Antibacterial Effects of Poly(2-(dimethylamino ethyl)methacrylate) against Selected Gram-Positive and Gram-Negative Bacteria

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Antimicrobial coatings can reduce the occurrence of medical device-related bacterial infections. Poly(2-(dimethylamino ethyl)methacrylate) (pDMAEMA) is one such polymer that is being researched in this regard. The aims of this study were to (1) elucidate pDMAEMA's antimicrobial activity against a range of Gram-positive and Gram-negative bacteria and (2) to investigate its antimicrobial mode of action. The methods used include determination of minimum inhibitory concentration (MIC) values against various bacteria and the effect of pH and temperature on antimicrobial activity. The ability of pDMAEMA to permeabilise bacterial membranes was determined using the dyes 1-*N*-phenyl-naphthylamine and calcein-AM. Flow cytometry was used to investigate pDMAEMA's capacity to be internalized by bacteria and to determine effects on bacterial cell cycling. pDMAEMA was bacteriostatic against Gram-negative bacteria with MIC values between 0.1−1 mg/mL. MIC values against Gram-positive bacteria were variable. pDMAEMA was active against Gram-positive bacteria around its pK_a and at lower pH values, while it was active against Gram-negative bacteria around its pK_a and at higher pH values. pDMAEMA inhibited bacterial growth by binding to the outside of the bacteria, permeabilizing the outer membrane and disrupting the cytoplasmic membrane. By incorporating pDMAEMA with erythromycin, it was found that the efficacy of the latter was increased against Gram-negative bacteria. Together, the results illustrate that pDMAEMA acts in a similar fashion to other cationic biocides.

Introduction

Microbial contamination is a major concern in areas such as food packaging and storage and water treatment. Growth of bacteria on implanted medical devices is a particular problem[1,2] and contamination can lead to development of severe infections. A potential method to prevent microbial contamination is to coat susceptible surfaces with antimicrobial agents including polymers, that inhibit growth of microorganisms.[1] Numerous polymers have been found to have antimicrobial activities against Gram-negative and Gram-positive bacteria, yeast, and viruses.[3,4] For this reason they have been investigated as potential antimicrobial coatings and inhibit bacterial growth when attached to glass, paper, plastic, and metal.[5−8] For example, methacryloyloxydodecylpyridinium was incorporated into dental resin composite and reduced microbial activity without leaching.[9] Polymers have also been used to deliver encapsulated antimicrobial agents allowing localized, controlled release of the drug into the target site.[10,11] In addition, in vivo experiments have recently demonstrated antimicrobial potential of selected polymers. For example, oral administration of a chitosan-oligosaccharide formulation over 7 days to mice subsequently challenged with *Staphylococcus aureus* by the intraperitoneal route resulted in a higher survival rate compared with that of controls.[12]

Poly(2-(dimethylamino ethyl)methacrylate) (pDMAEMA) is a mucoadhesive polymer, that is cationic if dissolved into acidified media or if quaternized by using an alkylating agent.[13,14] Recent advances in living radical polymerization (LRP)[15,16] and reversible addition−fragmentation transfer (RAFT) polymerization[17] allowed for the synthesis of pDMAEMA with tunable polymer chain length and macromolecular architecture, as well as a narrow molecular weight distribution. It is a thermoresponsive polymer and, at increased temperatures, the polymer phase separates from solution due to a breakdown in hydrogen bonding interactions.[18] The solubility of pDMAEMA in aqueous solution is also pH-dependent. At pH 7, pDMAEMA is partially charged (hydrophilic) and partially uncharged (hydrophobic), resulting in an amphiphilic molecule.[19] At lower pH the polymer becomes more positively charged, and at pH values greater than 7, it becomes mostly uncharged.[19] pDMAEMA has numerous potential uses that include use as a nonviral gene delivery vector[20,21] in water purification[22,23] and in drug delivery.[24] It is also used as a coating for soil-resistant surfaces,[25] as an ion exchange media for protein separation,[26] and to adapt the wettability of surfaces, including microfluidic devices.[27] In addition to this, pDMAEMA has been attached to glass,[28,29] filter paper,[28,30] polystyrene,[31] and polypropylene[32] and also as an antimicrobial surface coating to inhibit the growth of *Escherichia coli* (*E. coli*) and *Bacillus subtilis*. It has also been incorporated in antimicrobial copolymers to inhibit growth of *E. coli*[33] and *S. aureus*.[34] The monomer DMAEMA has been shown to decrease the binding of various coagulase negative and positive *Staphylococcus*, *Streptococcus pyogenes*, *E. coli*, and *P. aeruginosa* strains when attached to the surface of PVC

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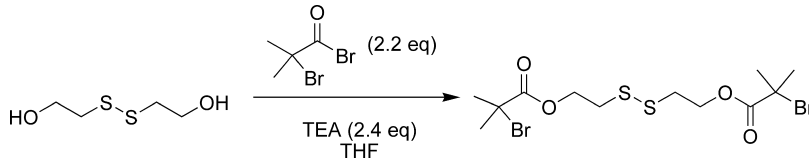
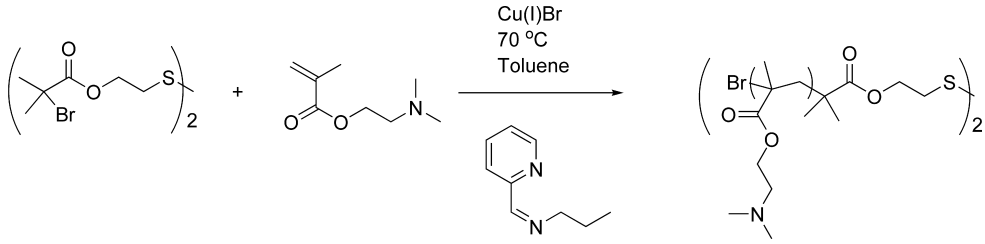
Scheme 1. Synthesis of Disulfide-Based Bifunctional Polymerisation Initiator, Bis[2-(2-bromoisobutyryloxy)ethyl] Disulfide $(BiBOE)_2S_2$



Scheme 2. Cu(I)Br Catalyzed Polymerization of Dimethyl Amino Ethyl Methacrylate (DMAEMA) Initiated by $(BiBOE)_2S_2$



catheters.[35] pDMAEMA has also been incorporated in an antiadherent coating on poly(methyl methacrylate) disks to inhibit binding of *E. coli*, macrophages, and fibroblasts.[25] Furthermore, pretreatment of human intestinal epithelial cell cultures with pDMAEMA led to reduced adhesion and invasion of *Salmonella* ser. Typhimurium.[36]

The mode of action of cationic biocides has been suggested to progress as follows: (1) adsorption onto the bacterial cell surface, (2) diffusion through the cell wall, (3) binding to the cytoplasmic membrane, (4) disruption of the cytoplasmic membrane, (5) release of cell cytoplasmic constituents, and (6) cell death.[37] pDMAEMA may work in a similar manner by adsorbing to the cell surface through electrostatic interactions and disrupting the cytoplasmic membrane through hydrophobic interactions.[28,30] The aims of the study were therefore to (1) investigate the range of pDMAEMA's antimicrobial activity against a wide group of pathogenic and commensal organisms using a range of pDMAEMA derivatives and (2) to investigate the antimicrobial mode of action against selected bacteria.

Materials and Methods

Materials. All chemical syntheses were carried out using standard Schlenk techniques under an inert atmosphere of oxygen-free nitrogen, unless otherwise stated. Copper(I) bromide (98%) was purified according to the method of Keller and Wycoff.[38] *N*-(*n*-Propyl)-2-pyridylmethanimine was prepared as described earlier.[15] Triethylamine (99%, Thermo Fischer Scientific, Waltham, MA) was stored over sodium hydroxide pellets. Anhydrous tetrahydrofuran (THF; "Hi-Dry", 99.99%, Romil, Cambridge, U.K.) was stored over activated 4 Å molecular sieves under dry nitrogen. All the other general chemicals and reagents used were of analytical grade and were obtained from Sigma-Aldrich Company Ltd. (Dorset, U.K.), unless otherwise stated.

Synthesis of Unconjugated and Hostasol-Conjugated pDMAEMA Polymers. Unconjugated pDMAEMA was tested to show antimicrobial effects of the polymer without any modifications and hostasol-conjugated pDMAEMA was also tested as it was used in some experiments to aid in visualization. Hostasol (thioxantheno[2,1,9-def]isochromene-1,3-dione) (389.5 Da, CAS Registry Number: 14121-49-4) was supplied by Clariant (Muttenz, Switzerland). Fluorescent hostasol-conjugated pDMAEMA was prepared, as described previously,[14] using ethyl-2-bromo isobutyrate as the polymerization initiator and lowering the pH of the final isolated polymer aqueous solution to pH 5.5 with 0.5 M aqueous HCl before freeze-drying. Hostasol was used as a fluorescent marker to track the polymer in imaging and flow cytometry studies. Nonfluorescent pDMAEMA (unconjugated pDMAEMA) was prepared in the same way, except that no hostasol methacrylate fluorescent comonomer was employed in the polymerization step. In addition to these molecules the following pDMAEMA polymers were also made for testing.

Synthesis of Reducible pDMAEMA. Reducible pDMAEMA has been reported to be less toxic than unconjugated pDMAEMA so it was also tested for its antimicrobial activity.[39]

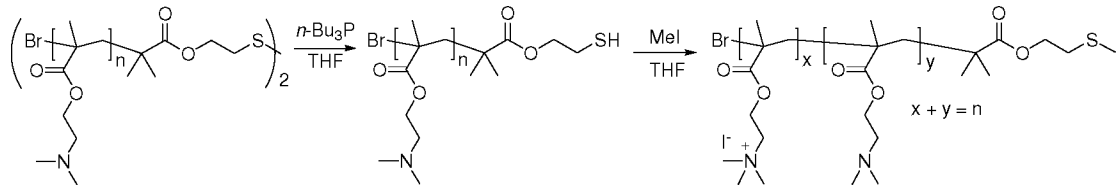
Bis(2-hydroxyethyl) disulfide (4.00 mL, 32.4 mmol) and an excess of triethylamine (9.9 mL, 71 mmol) was added to a 500 mL round-bottom flask, along with a magnetic stir bar, and was purged with nitrogen for 15 min on an ice bath. Anhydrous THF (150 mL) was then added, and the resulting solution was allowed to cool to 0 °C. Under a nitrogen atmosphere, 2-bromoisobutyryl bromide (8.4 mL, 68 mmol) was added dropwise via a degassed syringe (dropwise addition is essential to minimize the exotherm). The solution was allowed to reach ambient temperature and left to stir for 6 h. The resulting triethylammonium bromide salt was removed by filtration, and the solvent was removed under reduced pressure. The resulting pale yellow solution was stirred with 0.1 M aqueous Na_2CO_3 to hydrolyze any residual 2-bromoisobutyryl bromide. The crude product was then extracted three times with dichloromethane, and the organic layers were combined and dried over anhydrous magnesium sulfate, filtered, and the volatiles removed under reduced pressure, yielding the $(BiBOE)_2S_2$ initiator as a clear yellow oil (Scheme 1; 10.1 g, 22.0 mmol, 74.1%), which was stored at 4 °C. ^1H, $^{13}C[^1H]$, and CHN elemental analysis were in line with previously published data.[40]

Cu(I)Br (0.33 g, 2.3 mmol) was added to a clean, oven-dried Schlenk tube, along with a magnetic follower. The Schlenk tube was sealed with a suba-seal, evacuated, and filled with nitrogen. Toluene (25.0 mL), DMAEMA (25.0 mL, 148 mmol), and bis[2-(2-bromoisobutyryloxy)ethyl] disulfide initiator (0.25 mL, 1.2 mmol) were sequentially added via a degassed syringe and the resulting solution was degassed by five freeze−pump−thaw cycles (Scheme 2). The solution was heated to 70 °C in a thermostatically controlled oil bath, and then *N*-(*n*-propyl)-2-pyridylmethanimine (0.72 mL, 4.7 mmol) was added via a degassed syringe ($t = 0$). Samples were taken every 15 min via a degassed syringe for conversion and molecular weight analysis. Once the reaction had reached a satisfactory conversion, the suba-seal was removed, and air was bubbled through for 1 h. The polymer solution was passed through a basic alumina column and then precipitated in petroleum ether, 40−60 °C (1 L), at ambient temperature.

Synthesis of 50% Quaternized pDMAEMA. As pDMAEMA is a charged molecule, a 50% quaternized pDMAEMA polymer was used to investigate the impact of charge on antimicrobial activity.

The disulfide-containing polymer (3.0 g, 0.17 mmol) was dissolved in THF (20 mL) and placed in a round-bottom flask equipped with a stirring bar. Once heated to 40 °C, Bu_3P (210 μL, 0.87 mmol) was added to the system. The reduction was complete after 10 min, the

Scheme 3. Reduction of the Disulfide Bridge and Subsequent Quaternization with MeI



solution was cooled down to ambient temperature, passed through a short neutral alumina pad and the volatiles were removed under reduced pressure.

An aliquot of this reduced polymer (1.0 g, 6.4 mmol of quaternizable amine repeating units) was dissolved in THF (25 mL) and MeI (0.20 mL, 3.2 mmol) via syringe and stirred at ambient temperature for 48 h (Scheme 3). The solvent was then removed under reduced pressure and the solid residue was dissolved in 20 mL of deionized water and freeze-dried, affording the desired quaternized polymer.

Analysis of Polymers. Molar mass distributions were measured using size exclusion chromatography (SEC), on a system equipped with two PL gel 5 μm mixed D-columns (300 × 7.5 mm) and one PL gel 5 mm guard column (50 × 7.5 mm; Polymer Laboratories, suitable for molecular weights between 200 and 400000 g/mol) with differential refractive index detection, using THF/triethylamine 95:5 (v/v), at 1.0 mL/min, as the eluent. Poly(MMA) standards (200−3 × 10^5 g/mol) were used to calibrate the SEC. Analyte samples contained (0.2% vol) toluene as the flow marker. For purity determination, pDMAEMA polymers were subjected to SEC-HPLC with a Varian 920 HPLC using a BioSep-SEC-S-2000 column 300 × 7.8 mm (Phenomenex, U.K.). Samples were eluted with 50 mM phosphate buffer (pH 6.8) at a flow rate of 1 mL/min and monitored at a UV absorbance of 280 nm.

Bacterial Strains, Media, and Culture Conditions. *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* ser. Typhimurium) IMD 574[41] was obtained from Dr. Rebecca O'Mahony, Centre for Food Safety, School of Agriculture, Food Science and Veterinary Medicine, University College, Dublin. *Escherichia coli* (*E. coli*) ATCC 10536 and *Micrococcus luteus* (*M. luteus*) ATCC 9341 were obtained from Dr. Siobhan McClean, Institute of Technology, Tallaght, Dublin. *E. coli* equine isolate, *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*Salmonella* ser. Enteritidis) ATCC 13076, *Salmonella* ser. Typhimurium bovine isolate, *Lactobacillus salivarius* (*L. salivarius*) UCC118, *Listeria monocytogenes* (*L. monocytogenes*) NCTC 11994, *Listeria* spp. Wild type #28, and *Pseudomonas aeruginosa* (*P. aeruginosa*) QC strain were obtained from Dr. Denise Drudy, Centre for Food Safety, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin. *Bifidobacterium breve* (*B. breve*) DSMZ 20213, *Bifidobacterium bifidum* (*B. bifidum*) DSMZ 20456 and *Candida albicans* (*C. albicans*) C were obtained from Mr. Michael Folan, Westgate Biologicals Ltd., Donegal Town, Ireland. *Staphylococcus epidermidis* (*S. epidermidis*) 1457[42] was obtained from Dr. James O'Gara, School of Biomolecular and Biomedical Science, University College, Dublin. Aerobic bacterial strains, including facultative anaerobes, except *L. salivarius*, were cultured aerobically in tryptic soy broth (TSB) or agar (TSA) at 37 °C, unless otherwise stated. Anaerobic bacterial strains, including *L. salivarius*, were cultured in de Man Rogosa and Sharpe (MRS) media, supplemented with 0.05% L-cysteine-HCl, at 37 °C. *L. salivarius* was cultured aerobically and *B. bifidum* and *B. breve* were cultured under anaerobic conditions maintained using an AnaeroGen oxygen depleting system (Oxoid, Cambridge, U.K.) in an anaerobic chamber. *C. albicans* C was cultured aerobically in TSB supplemented with 0.5% yeast extract (TSBYE) at 37 °C. All organisms were grown from frozen stocks and subcultured at least twice before use in experiments to ensure normal growth patterns.

MIC. The MIC is defined as the lowest concentration of polymer to completely inhibit growth of the bacterial cultures examined. The method of MIC calculation for aerobic bacteria was adapted from the microdilution broth dilution procedure from the Clinical and Laboratory Standards Institute (formerly NCCLS) protocol.[43] For anaerobic bacteria the method was adapted from the microdilution broth dilution procedure from the NCCLS protocol,[44] and for yeast the method was adapted from the microdilution broth dilution procedure from the NCCLS protocol.[45] Aerobically- and anaerobically-grown bacterial cells were seeded in microtiter plates at 5 × 10^5 CFU/mL per well and 1 × 10^5 CFU/mL per well, respectively. Yeast cells were seeded at 0.5−2.5 × 10^3 CFU/mL per well. Cells were incubated with varying concentrations of pDMAEMA in fresh media. Aerobic bacteria plates were incubated at 37 °C for 18 h and microbial growth was determined using a microplate spectrophotometer (UVM340, Asys Hitech GmbH, Eugendorf, Austria) at 600 nm. Anaerobic bacteria and yeast plates were incubated at 37 °C for 48 h and quantitative cell growth was determined by eye. The results are presented as the mean of a minimum of three independent replicates.

Time-Kill Curves. Time-kill studies were adapted from the time-kill method for determining bactericidal activity as outlined in the NCCLS protocol.[46] The bacteria were inoculated into flasks at 5 × 10^5 CFU/mL and incubated for 90 min at 37 °C, 170 rpm. An initial sample was taken for serial dilution and colony counting and then polymer (unconjugated pDMAEMA) or media alone (as a control), were added to the flasks. Incubation was continued at 37 °C, 170 rpm, and samples taken at timed intervals up to 48 h for colony counts. Viable counts were calculated to give CFU/mL and time-kill curves were plotted with $\log_{10}$CFU/mL against time. A bactericidal effect was defined as a ≥3 $\log_{10}$ decrease in CFU/mL after 24 h. The results are presented as a mean ± standard error of the mean (SEM) of a minimum of three independent replicates.

Determination of Optimum pH and Temperature for Antibacterial Activity. pH and temperature optimums were determined by calculating MIC values at varying pH values and temperatures with increasing concentrations of polymer (unconjugated pDMAEMA and 50% quaternized pDMAEMA). Growth is represented as a percentage of cultures containing no polymer (100% growth). The results are presented as a mean ± SEM of a minimum of three independent replicates.

Estimation of pK_a Values for Unconjugated and 50% Quaternized pDMAEMAs. The pK_as of unconjugated pDMAEMA and 50% quaternized pDMAEMA were determined by pH titration. Briefly, 10 mL of 1 mg/mL polymer, in distilled water, was titrated against 0.01 or 1 M NaOH to the equivalence point (unconjugated pDMAEMA, pH = 9.3−9.8; 50% quaternized pDMAEMA, pH = 11.3−11.6) to obtain the basic form of the polymers. At this point, the titration was continued with 0.01 M HCl and the pH of the solution was monitored. The pH at equivalence of this curve is equal to the pK_a of the polymer. The results are presented as the average of three independent replicates.

Visualization of Hostasol-Conjugated pDMAEMA Binding to Bacteria. To visualize the interaction of the polymer with the bacteria, hostasol-conjugated pDMAEMA was incubated with 1 × 10^9 CFU/mL *Salmonella* ser. Typhimurium IMD 574 and *S. epidermidis* 1457 at concentrations of 1 and 0.1 mg/mL, respectively. At 30 and 120 min, samples were centrifuged and the pellet was washed 3 times in PBS to remove unbound polymer. Samples were resuspended in PBS, mounted on slides, and viewed on a Nikon Eclipse E400 fluorescent microscope (Nikon, Japan) at 60× magnification, using a FITC filter, excitation wavelength (Ex) = 465−495 nm, and emission wavelength (Em) = 515−555 nm. Samples containing no polymer were also viewed as controls and brightfield pictures were taken for comparisons. Pictures

were captured using QCapture Pro software, version 5.0 (QImaging Corporation, BC, Canada).

Internalization of pDMAEMA by Bacteria. To investigate the ability of pDMAEMA to enter bacteria overnight cultures of *Salmonella* ser. Typhimurium IMD 574 and *S. epidermidis* 1457 were grown. The cultures were incubated at approximately 5×10^8 CFU/mL with and without hostasol-conjugated pDMAEMA (10.7 kDa) at 1 and 0.1 mg/mL, respectively, for 0, 0.5, 2, and 4 h at 37 °C, 170 rpm. Cells were centrifuged at 3000 × *g* for 10 min at 4 °C, washed 3 times in PBS then resuspended in 1 mL PBS. A total of 50 μL from each time point was resuspended in 10 mL PBS at 2.5×10^6 CFU/mL and another 50 μL was resuspended in 10 mL of 1 mg/mL trypan blue (Beckman Coulter, CA) in PBS at 2.5×10^6 CFU/mL.[37] Samples were incubated at ambient temperature for at least 30 min but not more than 6 h to ensure bacterial survival. Samples were analyzed on a Cyan ADP flow cytometer (Beckman Coulter, CA) using Summit version 4.3 software (Beckman Coulter, CA). At least 10000 bacteria were analyzed per sample. The excitation laser was set at 488 nm. For analysis of hostasol-conjugated pDMAEMA fluorescence a 530/40 nm bandpass filter (FL 1) was used. After initial analysis, 100 μg/mL propidium iodide (PI) was added to samples, which were incubated at 4 °C for 5 min in the dark,[47] and then reanalyzed under the same conditions. PI stains DNA of the bacteria but is only able to enter dead cells so was used as an indicator of cell survival. PI fluorescence was determined using a 613/20 nm bandpass filter (PE, Texas Red, FL 3). Experiments were repeated on at least three different occasions and representative results are shown.

Outer Membrane Permeabilization. Fluorescence of the probe 1-*N*-phenyl-naphthylamine (NPN) increases when incorporated into the hydrophobic core of a bacterial cell membrane (after permeation) compared with the fluorescence of a nonpermeated bacterial cell.[48,49] *Salmonella* ser. Typhimurium IMD 574, *Salmonella* ser. Enteritidis ATCC 13076 and *E. coli* ATCC 10536 were grown to an optical density at 600 nm (OD_{600}) of 0.5. Cells were harvested and washed three times in PBS then resuspended in 10 mM sodium phosphate buffer with 100 mM NaCl, at a pH of 7.5. Unconjugated pDMAEMA was added to *Salmonella* ser. Typhimurium, *Salmonella* ser. Enteritidis and *E. coli* suspensions at concentrations of 2, 2, or 0.5 mg/mL, respectively. Experiments were also carried out at pH 6 and 7 for *Salmonella* ser. Typhimurium IMD 574. A total of 200 μL of bacterial suspension was added in five replicates to 96-well plates. A 20 μL aliquot of 0.2 mM NPN was added, and the increase in fluorescence measured on a fluorescent microplate spectrophotometer (Spectra Max Gemini, Molecular Devices, CA) every minute for 20 min with Ex = 350 nm and Em = 429 nm. The results are presented as a mean ± SEM of a minimum of three independent replicates.

Cytoplasmic Membrane Permeabilization. The method for determination of cytoplasmic membrane permeability was adapted from the methods of Essodaigui et al. and Edgerton et al.[50,51] Calcein-AM is a nonfluorescent derivative of the dye calcein.[50,52] It is lipid soluble and therefore able to transport across cell membranes into the cytoplasm where it is cleaved by cytoplasmic esterases to form the hydrophilic, fluorescent molecule calcein.[50,52] Once inside, fluorescent calcein is unable to transport back across the membrane, unless the membrane of the cell becomes permeabilized.[52] Stock solutions of calcein-acetoxymethylester (calcein-AM) (1 mM) were prepared in DMSO and stored at −20 °C. Overnight cultures of *Salmonella* ser. Typhimurium IMD 574 and *S. epidermidis* 1457 were centrifuged at 3000 × *g* for 10 min at 4 °C and washed 3 times in PBS. The cells were then resuspended in PBS containing 5 μM calcein-AM (Invitrogen Corporation, CA, USA) supplemented with 10% vol/vol Brain Heart Infusion broth at a final concentration of 5×10^7 CFU/mL. 180 μL of resuspended culture was incubated for 90 min at 37 °C in a 96-well tissue culture plate. Fluorescence, at Ex = 496 nm and Em = 517 nm, was monitored every 10 min in a fluorescent microplate spectrophotometer (Spectra Max Gemini, Molecular Devices, CA, USA). Twenty μL of 10 × final concentration of unconjugated pDMAEMA, diluted in PBS, was added to bacteria in the 96-well tissue culture plate and the fluorescence was continued to be monitored every 5 min for 2 h. Final concentrations added were 0.1 and 1 mg/mL for *S. epidermidis* and 2 mg/mL for *Salmonella* ser. Typhimurium. Controls included bacteria incubated without pDMAEMA and bacteria incubated with 0.1 and 1% Triton X-100. Bacteria incubated without pDMAEMA were considered 100% controls. The results are presented as a mean ± SEM of a minimum of three independent replicates.

Effect of pDMAEMA on Bacterial Cell Growth. One mL aliquots from overnight cultures of *Salmonella* ser. Typhimurium IMD 574 and *S. epidermidis* 1457 were incubated at approximately 5×10^8 CFU/mL with and without unconjugated pDMAEMA at concentrations of 1 and 0.1 mg/mL, respectively. They were incubated for 0, 0.5, and 4 h at 37 °C, 170 rpm. Cells were centrifuged at 3000 × *g* for 10 min at 4 °C, washed 3 times in PBS, then resuspended in 1 mL PBS. 50 μL resuspended sample was added to 1 mL of 70% ethanol and incubated at ambient temperature for 24 h. Fixed cells were centrifuged at 3000 × *g* for 10 min at 4 °C, washed 3 times in PBS and then resuspended in 1 mL PBS. 100 μL from each time point was resuspended in 1 mL PBS at 2×10^6 CFU/mL. Twenty μL RNase (10 mg/mL stock) and 10 μL PI (10 mg/mL stock) were added then samples were incubated at 37 °C for 30 min. Samples were analyzed on a Cyan ADP flow cytometer (Beckman Coulter, CA, USA) using Summit version 4.3 software (Beckman Coulter, CA, USA). At least 10,000 bacteria were analyzed per sample. The excitation laser was set at 488 nm. PI fluorescence was determined using a 613/20 nm bandpass filter (PE- Texas Red - FL 3). Experiments were repeated on at least 3 different occasions and representative results are shown.

Enhancement of the Efficacy of Erythromycin by pDMAEMA. To test whether pDMAEMA may be used to enhance the efficacy of other antimicrobial agents, it was added at concentrations of 4 and 10 times less than its MIC concentration to erythromycin MIC experiments against *Salmonella* ser. Typhimurium IMD 574, *S. epidermidis* 1457, *Salmonella* ser. Enteritidis ATCC 13076 and *E. coli* ATCC 10536. Growth of bacteria was determined visually. The results are presented as the mean of a minimum of three independent replicates.

Cytotoxicity Analysis: Sheep Red Blood Cell Hemolysis. The method for determination of hemolytic potential of pDMAEMA was adapted from the protocol outlined by Shin et al.[53] Briefly, 1 mL mechanically defibrinated sheep blood (TCS Biosciences, Buckingham, UK) was centrifuged (2000 × *g*, 5 min, 4 °C) and the pellet of erythrocytes was washed 3 times in PBS. The final pellet was resuspended in PBS (4% v/v) and 100 μL aliquots of the suspension were plated in 96-well microtiter plates. Cells were exposed to polymers at varying concentrations, incubated for 60 min at 37 °C, and then plates were centrifuged at 3000 rpm for 5 min. Aliquots (100 μL) of the supernatant were transferred to a fresh 96 well microtiter plate, where hemoglobin release was monitored spectrophotometrically at 414 nm using a microplate spectrophotometer (UVM340, Asys Hitech GmbH, Eugendorf, Austria). Percent hemolysis was calculated relative to that detected with 1% Triton X-100. The results are presented as the mean of a minimum of three independent replicates.

Cytotoxicity Analysis: MTT. This method is based on the reduction of the tetrazolium salt, methylthiazolyldiphenyl-tetrazolium bromide (MTT) into a crystalline formazan product by the cellular oxidoreductases of viable cells.[54,55] All cell culture reagents were from Invitrogen Corporation (CA, USA). Cell lines were obtained from the American Tissue Type Culture Collection (ATCC, MA, USA). Caco-2 human intestinal epithelial cells (ATCC: HTB-37, passage numbers 56−64) were cultured in Dulbecco's Modified Eagles Medium (DMEM) containing GlutaMAX, supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids (NEAA) and 1% penicillin/streptomycin (Pen-Strep). U937 human monocyte-like cells (ATCC: CRL-1593.2, passage numbers 11−17) were cultured using RPMI medium supplemented with FBS, NEAA, Pen-Strep and 1% L-glutamine. All cells were grown in a humidified 37 °C incubator, with 5% CO_2, in air. At 70−80% confluence, cells were seeded, at a density

of 2×10^4 and 2×10^5 cells/well, for Caco-2 and U937 cells respectively, in 96-well tissue culture plates and allowed to grow for 20 − 24 h. Caco-2 monolayers were rinsed with fresh growth medium and allowed to equilibrate at 37 °C, 5% CO_2 for 60 min. Media was removed after equilibration and 200 μL fresh supplemented DMEM media with or without pDMAEMA was added to the wells. For U937 cells, plates were centrifuged at 3000 rpm for 5 min then media was removed. 200 μL fresh supplemented RPMI media with or without pDMAEMA was added to the wells. Monolayers were incubated at 37 °C, 5% CO_2 for 1 and 24 h. Following incubation, 20 μL of MTT (5 mg/mL in PBS, pH 7.4) was added and cells were incubated for a further 3−4 h at 37 °C, 5% CO_2. The U937 plates were centrifuged at 3000 rpm, no centrifuge step was necessary for the Caco-2 cells, then the media was gently removed and 100 μL dimethyl sulphoxide (DMSO) was added to all wells. The plates were shaken for 2 − 5 min to dissolve the formazan crystals and the absorbance was read at 550 nm. 0.1% Triton X-100 was used as a positive control for cytotoxicity. The results are presented as the mean of a minimum of three independent replicates.

Statistical Analysis. Statistical analyses were carried out using one-way ANOVA with Bonferroni posthoc tests. The significance level was set at $\alpha = 0.05$ (95% confidence intervals).

Results

Characterization of pDMAEMA Polymers. SEC-HPLC analysis produced a single dominant, clear peak for each polymer, confirming purity (data not shown). The molecular weight (M_n), as determined by SEC-HPLC and the polydispersity index (PDi) of pDMAEMA polymers used in this report are described in the Supporting Information section. The M_n can also be calculated from ^{1}H NMR, however, the peaks from the polymer are broad and difficult to determine, see Supporting Information section. This was carried out for the reducible polymer to give two different values, depending on the peaks chosen and as both values are within reasonable agreement with the SEC results (SEC is usually quoted to give up to 10% error) SEC data was used. It is also noted that the apparent molecular weight of the polymers decreases upon quaternisation whereas one would expect an increase in mass, this is ascribed again to the differences in hydrodynamic volumes. The pK_a of unconjugated pDMAEMA was 7. This is in agreement with other reports in the literature where it is reported to be 7 − 7.5.[13,56] The pK_a of the 50% quaternized pDMAEMA was found to be approximately 11. The pK_a of the hostasol-conjugated and reducible polymers was not determined as these molecules were not used in pH determination experiments.

MIC's. In order to obtain an overview of how pDMAEMA interacts with bacteria, a screen of 13 bacteria and 1 yeast strain was carried out. pDMAEMA was found to have an antimicrobial effect against all of the Gram-negative bacteria tested in the range of 0.1 − 1 mg/mL. It had variable effects on the Gram-positive bacteria tested (MIC values range from 0.1 - > 18 mg/mL) and did not effect the growth of the yeast *Candida albicans* up to 10 mg/mL. The MIC screen results are described in the Supporting Information section.

From the results of the MIC screen, it appeared that pDMAEMA may generally be more effective against Gram-negative bacteria than Gram-positive. In order to investigate this further, we examined a number of different derivatives of pDMAEMA against two representative strains of Gram-positive and Gram-negative bacteria (Table 1). *Salmonella* ser. Typhimurium IMD 574 was chosen as a model Gram-negative bacterium as it is an infection-related food isolate that could represent a target organism for pDMAEMA as a food packaging coating.[41] *S. epidermidis* 1457 was chosen as a model Gram-positive bacterium as it is a catheter-related clinical isolate that could represent a target organism for pDMAEMA as a coating for indwelling medical devices, such as catheters.[42] All of the polymers gave comparable MIC values (Table 1), except for 50% quaternized pDMAEMA against *Salmonella* ser. Typhimurium, which was found to be much higher. It is possible that the more positive charge on the 50% quaternized polymer may influence its ability to inhibit the growth of Gram-negative bacteria. Hostasol alone was also tested for its antimicrobial activity (Table 1). It was not found to show any antimicrobial activity at concentrations up to 0.016 mg/mL. This concentration coincides to 8 times its concentration at MIC in hostasol-conjugated pDMAEMA, against *S. epidermidis* and 0.2 times its concentration at MIC in hostasol-conjugated pDMAEMA, against *Salmonella* ser. Typhimurium. Higher concentrations were not able to be tested due to insolubility in aqueous media.

Table 1. MIC Values (mg/mL) of pDMAEMA Polymers against *Salmonella* ser. Typhimurium IMD 574 and *S. epidermidis* 1457

Polymer	*Salmonella* ser. Typhimurium	*S. epidermidis*
Unconjugated	1	0.1
Reducible	1	0.05
50% Quaternized	>8[a]	0.1
Hostasol alone	>0.016	>0.016

[a] $P < 0.001$ compared with unconjugated pDMAEMA against *Salmonella* ser. Typhimurium.

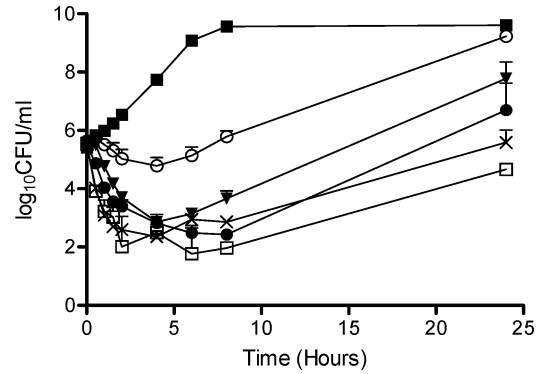


Figure 1. Time-kill curve of unconjugated pDMAEMA (12.8 kDa) against *S. epidermidis* 1457. ■ = Control, ○ = 0.05 mg/mL, ▼ = 0.1 mg/mL, • = 0.2 mg/mL, × = 0.4 mg/mL and □ = 0.8 mg/mL.

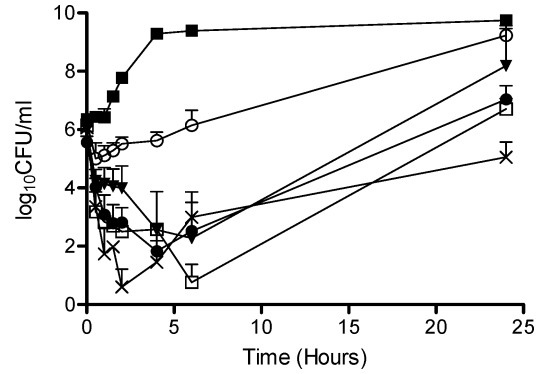


Figure 2. Time-kill curve of unconjugated pDMAEMA (12.8 kDa) against *Salmonella* ser. Typhimurium IMD 574. ■ = Control, ○ 0.5 mg/mL, ▼ = 1 mg/mL, • = 2 mg/mL, × = 4 mg/mL and □ = 8 mg/mL.

Time-kill Curves. Figures 1 and 2 show time-kill curves of the effect of unconjugated pDMAEMA against *S. epidermidis* 1457 and *Salmonella* ser. Typhimurium IMD 574, respectively. In both instances the polymer was bacteriostatic against the

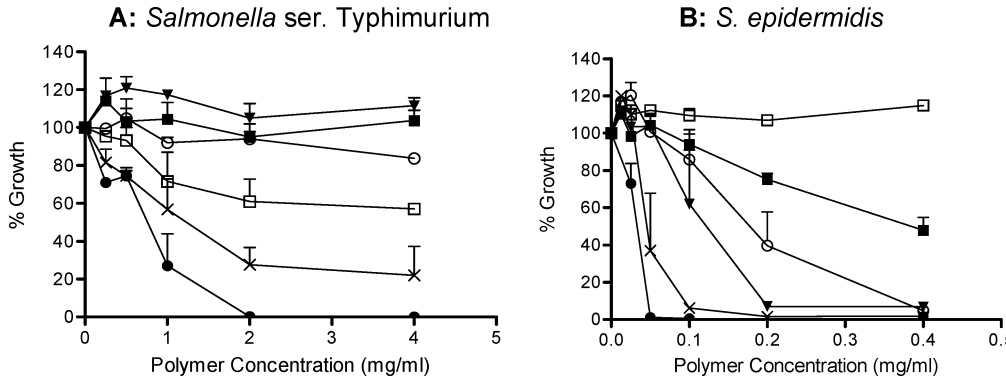


Figure 3. Effect of pH on antimicrobial activity. pH values ■ = 5, ○= 6, ▼ = 7, • = 7.5, × = 8 and □ = 9. *A* = unconjugated pDMAEMA (12.8 kDa) against *Salmonella* ser. Typhimurium IMD 574. *B* = unconjugated pDMAEMA (12.8 kDa) against *S. epidermidis* 1457. % Growth is compared to untreated control and is designated at 100%.

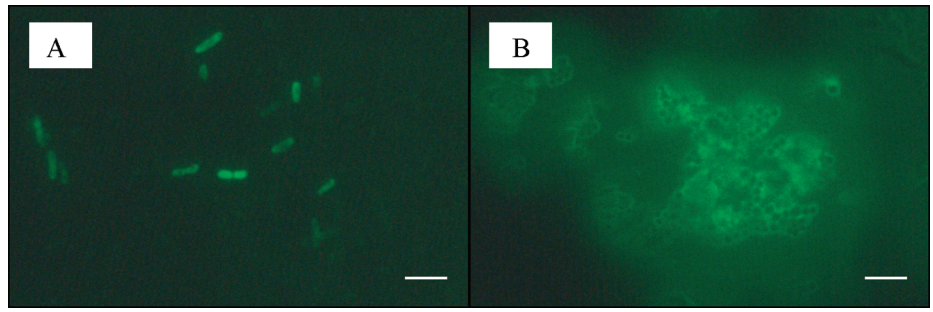


Figure 4. Fluorescent pictures of hostasol-conjugated pDMAEMA (10.7 kDa) interactions with bacteria. *A* = *Salmonella* ser. Typhimurium IMD 574, B =. *S. epidermidis* 1457 bar = 5 μM. Ex wavelength = 465−495 nm, Em wavelength = 515−555 nm.

bacteria, as after 24 h persister bacteria had regrown and there was no evidence of a•3 $\log_{10}$ reduction in CFU/mL.

Optimum pH and Temperature for Antimicrobial Activity. As was suggested by the high MIC of 50% quaternized pDMAEMA (Table 1), the charge on the polymer may influence its ability to inhibit bacterial growth. Therefore MIC's were carried out at different pH values. The optimum pH for antimicrobial activity for the two selected pathogens (Figure 3) showed a difference in the effect of unconjugated pDMAEMA against the Gram-positive compared with Gram-negative bacteria. Despite both bacteria showing an optimum for antimicrobial activity, of 7.5 − 8, against the Gram-negative bacteria, *Salmonella* ser. Typhimurium, pDMAEMA was effective at pH = 7.5 and above, while against the Gram-positive bacteria, *S. epidermidis*, pDMAEMA was only effective at pH = 8 and below. As mentioned previously, the pK_a of pDMAEMA is approximately 7 − 7.5 and for both bacteria tested, optimum antibacterial activity is around the pK_a. The effect of pH was also tested against the 50% quaternized pDMAEMA (9.7 kDa) using *Salmonella* ser. Typhimurium. We found that even at pH 9, 50% quaternized pDMAEMA did not have any effect against *Salmonella* ser. Typhimurium (data not shown). The bacteria did not grow above the pH values tested.

The optimum temperature for activity for unconjugated pDMAEMA (12.8 kDa) was 37 − 43 °C. At ambient temperature, the activity was reduced against Gram-negative *Salmonella* ser. Typhimurium (data not shown).

Hostasol-conjugated pDMAEMA Binding to Bacteria. Hostasol-conjugated pDMAEMA (10.7 kDa) was used to visualize the polymer binding to bacteria. In Figure 4, pDMAEMA can be seen binding to each of *Salmonella* ser. Typhimurium and *S. epidermidis* after 30 min incubation. A similar result was seen after 2 h incubation (data not shown).

Internalisation of pDMAEMA by Bacteria. In order to test whether pDMAEMA was able to be internalized, bacteria were incubated alone or with hostasol-conjugated pDMAEMA for 30 min, 2 and 4 h. The bacteria were analyzed by flow cytometry and live cells were selected based on PI staining. Trypan blue was added to quench the fluorescence outside the bacteria and the samples were reanalyzed. When the bacteria were incubated with the fluorescently conjugated pDMAEMA, the fluorescence peak shifted to the right compared with peaks for the bacteria alone (Figures 5A and 6A). This is due to increased fluorescence of the bound polymer. When trypan blue was added to the *S. epidermidis* samples, the increased fluorescence of the polymer was completely quenched, compared with control, i.e. all samples showed similar fluorescence peaks to bacteria with no polymer suggesting that the polymer is not internalized (Figure 5B). However, when trypan blue was added to the *Salmonella* ser. Typhimurium samples, some bacteria retained fluorescence (Figure 6B). This showed that some of the polymer is able to penetrate the Gram-negative bacteria.

Outer Membrane Permeabilisation. In order to test if pDMAEMA's ability to penetrate *Salmonella* ser. Typhimurium is due to the polymer's ability to permeabilise the outer membrane of Gram-negative bacteria, we incubated unconjugated pDMAEMA (12.8 kDa) with *Salmonella* ser. Typhimurium IMD 574 and used the fluorescent probe NPN to measure membrane permeabilisation. At pH 7.5 pDMAEMA permeabilized the outer membrane of this bacteria within 1 min of incubation (Figure 7). The outer membranes of *E. coli* ATCC 10536 and *Salmonella* ser. Enteritidis ATCC 13076 were also permeabilized within 1 min (data not shown). Controls of: 1) bacteria incubated with polymer, but no NPN, and 2) polymer incubated with NPN, but no bacteria, did not produce any increase in fluorescence (data not shown). At pH 7, the permeabilisation of *Salmonella* ser. Typhimurium by pDMAEMA was decreased and at pH 6 it was even lower compared to that at pH 7.5 (Figure 7).

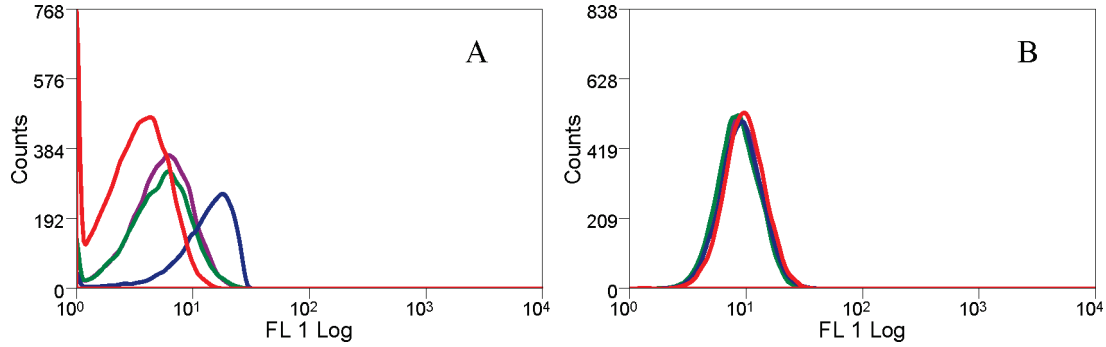


Figure 5. Flow cytometric analysis of *S. epidermidis* 1457 treated with 0.1 mg/mL hostasol-conjugated pDMAEMA (10.7 kDa) (+100 μg/mL PI to gate for live cells). *A* = No trypan blue, *B* = Cell treated with 1 mg/mL trypan blue. Red line = control, blue line = 30 min treatment, green line = 2 h treatment, purple line = 4 h treatment. FL 1: 530/40 nm filter. Results gated for single alive bacteria.

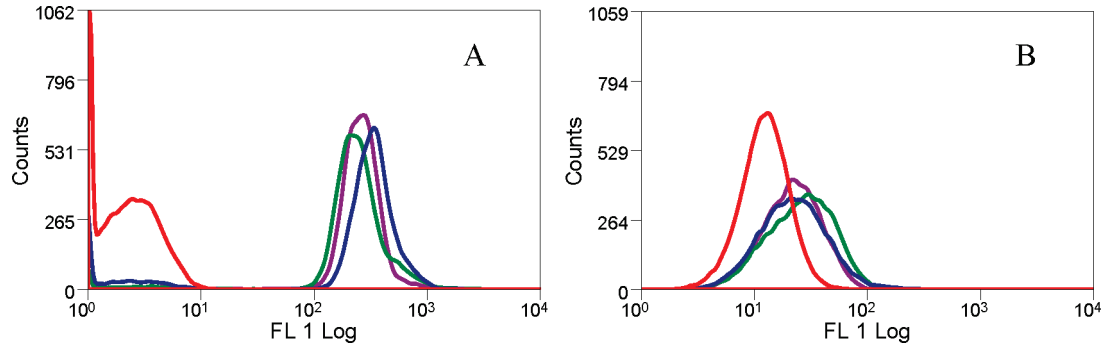


Figure 6. Flow cytometric analysis of *Salmonella* ser. Typhimurium IMD 574 treated with 1 mg/mL hostasol-conjugated pDMAEMA (10.7 kDa) (+100 μg/mL PI to gate for live cells). *A* = No trypan blue, *B* = Cell treated with 1 mg/mL trypan blue. Red line = control, blue line = 30 min treatment, green line = 2 h treatment, purple line = 4 h treatment. FL 1: 530/40 nm filter. Results gated for single alive bacteria.

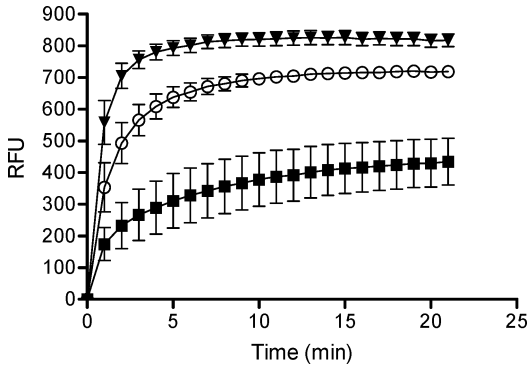


Figure 7. Permeabilisation of the outer membrane of *Salmonella*ser. Typhimurium IMD 574 by 2 mg/mL unconjugated pDMAEMA (12.8 kDa). ■ = pH 6, ○= pH 7, ▼ = pH 7.5. RFU = relative fluorescence units. Values compared to fluorescence of bacteria incubated with NPN, but no polymer (0 RFU).

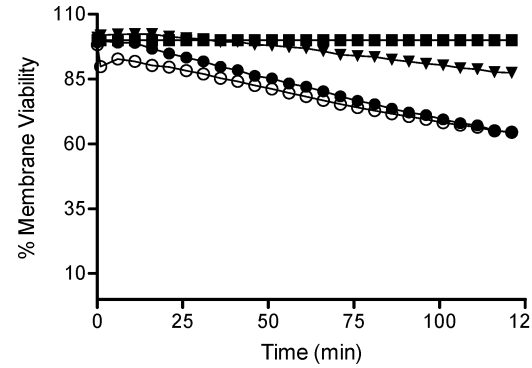


Figure 8. Permeabilisation of the cytoplasmic membrane of *Salmonella*ser. Typhimurium IMD 574 by unconjugated pDMAEMA (12.8 kDa). ■ = PBS, ○= 2 mg/mL pDMAEMA, ▼ = 0.1% Triton X-100, • = 1% Triton X-100.

Cytoplasmic Membrane Permeabilisation. pDMAEMA may also have an effect on the cytoplasmic (inner) membrane of bacteria. In order to test this hypothesis, both Gram-negative, *Salmonella* ser. Typhimurium and Gram-positive, *S. epidermidis*, were loaded with the dye calcein-AM and then incubated with unconjugated pDMAEMA. Permeabilisation of the cytoplasmic membrane was observed with both bacteria based on a decrease in calcein fluorescence relative to controls containing no polymer (Figures 8 and 9).

Effect of pDMAEMA on Bacterial Cell Growth. The DNA content of bacteria fluctuates as it goes through its life cycle. The DNA fluorescence, when stained with fluorescent dyes, is an indication of the number of chromosomes inside individual cells.[57] As the bacteria grow they replicate their chromosome in replicates of N1, N2, N3 etc., therefore, DNA staining will increase. This increase can be an indicator of bacterial cell growth.[57] Figures 10 and 11 demonstrate the effect of pDMAEMA on cell growth of *S. epidermidis* and *Salmonella* ser. Typhimurium, respectively. After incubation of the polymer with bacteria for both 30 min and 4 h a shift to the left of the peaks was observed when the bacteria were incubated with pDMAEMA, i.e. the N2 (replicating bacteria) peaks are reduced and N1 (unreplicating bacteria) peaks are larger in treated samples compared to untreated amples. This indicates that cell growth was inhibited, as the cellular DNA of the bacteria was not replicating as much as untreated controls, suggesting that the cells were not progressing through their normal cell cycle pattern.

Enhancement of the Efficacy of Erythromycin by pDMAEMA. Against all of the Gram-negative bacteria tested (*Salmonella* ser. Typhimurium IMD 574, *Salmonella* ser.

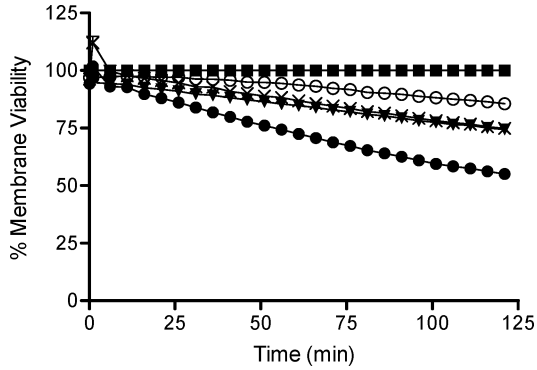


Figure 9. Permeabilisation of the cytoplasmic membrane of *S. epidermidis* 1457 by unconjugated pDMAEMA (12.8 kDa). ■ = PBS, ○= 0.1 mg/mL pDMAEMA, ▼ = 1 mg/mL pDMAEMA, × = 0.1% Triton X-100, • = 1% Triton X-100.

Enteritidis ATCC 13076 and *E. coli* ATCC 10536), incubation with unconjugated pDMAEMA, at concentrations 4 and 10 times lower than its MIC, reduced the MIC of erythromycin by 7 − 59 fold (Table 2). This effect was also seen as a 2-fold reduction in erythromycin MIC, using 4 times lower than MIC pDMAEMA concentration, against *S. epidermidis* 1457.

Cytotoxicity Analysis. The cytotoxicity of pDMAEMA polymers was tested against sheep erythrocytes. Up to 10 mg/mL, none of the pDMAEMA polymers tested were found to cause significant hemolysis (Table 3). Cytotoxicity against the human intestinal epithelial cell line, Caco-2, and the human monocytic cell line, U937, was assessed by MTT assay (Table 3). All polymers produced similar levels of cytotoxicity to the human cell lines, though all were more cytotoxic toward U937 cells than toward Caco-2 cells. Surprisingly the reducible polymer, that has previously been reported to be less toxic to U937 cells,[39] did not cause less cytotoxicity than unconjugated pDMAEMA.

Discussion

We have previously shown that pDMAEMA is able to inhibit the binding and uptake of *Salmonella* ser. Typhimurium to the human epithelial intestinal cell line, HT29-MTX-E12, and reduce the, *in vitro*, inflammatory response to bacterial cell and toxin challenge.[36] We also reported that pDMAEMA conjugated with hostasol, was bactericidal against *Salmonella* ser. Typhimurium IMD 574,[36] however, in the present work unconjugated pDMAEMA was found to be bacteriostatic. The hostasol conjugated to pDMAEMA does not appear to adjust the MIC concentration compared to other pDMAEMA derivatives including the unconjugated polymer, but it does confer a bactericidal ability on it, while having no effect on its own. It is unclear as to why this occurs, however, one hypothesis is that it is due to the increased size of the side arm when hostasol is attached to pDMAEMA, which may allow for the polymer to reach the cytoplasmic membrane of the bacteria more effectively.[30] Another hypothesis is that the increased hydrophobicity of the hostasol may increase the polymer's ability to enter bacterial cell membranes. Hostasol alone was not cytotoxic to sheep red blood cells, nor the human cell lines U937 and Caco-2 at concentrations up to 0.016 mg/mL. Using the hostasol-conjugated pDMAEMA, we screened an array of Gram-negative and Gram-positive bacteria as well as one yeast strain, to investigate the scope of pDMAEMA's antimicrobial activity. pDMAEMA was found to inhibit the growth of all the Gram-negative bacteria tested, with variable effects on the Gram-positive bacteria and no effect on the yeast. The selectivity for Gram-negative over Gram-positive bacteria has also been previously observed with other polymers.[58,59]

As pDMAEMA is a thermo- and pH-responsive polymer,[19,60] pH and temperature may influence its ability to inhibit the growth of bacteria. At higher temperatures, pDMAEMA phase-transitions out of solution. We therefore tested to see if it would retain its effectiveness at temperatures slightly above body temperature. pDMAEMA was effective at inhibiting growth up to 43 °C and remained in solution. This effect was seen with both the Gram-negative and Gram-positive bacteria. However, the effect of pH on growth inhibition was quite different for the Gram-negative compared with the Gram-positive bacteria. pDMAEMA only inhibited the Gram-negative bacterium, *Salmonella* ser. Typhimurium, when the polymer was around its pK_a or when in a more hydrophobic state, at higher pH values. When the polymer is more hydrophobic, less protonated, it may be able to interact more effectively with the outer membrane of the Gram-negative bacteria. This was confirmed by a decrease in permeabilisation of the outer membrane observed at pH 7 and 6 compared with pH 7.5. Against the Gram-positive bacteria, *S. epidermidis*, however, pDMAEMA was only effective at around its pK_a and when it is more highly protonated and more hydrophilic at lower pH values. The lack of the outer membrane, in Gram-positive bacteria, reduces the barriers for the polymer to access the cytoplasmic membrane. Therefore, although the polymer must retain some hydrophobic portions, as the ability to inhibit growth decreased with decreasing pH, hydrophobicity does not appear to be as important in the inhibition of the Gram-positive bacterium.

A range of pDMAEMA polymers were tested in order to investigate effects of modifications to the polymer on antimicrobial activity. Unconjugated pDMAEMA was tested to ensure that, without any modifications, the polymer retained activity. A polymer with reportedly lower cytotoxicity,[39] reducible pDMAEMA, was tested to investigate if the disulfide bond in this molecule interferes with antimicrobial activity. A more positively charged polymer, the 50% quaternized pDMAEMA, was tested to investigate the effect of charge on the polymer. All polymers had similar MIC values to each other except for the 50% quaternized pDMAEMA against *Salmonella* ser. Typhimurium (Table 1). Up to a pH value of 9, 50% quaternized pDMAEMA remained unable to inhibit growth of the Gram-negative bacteria. As the pK_a of this polymer is approximately 11, it is highly protonated at pH 9 and consequently hydrophilic. The results for the unconjugated polymer showed that pDMAEMA was only able to inhibit the growth of the Gram-negative bacteria when presented in a more hydrophobic state, at pH values around or above its pK_a. Therefore, as pH 9 is lower than the pK_a of quaternized pDMAEMA, the result for the 50% quaternized polymer against *Salmonella* ser. Typhimurium correlates with the unconjugated polymer data.

To date, most reports with pDMAEMA as an antimicrobial agent have used a quaternized derivative. The majority of these reports also use pDMAEMA as part of a copolymer with other polymers, which may be contributing to its antimicrobial activity. In contrast to our data, some studies have found quaternized pDMAEMA to be effective against the Gram-negative bacteria, *E. coli*.[30,32] In these reports the bacteria were only incubated with pDMAEMA for 1 h. However, in the present study pDMAEMA was incubated with the bacteria for 18 h. While it is possible that the quaternized polymer is able to inhibit growth of Gram-negative bacteria over short periods, over longer periods the bacteria are able to grow back. In

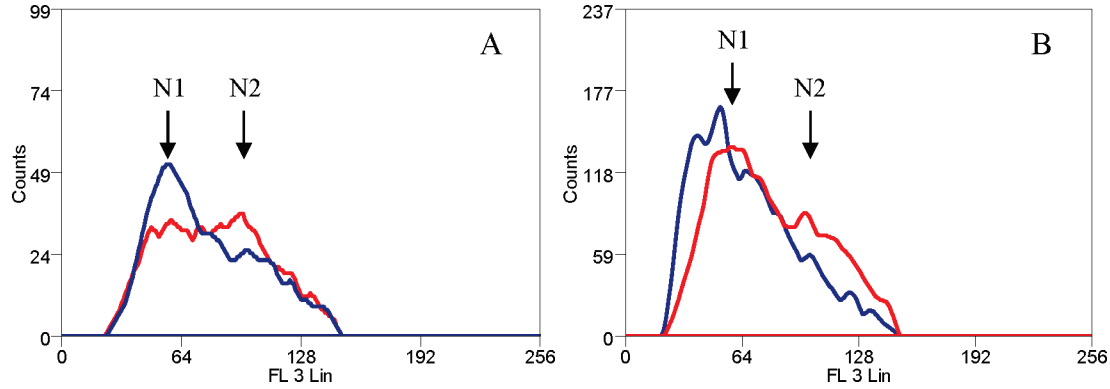


Figure 10. Effect of unconjugated pDMAEMA (12.8 kDa) on *S. epidermidis* 1457 cell cycling. *A* = cell growth after 30 min, *B* = cell growth after 4 h. Red line = control untreated bacteria, blue line = bacteria treated with 0.1 mg/mL pDMAEMA. FL 3: 613/20 nm filter. Results gated for single bacteria. Arrows represent N1 and N2 DNA replicates of actively growing bacteria.

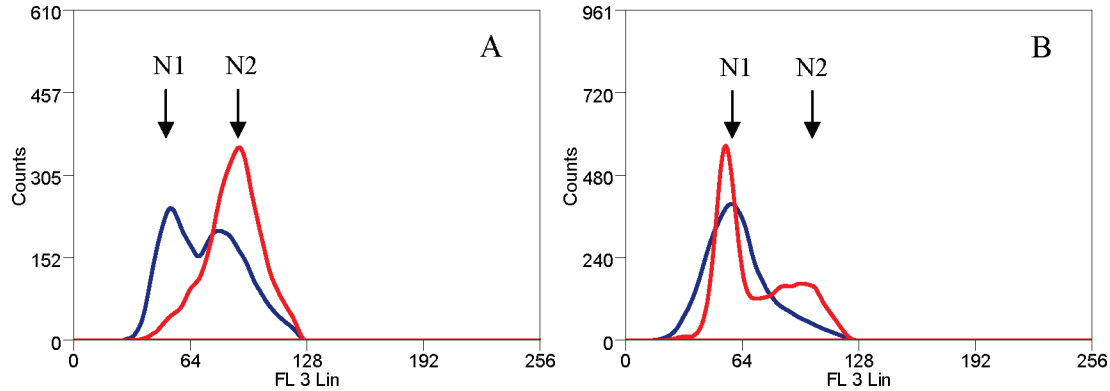


Figure 11. Effect of unconjugated pDMAEMA (12.8 kDa) on *Salmonella* ser. Typhimurium IMD 574 cell cycling. *A* = cell growth after 30 min, *B* = cell growth after 4 h. Red line = control untreated bacteria, blue line = bacteria treated with 1 mg/mL pDMAEMA. FL 3: 613/20 nm filter. Results gated for single bacteria. Arrows represent N1 and N2 DNA replicates of actively growing bacteria.

Table 2. Enhanced Antimicrobial Activity of Erythromycin against Bacteria by Addition of Unconjugated pDMAEMA. MIC's are Expressed in μg/mL

	Salmonella ser. Typhimurium		*Salmonella* ser. Enteritidis		*E. coli*		*S. epidermidis*	
Antimicrobial agent	MIC	Fold reduction	MIC	Fold reduction	MIC	Fold reduction	MIC	Fold reduction
Erythromycin (Ery)	125	-	23.4	-	5.86	-	0.24	-
pDMAEMA	2000	-	2000	-	0.5	-	0.1	-
Ery + pDMAEMA (0.25 × MIC)	3.9	32	2.32	10	0.1	59	0.12	2
Ery + pDMAEMA (0.1 × MIC)	15.6	8	3.17	7	0.13	45	0.24	0

Table 3. Cytotoxicity of pDMAEMA Polymers and Hostasol (mg/mL). IC_{50} = Concentration that Produces 50% Cytotoxicity Compared to 100% Trition X-100 Control

	RBC	Caco-2		U937	
Polymer	1 h IC_{50}	1 h IC_{50}	24 h IC_{50}	1 h IC_{50}	24 h IC_{50}
Unconjugated	>10	7.6 ± 0.37	2.1 ± 1.2	0.38 ± 0.03	0.19 ± 0.06
Reducible	>10	>10	0.68 ± 0.08	0.05 ± 0.004	0.05 ± 0.01
50% Quaternized	>10	>10	5.2 ± 1.2	1.1 ± 0.14[a]	1.0 ± 0.26[b]
Hostasol-conjugated	>10	4.9 ± 2.1	0.27 ± 0.03[c]	0.05 ± 0.02	0.06 ± 0.01
Hostasol[d]	>0.016	>0.016	>0.016	>0.016	>0.016

[a] $P < 0.05$. [b] $P < 0.001$ as compared to unconjugated polymer in the same column. [c] $P < 0.01$. [d] Maximum solubility of hostasol was obtained at 0.016 mg/mL.

addition, Yancheva et al.[33] found that unconjugated and 50% quaternized pDMAEMA had a similar inhibitory effect against *E. coli*, producing an MIC of 0.3 mg/mL. This value is similar to the MIC we found for the unconjugated polymer against *E. coli* ATCC 10536 (0.5 mg/mL). However, again, the bacteria were only exposed to the polymers for a short time (30 min). A longer time point may have produced a different result for the quaternized molecule.

The initial step in the mode of action of cationic biocides is adsorption onto the bacterial cell surface.[37] pDMAEMA, with a fluorescent tag, bound to both the Gram-negative, *Salmonella* ser. Typhimurium, and Gram-positive, *S. epidermidis*. To test if the polymer was gaining internal access to the bacteria, the fluorescence was quenched with trypan blue. All fluorescence around the Gram-positive bacteria was quenched showing that the polymer is acting at the cell surface. However, some fluorescence remained around the Gram-negative bacteria after quenching, suggesting that the polymer is getting inside the bacteria. pDMAEMA was shown to permeabilise the outer membrane of *Salmonella* ser. Typhimurium, *Salmonella* ser.

Enteritidis, and *E. coli*. It is possible that the remaining fluorescence seen is the polymer located inside the outer membrane.

The next steps in the mode of action of cationic biocides are the diffusion through the cell wall and binding to the cytoplasmic membrane. The variation in the effect on Gram-positive compared with Gram-negative bacteria may be partially explained at this step. Lienkamp et al.[58] synthesized an array of polymers to mimic the effects of antimicrobial peptides. They found that some of their polymers were also more effective against Gram-negative than Gram-positive bacteria. They related this effect to the molecular weight of the polymer. They suggested that a polymer of 10 kDa may be less effective against Gram-positive bacteria than a polymer of 3 kDa, as the larger polymer can form polyion complexes in the thick murein layer (cell wall) around the cytoplasmic membrane, which are more difficult to dissociate at higher molecular weights. Therefore the larger polymer may be unable to transfer across the cell wall to the membrane and therefore not able to disrupt the membrane and cause cell death. In the case of the Gram-negative bacteria, the murein layer is a thin layer between the outer membrane and the cytoplasmic membrane so it is potentially easier for the polymer to cross. This may also be the case with pDMAEMA against some of the Gram-positive bacteria as the polymers used here are large, approximately 7−18 kDa in size. In addition to size, the charge on the bacteria may also contribute to the difference in activity. Chung et al.[61] suggested that the antimicrobial polymer, chitosan, is more effective against Gram-negative bacteria than Gram-positive as the Gram-negative cells are more hydrophilic and have a more negative charge so the polymer is able to interact with them more than the Gram-positive. The reason for the variation in activity within the Gram-positive group is unclear, but it may also be related to charge, hydrophobicity and hydrodynamic volume.

The final steps in the mode of action of cationic biocides are the disruption of the cytoplasmic membrane, release of cytoplasmic constituents and cell death. pDMAEMA permeabilized the cytoplasmic membrane of both the Gram-positive and the Gram-negative bacteria. In addition, the polymer interfered with the growth cell cycle of the bacteria. Therefore, it is not only able to kill the bacteria, typical of cationic biocides, but it may also slow their growth. Due to the ability of pDMAEMA to disrupt the outer membrane of Gram-negative bacteria it may have potential to increase the activity of other antimicrobial agents that are less effective against these bacteria. Erythromycin is a hydrophobic, macrolide, antibiotic that is extensively used in treating Gram-positive bacterial infections.[62,63] However, it has only very limited use in the treatment of Gram-negative infections due to the outer membrane of these bacteria acting as a permeability barrier.[64] For this reason, agents that sensitize the outer membrane of Gram-negative bacteria are useful to increase the antimicrobial activity of antibiotics, including erythromycin.[62] In this study we found that, due to pDMAEMA's ability to disrupt the outer membrane of bacteria, it was able to increase the antibacterial activity of erythromycin by up to 59-fold at concentrations where pDMAEMA was not inhibitory by itself. The permeabilization of the outer membrane by pDMAEMA appears to remove the barrier for the antibiotic, allowing it to enter the bacteria and inhibit growth. This erythromycin-pDMAEMA mix may have use in clinical settings, particularly in the treatment of resistant bacteria where other antibiotics have become less effective. Further investigations into the use of pDMAEMA in combinatorial therapies are necessary to fully understand the potential of the polymer in this context.

To use pDMAEMA as an antimicrobial coating or treatment, it must be shown to be safe to humans. Reports on the cytotoxicity of pDMAEMA vary depending on the method of administration, quaternization of the polymer, other polymers attached, and size.[65−68] Moreau et al.[65] found that pDMAEMA caused little or no hemolysis to human red blood cells, however, when injected intravenously into the tail vein of rats, caused death at 5.1 mg/kg, but was tolerated at 2.1 mg/kg. Yancheva et al.[68] showed that despite the fact that pDMAEMA did not cause hemolysis of red blood cells it did encourage hemeagluttination. Here we have found that none of the pDMAEMA polymers caused any substantial hemolysis against sheep red blood cells. Cytotoxicity against Caco-2 and U937 cells was similar to concentrations found for 43 kDa pDMAEMA against human brain microvascular endothelial cells.[66] Cytotoxicity against the human cell lines was also similar to the antimicrobial polymer, chitosan, against B16F10 murine melanoma cell line and the cationic reference polymer, poly(L-lysine).[69] Against Caco-2 cells none of the polymers produced significantly different cytotoxicity concentrations than the unconjugated polymer, except for the hostasol-tagged polymer after 24 h. Against U937 cells the hostasol-tagged and reducible polymers produced similar cytotoxicity to the unconjugated polymer, while the 50% quaternized polymer was slightly less toxic. Reducible pDMAEMA has previously been reported to be less cytotoxic than unconjugated pDMAEMA.[39] Here the reducible polymer was not less cytotoxic than the unconjugated polymer. This may be due to slight variations in the structure of the polymer used here and in previous reports.

Cytotoxicity against the human intestinal cell line Caco-2 was found to be 5-fold greater than that found for the human monocyte-like cell line U937. Together with the lack of affect on red blood cells, these results show great variability depending on the cell type tested. In addition, pDMAEMA has been previously shown to have similar cytotoxicity to the natural polymer chitosan, which is considered nontoxic,[70] and when added to human epithelial cell layers, pDMAEMA prevents disruption of the cells after challenge with bacterial toxins.[36] Therefore, conclusions about the toxicity of this polymer must be made with caution, and although the in vivo work of Moreau et al.[65] showed a fairly low tolerance for intravenous injection of the polymer, perhaps as a topical or oral formulation, it could prove to be safe and effective.

Conclusions

The mode of action of the quaternized and protonated polymer pDMAEMA was similar to other cationic biocides and involved direct binding to bacteria, diffusion through the cell wall, disruption of the cytoplasmic membrane, and cell death. This effect was dependent on the bacteria type, as Gram-negative bacteria require the polymer to be in a less-charged/hydrophobic state to permeabilize the outer membrane. This antimicrobial effect was bacteriostatic in nature and may be useful for enhancing the efficacy of another antibiotic, erythromycin. The mucoadhesive polymer, pDMAEMA, therefore, appears to show promise as a potential antimicrobial coating, when formulated with other antimicrobial agents, due to its effective inhibitory action against *S. epidermidis*.

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Supporting Information Available. Molecular weight (M_n) and polydispersity index (PDi) values for pDMAEMA polymers, as determined by SEC-HPLC. NMR trace of reducible pDMAEMA. A table showing MIC values of pDMAEMA against numerous bacteria and yeast is also presented. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Kenawy, E.-R.; Worley, S. D.; Broughton, R. *Biomacromolecules* **2007**, *8*, 1359–1384.
(2) Patel, M. B.; Patel, S. A.; Ray, A.; Patel, R. M. *J. Appl. Polym. Sci.* **2003**, *89*, 895–900.
(3) Kenawy, E.-R.; Abdel-Hay, F. I.; El-Magd, A. A.; Mahmoud, Y. *J. Bioact. Compat. Polym.* **2005**, *20*, 95–111.
(4) Koplin, S. A.; Lin, S.; Domanski, T. *Biotechnol. Prog.* **2008**, *24*, 1160–1165.
(5) Huh, M. W.; Kang, I.-K.; Lee, D. H.; Kim, W. S.; Lee, D. H.; Park, L. S.; Min, K. E.; Seo, K. H. *J. Appl. Polym. Sci.* **2001**, *81*, 2769–2778.
(6) Makal, U.; Wood, L.; Ohman, D. E.; Wynne, K. J. *Biomaterials* **2006**, *27*, 1316–1326.
(7) Chun, M. J.; Shim, E.; Kho, E. H.; Park, K. J.; Jung, J.; Kim, J. M.; Kim, B.; Lee, K. H.; Cho, D. L.; Bai, D. H.; Lee, S. I.; Hwang, H. S.; Ohk, S. H. *Angle Orthod.* **2007**, *77*, 483–488.
(8) Pasquier, N.; Keul, H.; Heine, E.; Moeller, M. *Biomacromolecules* **2007**, *8*, 2874–2882.
(9) Imazato, S.; Torii, M.; Tsuchitani, Y.; McCabe, J. F.; Russell, R. R. *J. Dent. Res.* **1994**, *73*, 1437–1443.
(10) Leung, W. K.; Lau, A. P.; Yeung, K. L. *J. Appl. Microbiol.* **2009**, *106*, 1463–1472.
(11) Shinsako, K.; Okui, Y.; Matsuda, Y.; Kunimasa, J.; Otsuka, M. *Biomed. Mater. Eng.* **2008**, *18*, 377–385.
(12) Moon, J. S.; Kim, H. K.; Koo, H. C.; Joo, Y. S.; Nam, H. M.; Park, Y. H.; Kang, M. I. *Appl. Microbiol. Biotechnol.* **2007**, *75*, 989–998.
(13) Butun, V.; Armes, S. P.; Billingham, N. C. *Polymer* **2001**, *42*, 5993–6008.
(14) Limer, A. J.; Rullay, A. K.; Miguel, V. S.; Peinado, C.; Keely, S.; Fitzpatrick, E.; Carrington, S. D.; Brayden, D. J.; Haddleton, D. M. *Funct. Polym.* **2006**, *66*, 51–64.
(15) Haddleton, D. M.; Crossman, M. C.; Dana, B. H.; Duncalf, D. J.; Heming, A. M.; Kukulj, D.; Shooter, A. J. *Macromolecules* **1999**, *32*, 2110–2119.
(16) Zhang, X.; Matyjaszewski, K. *Macromolecules* **1999**, *32*, 1763–1766.
(17) Chiefari, J.; Chong, Y. K. B.; Ercole, F.; Kristina, J.; Jeffery, J.; Le, T. P. T.; Mayadunne, R. T. A.; Meijs, G. F.; Moad, C. L.; Moad, G.; Rizzardo, E.; Thang, S. H. *Macromolecules* **1998**, *31*, 5559–5562.
(18) Jana, S.; Rannard, S. P.; Cooper, A. I. *Chem. Commun.* **2007**, *28*, 2962–2964.
(19) Liu, G.; Wu, D.; Ma, C.; Zhang, G.; Wang, H.; Yang, S. *ChemPhysChem* **2007**, *8*, 2254–2259.
(20) van de Wetering, P.; Cherng, J. Y.; Talsma, H.; Hennink, W. E. *J. Controlled Release* **1997**, *49*, 59–69.
(21) Gu, Z.; Yuan, Y.; He, J.; Zhang, M.; Ni, P. *Langmuir* **2009**, *25*, 5199–5208.
(22) Hoogeveen, N. G.; Cohen Stuart, M. A.; Fleer, G. J. *Colloids Surf., A* **1996**, *117*, 77–88.
(23) Zhu, S.; Yang, N.; Zhang, D. *Mater. Chem. Phys.* **2009**, *113*, 784–789.
(24) Keely, S.; Ryan, S. M.; Haddleton, D. M.; Limer, A.; Mantovani, G.; Murphy, E. P.; Colgan, S. P.; Brayden, D. J. *J. Controlled Release* **2009**, *135*, 35–43.
(25) Lowe, A. B.; Vamvakaki, M.; Wassall, M. A.; Wong, L.; Billingham, N. C.; Armes, S. P.; Lloyd, A. W. *J. Biomed. Mater. Res.* **2000**, *52*, 88–94.
(26) Kusumo, A.; Bombalski, L.; Lin, Q.; Matyjaszewski, K.; Schneider, J. W.; Tilton, R. D. *Langmuir* **2007**, *23*, 4448–4454.
(27) Zhang, Q.; Xia, F.; Sun, T.; Song, W.; Zhao, T.; Liu, M.; Jiang, L. *Chem. Commun.* **2008**, *10*, 1199–1201.
(28) Lee, S. B.; Koepsel, R. R.; Morley, S. W.; Matyjaszewski, K.; Sun, Y.; Russell, A. J. *Biomacromolecules* **2004**, *5*, 877–882.
(29) Huang, J.; Koepsel, R. R.; Murata, H.; Wu, W.; Lee, S. B.; Kowalewski, T.; Russell, A. J.; Matyjaszewski, K. *Langmuir* **2008**, *24*, 6785–6795.
(30) Roy, D.; Knapp, J. S.; Guthrie, J. T.; Perrier, S. *Biomacromolecules* **2008**, *9*, 91–99.
(31) Lenoir, S.; Pagnoulle, C.; Detrembleur, C.; Galleni, M.; Jerome, R. *e-Polym.* **2005**, *074*, 1–11.
(32) Huang, J.; Murata, H.; Koepsel, R. R.; Russell, A. J.; Matyjaszewski, K. *Biomacromolecules* **2007**, *8*, 1396–1399.
(33) Yancheva, E.; Paneva, D.; Maximova, V.; Mespouille, L.; Dubois, P.; Manolova, N.; Rashkov, I. *Biomacromolecules* **2007**, *8*, 976–984.
(34) Ward, M.; Sanchez, M.; Elasri, M. O.; Lowe, A. B. *J. Appl. Polym. Sci.* **2006**, *101*, 1036–1041.
(35) Yousefi Rad, A.; Ayhan, H.; Kisa, U.; Piskin, E. *J. Biomater. Sci., Polym. Ed.* **1998**, *9*, 915–929.
(36) Keely, S.; Rawlinson, L. A.; Haddleton, D. M.; Brayden, D. J. *Pharm. Res.* **2008**, *25*, 1193–1201.
(37) Ikeda, T.; Yamaguchi, H.; Tazuke, S. *Antimicrob. Agents Chemother.* **1984**, *26*, 139–144.
(38) Keller, R. N.; Wycoff, H. D. *Inorg. Synth.* **1946**, *II*, 1–4.
(39) You, Y. Z.; Manickam, D. S.; Zhou, Q. H.; Oupicky, D. *J. Controlled Release* **2007**, *122*, 217–225.
(40) Shah, R. R.; Merreceyes, D.; Husemann, M.; Rees, I.; Abbott, N. L.; Hawker, C. J.; Hedrick, J. L. *Macromolecules* **2000**, *33*, 597–605.
(41) O'Mahony, R.; Quinn, T.; Drudy, D.; Walsh, C.; Whyte, P.; Mattar, S.; Fanning, S. *Microb. Drug Resist.* **2006**, *12*, 269–277.
(42) Mack, D.; Siemssen, N.; Laufs, R. *Infect. Immun.* **1992**, *60*, 2048–2057.
(43) *CLSI. Approved Standard: M7-A7*, 7th ed.; CLSI: Wayne, PA, 2006.
(44) *NCCLS. Approved Standard: M11-A5*, 5th ed.; NCCLS: Wayne, PA, 2001.
(45) *NCCLS. Approved Standard: M27-A2*, 2nd ed.; NCCLS: Wayne, PA, 2002.
(46) *NCCLS. Approved Guideline: M26-A*; NCCLS: Wayne, PA, 1999.
(47) Mattiuzzo, M.; Bandiera, A.; Gennaro, R.; Benincasa, M.; Pacor, S.; Antcheva, N.; Scocchi, M. *Mol. Microbiol.* **2007**, *66*, 151–163.
(48) Je, J. Y.; Kim, S. K. *J. Agric. Food Chem.* **2006**, *54*, 6629–6633.
(49) Ibrahim, H. R.; Sugimoto, Y.; Aoki, T. *Biochim. Biophys. Acta* **2000**, *1523*, 196–205.
(50) Essodaigui, M.; Broxterman, H. J.; Garnier-Suillerot, A. *Biochemistry* **1998**, *37*, 2243–2250.
(51) Edgerton, M.; Koshlukova, S. E.; Lo, T. E.; Chrzan, B. G.; Straubinger, R. M.; Raj, P. A. *J. Biol. Chem.* **1998**, *273*, 20438–20447.
(52) Hollo, Z.; Homolya, L.; Davis, C. W.; Sarkadi, B. *Biochim. Biophys. Acta* **1994**, *1191*, 384–388.
(53) Shin, S. Y.; Lee, S. H.; Yang, S. T.; Park, E. J.; Lee, D. G.; Lee, M. K.; Eom, S. H.; Song, W. K.; Kim, Y.; Hahm, K. S.; Kim, J. I. *J. Pept. Res.* **2001**, *58*, 504–514.
(54) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55–63.
(55) Maher, S.; McClean, S. *Biochem. Pharmacol.* **2006**, *71*, 1289–1298.
(56) van de Wetering, P.; Moret, E. E.; Schuurmans-Nieuwenbroek, N. M.; van Steenbergen, M. J.; Hennink, W. E. *Bioconjugate Chem.* **1999**, *10*, 589–597.
(57) Muller, S. *Cell Proliferation* **2007**, *40*, 621–639.
(58) Lienkamp, K.; Madkour, A. E.; Musante, A.; Nelson, C. F.; Nusslein, K.; Tew, G. N. *J. Am. Chem. Soc.* **2008**, *130*, 9836–9843.
(59) Endo, Y.; Tani, T.; Kodama, M. *Appl. Environ. Microbiol.* **1987**, *53*, 2050–2055.
(60) Cho, S. H.; Jhon, M. S.; Yuk, S. H.; Lee, H. B. *J. Polym. Sci., Part B: Polym. Phys.* **1997**, *35*, 595–598.
(61) Chung, Y. C.; Su, Y. P.; Chen, C. C.; Jia, G.; Wang, H. L.; Wu, J. C.; Lin, J. G. *Acta Pharmacol. Sin.* **2004**, *25*, 932–936.
(62) Saha, S.; Savage, P. B.; Bal, M. *J. Appl. Microbiol.* **2008**, *105*, 822–828.
(63) Champney, W. S.; Tober, C. L. *Curr. Microbiol.* **2000**, *41*, 126–135.
(64) Vaara, M. *Antimicrob. Agents Chemother.* **1993**, *37*, 354–356.
(65) Moreau, E.; Domurado, M.; Chapon, P.; Vert, M.; Domurad, D. *J. Drug Target.* **2002**, *10*, 161–173.
(66) Layman, J. M.; Ramirez, S. M.; Green, M. D.; Long, T. E. *Biomacromolecules* **2009**, *10*, 1244–1252.
(67) Jones, R. A.; Poniris, M. H.; Wilson, M. R. *J. Controlled Release* **2004**, *96*, 379–391.
(68) Yancheva, E.; Paneva, D.; Danchev, D.; Mespouille, L.; Dubois, P.; Manolova, N.; Rashkov, I. *Macromol. Biosci.* **2007**, *7*, 940–954.
(69) Carreño-Gómez, B.; Duncan, R. *Int. J. Pharm.* **1997**, *148*, 231–240.
(70) Keely, S.; Rullay, A.; Wilson, C.; Carmichael, A.; Carrington, S.; Corfield, A.; Haddleton, D. M.; Brayden, D. J. *Pharm. Res.* **2005**, *22*, 38–49.

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