catheter samples were immersed in 10% (w/v) PCL solution, containing 10 mg/mL HHC36, for 10 s. The catheter segments were withdrawn slowly and left to dry at room temperature (25°C) for 24 h. The single layer HHC36-laden PCL(P)-coated catheter was then dipped into 26 mM POPC solution (in ethanol) for 3 s. Catheters were subsequently withdrawn rapidly and dried under atmospheric condition (25°C, 24 h) to form the peptide impregnated dual layer coated catheter (CAT-PCL(P)-POPC(P)).

2.9. Cat-PCL(P)-POPC(P) mechanical analysis

Mechanical properties (i.e. elasticity and tensile strength) of Cat-PCL(P)-POPC(P) were analyzed using a TA.XTPlus texture analyzer (Stable Micro Systems, UK). The equipment was attached with a 50 kg load cell, equipped with a A/MTG mini tensile grip accessory. Cat-PCL(P)-POPC(P) and respective control catheters were fixed onto the two tensile grips with

an initial separating distance of 10 mm between the two grips. The tests were conducted under the “tension” mode. The respective catheter samples were being stretched at a speed of 3.0 mm/s until a total strain of 50% is achieved, after which the probe will return to the initial starting position. The applied stress was recorded as a function of strain. Tensile strength of the respective catheters was calculated with software Texture Exponent 32.

2.10. Cat-PCL(P)-POPC(P) antimicrobial and anti-biofilm characterization

Cat-PCL(P)-POPC(P), Dover catheter and uncoated silicone catheter (Cat) were subjected to the following antimicrobial assay. 500 μ L of *E. coli* culture (5×10^4 CFU) in nutrient rich *Mueller Hinton* (MH) broth was added to 2.0 mL microtubes containing the respective catheter samples and incubated at 37°C for 24 h. CFU enumeration of the resulting overnight bacteria suspensions were conducted to determine bacteria growth. The antimicrobial assay was repeated for testing with *S. aureus* and *P. aeruginosa*.

The anti-biofilm property of Cat-PCL(P)-POPC(P) and control catheters were evaluated by similar bacteria adherence assay. Briefly, the catheter samples were immersed into microtubes containing 500 μ L of $\sim 1 \times 10^7$ CFU/mL *E. coli* in biofilm promoting medium. The samples were incubated at 37°C for 48 h and 168 h respectively. After incubation, catheter samples were removed and rinsed thrice with fresh PBS solution. A portion of the samples were fixed with glutaraldehyde, treated and imaged using SEM (JSM 5600LV, JEOL, Japan), while the rest of the samples were re-immersed in 1.0 mL fresh PBS, sonicated and vortexed to remove the adherent bacteria cells for CFU enumeration.

2.11. Cat-PCL(P)-POPC(P) biocompatibility determination

Hemocompatibility assay

Cytotoxicity of Cat-PCL(P)-POPC(P) samples against human red blood cells (hRBCs) was investigated using a stringent hemolytic assay (Lim et al., 2013, 2015). Cat-PCL(P)-POPC(P) and control catheters were immersed in 1.0 mL 5% (v/v) hRBCs in PBS, and subsequently

incubated for 24 h at 37°C. Intact hRBCs were spun down by centrifugation for 5 min at 800 g and supernatant was withdrawn for absorbance measurement at 540 nm. Erythrocytes incubated with PBS and 1% Triton X were used as negative and positive controls respectively.

Uroepithelial cell viability assay

Cat-PCL(P)-POPC(P) and control catheter samples were tested for cytotoxicity against uroepithelial cell-line (SV-HUC-1) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. SV-HUC-1 cells were cultured in F-12K medium, supplemented with 10% FBS (Hyclone GE Healthcare Life Sciences, USA) in a 37°C humidified incubator. Cells were seeded into wells of a 24-well tissue culture plate at a concentration of $\sim 4 \times 10^5$ cells per well. Overnight cell cultures, presenting 80% confluency, were washed once with PBS followed by addition of respective catheter samples to the cell cultures and supplementing with 1.0 mL of fresh F-12 K medium to the wells. After 24 h incubation, samples were removed by aspiration and cells were washed once with PBS, followed by the addition of 1 x MTT reagent (0.5 mg/mL, 500 μ L) and incubation at 37°C for 1 h. MTT reagent was subsequently removed and cells were washed once with PBS, followed by cell lysis with 500 μ L of dimethylsulfoxide (DMSO). Violet formazan crystals were allowed to dissolve in the DMSO, with gentle agitation on a rocker for 30 mins. Optical absorbance of the samples was measured at 570 nm. Uroepithelial cells incubated with PBS and 1% triton X were used as negative and positive controls, respectively.

2.12. Statistical analysis

All experiments were conducted in triplicate (unless otherwise stated), with average and standard deviation calculated for all measurements. The non-parametric Mann-Whitney U-test was applied for analyzing differences in parameters between the PCL(P)-POPC(P) and respective control groups. Statistical data analysis was conducted using Graphpad Prism (Version 6, Graphpad Software Inc., USA).

3. Results

3.1. Development of dual layer AMP-impregnated polymer coating

Peptide HHC36 was directly solvated in TFE-based PCL suspension. Good peptide-solvent compatibility was achieved, yielding a clear suspension. The AMP-polymer suspension was aliquoted into 96-well plate wells and left to dry under atmospheric condition, which formed a single layer HHC36-laden PCL polymer coating (PCL(P)). The PCL(P) was then layered with an additional POPC thin film to yield a dual layer PCL(P)-POPC(P) coating with a thickness of $115 \pm 46 \mu\text{m}$ (Figure 1).

3.2. Characterization of the PCL(P)-POPC(P) coating

Surface morphologies of the PCL(P) and PCL(P)-POPC(P) coatings were studied using scanning electron microscopy (SEM). The PCL(P) coating layer had an uneven surface morphology (Figure 2a, top), with relatively large circular indentations measuring $143.63 \pm 24.15 \mu\text{m}^2$. The addition of a POPC layer covered the indentation, presenting the dual layer coating with a smooth overall surface morphology (Figure 2a, bottom and Figure S1). EDS was employed to verify the chemical composition of the dual layer coating. Surface elemental scanning showed abundance of carbon ($46.28 \pm 3.63\%$) and oxygen ($11.08 \pm 2.83\%$) on the PCL(P)-POPC(P) dual layer coating (Table S1). The carbon and oxygen contents were largely contributed by the caprolactone monomer, POPC and peptides components, which made up the dual layer coating. Nitrogen ($1.93 \pm 0.66\%$) was also detected on the PCL(P)-POPC(P) coating, but not on the non-AMP-laden PCL-POPC control coating. While the top POPC film might have contributed to a minor amount of nitrogen content detected, most of the nitrogen should originate from the multiple amide groups present on the side chain of arginines as well as amide bonds within the impregnated peptides. Detection of nitrogen confirmed successful impregnation of peptide within the dual layer coating. Surface nitrogen mapping demonstrated that peptides were uniformly distributed across the PCL(P)-POPC(P) coating, indicating

successful and homogeneous solvation of target HHC36 into the polymer solution during the prior synthesis process (Figure 2b).

The ATR-FTIR spectrum of the PCL(P)-POPC(P) coating is shown in Figure 2c. Distinctive peaks corresponding to POPC were observed in the spectrum (Kazemzadeh-Narbat et al., 2013). Two additional transmittance peaks, typically absent in the POPC spectrum, were observed at 1536 cm^{-1} and 1675 cm^{-1} . These transmittance peaks are characteristic amide I and II bands, which indicates the presence of peptides within the coating network (Zhao & Tamm, 2003). The ATR-FTIR result further confirmed the successful impregnation of target AMPs within the dual layer PCL(P)-POPC(P) coating.

3.3. AMP release kinetics from dual layer PCL(P)-POPC(P) coating

Upon exposure to aqueous medium, the HHC36 impregnated within the PCL(P)-POPC(P) dual layer coating was gradually released. Figure 3 shows the HHC36 release profile of the PCL(P) and PCL(P)-POPC(P) coatings over a period of 14 days. Both coatings illustrated similar peptide elution profiles, with a sharp initial burst release followed by subsequent continuous release. The PCL(P)-POPC(P) coating exhibited a moderated burst release (10% of total loaded HHC36s) in the initial 24 h, followed by a continuous sustained release ($\geq 2 \mu\text{g/day}$) for the next 13 days. In contrast, the single layer PCL(P) coating showed a steeper burst release, coupled with a less sustainable subsequent release, with peptide release ceasing after day 6.

3.4. Characterization of released HHC36 peptide

Antibacterial activities of AMPs are often strongly correlated to their structure. It is imperative that the peptide encapsulation and subsequent release processes do not compromise peptide integrity, adversely affecting the biofunctionalities. The stability of the impregnated peptides was investigated using a variety of spectroscopic analysis including CD spectroscopy, 1D NMR analysis, and fluorescence emission spectroscopy. High degree of similarities between

the released and native peptides suggested conservation of peptide structural integrity and biofunctional features during coating impregnation and subsequent release process.

CD spectroscopy was used to investigate the secondary structures of the released HHC36 in different environments, neutral deionized water (Figure 4a) and 50% TFE (Figure 4b). The released and native peptides showed similar CD spectroscopic fingerprints in different solvent environments. The absence of prominent peak maxima or minima for both released and native HHC36 in deionized water, suggested the adoption of a random uncoiled conformation by the peptides. Upon exposure to 50% TFE solution, both peptides adopted a turned conformation, characterized by a prominent negative trough at 220-225 nm, caused by interaction and stacking of the indole rings of tryptophan residues at position 4 and 7 (Nichols et al., 2013).

¹D-NMR analysis of the released peptide revealed preservation of functional groups, which is crucial for its antimicrobial activity. Down-field shifted indole protons of the four tryptophan residues (10.00 - 10.20 ppm), and backbone, along with sidechain amine groups (7.0 – 9.0 ppm), were readily identifiable in both the released and native HHC36 spectrum (Figure 4c and 4d). Released and native HHC36 peptides were also analysed using tandem mass spectroscopy (MS/MS), where similarity in fragmentation pattern and multitude of corresponding peaks were clearly comparable for both native and released peptides (data not shown).

Fluorescence emission spectra were recorded to determine HHC36 association pattern with the bacteria membrane. The emission spectra of the released peptide in both neutral deionized water and 10 µM gram negative membrane component LPS, were comparable to those of the native peptide (Figures 5a and 5b). Both released and native HHC36 registered fluorescence maxima at ~357 nm and ~353 nm in deionized water and LPS respectively. A blue shift of the same magnitude (i.e. 4 nm) was observed for both peptides. The small differences in respective peak height could be attributed to small peptide concentration differences. Similarities in

emission spectra suggested a similar pattern of membrane interaction for the released and native HHC36.

3.5. Antimicrobial potency and sustainability of PCL(P)-POPC(P) coating

Antimicrobial activities of the PCL(P)-POPC(P) dual layer coating and the respective non peptide-impregnated controls (i.e. PCL, POPC and PCL-POPC) were evaluated (Figure S2). Except for PCL(P)-POPC(P) coating, which exhibited total killing of the inoculated 5×10^4 CFU *E. coli*, none of the controls showed significant bactericidal action. This result indicated that the antimicrobial activity observed from the PCL(P)-POPC(P) coating, was attributed to the bactericidal property of the released peptides.

To investigate the antibacterial sustainability of the coating, the PCL(P)-POPC(P) coating was subjected to further cycles of repeated bacterial inoculations at 24 h intervals. Bacterial growth was significantly inhibited up to 13 inoculation cycles (Figure 6). The result corresponded well with the peptide release profile of PCL(P)-POPC(P) (Figure 4), where significant peptide release was observed over approximately 14 days (Section 3.3).

3.6. PCL(P)-POPC(P) *in vivo* antibacterial efficacy

In vivo antibacterial performance of the dual layer PCL(P)-POPC(P) coating was evaluated using an established mouse wounding model (Chong et al., 2017b; Keogh et al., 2017). High titer *E. coli* wound infection was established in ~44% of control mice (treated with non-peptide impregnated PCL-POPC), with a median burden of 2.80×10^4 CFU and a maximum burden of 10^8 CFU at 24 hpi. By contrast, PCL(P)-POPC(P) treatment significantly prevented high titer infection, with a lower median wound microbial count of 1.52×10^3 CFU ($P < 0.005$) (Figure 7a). Importantly, CFU enumeration of the PCL(P)-POPC(P) coatings retrieved from the infected wounds showed significantly lower bacterial adherence (median: 2.00×10^2 CFU), in contrast to the non AMP-laden controls (median: 2.40×10^7 CFU) ($P < 0.005$) (Figure 7b). The

results highlighted the capability of the HHC36-impregnated dual layer coating to maintain its anti-infective and anti-biofilm properties under dynamic physiological conditions.

3.7. Application of PCL(P)-POPC(P) coating onto urinary catheter surfaces

To test its applicability on commercial medical devices, the PCL(P)-POPC(P) coating was applied onto commercial silicone urinary catheter surface. The coating process was done *via* simple dip-coating procedures.

3.8. Tensile strength of Cat-PCL(P)-POPC(P)

Effect of applying the dual layer coating on the mechanical properties of urinary catheter was assessed. No significant difference in the stress-strain profile (Figure 8a) and absolute tensile strength (figure 8b) between the cat-PCL(P)-POPC(P) and respective control catheters were observed. Peptide impregnation and application of reported PCL(P)-POPC(P) coating does not adversely affect the mechanical properties of the silicone urinary catheter.

3.9. Antibacterial efficacy, anti-biofilm property and biocompatibility assessment of PCL(P)-POPC(P)-coated urinary catheters

The antibacterial efficacy of the PCL(P)-POPC(P)-coated urinary catheter (Cat-PCL(P)-POPC(P)) was tested against common CAUTI etiological agents, *E. coli*, *P. aeruginosa* and *S. aureus*. The Cat-PCL(P)-POPC(P) significantly inhibited planktonic growth of the respective bacteria strains, relative to the uncoated silicone catheters (Figure 9a). Bactericidal performance of the Cat-PCL(P)-POPC(P) also exceeds the Dover antimicrobial catheter.

MIC of native HHC36 against high initial bacteria ($\sim 1 \times 10^8$ CFU/mL) has been determined to be 125.00 ± 0.01 $\mu\text{g/mL}$ (Table S2). Upon exposure to 1×10^8 CFU/mL of bacteria, in which the peptide release from the polymer coating was insufficient to completely target all the planktonic microbes, negligible amount of biofilm was detected on the treated catheter surface (Figure 9b). Imaging of the catheter surface showed only a small amount of bacteria on the Cat-PCL(P)-POPC(P) surface (Figure 9c). By contrast, untreated silicone catheter (Cat)

1 allowed exponential proliferation of planktonic microbes and exhibited a surface colonized
2 with a dense layer of biofilm. Cat-PCL(P)-POPC(P) clearly outperformed the Dover catheter
3 in preventing bacteria adherence under such bacteria-rich condition, where 100.0 ± 0.0 %
4 reduction in bacteria adherence for Cat-PCL(P)-POPC(P) was observed as compared to 11.1
5 ± 3.2 % reduction for the Dover catheter. Upon 7 days of incubation, the cat-PCL(P)-POPC(P)
6 maintained its anti-biofilm properties. All measurements were relative to untreated catheters.
7 Biocompatibility of Cat-PCL(P)-POPC(P) against mammalian cells was also assessed (Figure
8 9d and 9e). No severe cytotoxic effect was observed upon prolonged incubation (24 h) with
9 hRBCs and uroepithelial cells, where hemolytic rate (2.12 ± 0.10 %) and uroepithelial cell
10 viability (~ 100 %) were maintained within the acceptable clinical level.

11 **4. Discussion**

12 The reliance on systemic administration of antibiotics to treat nosocomial infections is not
13 sustainable as the method is impeded by challenges such as inefficient drug delivery to target
14 site, bacteria resistance development as well as potential systemic toxicity (Kazemzadeh-
15 Narbat et al., 2013; Zilberman & Elsner, 2008). This is especially true for device associated
16 infections such as CAUTI, where infection occurs in the urinary tract with an indwelling device,
17 which bypasses normal host defenses. An efficient antibacterial coating with bactericidal
18 functionality is needed to overcome the aforementioned challenges (Zilberman & Elsner, 2008).
19 This can be achieved through the use of a targeted delivery coating that can maintain localized
20 and sustained release of antimicrobial compound. An optimal depot coating formulation should
21 exhibit high initial release for site sterilization, during the early post-implantation period when
22 risk of infection is high, followed by a sustained and uninterrupted biocide release as
23 prophylaxis (Kazemzadeh-Narbat et al., 2013; Vasilev, Cook, & Griesser, 2009).
24 Hydrogels are commonly chosen as candidate materials for controlled release purpose. While
25 the aqueous-based material may offer numerous advantages, hydrogel coatings still suffer from

many limitations including low mechanical strength (Hoffman, 2002), uncontrollable release profile (Hoare & Kohane, 2008), problematic applicability to biomedical devices with non-standard contour (Hoare & Kohane, 2008), and requirement for minimum thickness. These issues severely restrict practical usage of hydrogel-based coatings for biomedical devices.

An anhydrous controlled release polymer coating can potentially overcome the aforementioned limitations of hydrogel coatings. These anhydrous polymers generally possess robust mechanical properties, low porosity to maintain slow release kinetic and simple synthesis processes. PCL, in particular has been frequently reported in a variety of biomedical and biotechnology applications (Low et al., 2013; Luong-Van et al., 2006; Woodruff & Hutmacher, 2010). *Chang et al.* showed that by encapsulating antibiotic (gentamycin) in PCL, a sustained drug release for up to 14 days could be achieved (Chang, Perrie, & Coombes, 2006). However, the use of PCL polymer as coating material is not without its drawbacks. The harsh organic condition required for PCL ring opening polymerization reaction often presents a challenge for encapsulating sensitive therapeutic biological entities, such as proteins and peptides (Pace, Treviño, Prabhakaran, & Scholtz, 2004; van de Weert, Hennink, & Jiskoot, 2000). To preserve the functionalities of these biomolecules, extra protection steps (e.g. PEGylation and nanoprecipitation) are required prior to contact with the common organic solvents (Diwan & Park, 2001; Morales-Cruz et al., 2012). While widely reported, such protection techniques are often associated with lowered encapsulation efficacy, complicated synthesis and high cost.

This study reports the development of a novel coating platform comprising a dual layer thin film assembly, i.e. a PCL basal layer topped with a thin POPC film, that allows direct impregnation and facilitates sustained release of a potent AMP candidate (HHC36). The 9-mer synthetic peptide is reported to possess good bactericidal properties against a broad spectrum of microbes (Cherkasov et al., 2008), serving as an excellent candidature antimicrobial compound for the coating. HHC36 was directly added and solvated into the 10% PCL polymer

suspension, with TFE as solvent for AMP solvation and reaction medium for ring opening polymerization reaction. The solvent was eventually removed through vaporization to form the monolayer PCL(P). The use of TFE was critical for preserving peptide integrity and antimicrobial functionality during the coating synthesis process. TFE has been reported to protect the integrity and functionality of these biomolecules against extreme pH, temperature and proteolytic agents (Hackl, 2014; Nichols et al., 2013). In this study, TFE is hypothesized to stabilize the peptide, by inducing it to adopt highly stable turned configuration with sensitive functional group folded inwards, conserving peptide structure and protecting important side-chain functional groups. Peptide characterization assays indicated corresponding physical and structural properties between the released and native HHC36 peptides. Secondary structural adaptation, membrane interaction and functional groups were well-preserved throughout the coating synthesis and subsequent release process.

While the single layer PCL(P) coating exhibited significant initial release, the release profile was not sustainable, ceasing after 6 rounds of daily inoculation (Figure 3). The efficacy of an antimicrobial coating for clinical application requires a sustainable bactericidal effect for at least 7 days post-implantation, as it represents the duration that infection is most likely to occur (Librach, 2007). To enhance sustainability of peptide release and prolonged antibacterial action, a secondary diffusion boundary film, constituting of POPC phospholipids, was applied onto the basal PCL(P) forming the dual layer PCL(P)-POPC(P) coating. The additional POPC film effectively modulated initial HHC36 burst release (from ~15% of total peptide impregnated in PCL(P) to ~10% in PCL(P)-POPC(P) film). The sustained daily release of peptide at concentrations above the MIC concentration ($5.38 \pm 0.01 \mu\text{M}$) (Table S2) was also enhanced to 14 days in dual layer PCL(P)-POPC(P) (Figure 3). The use of POPC as a diffusion boundary to modulate release kinetics has been widely reported and applied in a variety of coatings (Kazemzadeh-Narbat et al., 2013). The additional POPC layer moderated initial AMP burst

1 release by acting as a physical diffusion boundary, where the peptides are first physically
2 trapped and gradually released, with film degradation over time. Current studies underway
3 show that the remaining entrapped peptides will be released gradually over a period of 2 to 3
4 months, with progressive degradation of the PCL polymer (Gunatillake & Adhikari, 2003; Lan
5 et al., 2017). Despite the differences in release kinetics, both PCL(P) and PCL(P)-POPC(P)
6 coating showed diffusion driven release kinetics, with an initial burst release followed by a
7 gradual elution of deeply impregnated peptide molecules. The initial burst release was likely a
8 combination of the diffusion of HHC36 at or near the coating interface, and the release of
9 previously POPC-entrapped peptides due to swelling of the superficial POPC layer upon
10 exposure to the aqueous medium (Kazemzadeh-Narbat et al., 2013). The subsequent sustained
11 release phase is due to the remainder of the encapsulated peptide being progressively released
12 due to slow internal diffusion of peptides through the dense PCL polymer network. The 2-
13 phase release profile is ideal for prolonged implant protection against bacterial infection. The
14 moderate burst release in the first 24 h ensures thorough sterilization of the implant site post-
15 implantation, while subsequent controlled and sustained peptide elution that follows will
16 prevent against subsequent bacterial invasion and colonization of implant surface.

17 Antimicrobial functionality assessment of the PCL(P)-POPC(P) coating was first conducted in
18 96-well plates, to facilitate tight control of experimental parameters and consistency in sample
19 preparation. *In vitro* antimicrobial result corresponded well with the peptide release profile,
20 with the PCL(P)-POPC(P) coating demonstrating significant antimicrobial potency against
21 virulent *UTI89 E. coli* exposures up to 13 days (Figure 6). The results also verified that
22 bactericidal functionality of the encapsulated HHC36 peptide was preserved throughout the
23 coating synthesis process, with the released peptide showing potent antimicrobial action.

24 Antimicrobial performance of the PCL(P)-POPC(P) coating under dynamic physiological
25 condition was examined using an established wound infection model (Figure 7). Microbial

proliferation at the site of infection was inhibited with approximately 1 log lower CFU count at the site of infection 24 hpi, in comparison to the control. The presence and constant elution of peptides from the PCL(P)-POPC(P) coating also acted as a deterrence against bacterial adhesion to the coating surface. This is crucial as microbial attachment and eventual colonization of coating surface represent one of the main causes of failure for current device coatings (Nejadnik, van der Mei, Norde, & Busscher, 2008). Further *in vivo* testing in small animal models using mouse catheters was limited by several factors, including limited coating area due to miniature mouse catheter size and undesired urethra injury during delicate catheterization process, compromising the reliability of the result. The use of larger animals for safety and efficacy studies of our coated catheters, in the future, will be more appropriate to overcome these aforementioned challenges.

As a preliminary means to demonstrate translatability, the dual layer PCL(P)-POPC(P) coating was applied to commercial silicone urinary catheter surfaces using established dip-coating strategy. Application of the PCL(P)-POPC(P) coating onto silicone urinary catheters did not compromise the coating's antimicrobial efficacy, demonstrating potent, broad-spectrum antimicrobial activities and anti-biofilm properties. This can be attributed to successful impregnation and elution of the HHC36 peptides, which has been previously reported to demonstrate good antibacterial against a variety of gram positive and gram negative microbes (Cherkasov et al., 2008). To complement its antimicrobial potency, the PCL(P)-POPC(P) coating also demonstrated high biocompatibility with mammalian cells, highlighting its potential for clinical application. **Additionally, application of the coating does not compromise the elasticity and tensile strength of the silicone catheter (Figure 8).** The relatively thin (10 μ m) coating is also ideal to ensure patient comfort throughout the implantation cycle.

Compared to the commercial Dover antimicrobial catheter, which elutes silver phosphate ions from a loaded hydrogel coating, the PCL(P)-POPC(P) coated catheter showed better

antimicrobial efficiency against both planktonic and adherent bacteria. The inconsistent performance of ‘silver ions’-impregnated catheters in the *in vitro* test and clinical trials (Riley et al., 2017; Srinivasan, Karchmer, Richards, Song, & Perl, 2006) could be attributed to the bacteriostatic nature of silver ions, which allows further proliferation of the microbes upon longer exposure (Randall, Oyama, Bostock, Chopra, & O’Neill, 2013), and non-specific binding of serum protein to the eluted silver ions, which detracts the antimicrobial activity (Gnanadhas, Ben Thomas, Thomas, Raichur, & Chakravorty, 2013). In contrast, AMPs such as HHC36, which have membrane perforating mode of bactericidal action (Nichols et al., 2013), can permanently incapacitate microbes, obliterating the possibility for further bacteria proliferation. While susceptibility to resistance against silver-based biocides (Silver, 2003) and conventional antibiotics (Walsh, 2000) is a common problem, acquisition of resistance by a sensitive microbial strain to membrane lytic peptides is relatively improbable (Lavery, Gorman, & Gilmore, 2011; Zasloff, 2002). This further justifies the development of the AMP-based PCL(P)-POPC(P) coating as a potential antibacterial coating for biomedical devices. While numerous AMP-based antimicrobial coatings had been developed over the years, few were successfully translated onto medical devices due to limitation factors such as complicated synthesis chemistry, requirement for specialty chemicals and sophisticated equipment, stringent requirement for substrate form and diminishing long term potency. The AMP-impregnated PCL(P)-POPC(P) coating developed in this study overcomes the aforementioned drawbacks by providing an antibacterial coating with potent and sustainable antibacterial activity, which can be achieved using simple synthesis methodology. Preliminary translation studies on silicone catheters demonstrated positive antimicrobial and antibiofilm results. However, further optimization of the coating formulation and application methodology to enhance sustainability of antibacterial and anti-biofilm properties beyond 14 days are needed to strengthen the commercialization reality of the reported coating.

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List of figure caption

Figure 1. Schematic of dual layer HHC36-impregnated PCL(P)-POPC(P) controlled release coating.

Figure 2. Surface characterization of PCL(P)-POPC(P) coating. (a) SEM micrographs of single layer PCL(P) (top) and dual layer PCL(P)-POPC(P) (bottom). In contrast to the PCL(P), which illustrated a highly uneven surface morphology, the PCL(P)-POPC(P) had a smooth surface. (b) EDS surface elemental mapping of carbon (left) and nitrogen (right). Carbon was found abundantly on the surface, while nitrogen, although sparingly present, was found homogeneously distributed across the PCL(P)-POPC(P) surface.

Figure 3. Kinetics of HHC36 release from PCL(P) and PCL(P)-POPC(P) coatings over 14 days. The dual layer coating showed a better moderated initial burst release and more sustainable subsequent release, in comparison to the single layer PCL(P). The PCL(P)-POPC(P) coating illustrated a significant sustained release with a consistent daily elution of ~ 2 $\mu\text{g/day}$ from Day 9 onward (inset).

Figure 4. Far-UV CD spectra of native (○) and released (●) HHC36 from the dual layer PCL(P)-POPC(P) coating in (a) deionised water and (b) 50% TFE. 1D NMR spectroscopy of (c) native and (d) released HHC36 in 90% $\text{H}_2\text{O}/\text{D}_2\text{O}$.

Figure 5. Fluorescence emission spectra of native (blue line) and released (solid line) peptides from the PCL(P)-POPC(P) coating in (a) deionised water and (b) 10 μM LPS, recorded with excitation wavelength at 280 nm.

Figure 6. Sustained antimicrobial assessment of the dual layer PCL(P)-POPC(P) coating upon subjected to repeated bacterial inoculations, up to 14 cycles. AMP release coating illustrated potent and sustainable antibacterial activity, significantly inhibiting bacterial proliferation up to 13 cycles of inoculations. * indicates $p < 0.05$ in comparison to negative controls.

Figure 7. *In vivo* safety and efficacy studies of HHC36 impregnated PCL(P)-POPC(P) coating. (a) The PCL(P)-POPC(P) dual layer coating prevented high titer wound infection ($>10^6$ CFU/wound), whereas the control coating resulted in $\sim 44\%$ of the animals manifesting high titer infection. (b) Anti-adherence capability of the coating was investigated. The PCL(P)-POPC(P) peptide-impregnated coating was significantly more resistant against *E. coli* attachment, in contrast to non-impregnated controls with high concentration of bacteria attached. Each circle indicates one animal and the thick line indicates the median of the group. The experiment was repeated two times, with five mice per group per experiment. *** indicates $p < 0.005$ in comparison to negative controls.

Figure 8. Elasticity of Cat-PCL(P)-POPC(P). (a) Cat-PCL(P)-POPC(P) showed similar elasticity and tensile strength as uncoated silicone catheter and non-peptide impregnated cat-PCL-POPC controls. ● loading and ○ unloading of uncoated silicone catheter (cat); ■ loading and □ unloading of non peptide-impregnated cat-PCL-POPC; ▲ loading and Δ unloading of HHC36-impregnated cat-PCL(P)-POPC(P). (b) Tensile strength of cat-PCL(P)-POPC(P) and respective controls.

Figure 9. (a) Potency of Cat-PCL(P)-POPC(P) was assessed against a variety of clinically relevant pathogens. CFU enumeration illustrated that the Cat-PCL(P)-POPC(P) was efficacious against both gram positive and gram negative microbes. (b) Anti-biofilm

1 assessment of Cat-PCL(P)-POPC(P). Coated and untreated catheters were exposed to high
2 concentration of *E. coli* ($\sim 10^8$ CFU/mL) and incubated at 37°C for 48 h and 168 h respectively.
3 Extent of biofilm development was quantitated through CFU enumeration. (c) SEM
4 micrographs of coated and untreated catheters upon prolong exposure (48 h) to high bacteria
5 concentration. Both CFU counts and SEM demonstrated good anti-biofilm properties with
6 negligible amount of bacteria adhered. Biocompatibility assay of Cat-PCL(P)-POPC(P)
7 showed (d) low degree of hemolysis against hRBS and (e) minimal adverse effect on
8 uroepithelial cell viability. * indicates $p < 0.05$ in comparison to negative untreated catheter
9 controls.

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1 **Figure list**

2

3 **Figure 1.** Schematic of dual layer HHC36-impregnated PCL(P)-POPC(P) controlled release
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Figure 2. Surface characterization of PCL(P)-POPC(P) coating. (a) SEM micrographs of single layer PCL(P) (top) and dual layer PCL(P)-POPC(P) (bottom). In contrast to the PCL(P), which illustrated a highly uneven surface morphology, the PCL(P)-POPC(P) had a smooth surface. (b) EDS surface elemental mapping of carbon (left) and nitrogen (right). Carbon was found abundantly on the surface, while nitrogen, although sparingly present, was found homogeneously distributed across the PCL(P)-POPC(P) surface.

Figure 3. Kinetics of HHC36 release from PCL(P) and PCL(P)-POPC(P) coatings over 14 days. The dual layer coating showed a better moderated initial burst release and more sustainable subsequent release, in comparison to the single layer PCL(P). The PCL(P)-POPC(P) coating illustrated a significant sustained release with a consistent daily elution of ~2 µg/day from Day 9 onward (inset).

Figure 4. Far-UV CD spectra of native (○) and released (●) HHC36 from the dual layer PCL(P)-POPC(P) coating in (a) deionised water and (b) 50% TFE. 1D NMR spectroscopy of (c) native and (d) released HHC36 in 90% H₂O/D₂O.

Figure 5. Fluorescence emission spectra of native (blue line) and released (solid line) peptides from the PCL(P)-POPC(P) coating in deionised water (a) and 10 µM LPS (b), recorded with excitation wavelength at 280 nm.

Figure 6. Sustained antimicrobial assessment of the dual layer PCL(P)-POPC(P) coating upon subjected to repeated bacterial inoculations, up to 14 cycles. AMP release coating illustrated potent and sustainable antibacterial activity, significantly inhibiting bacterial proliferation up to 13 cycles of inoculations. * indicates $p < 0.05$ in comparison to negative controls.

Figure 7. *In vivo* safety and efficacy studies of HHC36 impregnated PCL(P)-POPC(P) coating. (a) The PCL(P)-POPC(P) dual layer coating prevented high titer wound infection ($>10^6$ CFU/wound), whereas the control coating resulted in ~44% of the animals manifesting high titer infection. (b) Anti-adherence capability of the coating was investigated. The PCL(P)-POPC(P) peptide-impregnated coating was significantly more resistant against *E. coli* attachment, in contrast to non-impregnated controls where high concentration of bacteria can be found attached. Each circle indicates one animal and the thick line indicates the median of the group. The experiment was repeated two times, with five mice per group per experiment. *** indicates $p < 0.005$ in comparison to negative controls.

(b)

Samples	Strength (MPa)
Cat	1.17 ± 0.06
Cat-PCL-POPC	1.00 ± 0.09
Cat-PCL(P)-POPC(P)	1.07 ± 0.01

Figure 8. Elasticity of Cat-PCL(P)-POPC(P). (a) Cat-PCL(P)-POPC(P) showed similar elasticity and tensile strength as uncoated silicone catheter and non-peptide impregnated cat-PCL-POPC controls. ● loading and ○ unloading of uncoated silicone catheter (cat); ■ loading and □ unloading of non peptide-impregnated cat-PCL-POPC; ▲ loading and Δ unloading of HHC36-impregnated cat-PCL(P)-POPC(P). (b) Tensile strength of cat-PCL(P)-POPC(P) and respective controls.

Figure 9. (a) Potency of Cat-PCL(P)-POPC(P) was assessed against a variety of clinically relevant pathogens. CFU enumeration illustrated that the Cat-PCL(P)-POPC(P) was efficacious against both gram positive and gram negative microbes. (b) Anti-biofilm assessment of Cat-PCL(P)-POPC(P). Coated and untreated catheters were exposed to high concentration of *E. coli* ($\sim 10^8$ CFU/mL) and incubated at 37°C for 48 h and 168 h respectively. Extent of biofilm development was quantitated through CFU enumeration. (c) SEM micrographs of coated and untreated catheters upon prolonged exposure (48 h) to high bacteria concentration. Both CFU counts and SEM demonstrated good anti-biofilm properties with negligible amount of bacteria adhered. Biocompatibility assay of Cat-PCL(P)-POPC(P) showed (d) low degree of hemolysis against hRBCs and (e) minimal adverse effect on uroepithelial cell viability. * indicates $p < 0.05$ in comparison to negative untreated catheter controls.

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Novel anhydrous polymer-based antimicrobial peptide-impregnated coating, consisting of poly(caprolactone) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, is developed. Use of helix stabilizing 2,2,2-trifluoroethanol as polymerization medium and solvent enables direct impregnation of antimicrobial peptide into the polymer, without requiring additional protection steps, enhancing loading efficacy. The coating demonstrated an ideal peptide release profile with prolonged antimicrobial and anti-biofilm potency. Easy application onto commercial urinary catheter demonstrated excellent translatability of reported coating.

Keyword: Antimicrobial peptides, trifluoroethanol solvent, anhydrous polymer coating, controlled release, urinary catheter

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Anhydrous polymer-based device coating with sustainable controlled release functionality for facile, efficacious impregnation and delivery of antimicrobial peptides

Figure S1. High resolution FESEM image of PCL(P)-POPC(P) dual layer coating surface morphology. Similar to the result from SEM, the PCL(P)-POPC(P) coating exhibited a smooth surface morphology.

Figure S2. Antimicrobial assessment of the peptide-impregnated PCL(P)-POPC(P) and respective non-peptide impregnated intermediates. Antimicrobial property from the dual layer PCL(P)-POPC(P) is due to the bactericidal action of the released peptides.

Table S1. PCL(P)-POPC(P) surface atomic composition.

Element	Surface atomic composition (%)
Platinum (Pt)	39.70 ± 5.90
Carbon (C)	46.28 ± 3.63
Oxygen (O)	11.08 ± 2.83
Nitrogen (N)	1.93 ± 0.66
Sulfur (S)	0.82 ± 0.28

Table S2. MIC of HHC36 against different initial bacteria densities.

Bacteria concentration (CFU/mL)	Minimum inhibitory concentration of HHC36 ($\mu\text{g/mL}$)
1×10^6	8.00 ± 0.01
1×10^7	16.00 ± 0.01
1×10^8	125.00 ± 0.01