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Antimicrobial activity of novel biocompatible wound dressings based on triblock copolymer hydrogels

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Abstract Wound infection is a common complication often resulting in delayed healing with adverse clinical and financial consequences. Current antimicrobial treatments are far from ideal, side effects can include both bacterial resistance and toxicity. As a result, a great deal of effort over the last 20 years has been spent on investigating new forms of antimicrobial dressings. Here, we report the unexpected antimicrobial activity of a relatively new biocompatible thermo-responsive PHPMA–PMPC–PHPMA triblock copolymer gelator [where PHPMA denotes poly (2-hydroxypropyl methacrylate) and PMPC denotes poly (2-(methacryloyloxy)ethyl phosphorylcholine)]. In a radial diffusion assay, a 20% w/v copolymer gel produced an inhibitory zone up to six times greater than the corresponding control against *Staphylococcus aureus*. Similarly, in a broth inhibition assay the same copolymer reduced bacterial growth by 45% compared with control experiments conducted in the absence of any copolymer. Moreover, addition of the copolymer to a 3D-infected skin model reduced bacterial recovery by 38% compared to that of controls over 24–48 h. This is particularly relevant since these antimicrobial triblock copolymers were recently shown to be non-toxic when exposed to a tissue-engineered skin model. This antimicrobial activity was also successfully immobilised by grafting PMPC–PHPMA diblock copolymer brushes onto silicon wafers. Our results indicate that both PMPC–PHPMA diblock and PHPMA homopolymer brushes exhibit antimicrobial activity. Our hypothesis for the mode of action is that the moderately hydrophobic PHPMA chains penetrate the bacterial membrane, causing leakage of the cell contents. In summary, these gels and surfaces offer a promising new approach to antimicrobial dressings.

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Introduction

With an ever-increasing ageing population in the western world, the incidence of chronic non-healing wounds has increased dramatically over the last decade [1]. Wound healing in healthy individuals is a highly organised process dependent on the migration and proliferation of cells that ultimately results in the re-epithelisation of wounds [2]. However, the combination of poor nutrition, slow vascularisation and weak immune systems associated with infection in the elderly can cause severe delays in wound closure due to pathogens prolonging the inflammatory phase and destroying the newly formed tissue [3]. Hence, a great deal of effort over the last 20 years has been dedicated to investigating new forms of antimicrobial dressings.

Due to the recent emergence of bacterial resistance, there is an understandable reluctance to use systemic or topical antibiotics. This is particularly the case for the management of chronic wounds that might persist for several years, thereby increasing the risk of development of microbial resistance. Topical antimicrobials are the treatment of choice for superficial wounds because the whole body commensal microbiota are not exposed to the antimicrobial agent [4]. One of the most commonly used topical antimicrobials is silver. This element has a broad spectrum of activity [5] and various silver-based formulations are in use, such as silver-impregnated dressings, silver nitrate and sulfadiazine cream (commonly used for burns patients). However, in order to be effective, silver must be delivered in a soluble form. Unfortunately, high local concentrations of reactive silver can retard the healing process [6]. In addition, these formulations may also be associated with various side effects, such as hypotonia, leukopenia, allergic reactions and discoloration (which affects the assessment of the wound bed) [5, 7]. On the other hand, if the concentration is too low then bacterial resistance is likely to emerge [6]. Hence, using these dressings for long-term management of chronic wounds requires experienced clinical judgement to obtain antimicrobial activity without adversely affecting wound healing. Consequently, the development of alternatives to silver-based preparations is desirable.

Naturally occurring peptides exhibiting antimicrobial activity vary widely in their structure as well as the potency of their activity. However, some aspects remain crucial, such as cationic charge density and hydrophobicity. Such peptides interact with the negatively charged and hydrophobic components of the bacterial membranes. This interaction results in disruption of the membrane and aggregation of the peptides at this particular site of the microorganism causing its permeabilization [8]. Inspired by these antimicrobial peptides, chemists have focused on designing synthetic amphiphilic cationic copolymers with some hydrophobic character. Examples of such synthetic mimics of antimicrobial peptides include: polyoxanorbornene containing various quaternary alkylpyridinium side chains [9] and polymethacrylate derivatives [10].

ABA triblock copolymers (where A = poly(2-hydroxypropyl methacrylate) or PHPMA and B = 2-(methacryloyloxy)ethyl phosphorylcholine or PMPC) were recently demonstrated to self-assemble to form soft, free-standing, transparent copolymer gels in aqueous solution [11, 12]. Cell viability experiments confirmed that such micellar gels exhibit excellent biocompatibility and hence may offer some potential for topical drug delivery into chronic wounds [12]. During the course of our investigations, we serendipitously observed that these hydrogels also appeared to have antimicrobial properties. Accordingly, the aim of the current study was to assess the antimicrobial activity of such hydrogels using a range of assays and to investigate their effect on a recently developed bacterially infected tissue-engineered skin (TE skin) model [13]. Finally, we grew PMPC–PHPMA diblock copolymer, PMPC homopolymer and PHPMC homopolymer brushes from planar silicon wafers so as to learn more about the antimicrobial mechanism.

Materials and methods

Materials

The 2-(methacryloyloxy)ethyl phosphorylcholine monomer (MPC, 99.9% purity) was kindly donated by Biocompatibles UK Ltd (Farnham, UK). The 2-hydroxypropyl methacrylate (HPMA) was kindly donated by Cognis Performance Chemicals (Hythe, UK). Bis(2-hydroxyethyl)disulphide (98%), 2-bromoisobutyryl bromide (98%), basic alumina (Brockmann I, standard grade, 150 mesh, 58 Å), anhydrous methanol (MeOH 99.8%), copper bromide [Cu(I)Br, 99.9%] and 2,2′-bipyridine (bpy, 99%), triethylamine (99%) and diethyl meso-2,5-dibromoadipate (DEDBA, 98%) were purchased from Sigma–Aldrich UK (Dorset, UK). All were used as received apart from the triethylamine, which was refluxed over potassium hydroxide and kept sealed over potassium hydroxide. The silica gel 60 (0.063–0.200 μm) used to remove the spent ATRP catalyst was purchased from E. Merck (Darmstadt, Germany). Propan-1,3-diol was purchased from Hopkins and Williams (London, UK). Methanol and tetrahydrofuran were HPLC-grade and obtained from Fisher Scientific (Loughborough, UK).

Boron-doped silicon wafers (⟨100⟩ orientation, boron doped, 0–100 Ω cm) were purchased from Compart Technology (Peterborough, UK). 3-Aminopropyl triethoxysilane (APTES, >98%) was purchased from Fluka. Deionised water was obtained using an Elga Elgastat Option 3 system.

The following microorganisms were obtained from stocks held in the Department of Oral Pathology in the School of Clinical Dentistry at the University of Sheffield: *Staphylococcus aureus* NCTC 6571 (Oxford), *Staphylococcus epidermidis* (clinical isolate), *Candida albicans* NCPF 3091, *Enterococcus faecalis* (clinical isolate) and *Pseudomonas aeruginosa* (clinical isolate). Brain heart infusion (BHI) broth and agar were purchased from Oxoid (Basingstoke, UK). Image J software was used to determine zones of inhibition. Live/dead staining was performed using propidium iodide (PI) and Syto 9, both of which were purchased from Invitrogen (Paisley, UK). The microorganisms on the

silicon wafers were viewed using a ZEISS LSM 510 M confocal microscope.

Dulbecco's modified Eagle's medium (DMEM), cholera toxin, epidermal growth factor, adenine, insulin, sodium chloride, triiodothyronine and EDTA were all purchased from Sigma–Aldrich (Poole, Dorset, UK). Foetal calf serum (FCS) was purchased from Labtech (Ringmer, UK). Ham's F12 medium, glutamine, amphotericin B, penicillin and streptomycin were purchased from Gibco (Paisley, UK). Hydrocortisone was purchased from Novabiochem (Nottingham, UK). Trypsin was purchased from Difco Laboratories (Detroit, MI, USA). Collagenase A was purchased from Boehringer–Mannheim (East Sussex, UK). ThinCerts were purchased from Greiner Bio-one (Stonehouse, UK). Stainless steel rings and grids were supplied by the Royal Hallamshire Hospital (Sheffield, UK).

Methods

1. Investigation of the antibacterial activity of ($PHPMA_{88}$–$PMPC_{200}$–S)$_2$ polymer gels

1.1 Initiator synthesis

1.1.1 Synthesis of the disulphide-based bifunctional ATRP initiator, bis[2-(2-bromoisobutyryloxy)ethyl] disulphide ($(BiBOE)_2S_2$)

The disulphide-based bifunctional ATRP initiator $(BiBOE)_2S_2$ was synthesised according to a literature protocol [14, 15].

1.1.2 Synthesis of the propandiol-based bifunctional ATRP initiator, 1,3-bis (2-bromoisobutyryloxy) propane ($(BiB)_2P$)

Propan-1,3-diol (2.014 g, 0.026 mol) was dispersed in 10 mL methylene chloride. To this was added triethylamine (8.3 mL, 6.0 g, 0.06 mol) and *N*,*N*′-dimethylaminopyridine (0.7355 g, 0.0060 mol). The resulting solution was placed under nitrogen and cooled on ice. 2-Bromoisobutyryl bromide (7.45 mL, 13.9 g, 0.060 mol) in 25 mL methylene chloride was added dropwise over 20 min. The reaction mixture was stirred for 72 h, followed by addition of methylene chloride (150 mL). The organic phase was washed with water (2 × 50 mL), saturated sodium hydrogen carbonate (2 × 50 mL) and water (2 × 50 mL). After drying over anhydrous magnesium sulphate, filtration and solvent removal at 50 °C yielded a yellow oil. This was dissolved in methylene chloride and passed through a basic alumina column. The isolated mass was 4.66 g, which corresponded to a 47% yield.

^{1}H NMR ($CDCl_3$) δ 4.29 (t, 4H, $J = 6.3$ Hz), 2.09 (m, 2H), 1.93 (s, 12H) ppm.

^{13}C NMR ($CDCl_3$) δ 171.5, 62.1, 55.6, 30.7, 27.5 ppm.

ESMS, 397 $(M + Na)^+$.

Elemental microanalyses gave Br = 42.63% (theory 42.72%) suggesting that the initiator purity exceeded 99%.

1.1.3 Synthesis of rhodamine 6G bis(2-(2-bromoisobutyryl)ethyl)amide ATRP initiator ($RH(EtOBr)_2$)

In a round-bottomed flask, rhodamine 6G (10.0 g, 0.021 mol) was dissolved in *N*,*N*′-diethanolamine (10.0 g, 0.095 mol). The flask was fitted with a reflux condenser, placed under nitrogen and heated to 90 °C for approximately 24 h. After cooling, the solution was dissolved in the minimum amount of methanol and poured into 500 mL water. After filtering, the aqueous solution was saturated with sodium chloride and extracted with 50 mL aliquots of a 2:1 isopropanol:methylene chloride mixture until only a faint colour remained in the aqueous phase. The combined organic phases were dried over anhydrous sodium sulphate, filtered and evaporated. The resulting solid was recrystallized from methanol. The hydrobromide salt was prepared by dissolving this solid in water, adding 1.1 molar equivalents of the corresponding acid and freeze-drying the aqueous solution overnight to give rhodamine 6G bis(2-hydroxyethyl)amide, $RH(EtOH)_2$ in 75% yield.

^{1}H NMR (400 MHz, 3:1 $CDCl_3$: CD_3OD) δ 7.66 (m, 1H), 7.26 (m, 2H), 7.19 (m, 1H), 6.80 (s, 2H), 6.56 (s, 2H), 3.47 (t, 2H, $J = 5.50$ Hz), 3.33 (q, 4H, $J = 7.21$ Hz), 3.15 (m, 8H), 1.99 (s, 6H), 1.20 (m, 6H, $J = 7.21$ Hz) ppm.

^{13}C NMR (400 MHz, 3:1 $CDCl_3$:CD_3OD) δ 173.24, 160.42, 159.16, 158.90, 139.07, 133.84, 132.90, 132.56, 131.48, 130.33, 128.15, 116.71, 96.81, 62.26, 61.84, 55.46, 50.36, 41.43, 20.01, 16.44 ppm.

ESMS, 502 $(M + H)^+$.

$RH(EtOH)_2$ (Cl-salt, 1.030 g, 1.914 mmol) was dissolved in 50 mL water. 2-Bromoisobutyric anhydride (1.0439 g, 3.304 mmol) [16] was dissolved in methylene chloride (9 mL) and transferred to the reaction mixture with methylene chloride (11 mL). Further, methylene chloride (10 mL) was then added. After 47 h, the reaction mixture was transferred to a separating funnel with saturated sodium hydrogen carbonate. The aqueous phase was extracted with methylene chloride (4 × 50 mL). The combined organic phases were washed with water (3 × 50 mL), then with brine (50 mL). After drying over sodium sulphate and filtering, the solution was concentrated at 50 °C, cooled to room temperature and precipitated with diethyl ether. After filtration, the solid was redissolved in methylene chloride and precipitated with diethyl ether and this procedure was repeated until no more acid could be detected by NMR. The resulting dark red solid was dried in vacuum overnight to give 0.22 g (14%) of neutral product.

^{1}H NMR (CD_3OD) δ 7.64 (m, 3H), 7.29 (m, 1H), 6.82 (s, 2H), 6.56 (s, 2H), 4.09 (m, 2H), 3.65 (m, 2H), 3.45 (m, 8H), 2.19 (s, 6H), 1.81 (s, 6H), 1.71 (s, 6H), 1.28 (s, 6H) ppm.

ES-MS 800 $(M + H)^+$.

1.2 Copolymer synthesis and purification

The PHPMA–PMPC–PHPMA triblock copolymers were prepared via one-pot copolymer syntheses as reported previously [12]. These were conducted using sequential monomer addition without purification of the intermediate PMPC macro-initiator. A typical synthesis was carried out as follows: MPC (10.002 g, 33.9 mmol, 250 eq.) was placed under nitrogen. $(BiBOE)_2S_2$ (61.2 mg, 0.135 mmol, 1 eq.) was dissolved in anhydrous methanol (12 mL) and added to the MPC using a cannula. The reaction mixture was purged with nitrogen for 30 min. 2,2′-Bipyridine (83.8 mg, 0.537 mmol, 4.0 eq.) and Cu(I)Br (38.6 mg, 0.269 mmol, 2.0 eq.) was added to commence the first-stage polymerisation. After 6 h, HPMA (1.9533 g, 13.5 mmol, 100 eq.) was added to the dark brown viscous solution by cannula and the reaction mixture was stirred for a further 70–100 h until no vinyl signals were observed in the ^{1}H NMR spectrum. After this time period, the reaction mixture was diluted with methanol and passed through a silica column to remove the spent catalyst. The solution was partly evaporated and precipitated into excess THF (500 mL) to remove residual monomer and traces of 2,2′-bipyridine. After filtration, residual THF was removed by co-evaporation with three 50 mL portions of methanol. To the solid residue was added water (200 mL) and this was stirred until a uniform mixture was obtained. The water was evaporated at 50–60 °C under reduced pressure to obtain a solution volume of approximately 50 mL prior to addition of 150 mL water. Approximately 150 mL of water was again removed under vacuum and the resulting solution was freeze-dried overnight. Finally, the copolymer was dried at 80 °C under vacuum for 48 h, then for 5–6 h at 90 °C. These additional co-evaporation steps were essential for the cell studies, since it was found that traces of cytotoxic methanol were very difficult to remove by simply drying the copolymer in a vacuum oven. In contrast, repeated co-evaporation of residual methanol with water under reduced pressure proved to be a reliable means of ensuring sufficient purification to achieve biocompatibility. This protocol produced 9–10 g of purified triblock copolymer (75–83% yield).

The triblock copolymer based on the bifunctional rhodamine initiator, $RH(EtOBr)_2$, was prepared using the same protocol. Targeting a composition of $(PHPMA_{50}–PMPC_{125}–RH(EtO))_2$ gave a copolymer with a GPC number-average molecular weight close to that found for $(PHPMA_{88}–PMPC_{200}–S)_2$ but significantly higher than that found for $(PHPMA_{43}–PMPC_{125}–S)_2$. This suggested that the efficiency of this particular initiator was around 65% of the $(BiBOE_2)S_2$ initiator. Therefore, the composition of this copolymer was assessed to be $(PHPMA_{75}–PMPC_{190}–RH(EtO))_2$.

1.3 Microorganism culture

Microorganisms were each cultured in 20 mL BHI broth, and maintained by subculture on BHI agar plates.

1.4 Preparation of gels

The thermo-responsive copolymers were dissolved in sterile phosphate-buffered saline (PBS) at the appropriate concentration and incubated for 24–48 h at 4 °C prior to use to ensure their complete dissolution. When fluorescently labelled copolymers were used, 6.0% w/w of $(PHPMA_{75}–PMPC_{190}–RH(EtO))_2$ was mixed with 94% w/w of $(PHPMA_{88}–PMPC_{200}–S)_2$ to produce a 20% w/v final copolymer concentration.

1.5 Investigation of the effects of $(PHPMA_{88}–PMPC_{200}–S)_2$ polymers on microbial growth using standard antimicrobial assays

Four techniques were used to investigate the effect of the copolymers on microbial growth, two on agar cultures and two in liquid suspension.

1.5.1 "Drop on" assay

A single colony from an overnight BHI agar culture was suspended in sterile distilled water (20 mL) to a density of $\sim 5 \times 10^7$ colony forming units (CFU) per mL. Uniform lawns of inoculae were prepared by swabbing the bacterial suspensions evenly on BHI agar plates. Then a 100 μL aliquot of 20% w/v $(PHPMA_{88}–PMPC_{200}–S)_2$ in aqueous solution was placed directly onto each lawn. After incubation overnight at 37 °C, any inhibition was indicated by areas of either no growth or significantly reduced bacterial growth. The following copolymers were also examined: $(PHPMA_{88}–PMPC_{200}–S)_2/(PHPMA_{75}–PMPC_{190}–RH(EtO))_2$ (20% w/v, 94% w/w/6% w/w), $PHPMA_{71}–PMPC_{400}–PHPMA_{71}$ (20% w/v), $(PMPC_{125}–S)_2$ (20% w/v), $[(PHPMA_{69}–PMPC_{125}–S)_2$ (20% w/v)], $[(PHPMA_{44}–PMPC_{125}–S)_2$, (20% w/v)]. The control for each was PBS dropped onto the bacterial lawns. Finally, when fluorescently labelled copolymers were investigated, the bacterial plates were viewed in a Syngene G: Box Chemi HR16 using a 20 × 30 filter size with a wavelength of 302 nm. Digital photographs were recorded to monitor the extent of copolymer diffusion on BHI agar plates.

1.5.2 Radial diffusion assay

Microbial lawns on BHI agar plates were prepared as described above and wells with an area of 0.2 cm^2 were punched into the agar. Aliquots ($\sim$50 μL) of $(PHPMA_{88}–PMPC_{200}–S)_2$ copolymers of varying concentration (0, 10 and 20% w/v) were placed in these wells and after overnight incubation at 37 °C the zones of inhibition around the wells were measured using Image J software. The control was PBS.

1.5.3 Growth assays

Copolymers were dissolved in BHI broth to 20% w/v and each inoculated with $\sim 5 \times 10^7$ CFU of *S. aureus*. Growth within the gels was compared to growth within the BHI broth without copolymer. Bacterial growth was assessed by optical density (A_{450nm}), measured at hourly intervals for 6 h. Absorbance readings resulting from the

gel and broth alone without bacteria were subtracted each time to obtain the absorbance produced by the bacteria.

1.5.4 Bacteriocidal assay

Staphylococcus aureus (10^5 CFU) was added to 100 μL aliquots of either 20% w/v ($PHPMA_{88}$–$PMPC_{200}$–S)$_2$ gel or 30% w/v Pluronics F127 gels both of which were prepared in PBS (in the absence of any nutrients). The control used for this experiment was PBS. After exposure to the polymer gels for 0 and 2 h the mixtures (gels + bacteria and bacteria + PBS) were diluted in 5 mL of cold PBS to allow gel dissolution and the release of bacteria. Serial dilutions of the suspension were performed and the number of surviving CFU was assessed on BHI agar (following 16 h incubation at 37 °C). Data were expressed as the percentage CFU of the PBS control.

1.6 Preparation of bacterially infected 3D TE skin model

1.6.1 Cell culture

Skin was obtained from patients undergoing breast reductions and abdominoplasty elective surgical procedures. Patients gave informed consent for skin not required for their treatment to be used for experimental purposes under a protocol approved by the Ethical Committee of the Northern General Hospital Trust (NHS), Sheffield, UK. For further details relating to cell culture, see [12].

1.6.2 Preparation of 3D TE skin model

Tissue-engineered skin (TE skin) was prepared as detailed by Ghosh et al. [17], except that the dermis was not sterilised for the current experiments. De-epidermized dermis (DED) was prepared by incubating the skin in 1.0 M NaCl for 24 h at 37 °C to remove the epidermis. Composites were cultured in tissue culture inserts as previously described [13].

Briefly, rings of DED of 15 mm diameter were cut using a sterile cork borer and placed within 12 mm tissue culture inserts (ThinCerts) with 4 μm pores in the membrane (Greiner) (the DED was cut slightly larger than the inserts to allow for skin contraction on burning). Inserts were suspended from the edges of 12 well plates into the wells. A total of 10% Green's medium was added to the bottom of the wells so that it lapped the under surface of the DED. The DED was then seeded with 1×10^5 fibroblasts and 5×10^5 keratinocytes, each in 250 μL of Green's medium. After 24 h at 37°C, the seeding medium was replaced with fresh Green's medium and 24 h later the medium was removed from inside the inserts to allow the composites to be at an air–liquid interface. Subsequently, Green's medium was replaced every 24 h and the models were used for experimentation after 10–14 days culture at an air–liquid interface.

1.6.3 Preparation of ($PHPMA_{88}$–$PMPC_{200}$–S)$_2$ copolymer gels for 3D TE skin

The ($PHPMA_{88}$–$PMPC_{200}$–S)$_2$ copolymer gel was dissolved in antibiotic-free Green's medium at 4 °C for 24–48 h to produce a concentration of 20% w/v copolymer gel before addition to TE skin.

1.6.4 Preparation of bacteria for TE skin infection

A clinical isolate of *P. aeruginosa* (SOM1) was cultured in 20 mL BHI (Oxoid) broth at 37 °C for 24 h from stock plates prior to use. Broths were centrifuged and the resulting pellet washed and resuspended in PBS to a bacterial concentration of 1×10^{10} CFU/mL. Immediately prior to use, bacteria were centrifuged and resuspended in BHI broth.

1.6.5 TE skin infection

Skin composites were infected as recently described [13]. Essentially, TE skin was washed in antibiotic-free Green's medium for 72 h prior to infection. The models were then burnt by the application of a heated metal rod (4 mm diameter) for 6 s immediately prior to infection in order to provide the bacteria a mode of entry into the skin. A total of 1×10^7 *P. aeruginosa* cells in 100 μL BHI broth per model were pipetted into the inserts covering the epidermal surface. A total of 600 μL of 20% w/v of ($PHPMA_{88}$–$PMPC_{200}$–S)$_2$ copolymer gels or antibiotic-free Green's medium were then added to test TE skin (bacteria + gel) and control TE skin (bacteria), respectively. Other relevant burnt and infected controls in all combinations were used for histological investigations of this TE skin model.

The models were then incubated in antibiotic-free Green's medium at 37 °C/5% CO_2 for 24 h after infection before immersion in antibiotic-free medium for a further 24 h to allow gel dissolution. The skin was then sacrificed for analysis and bisected using sterile scalpels. Half of each model was fixed in 10% formalin for more than 24 h, it was then processed and embedded in paraffin for histological analysis. In the case of burnt and infected (control) and burnt, infected and exposed to 20% w/v of ($PHPMA_{88}$–$PMPC_{200}$–S)$_2$ gel (test) samples, the other half was weighed and then the tissue was homogenised in 1 mL BHI broth. The resulting homogenates were diluted serially and used to perform viable counts of bacteria in the samples.

1.6.6 Histology

Formalin-fixed, paraffin-embedded samples were sectioned to 4 μm thick on a microtome. Sections were stained with haematoxylin and eosin using standard techniques.

2. Investigation of the antibacterial activity of polymer grafted silicon surfaces

2.1 Formation of self-assembled monolayers (SAMs)

A silicon wafer (ca. 25 cm^2) was washed with acetone, 2-propanol and water. This was then immersed in a bath containing a mixture of ammonia solution (28 mL, 35 wt%), hydrogen peroxide solution (28 mL, 30 wt%) and water (142 mL) at 70 °C for 15 min. Next, any residue on the water surface was removed by overflowing the bath with deionised water three times. The silicon wafer was

removed, washed with water and dried under a stream of nitrogen gas. The wafer was then amine-functionalised by exposure to APTES vapour under reduced pressure (in a chamber containing APTES pumped down to 0.2 mbar and then sealed) for 30 min and then annealed for a further 30 min at 110 °C. The wafer was then immersed in dry THF (100 mL) under a nitrogen atmosphere. Triethylamine (1.39 mL) was added followed by BIBB (1.24 mL) and left for 3 h without stirring at 20 °C. The wafer was removed, washed with THF, water, methanol and acetone, and then dried under a nitrogen stream. The total thickness of the SAM on the silicon wafer (i.e. the SiO_2 oxide layer plus the initiator layer) was found to be approximately 2.5–3.0 nm by ellipsometry.

2.2 Preparation of PMPC-grafted silicon wafers via ATRP

Methanol was de-oxygenated using a nitrogen purge for 30 min. MPC (4.00 g, 0.0136 mol) was added to a flask and placed under nitrogen using pump-refill cycling. Methanol (4.0 mL) was added and the mixture was stirred to aid dissolution. Meanwhile, the ATRP initiator-functionalised silicon wafer was placed under nitrogen using pump-refill cycling in a customised polymerisation vessel. Cu(I)Br (32.3 mg, 0.225 mmol), Cu(II)Br (15.1 mg, 0.0676 mmol) and bpy (98.2 mg, 0.628 mmol) were added to the MPC solution (relative molar ratios were MPC: Cu(I)Br:Cu(II)Br_2:bpy = 60:1:0.3:2.8) with sonication and stirring to aid dissolution. The reaction solution was then pumped into the polymerisation chamber using a syringe pump at a constant rate of 0.90 mL h^{-1} and maintained at 20 °C. Once the polymerising solution was 1 mm from the top of the wafer, the wafer was removed and washed with methanol, water and once again with methanol to remove unreacted MPC monomer and spent ATRP catalyst. The wafers were then soaked in methanol overnight to remove any ungrafted polymer chains and then dried under a nitrogen stream.

2.3 Preparation of PMPC–PHPMA diblocks via ATRP

Water, methanol and HPMA (12.5 g, 0.0867 mol) were separately de-oxygenated using a nitrogen purge for 30 min. The PMPC library was cut evenly in the direction of the PMPC brush gradient to produce five identical strips (ca. 5 cm^2). The wafers were placed under nitrogen in horizontal Schlenk tubes using pump-refill cycling. Water (6.25 mL) and methanol (6.25 mL) were added to the HPMA and this solution was stirred at 20 °C. Cu(I)Cl (143.5 mg, 1.449 mmol), Cu(II)Br (97 mg, 0.434 mmol), and bpy (635 mg, 4.0658 mmol) were added and the resulting mixture was stirred and sonicated to aid dissolution. The polymerising solution was then distributed evenly in each of the Schlenk tubes so as to completely cover the wafers. These wafers were immersed in these reaction solutions at 20 °C and removed at different desired times to produce various PHPMA brush thicknesses on each strip. The wafers were subjected to the same washing process as stated above, before soaking in methanol overnight and drying in a nitrogen stream the next day. The strips were then cut into ca. 1 × 1 cm^2 pieces, producing the final PMPC–PHPMA diblock copolymer brush library.

2.4 Surface characterisation

A Jobin–Yvon UVISEL spectroscopic ellipsometer was used to determine the thickness of the ATRP initiator SAMs, the PMPC homopolymer brushes and the PMPC–PHPMA diblock copolymer brushes on the silicon wafers at a 70° incident angle under ambient conditions. Measurements were recorded at incident wavelengths ranging from 300 to 500 nm. The ellipsometric data were then modelled and fitted to give film thickness (using a previously determined refractive index for the polymer layers) using WVASE32 software (J. A. Woolam Co., USA). The thickness of the PMPC homopolymer brush layer alone was determined by subtracting the SAM layer thickness. The PHPMA brush thickness was determined by subtracting the PMPC thickness from the PMPC–PHPMA diblock copolymer brush thickness.

2.5 Investigation of the antimicrobial effects of various polymers (PMPC, PHPMA and PMPC–PHPMA diblocks) grafted on silicon surfaces

The silicon surfaces were first rinsed in alcohol and allowed to dry in a sterile cell culture hood overnight. *Staphylococcus aureus* (10^6) in 5 μL of PBS was placed on silicon wafers onto which various polymers were grafted. "Clean" silicon wafers (with no polymer) were used as controls for these experiments. A sterile cover slip was then placed on top of the wafers to maximise the spread of the bacterial suspension onto the polymer surfaces and subsequently enhance bacterial contact with the polymers. Wafers plus cover slips were then incubated for 2 h within a humidified environment at 37 °C then dropped into 5.0 mL of PBS, separated with forceps and vortexed for approximately 1 min. Serial dilutions of the suspension were performed and the number of surviving CFU was assessed on BHI agar (after 16 h incubation at 37°C). Data were expressed as a percentage CFU of the control surfaces.

2.6 Confocal microscopy

A similar protocol to that described in Item 2.5 was used. Following incubation with *S. aureus* the cover slip was removed and 100 μL of Syto9/PI (5 μM/10 μg/mL) mixture was added and the cover slip placed back on the wafer and incubated for 20 min in the dark, before being thoroughly rinsed twice with PBS. The various silicon wafers were then imaged using a ZEISS LSM 510 M instrument.

3. Statistical analysis

Statistical analysis was performed using the paired Student *t*-test, in which a p value of <0.05 was considered

to indicate a significant difference. Experiments were repeated three times using triplicates for each experiment. Data within each experimental condition was presented as mean ± standard error of the mean (SEM).

Results and discussion

The "drop on" assay was used as an initial screening method for assessing the antibacterial properties of $(PHPMA_{88}–PMPC_{200}–S)_2$ against a range of five micro-organisms: *S. aureus*, *S. epidermidis*, *E. faecalis*, *P. aeruginosa* and *C. albicans*. These represented Gram-positive and Gram-negative bacteria and a fungus, respectively. Figure 1 shows the sensitivity of *S. aureus* and *S. epidermidis* towards a 20% w/v $(PHPMA_{88}–PMPC_{200}–S)_2$ copolymer gel: zones of growth inhibition where the drop of copolymer was placed directly on the bacteria compared with bacterial growth on the surrounding medium.

Table 1 summarises the sensitivities of the five micro-organisms tested. The gel showed a broad spectrum of activity, affecting all except *E. faecalis*. This suggests the possible broad application of these copolymer gels as topical antibacterial wound dressings: *S. aureus* is one of the leading causes of nosocomial infections [18] while *S. epidermidis* has been reported to be the most frequent isolate within a covered post-operative incision and a common pathogen in necrotizing soft tissue infections [19, 20]. *Pseudomonas aeruginosa* is one of the most abundant and troublesome pathogens in thermally injured patients

Table 1 Sensitivity of different bacteria to the antimicrobial activity of 20% w/v $(PHPMA_{88}–PMPC_{200}–S)_2$ copolymer gels using the "drop on" assay

Organism name	Type	Sensitivity	
		Yes	No
Staphylococcus aureus	Gram-positive bacteria	X	
Staphylococcus epidermidis	Gram-positive bacteria	X	
Enterococcus faecalis	Gram-positive bacteria		X
Pseudomonas aeruginosa	Gram-negative bacteria	X	
Candida albicans	Fungus	X	

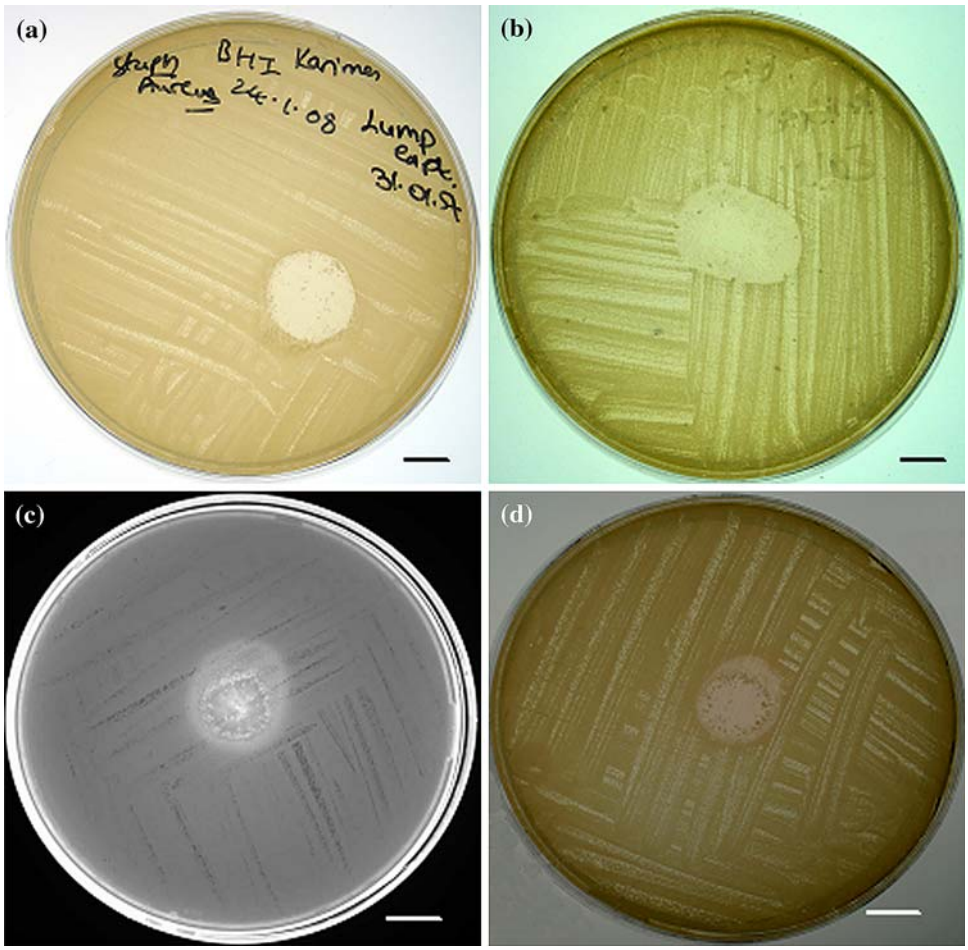

Fig. 1 Photographs showing the sensitivity of: **a** *Staphylococcus aureus* and **b** *Staphylococcus epidermidis* towards 20% w/v of $(PHPMA_{88}–PMPC_{200}–S)_2$ copolymer gels using the "drop on" assay. **c**, **d** $(PHPMA_{88}–PMPC_{200}–S)_2$ copolymer gel diffusion during a "drop on" assay using *S. aureus* on BHI agar plate highlighted by fluorescent copolymers $(PHPMA_{75}–PMPC_{190}–RH(EtO))_2$. **a** Under UV light. **b** Under normal light (Scale bar = 1 cm)

[21]. However, although the copolymer showed significant activity against *P. aeruginosa*, there were signs of incomplete inhibition within the zone. This slightly reduced activity might reflect a more resistant sub-population with a more hydrophobic outer membrane, which has been previously found to mediate resistance towards amphoteric and quaternary ammonium compounds in *Pseudomonas* before [22].

In order to determine the extent of diffusion taking place during the "drop on" assay, a shorter rhodamine-labelled triblock copolymer ($PHPMA_{75}$–$PMPC_{190}$–$RH(EtO))_2$ was then mixed with the "active" triblock ($PHPMA_{88}$–$PMPC_{200}$–$S)_2$ in order to highlight any copolymer diffusion that might occur following incubation with bacteria. The bacterial plate was then viewed under a UV lamp (see Fig. 1c, d). As expected, some copolymer diffusion was observed: the original gel droplet is highlighted by an intense fluorescent contour, while the diffused copolymer around the drop is less intense.

Next, an attempt was made to quantify the inhibitory activity observed in the preliminary screen of the "drop on" assay. For this, *S. aureus* was used because of its particular clinical relevance.

Figure 2a, b shows the results of a radial diffusion assay and compares the inhibitory zones produced by PBS, 10 and 20% w/v ($PHPMA_{88}$–$PMPC_{200}$–$S)_2$ copolymer gels (dissolved in PBS) compared with the area of a punched hole without additions. The area of the well was 0.23 ± 0.003 cm^2, which was not increased by PBS. A three-fold increase in zone area was obtained with a 10% w/v copolymer gel (0.70 ± 0.06 cm^2) and a six-fold increase was obtained with the 20% w/v copolymer gel (1.37 ± 0.05 cm^2). These zones are still relatively small and may be due to the relatively high copolymer molecular weight ($\sim$150 kDa) or the copolymer gelation that occurs at 37 °C.

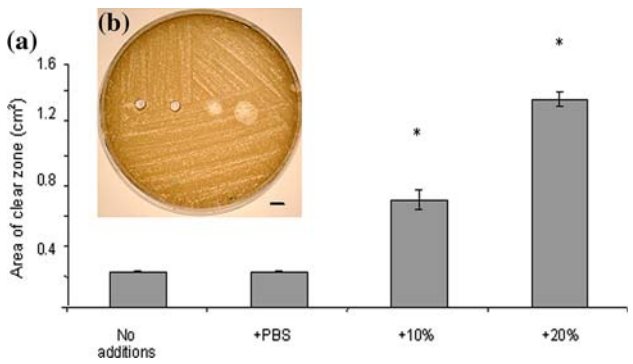

Fig. 2 **a** Inhibitory zones created by ($PHPMA_{88}$–$PMPC_{200}$–$S)_2$ copolymer gels on *S. aureus* growth. (mean ± SEM, $n = 3$ replicates). **b** Photograph of the inhibitory zone created by (from *left* to *right*): punch hole device, PBS, 10% and 20% w/v polymer gels. *$p < 0.05$ compared with control (Scale bar = 5 mm)

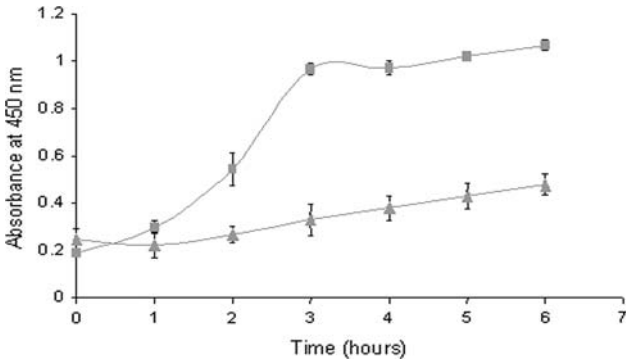

Fig. 3 Growth of *S. aureus* in control BHI broth (*filled square*) versus 20% w/v ($PHPMA_{88}$–$PMPC_{200}$–$S)_2$ copolymer gels (*filled triangle*) dissolved in BHI broth (mean ± SEM, $n = 3$ replicates)

As well as agar diffusion, the antimicrobial activity of the copolymer was also tested in liquid cultures (Fig. 3). Growth of *S. aureus* in BHI broth was assessed by monitoring the increase in optical density (450 nm) in the presence of 20% w/v ($PHPMA_{88}$–$PMPC_{200}$–$S)_2$ copolymer gels and was compared with that in broth alone (control). The growth of *S. aureus* increased exponentially over the first 3 h, before reaching a plateau as the stationary phase was entered. However, growth in the presence of the 20% w/v ($PHPMA_{88}$–$PMPC_{200}$–$S)_2$ copolymer gel was significantly retarded over the same period, attaining only 45% of the growth observed in the control broth after 6 h. While these data demonstrated inhibition of growth, it was not clear whether the copolymer was actually bacteriocidal or just bacteriostatic.

Consequently, *S. aureus* was incubated for 2 h with either 20% w/v ($PHPMA_{88}$–$PMPC_{200}$–$S)_2$ copolymer gel dissolved in PBS, 30% w/v Pluronic F127 dissolved in PBS (control copolymer) or with PBS alone (control). After 2 h exposure, *S. aureus* was released from the gels by copolymer dissolution in cold water and their viability was determined by colony counting. After 2 h, the 20% w/v copolymer gel yielded only 8.5 ± 2.3% of the PBS CFU count, whereas the control copolymer yielded 108 ± 2% of the PBS CFU. Thus, the copolymer gels are bacteriocidal for *S. aureus* after 2 h exposure but it seems that the presence of growth nutrients reduces their potency (as shown in Fig. 3). This may indicate protein binding to the copolymer and consequent reduction in efficacy.

To gain an insight into the antimicrobial mode(s) of action of these copolymers, a range of copolymers were synthesised with varying PHPMA and PMPC compositions. These were assessed for their antimicrobial activity using the "drop on" technique. Table 2 lists the copolymers tested and summarises the sensitivity of *S. aureus* towards each of them.

Table 2 Sensitivity of *S. aureus* growth to various 20% w/v copolymers using the drop on technique

Polymers	Sensitivity	
	Yes	No
$(PHPMA_{88}–PMPC_{200}–S)_2$	X	
$PHPMA_{71}–PMPC_{400}–PHPMA_{71}$	X	
$(PMPC_{125}–S)_2$		X
$(PHPMA_{69}–PMPC_{125}–S)_2$		X
$(PHPMA_{44}–PMPC_{125}–S)_2$		X

The in situ cleavage of the central disulphide bond in some of these copolymers to produce reactive sulphur-containing functional groups was initially considered as a possible mechanism of antimicrobial action. However, a 20% w/v of $PHPMA_{71}–PMPC_{400}–PHPMA_{71}$ gel contained no disulphide bonds and also inhibited bacterial growth, as shown in Fig. 4a, b. This is particularly noticeable with *P. aeruginosa*, for which $(PHPMA_{88}–PMPC_{200}–S)_2$ was only weakly active, while the $PHPMA_{71}–PMPC_{400}–PHPMA_{71}$ gel produced a clearer zone at the same volume and concentration. In addition, the two lower molecular weight triblock copolymers, $(PHPMA_{44}–PMPC_{125}–S)_2$ and $(PHPMA_{69}–PMPC_{125}–S)_2$, and also the $(PMPC_{125}–S)_2$ homopolymer, all of which contain a central disulphide bond, did not exhibit any significant antimicrobial effect in these assays. Thus, this "reactive disulphide" hypothesis was discarded.

The MPC monomer is zwitterionic, so the central PMPC block in these copolymers carries both cationic and anionic charge. Although there is no net charge at physiological pH, it was conceivable that the quaternary amine groups might be responsible for the antimicrobial activity, since cationic polyelectrolytes are well-known to be highly toxic towards most cell types and also towards a wide range of bacteria [9, 23]. This hypothesis was evaluated by examining a 20% w/v aqueous solution containing $(PMPC_{125}–S)_2$ homopolymer. However, this non-gelling control sample exhibited no discernible effect on bacterial growth (Table 2). These results suggest that the relatively hydrophobic PHPMA block may be an essential structural motif for antimicrobial activity.

Although the PMPC homopolymer did not have any discernible effect on bacterial growth, the antibacterial activity of PHPMA–PMPC–PHPMA triblock copolymer gels seemed to be dependent on the length of PMPC. Triblocks with mean degrees of polymerisation (DP_n) for the central PMPC block of less than 400 do not seem to exhibit any antibacterial activity. A longer PMPC block length might possibly be a contributory factor, which would perhaps explain the greater antimicrobial activity of $PHPMA_{71}–PMPC_{400}–PHPMA_{71}$ towards *P. aeruginosa* (see Fig. 4b). The disulphide bridge in $(PHPMA_{88}–PMPC_{400}–S)_2$ copolymer is known to be prone to cleavage by reduction or oxidation [12]. Such in situ degradation would inevitably lead to shorter PMPC blocks. On the other hand, $PHPMA_{71}–PMPC_{400}–PHPMA_{71}$ cannot be readily degraded, since it does not contain a disulphide bridge. Hence, it is possible that the hydrophilic/hydrophobic block ratio may be important: this effect has been reported for Poloxamer F-68, which comprises 80 wt% hydrophilic poly(ethylene oxide, EO). A dramatic reduction in the gross infection of *S. aureus* inoculated wounds in guinea pigs was observed when using this copolymer in combination with iodine as a surgical scrub solution [24]. In seeking to understand the relationship between the structure of the Poloxamer and its potential use for the efficient delivery of iodine, some Poloxamer compositions were investigated on their own but these formulations appeared to be only weakly antimicrobial. The levels of gross infection were reduced when a high hydrophilic to hydrophobic block ratio was used [4:1 (EO:propylene oxide PO) compared to 1:4 EO:PO]. However, significant infection of the wounds was still observed. Indeed, it was found that the formulation having the highest hydrophilic

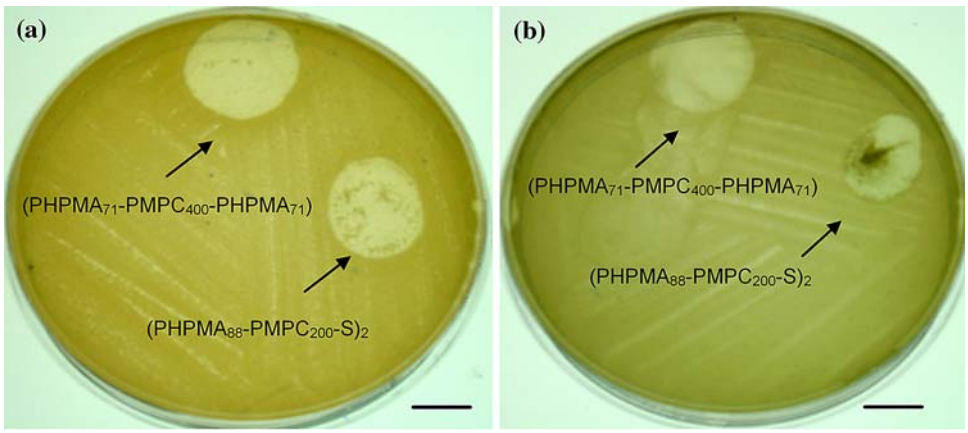

Fig. 4 Sensitivity of *P. aeruginosa* and *S. aureus* towards 20% w/v of $(PHPMA_{88}–PMPC_{200}–S)_2$ and $(PHPMA_{71}–PMPC_{400}–PHPMA_{71})$ copolymer gels (Scale bar = 1 cm)

to hydrophobic block ratio exhibited the lowest rate of infection at relatively low inoculae. The investigators interpreted the reduced incidence of infection as being due to Poloxamers having less tissue toxicity, rather than having any inherent antimicrobial activity [24]. Pluronic F-68 was also shown to prevent the development of infection when contaminated wounds in rabbits were scrubbed with a sponge soaked in these polymers [25]. Poloxamer F-68 has been approved by the FDA for use as a skin wound cleanser with no reported side effects. However, in contrast to the new copolymers reported in this article, these polymers do not have any intrinsic antibacterial activity [26].

Despite the relatively high bacterial inoculae used in the present study compared to those used in other antimicrobial wound dressing studies [27–29], the copolymer gels maintained their antimicrobial activity. Clearly, it would be of interest to examine the performance of these copolymer gels in the presence of lower numbers of bacteria. Various studies have reported conflicting results concerning the correlation between bacterial counts and sepsis. Indeed, it might be the degree of invasion within the tissue, rather than the bacterial density that determines the extent of infection [30].

Next the question of whether these ($PHPMA_{88}$–$PMPC_{400}$–$S)_2$ copolymer gels would be capable of exhibiting antimicrobial activity when in contact with infected skin was investigated using a 3D TE skin model that we have recently developed [13]. Here the TE skin was infected with *P. aeruginosa* and the antimicrobial activity of 20% w/v ($PHPMA_{88}$–$PMPC_{200}$–$S)_2$ was compared with infected skin not exposed to these copolymer gels. The degree of infection was assessed by histological studies using haematoxylin and eosin (H and E) staining (see Fig. 5) and by viable bacterial counts from the skin after 24–48 h exposure to the copolymer gel (see Fig. 6). Again, these copolymer gels exhibited potent antimicrobial activity, as shown by the statistically significant lower numbers of viable bacteria recovered from gel-exposed infected TE skin ($38.5 \pm 4.9\%$, $n = 3$ experiments)

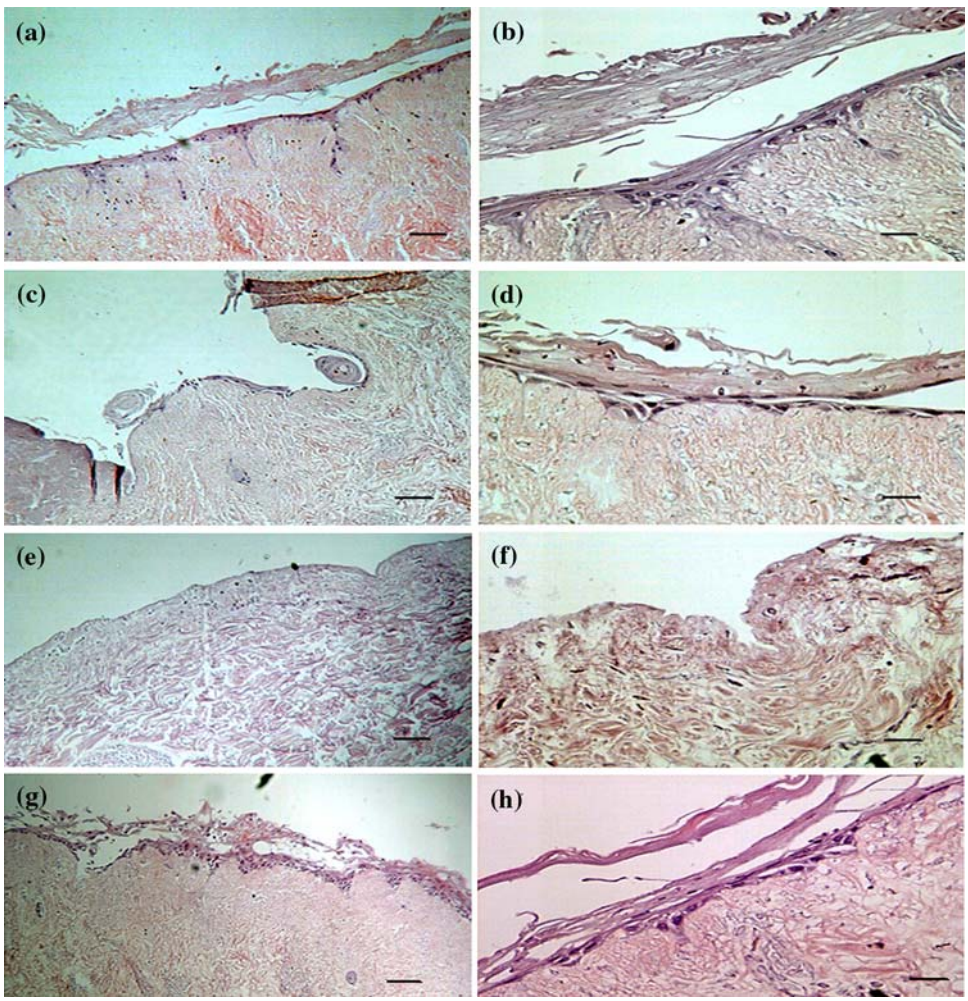

Fig. 5 Haematoxylin and eosin staining of: non-infected TE skin (**a**, **b**), Burnt TE skin (**c**, **d**), Burnt and infected TE skin with *P. aeruginosa* (**e**, **f**), Burnt and infected TE skin with *P. aeruginoasa* and exposed to 20% w/v of ($PHPMA_{88}$–$PMPC_{200}$–$PHPMA_{88}$–$S)_2$ copolymer gels for 24–48 h (**g**, **h**). Scale bar (10× = 500 μm, 40× = 45 μm)

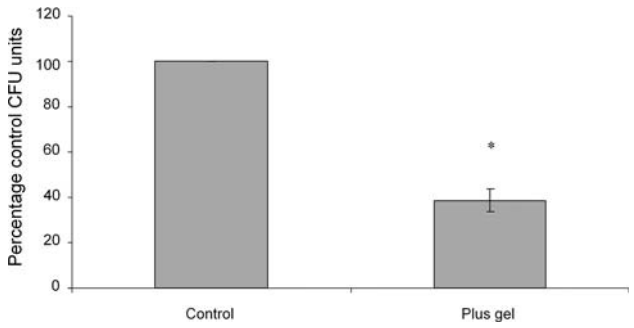

Fig. 6 Percentage control CFU of *P. aeruginosa* on infected TE skin model treated with 20% w/v $(PHPMA_{88}–PMPC_{200}–S)_2$ copolymer gels (mean ± SEM, $n = 3$ replicates)

compared to the untreated TE skin control (100 ± 0%, see Fig. 6). We have not yet tested the effect of the copolymer gels on *S. aureus*-infected skin because this organism does not markedly invade the tissue model when the latter was developed [13].

Figure 5 shows the non-burnt non-infected TE skin. This sample exhibits some features of normal skin with a basal layer of well-attached proliferating keratinocytes and upper layers that have progressively differentiated with loss of nuclei to form the skin barrier (Fig. 5a, b). To enable bacterial invasion in this model we found that the barrier had to be destroyed by heat treatment [13]. Figure 5c shows the effect of such heat treatment on the model: the epidermal layer is completely removed at the site where heat was applied while the rest of the adjacent TE skin exhibits normal features, as shown in Fig. 5d. When the model was burnt and infected with *P. aeruginosa*, there was complete detachment of the epidermal layer from the whole of the TE skin (see Fig. 5e, f). This observation is in agreement with our recent studies [13]. However, when the burnt, infected model was exposed to the copolymer, the gel not only reduced the residual bacterial count in the TE skin (see Fig. 6) but also the bacteria-induced loss of the epithelial layer on non-burnt areas was significantly suppressed, as shown in Fig. 5g, h. This shows that the hydrogel can reduce bacterial damage substantially in this physiologically relevant 3D-infected skin wound model.

In the final set of investigations we attempted to immobilise the antimicrobial activity of the triblock copolymers by growing PMPC–PHPMA diblock "brushes" (layers of densely grafted polymer chains) from planar silicon wafers. This allowed us to vary the thickness of PMPC blocks from 0.8 to 13 nm and to have PHPMA blocks up to 2.4 nm. All of these PMPC–PHPMA diblock brush-coated wafers exhibited antimicrobial activity. Even for the least activity, bacterial growth was reduced by 70% compared to that of a control surface (Fig. 7). However, no obvious trends or correlations were obtained from a library

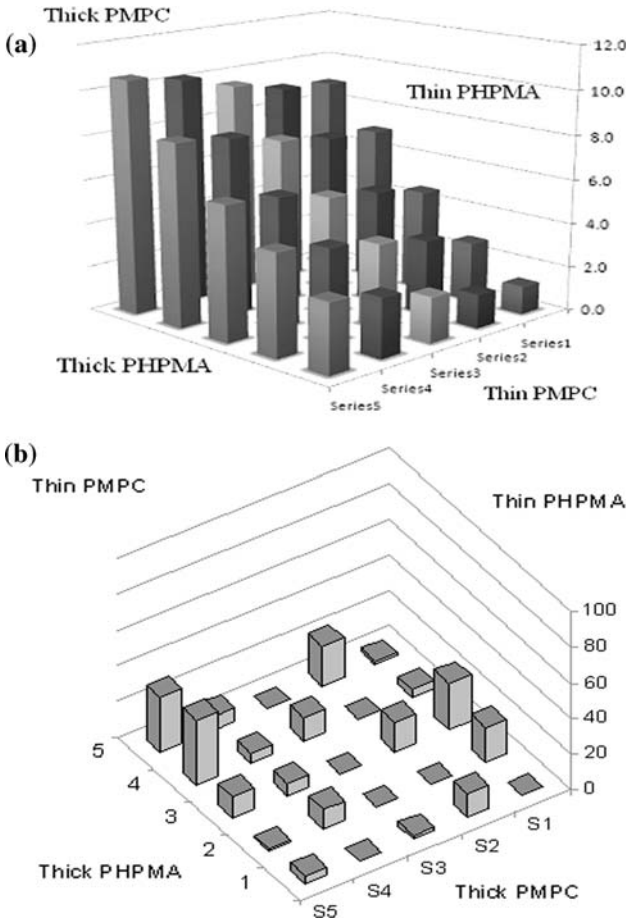

Fig. 7 **a** Library of brush thicknesses obtained by ellipsometry showing 2D gradient in two directions obtained for PMPC and PHPMA brushes. **b** Percentage control (clean silicon surfaces) *SA* growth versus the different surfaces

of 25 different substrates coated with varying lengths of PMPC and PHPMA chains (data not shown). The key feature appears to be that all such surfaces were reasonably effective.

We also evaluated different thicknesses of PMPC and PHPMA homopolymer brushes in a series of control experiments. Surfaces coated with PMPC with thicknesses varying from 0.6 to 7.2 nm showed no significant reduction in bacterial survival (Fig. 8). This is in agreement with the results obtained in solution, so that the $(PMPC_{125}–S)_2$ homopolymer did not exhibit any antimicrobial activity. In an aqueous environment, it is difficult to study the antimicrobial activity of PHPMA block without compromising the solubility of the polymer studied. Hence the advantage of studying the effects of hydrophobic blocks when the latter is grafted onto surfaces. PHPMA-coated surfaces exhibited significant antibacterial activity. Moreover, this activity appears to be related to the grafted brush thickness: 0.9 nm PHPMA-coated surfaces gave an approximate 50%

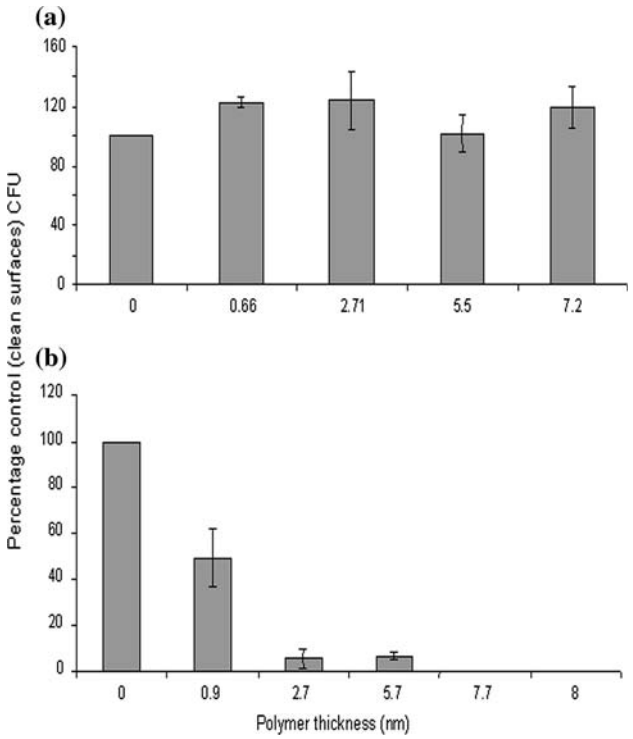

Fig. 8 Percentage control (clean silicon surfaces) *SA* growth versus polymer layer thickness. **a** PMPC surfaces, **b** PHPMA surfaces. (mean ± SEM, $n = 3$ replicates)

reduction in bacterial activity but surfaces coated with 2.7 nm PHPMA and above gave a more than 90% reduction in bacterial CFUs (Fig. 8). This is in accordance with several studies that have demonstrated that an increase in the hydrophobicity of antimicrobial amphiphilic copolymers was associated with an increase in antimicrobial activity. However, the selectivity of these copolymers towards bacteria (rather than mammalian cells) was also reduced [8–10, 31]. We have also recently published data on micellar solutions of PMPC–PHPMA diblock copolymers showing that PMPC–PHPMA diblock copolymers containing longer PHPMA blocks achieve relatively fast intracellular delivery. This suggests a more efficient interaction of these copolymers with cellular membranes [32]. The hydrophobic component of the copolymer may interact with both mammalian and bacterial membranes, while the cationic charge on the zwitterionic PMPC block may confer selectivity towards bacterial membranes. It is possible that, once a sufficiently high local concentration of copolymer is attained within the bacterial membranes, then disruption occurs, causing a breakdown of the transmembrane potential, leakage of cytoplasmic contents and ultimately cell death [31]. This may also explain the lack of any antibacterial activity observed for the 20% w/v $(PMPC_{125}–S)_2$ solutions, which lack the hydrophobic PHPMA component that appears to be essential for interacting with the bacterial membranes.

Bacterial cells are known to preferably adhere to hydrophobic rather than hydrophilic surfaces [33, 34]. Our next experiment aimed to establish whether bacteria incubated with PHPMA surfaces were dead or alive. We performed a live/dead staining assay on *S. aureus* incubated with surfaces containing the thickest PHPMA homopolymer layer (8 nm) and imaged the fluorescence obtained using a ZEISS LSM 510 M instrument (Fig. 9). Syto 9 is a green fluorescent dye that is able to stain all cells (whether viable or dead) while PI is a red fluorescent dye that can only enter organisms with compromised membranes. Confocal microscopy revealed that very low numbers of live bacteria remained attached to PHPMA surfaces compared to their growth on the non-grafted silicon surfaces on which most of the bacteria were viable (green). Taking these results together with the counts of CFU following incubation of such surfaces (Fig. 8), it can be concluded that *S. aureus* does not survive prolonged contact with PHPMA-coated surfaces. What remains uncertain is whether the death of these microorganisms led to their ready detachment from the surfaces by rinsing as the surfaces exhibit only a relatively small number of bacteria.

Conclusions

In summary, serendipitous antimicrobial effect of aqueous gels $(PHPMA_{88}–PMPC_{200}–S)_2$ triblock copolymer gels has been demonstrated using a range of well-established assays and a physiologically relevant 3D model of bacterially infected human skin. Such copolymers have the profile of a mid-range antimicrobial agent. The inherent antimicrobial activity of these gels appears to be unique: they are of potential major importance since they are unlikely to induce the problems of bacterial resistance. In addition they are shown to be non-toxic to skin, which is a problem that has sometimes been encountered with other commonly used topical antimicrobials. Further studies of these copolymer gels suggested that minimum block lengths for both PMPC and PHPMA were required to ensure significant antimicrobial activity and they could be grown as PMPC–PHPMA diblock copolymer brushes from planar silicon wafers and retain antimicrobial activity. Surprisingly, PHPMA homopolymer brushes alone showed significant antimicrobial activity towards *S. aureus*. Given the relatively high cost of the MPC monomer, this may be an important finding.

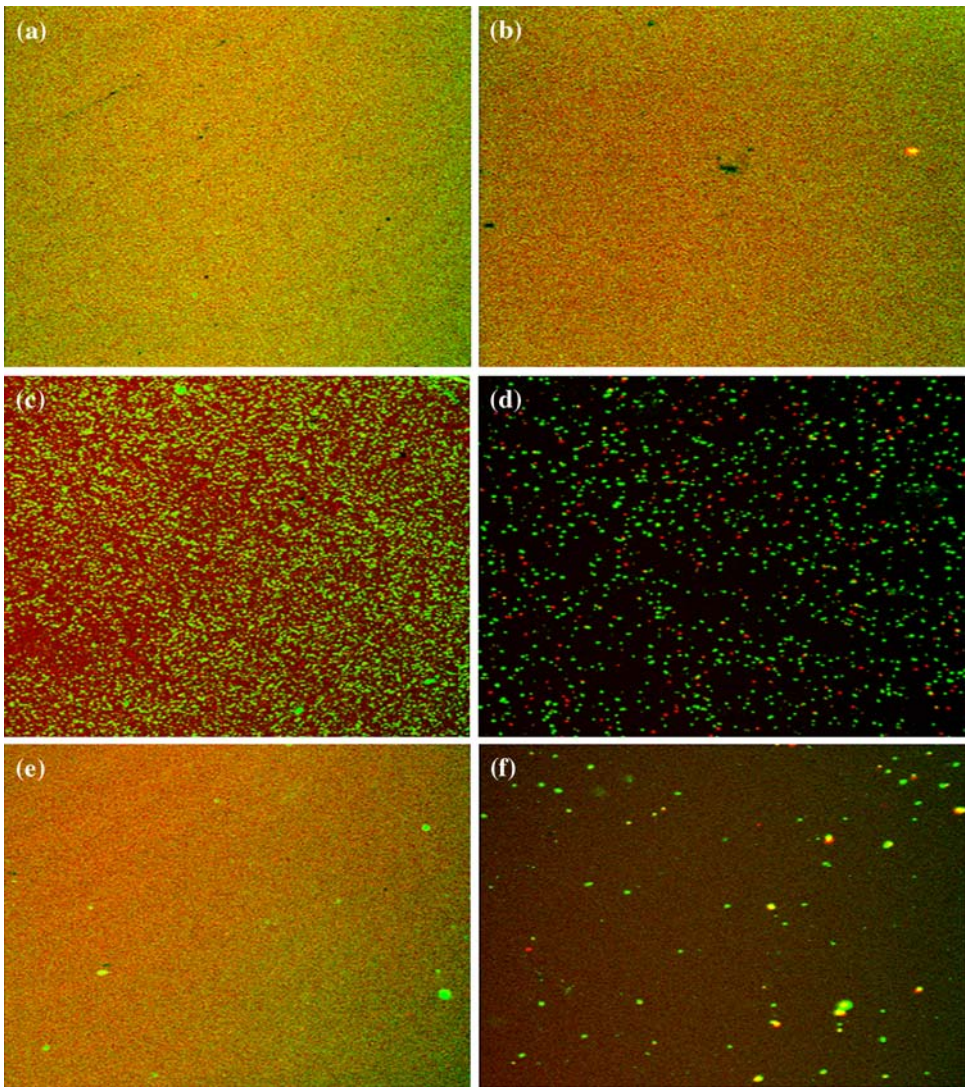

Fig. 9 Live/dead staining of *S. aureus* incubated in contact with various surfaces at 10× magnification (**a**, **c**, **e**) and 40× magnification (**b**, **d**, **f**). Control surfaces with no bacteria (**a**, **b**), control surfaces with bacteria (**c**, **d**), HPMA surfaces with 8.3 nm thickness (**e**, **f**)

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