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# **Anti-bacterial effects of poly(2-(dimethylamino ethyl)methacrylate)) against selected Gram-positive and Gram-negative bacteria**

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## **Abstract**

Anti-microbial 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 (NPN) and Calcein-AM. Flow cytometry was used to investigate pDMAEMA's capacity to be internalised by bacteria and to determine effects on bacterial cell cycling. pDMAEMA was bacteriostatic against Gram-negative bacteria with MIC values between 0.1–10 mg/ml. MIC values against Gram-positive bacteria were variable. pDMAEMA was active against Gram-positive bacteria around its pKa and at lower pH values, while it was active against Gram-negative bacteria around its pKa and at higher pH values. pDMAEMA inhibited bacterial growth by binding to the outside of the bacteria, permeabilising 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<sup>1,2</sup> 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<sup>1</sup>.

Numerous polymers have been found to have antimicrobial activities against Gram-negative and Gram-positive bacteria, yeast and viruses<sup>3,4</sup>. For this reason they have been investigated as potential antimicrobial coatings and inhibit bacterial growth when attached to glass, paper, plastic and metal<sup>5-8</sup>. For example, methacryloyloxydodecylpyridinium, was incorporated into dental resin composite and reduced microbial activity without leaching<sup>9</sup>. Polymers have also been used to deliver encapsulated antimicrobial agents allowing localized, controlled release of the drug into the target site<sup>10,11</sup>. 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 intra-peritoneal route resulted in a higher survival rate compared with that of controls<sup>12</sup>.

Poly(2-(dimethylamino ethyl)methacrylate) (pDMAEMA) is a mucoadhesive polymer, that is cationic if dissolved into acidified media or if quaternised by using an alkylating agent<sup>13,14</sup>. Recent advances in living radical polymerization (LRP)<sup>15,16</sup> and reversible addition-fragmentation transfer (RAFT) polymerisation<sup>17</sup> allowed for the synthesis of

pDMAEMA with tuneable polymer chain length and macromolecular architecture, as well as a narrow molecular weight distribution. It is a thermo-responsive polymer and at increased temperatures, the polymer phase-separates from solution due to a breakdown in hydrogen bonding interactions<sup>18</sup>. 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<sup>19</sup>. At lower pH the polymer becomes more positively charged and at pH values greater than 7 it becomes mostly uncharged<sup>19</sup>. pDMAEMA has numerous potential uses which include use as a non-viral gene delivery vector<sup>20,21</sup>, in water purification<sup>22,23</sup>, and in drug delivery<sup>24</sup>. It is also used as a coating for soil-resistant surfaces<sup>25</sup>, as an ion exchange media for protein separation<sup>26</sup>, and to adapt the wettability of surfaces including microfluidic devices<sup>27</sup>. In addition to this, pDMAEMA has been attached to glass<sup>28,29</sup>, filter paper<sup>28,30</sup>, polystyrene<sup>31</sup> and polypropylene<sup>32</sup> 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*<sup>33</sup> and *S. aureus*<sup>34</sup>. 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 catheters<sup>35</sup>. pDMAEMA has also been incorporated in an anti-adherent coating on poly(methyl methacrylate) disks to inhibit binding of *E. coli*, macrophages and fibroblasts<sup>25</sup>. Furthermore, pre-treatment of human intestinal epithelial cell cultures with pDMAEMA led to reduced adhesion and invasion of *Salmonella* ser. Typhimurium<sup>36</sup>.

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<sup>37</sup>. pDMAEMA may work in a similar manner by adsorbing to the cell surface through electrostatic interactions and disrupting the cytoplasmic membrane through hydrophobic interactions<sup>28,30</sup>. 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 different pDMAEMA analogues 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<sup>38</sup>. *N*-(*n*-Propyl)-2-pyridylmethanimine was prepared as described earlier<sup>15</sup>. Triethylamine (99%, Thermo Fischer Scientific, Waltham, MA, USA) was stored over sodium hydroxide pellets. Anhydrous tetrahydrofuran (THF) (“Hi-Dry”, 99.99%, Romil, Cambridge, UK) 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, UK), unless otherwise stated.

### Synthesis of unconjugated and hostasol-conjugated pDMAEMA analogues.

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 visualisation. Hostasol (thioxantheno[2,1,9-def]isochromene-1,3-dione) (389.5 Da, CAS Registry Number: 14121-49-4) was supplied by Clariant (Muttens, Switzerland). Fluorescent hostasol-conjugated pDMAEMA was prepared as described previously<sup>14</sup>, using ethyl-2-bromo isobutyrate as the polymerisation 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.

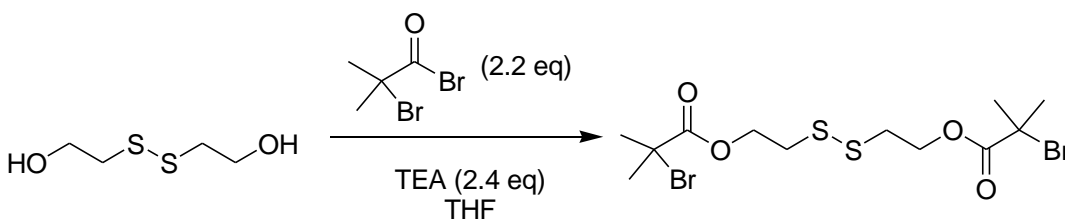
Analogous non-fluorescent pDMAEMA (unconjugated) materials were prepared in the same way, except that no hostasol methacrylate fluorescent co-monomer was employed

in the polymerisation step. In addition to these molecules the following pDMAEMA analogues 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<sup>39</sup>.

### Scheme 1: Synthesis of disulphide-based bifunctional polymerisation initiator, bis[2-(2-bromoisobutyryloxy)ethyl] disulphide (BiBOE)2S2

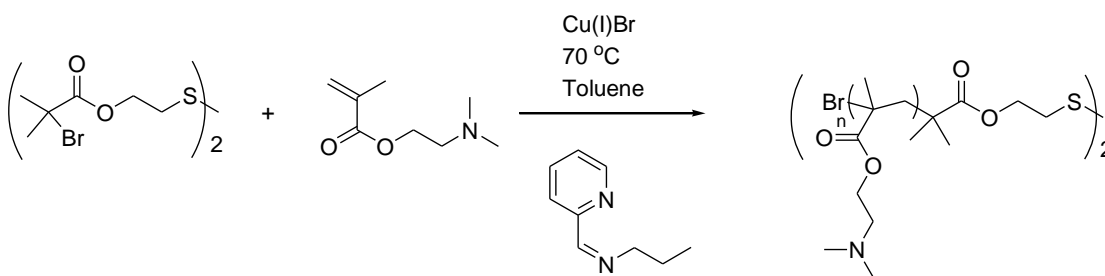


Bis(2-hydroxyethyl) disulphide (4.00 ml, 32.4 mmol) and an excess of triethylamine (9.9 ml, 71 mmol) was added to a 500 ml round bottom flask, along with a magnetic stir bar and was purged with nitrogen for 15 minutes on an ice bath. Anhydrous THF (150 ml) was then added and the resulting solution allowed to cool to 0°C. Under a nitrogen atmosphere, 2-bromoisobutyryl bromide (8.4 ml, 68 mmol) was added drop wise via a degassed syringe (dropwise addition is essential in order to minimise the exotherm). The solution was allowed to reach ambient temperature, and left to stir for 6 hours. The resulting triethylammonium bromide salt was removed by filtration, and solvent was



removed under reduced pressure. The resulting pale yellow solution was stirred with 0.1 M aqueous  $\text{Na}_2\text{CO}_3$  to hydrolyze any residual 2-bromoisobutyryl bromide. The crude product was then extracted three times with dichloromethane and the organic layers, combined, were dried over anhydrous magnesium sulphate, filtered, and the volatiles removed under reduced pressure, yielding the  $(\text{BiBOE})_2\text{S}_2$  initiator as a clear yellow oil (10.1 g, 22.0 mmol, 74.1 %) which was stored at 4 °C.  $^1\text{H}$ ,  $^{13}\text{C}$ [ $^1\text{H}$ ] and CHN elemental analysis were in line with previously published data <sup>40</sup>.

**Scheme 2: Cu(I)Br catalysed polymerisation of dimethyl amino ethyl methacrylate (DMAEMA) initiated by  $(\text{BiBOE})_2\text{S}_2$**



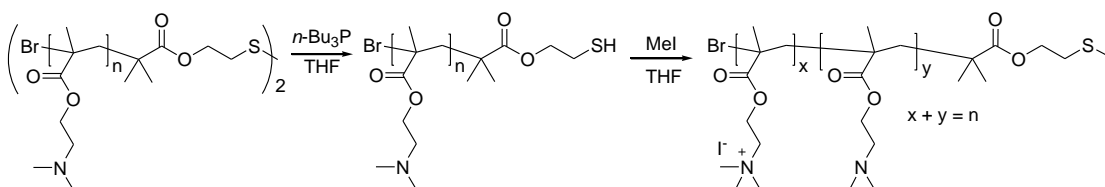
$\text{Cu(I)Br}$  (0.33 g, 2.3 mmol) was added to a clean, oven dried Schlenk tube, along with a magnetic follower. The Schlenk tube was sealed with a suba-seal, evacuated and filled with nitrogen. Toluene (25.0 ml), DMAEMA (25.0 ml, 148 mmol) and bis[2-(2-bromoisobutyryloxy)ethyl] disulphide initiator (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. The solution was heated to  $70\text{ }^\circ\text{C}$  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 minutes 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 one hour. 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 % quaternised pDMAEMA.

As pDMAEMA is a charged molecule, the 50 % quaternised analogue was used to investigate the impact of charge on antimicrobial activity.

### Scheme 3: Reduction of the disulphide-bridge and subsequent quaternisation with MeI



The disulphide-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,  $\text{Bu}_3\text{P}$  (210  $\mu\text{l}$ , 0.87 mmol) was added to the system. The reduction was complete after 10 min, the 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 quaternisable 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. The solvent was then removed under reduced pressure and the solid residue was dissolved in 20 ml of deionised water and freeze-dried, affording the desired quaternised polymer.

**Analysis of polymers.** Molar mass distributions were measured using size exclusion chromatography (SEC), on a system equipped with two PL gel 5  $\mu\text{m}$  mixed D-columns (300 x 7.5 mm) and one PL gel 5 mm guard column (50 x 7.5 mm) (Polymer Laboratories, suitable for molecular weights between 200 and 400,000 g/mol) with differential refractive index detection, using THF/triethylamine 95:5 (vol/vol), at 1.0 ml/min, as the eluent. Poly(MMA) standards (200-3 $\cdot$ 10<sup>5</sup> g/mol) were used to calibrate the SEC. Analyte samples contained (0.2 % vol) toluene as the flow marker. For purity determination, pDMAEMA analogues were subjected to SEC-HPLC with a Varian 920 HPLC using a BioSep-SEC-S-2000 column 300 $\times$ 7.8 mm (Phenomenex, UK). 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<sup>41</sup> 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<sup>42</sup> 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, UK) 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<sup>43</sup>. For anaerobic bacteria the method was adapted from the microdilution broth dilution procedure from the NCCLS protocol<sup>44</sup>, and for yeast the method was adapted from the microdilution broth dilution procedure from the NCCLS protocol<sup>45</sup>. Aerobically- and anaerobically-grown bacterial cells were seeded in microtitre plates at  $5 \times 10^5$  CFU/ml per well and  $1 \times 10^5$  CFU/ml per well, respectively. Yeast cells were seeded at  $0.5\text{-}2.5 \times 10^3$  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 hours and microbial growth was determined using a microplate spectrophotometer (UVM340, Asys Hitech GmbH, Eugendorf, Austria) at 600nm. Anaerobic bacteria and yeast plates were incubated at 37°C for 48 hours 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<sup>46</sup>. The bacteria were inoculated into flasks at  $5 \times 10^5$  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 hours 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  $\geq 3 \log_{10}$  decrease in CFU/ml after 24 hours. The results are presented as a mean  $\pm$  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 % quaternised pDMAEMA). Growth is represented as a percentage of cultures containing no polymer (100 % growth). The results are presented as a mean  $\pm$  SEM of a minimum of three independent replicates.

**Estimation of pKa values for unconjugated and 50 % quaternised pDMAEMAs.**

The pKa's of unconjugated pDMAEMA, and 50 % quaternised pDMAEMA were determined by pH titration. Briefly, 10 ml of 1 mg/ml polymer, in distilled water, was titrated against 0.01 M or 1 M NaOH to the equivalence point (unconjugated pDMAEMA, pH = 9.3 - 9.8; 50 % quaternised 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 pH of the solution was monitored. The pH at equivalence of this curve is equal to the pKa of the polymer. The results are presented as the average of three independent replicates.

**Visualisation of hostasol-conjugated pDMAEMA binding to bacteria.** In order to visualise the interaction of the polymer with the bacteria, hostasol-conjugated pDMAEMA was incubated with  $1 \times 10^9$  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 x magnification, using a FITC filter, Ex = 465 - 495 nm, 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).

**Internalisation of pDMAEMA by bacteria.** In order 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  $5 \times 10^8$  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 hours at 37°C, 170 rpm. Cells were centrifuged at 3000 x g for 10 min at 4°C, washed 3 times in PBS then resuspended in 1 ml PBS. 50 µl from each time point was resuspended in 10 ml PBS at  $2.5 \times 10^6$  CFU/ml and another 50 µl was resuspended in 10 ml of 1 mg/ml trypan blue (Beckman Coulter, CA, USA) in PBS at  $2.5 \times 10^6$  CFU/ml<sup>37</sup>. Samples were incubated at ambient temperature for at least 30 min, but not more than 6 hours to ensure bacterial survival. Samples were analysed 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 analysed 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<sup>47</sup>, then reanalysed 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 3 different occasions and representative results are shown.

**Outer membrane permeabilisation.** 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 non-permeated bacterial cell<sup>48, 49</sup>. *Salmonella* ser. Typhimurium IMD 574, *Salmonella* ser. Enteritidis ATCC 13076 and *E. coli* ATCC 10536 were grown to an OD<sub>600</sub> of 0.5. Cells were harvested and washed 3 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. 200 µl of bacterial suspension was added in 5 replicates to 96-well plates. 20 µl 0.2 mM NPN was added and the increase in fluorescence measured on a fluorescent microplate spectrophotometer (Spectra Max Gemini, Molecular Devices, CA, USA) every minute for 20 minutes with



Ex = 350 nm and Em = 429 nm. The results are presented as a mean  $\pm$  SEM of a minimum of three independent replicates.

**Cytoplasmic membrane permeabilisation.** The method for determination of cytoplasmic membrane permeability was adapted from the methods of Essodaigui *et. al.*, Koo *et. al.* and Edgerton *et. al.*<sup>50,51</sup>. Calcein-AM is a non-fluorescent derivative of the dye calcein<sup>50,52</sup>. 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<sup>50,52</sup>. Once inside, fluorescent calcein is unable to transport back across the membrane, unless the membrane of the cell becomes permeabilised<sup>52</sup>. 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 x g for 10 min at 4°C and washed 3 times in PBS. The cells were then resuspended in PBS containing 5  $\mu$ M calcein-AM (Invitrogen Corporation, CA, USA) supplemented with 10 % vol/vol Brain Heart Infusion broth at a final concentration of  $5 \times 10^7$  CFU/ml. 180  $\mu$ l of resuspended culture was incubated for 90 min at 37°C in a 96-well tissue culture plate. Fluorescence, at an excitation wavelength (Ex) = 496 nm and an emission wavelength (Em) = 517 nm, was monitored every 10 min in a fluorescent microplate spectrophotometer (Spectra Max Gemini, Molecular Devices, CA, USA). 20  $\mu$ l of 10 x 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 hours. Final concentrations added were 0.1 and 2 mg/ml for *S. epidermidis* and *Salmonella* ser.

Typhimurium, respectively. 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  $\pm$  SEM of a minimum of three independent replicates.

**Effect of pDMAEMA on bacterial cell growth.** 1 ml aliquots from overnight cultures of *Salmonella* ser. Typhimurium IMD 574 and *S. epidermidis* 1457 were incubated at approximately  $5 \times 10^8$  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 hours at 37°C, 170 rpm. Cells were centrifuged at 3000 x g for 10 min at 4°C, washed 3 times in PBS, then resuspended in 1 ml PBS. 50  $\mu$ l resuspended sample was added to 1 ml of 70 % ethanol and incubated at ambient temperature for 24 hours. Fixed cells were centrifuged at 3000 x g for 10 min at 4°C, washed 3 times in PBS and then resuspended in 1 ml PBS. 100  $\mu$ l from each time point was resuspended in 1 ml PBS at  $2 \times 10^6$  CFU/ml. 20  $\mu$ l RNase (10 mg/ml stock) and 10  $\mu$ l PI (10 mg/ml stock) were added then samples were incubated at 37°C for 30 min. Samples were analysed 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 analysed 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 by eye. The results are presented as the mean of a minimum of three independent replicates.

**Cytotoxicity analysis: Sheep red blood cell haemolysis.** The method for determination of haemolytic potential of pDMAEMA was adapted from the protocol outlined by Shin *et al*<sup>53</sup>. Briefly, 1 ml mechanically defibrinated sheep blood (TCS Biosciences, Buckingham, UK) was centrifuged (2000 x 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 microtitre 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 mins. Aliquots (100 µl) of the supernatant were transferred to a fresh 96 well microtitre plate, where haemoglobin release was monitored spectrophotometrically at 414 nm using a microplate spectrophotometer (UVM340, Asys Hitech GmbH, Eugendorf, Austria). Percent haemolysis 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<sup>54,55</sup>. 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 % foetal bovine serum (FBS), 1 % non-essential 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<sub>2</sub>, in air. At 70-80 % confluence, cells were seeded, at a density of 2 x 10<sup>4</sup> and 2 x 10<sup>5</sup> cells/well, for Caco-2 and U937 cells respectively, in 96-well tissue culture plates and allowed to grow for 20 - 24 hours. Caco-2 monolayers were rinsed with fresh growth medium and allowed to equilibrate at 37°C, 5% CO<sub>2</sub> 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<sub>2</sub> for 1 and 24 hours. 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 hours at 37°C, 5 % CO<sub>2</sub>. 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 post-hoc tests. The significance level was set at  $\alpha = 0.05$  (95 % confidence intervals).

## Results

**Characterisation of pDMAEMA analogues.** 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, the polydispersity index (PDi) and the pKa of pDMAEMA analogues used in this report are shown in Table 1. The pKa of unconjugated pDMAEMA was 7 (Table 1). This is in agreement with other reports in the literature where it is reported to be 7 - 7.5<sup>13,56</sup>. The pKa of the 50 % quaternised pDMAEMA was found to be approximately 11 (Table 1). The pKa of the hostasol-conjugated and reducible analogues was not determined as these molecules were not used in pH determination experiments.

**Table 1: Molecular weight, polydispersity index and pKa of pDMAEMA analogues.**

pDMAEMA Analogue	$M_n$ (kDa)	PDi	pKa
Unconjugated	12.8	1.16	7
50% Quaternised	9.7	1.31	11
Hostasol-conjugated	6.6-10.7	1.13	ND
Reducible	17.3	1.17	ND

ND = not determined.

**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 and did not effect the growth of the yeast *Candida albicans* up to 10 mg/ml (Table 2).

**Table 2: MIC concentrations (mg/ml) for pDMAEMA against a range of Gram-negative (-) and Gram-positive (+) bacteria and the yeast *Candida albicans*.**

Organism	Gram	MIC
<i>Salmonella</i> ser. Typhimurium IMD 574	-	1*
<i>Salmonella</i> ser. Typhimurium bovine isolate	-	1*
<i>E. coli</i> ATCC 10536	-	0.1
<i>E. coli</i> equine isolate	-	0.1
<i>Salmonella</i> ser. Enteritidis ATCC 13076	-	0.1
<i>P. aeruginosa</i> QC strain	-	1
<i>M. luteus</i> ATCC 9341	+	>18
<i>L. salivarius</i> UCC 118	+	>18
<i>L. monocytogenes</i> NCTC 11994	+	10
<i>Listeria</i> spp. wild type #28	+	1
<i>B. breve</i> DSMZ 20213	+	10
<i>B. bifidum</i> DSMA 20456	+	10
<i>S. epidermidis</i> 1457	+	0.1
<i>C. albicans</i> C	yeast	>10

\* Results published previously<sup>21</sup>. pDMAEMA tested with hostasol attached.

From the results of the MIC screen, it appeared that the polymer may generally be more effective against Gram-negative bacteria than Gram-positive. In order to investigate this further, we examined a number of different analogues of pDMAEMA against two representative strains of Gram-positive and Gram-negative bacteria (Table 3).

*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<sup>41</sup>. *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<sup>42</sup>. All of the analogues gave comparable MIC values (Table 3), except for the 50 % quaternised polymer against *Salmonella* ser. Typhimurium, which was found to be much higher. It is possible that the more positive charge on the 50 % quaternised polymer may influence its ability to inhibit the growth of Gram-negative bacteria.

Hostasol alone was also tested for its antimicrobial activity (Table 3). 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.

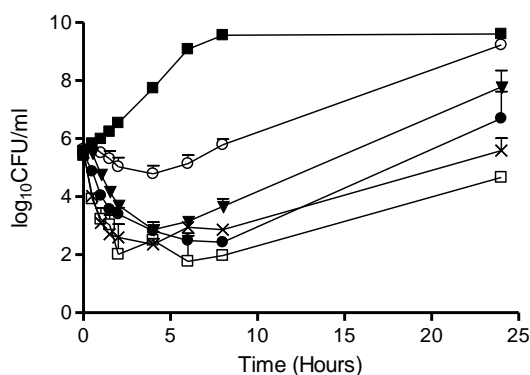


**Table 3: MIC values (mg/ml) of pDMAEMA analogues against *Salmonella ser. Typhimurium* IMD 574 and *S. epidermidis* 1457.**

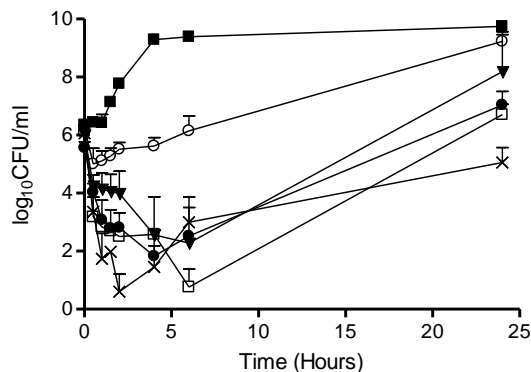
Polymer (MW)	<i>Salmonella ser. Typhimurium</i>	<i>S. epidermidis</i>
Unconjugated	1	0.1
Reducible	1	0.05
50% Quaternised	> 8 ***	0.1
Hostasol alone	> 0.016	> 0.016

\*\*\* P < 0.001 compared with unconjugated pDMAEMA analogue against *Salmonella ser. Typhimurium*.

**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 bacteria, as after 24 hours the bacteria had regrown and there was no evidence of a  $\geq 3 \log_{10}$  reduction in CFU/ml.



**Figure 1: Time-kill curve of pDMAEMA (12.8 kDa, mg/ml) 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.**

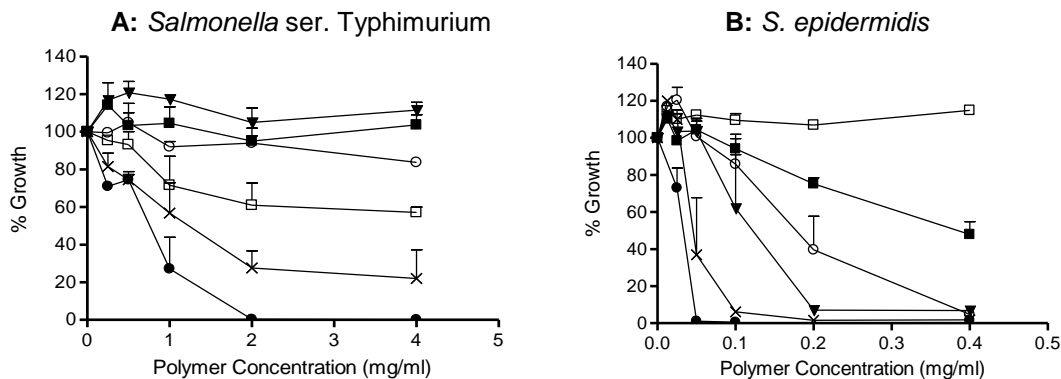


**Figure 2: Time-kill curve of pDMAEMA (12.8 kDa, mg/ml) against *Salmonella ser. Typhimurium* IMD 574. ■ = Control, ○ = 0.5 mg/ml, ▼ = 1 mg/ml, ● = 2 mg/ml, × = 4 mg/ml and □ = 8 mg/ml.**

**Optimum pH and temperature for antimicrobial activity.** As was suggested by the high MIC of the 50 % quaternised pDMAEMA analogue (Table 3), 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 pKa of pDMAEMA is approximately 7 - 7.5 and for both bacteria tested, optimum anti-bacterial activity is around the pKa. The effect of pH was also tested against the 50 % quaternised pDMAEMA (9.7 kDa) using *Salmonella ser. Typhimurium*. We found that even at pH 9,

the quaternised pDMAEMA did not have any effect against *Salmonella ser.*

Typhimurium (data not shown). The bacteria did not grow above the pH values tested.

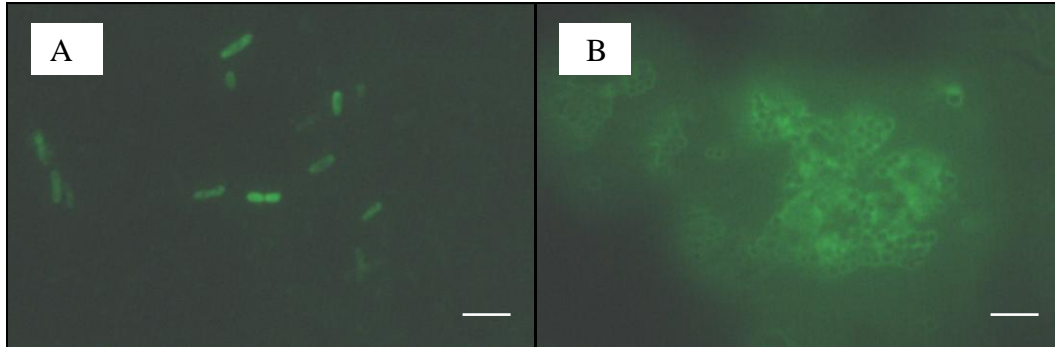


**Figure 3: Effect of pH on antimicrobial activity. pH values ■ = 5, ○ = 6, ▼ = 7, ● = 7.5, × = 8 and □ = 9. A = pDMAEMA (12.8 kDa) against *Salmonella ser.***

**Typhimurium IMD 574. B = pDMAEMA (12.8 kDa) against *S. epidermidis* 1457. % Growth is compared to untreated control and is designated at 100 %.**

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

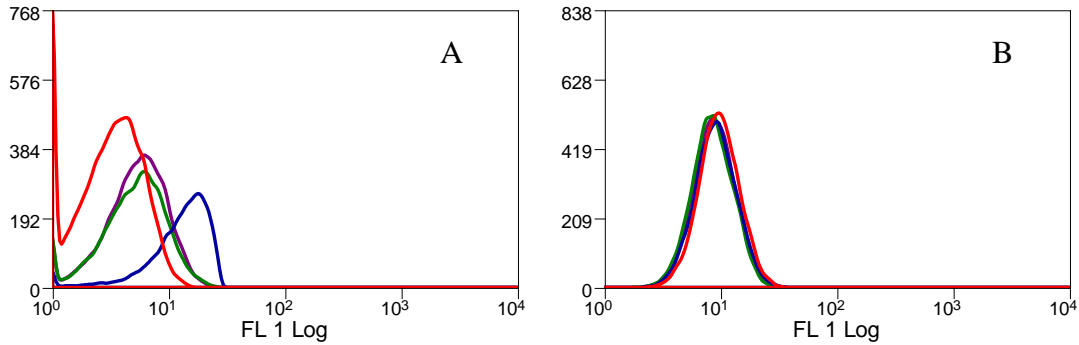
**Hostasol-conjugated pDMAEMA binding to bacteria.** The hostasol-conjugated pDMAEMA (10.7 kDa) analogue was used to visualise the polymer binding to bacteria. In Figure 4, pDMAEMA can be seen binding to each of *Salmonella ser.* Typhimurium and *S. epidermidis* after 30 min incubation. A similar result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



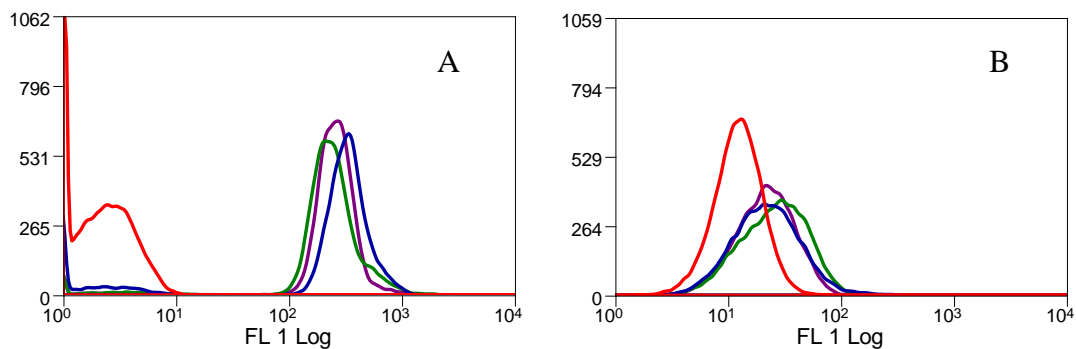
**Figure 4: Fluorescent pictures of hostasol-conjugated pDMAEMA (10.7 kDa) interactions with bacteria. A = *Salmonella* ser. Typhimurium IMD 574, B = *S. epidermidis* 1457 Bar = 5  $\mu$ M. Ex wavelength = 465-495 nm, Em wavelength = 515-555 nm.**

**Internalisation of pDMAEMA by bacteria.** In order to test whether pDMAEMA was able to be internalised, bacteria were incubated alone or with hostasol-conjugated pDMAEMA for 30 min, 2 and 4 hours. The bacteria were analysed 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 re-analysed. 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 internalised (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.



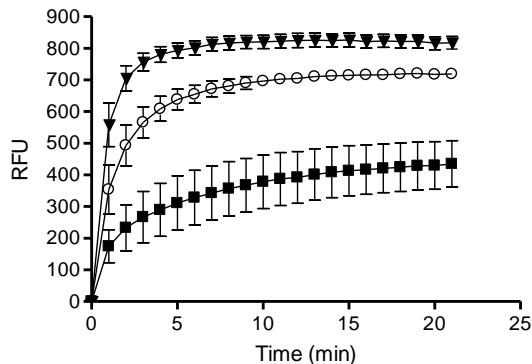
**Figure 5: Flow cytometric analysis of *S. epidermidis* 1457 treated with 0.1 mg/ml hostasol-conjugated pDMAEMA (+ 100 µg/ml PI to gate for live cells). A = No trypan blue, B = Cell treated with 1 mg/ml trypan blue. Red line = control, blue line = 30 min treatment, green line = 2 hours treatment, purple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

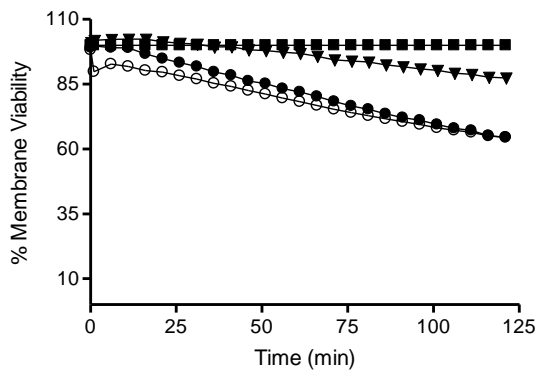
**Outer membrane permeabilisation.** In order to test if pDMAEMA's ability to penetrate *Salmonella* ser. Typhimurium is due to the polymer's ability to permeabilise the outer membrane of Gram-negative bacteria, we incubated pDMAEMA (12.8 kDa) with *Salmonella* ser. Typhimurium IMD 574 and used the fluorescent probe NPN to measure membrane permeabilisation. At pH 7.5 pDMAEMA permeabilised 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 permeabilised 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).



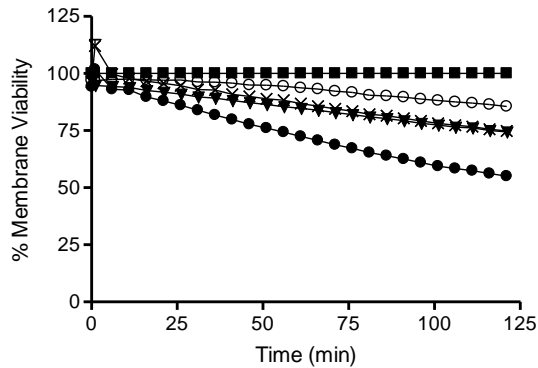
**Figure 7: Permeabilisation of the outer membrane of *Salmonella* ser. Typhimurium IMD 574 by pDMAEMA (12.8 kDa). ■ = pH 6, ○ = pH 7, ▼ = pH 7.5. RFU = relative fluorescence units. Values compared to fluorescence of bacteria incubated with NPN, but no polymer (0 RFU).**

**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).



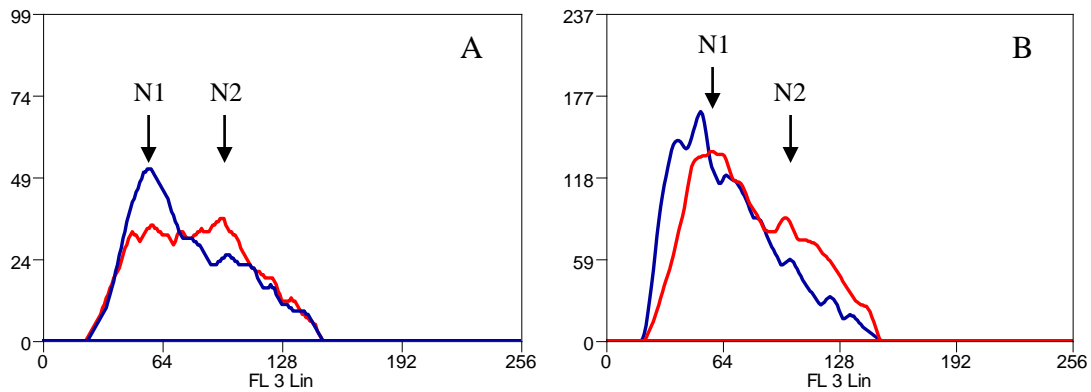
**Figure 8: Permeabilisation of the cytoplasmic membrane of *Salmonella* ser. Typhimurium IMD 574 by pDMAEMA. ■ = PBS, ○ = 2 mg/ml pDMAEMA, ▼ = 0.1 % Triton X-100, ● = 1 % Triton X-100.**



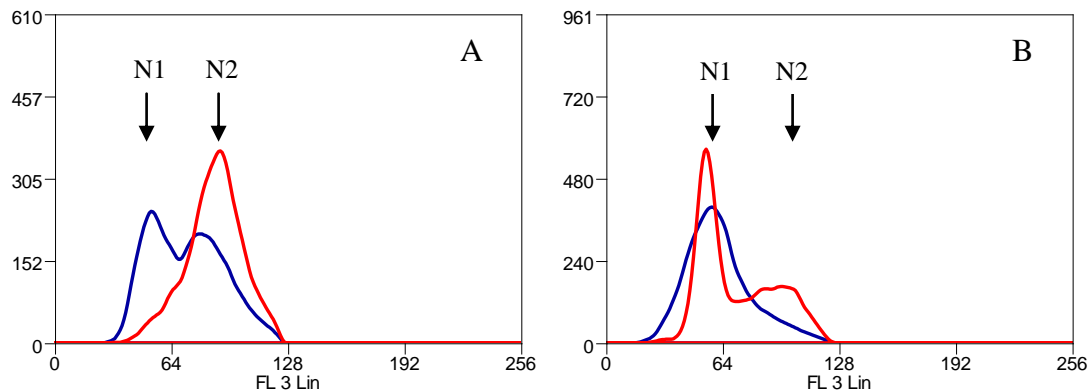
**Figure 9: Permeabilisation of the cytoplasmic membrane of *S. epidermidis* 1457 by pDMAEMA. ■ = PBS, ○ = 0.1 mg/ml pDMAEMA, ▼ = 1 mg/ml pDMAEMA, × = 0.1 % Triton X-100, ● = 1 % Triton X-100.**

**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<sup>57</sup>. 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<sup>57</sup>. 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 hours 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 samples. 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.





**Figure 10: Effect of pDMAEMA on *S. epidermidis* 1457 cell cycling. A = cell growth after 30 min, B = cell growth after 4 hours. 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 pDMAEMA on *Salmonella* ser. Typhimurium IMD 574 cell cycling. A = cell growth after 30 min, B = cell growth after 4 hours. 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.**

**Enhancement of the efficacy of erythromycin by pDMAEMA.** Against all of the Gram-negative bacteria tested (*Salmonella* ser. Typhimurium IMD 574, *Salmonella* ser. 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 4). 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.

**Table 4: Enhanced antimicrobial activity of erythromycin against bacteria by addition of unconjugated pDMAEMA. MIC's are expressed in µg/ml.**

Antimicrobial agent	<i>Salmonella</i> ser. Typhimurium		<i>Salmonella</i> ser. Enteritidis		<i>E. coli</i>		<i>S. epidermidis</i>	
	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 x MIC)	3.9	32	2.32	10	0.1	59	0.12	2
Ery + pDMAEMA (0.1 x MIC)	15.6	8	3.17	7	0.13	45	0.24	0

**Cytotoxicity analysis:** The cytotoxicity of pDMAEMA analogues was tested against sheep erythrocytes. Up to 10 mg/ml, none of the pDMAEMA analogues tested were found to cause significant haemolysis (Table 5). Cytotoxicity against the human

intestinal epithelial cell line, Caco-2, and the human monocytic cell line, U937, was assessed by MTT assay (Table 5). All analogues produced similar levels of cytotoxicity to the human cell lines, though all were more cytotoxic towards U937 cells than towards Caco-2 cells. Surprisingly the reducible polymer, that has previously been reported to be less toxic to U937 cells<sup>39</sup>, did not cause less cytotoxicity than unconjugated pDMAEMA.

**Table 5: Cytotoxicity of pDMAEMA analogues and hostasol (mg/ml). IC<sub>50</sub> = concentration that produces 50 % cytotoxicity.**

Polymer	RBC	Caco-2		U937	
	1hr IC <sub>50</sub>	1hr IC <sub>50</sub>	24hr IC <sub>50</sub>	1hr IC <sub>50</sub>	24hr IC <sub>50</sub>
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% Quaternised	>10	>10	5.2 ± 1.2	1.1 ± 0.14 *	1.0 ± 0.26 ***
Hostasol-conjugated	>10	4.9 ± 2.1	0.27 ± 0.03 **	0.05 ± 0.02	0.06 ± 0.01
Hostasol †	>0.016	>0.016	>0.016	>0.016	>0.016

\* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 as compared to unconjugated polymer in the same

column. † Maximum solubility of hostasol was obtained at 0.016 mg/ml.

## 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<sup>36</sup>. We also reported that pDMAEMA conjugated with hostasol, was bactericidal against *Salmonella* ser. Typhimurium IMD 574<sup>36</sup>, 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 analogues 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<sup>30</sup>. 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<sup>58, 59</sup>.

As pDMAEMA is a thermo- and pH-responsive polymer<sup>19, 60</sup>, 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 pKa 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 pKa 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.

Analogues of pDMAEMA were tested in order to investigate effects of modifications to the polymer on antimicrobial activity. The unconjugated polymer was tested to ensure that, without any modifications, the polymer retained activity. A polymer with reportedly lower cytotoxicity<sup>39</sup>, a reducible analogue, was tested to investigate if the disulphide bond in this molecule interferes with antimicrobial activity. A more positively charged polymer, the 50 % quaternised analogue, was tested to investigate the effect of charge on the polymer. All analogues had similar MIC values to each other except for the 50 % quaternised polymer against *Salmonella* ser. Typhimurium (Table 3). Up to a pH value of 9, the 50 % quaternised polymer remained unable to inhibit growth of the Gram-negative bacteria. As the pKa of this analogue is approximately 11, the polymer 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 pKa. Therefore, as pH 9 is lower than the pKa of quaternised pDMAEMA, the result for the 50 % quaternised analogue against *Salmonella* ser. Typhimurium correlates with the unconjugated polymer data.

To date, most reports with pDMAEMA as an antimicrobial agent have used a quaternised 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 quaternised pDMAEMA to be effective against the Gram-negative bacteria, *E. coli*<sup>30, 32</sup>. In these reports the bacteria were only incubated with pDMAEMA for 1 hour. However, in the present study pDMAEMA was incubated

with the bacteria for 18 hours. While it is possible that the quaternised polymer is able to inhibit growth of Gram-negative bacteria over short periods, over longer periods the bacteria are able to grow back. In addition, Yancheva *et al.*<sup>33</sup> found that unconjugated and 50 % quaternised 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 minutes). A longer time point may have produced a different result for the quaternised molecule.

The initial step in the mode of action of cationic biocides is adsorption onto the bacterial cell surface<sup>37</sup>. 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.*<sup>58</sup> 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 analogues 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.*<sup>61</sup> 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 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



permeabilised 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<sup>62,63</sup>. 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<sup>64</sup>. For this reason, agents that sensitise the outer membrane of Gram-negative bacteria are useful to increase the antimicrobial activity of antibiotics, including erythromycin<sup>62</sup>. 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 permeabilisation 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.

In order 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, quaternisation of the polymer, other polymers attached and size<sup>65-68</sup>. Moreau *et al.*<sup>65</sup> found that pDMAEMA caused little or no haemolysis 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.*<sup>68</sup> showed that despite the fact that pDMAEMA did not cause haemolysis of red blood cells it did encourage haemagglutination. Here we have found that none of the pDMAEMA polymers caused any substantial haemolysis 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<sup>66</sup>. 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)<sup>69</sup>. Against Caco-2 cells none of the analogues produced significantly different cytotoxicity concentrations than the unconjugated polymer, except for the hostasol-tagged polymer after 24 hours. Against U937 cells the hostasol-tagged and reducible analogues produced similar cytotoxicity to the unconjugated polymer, while the 50 % quaternised polymer was slightly less toxic. Reducible pDMAEMA has previously been reported to be less cytotoxic than unconjugated pDMAEMA<sup>39</sup>. Here the reducible polymer was not less cytotoxic than the unconjugated analogue. This may be 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. Therefore, conclusions about the toxicity of this polymer must be made with caution and although the *in vivo* work of Moreau *et al*<sup>65</sup> 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 quaternised and/or 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 in order to permeabilise 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 due to its effective inhibitory action against *S. epidermidis*.

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## References

1. Kenawy, E.-R.; Worley, S. D.; Broughton, R., The chemistry and applications of antimicrobial polymers: a state-of-the-art review. *Biomacromolecules* **2007**, 8, (5), 1359-84.
2. Patel, M. B.; Patel, S. A.; Ray, A.; Patel, R. M., Synthesis, characterization, and antimicrobial activity of acrylic copolymers. *J Appl Polym Sci* **2003**, 89, (4), 895-900.
3. Kenawy, E.-R.; Abdel-Hay, F. I.; El-Magd, A. A.; Mahmoud, Y., Biologically active polymers: Modification and anti-microbial activity of chitosan derivatives. *J Bioact Compat Polym* **2005**, 20, (1), 95-111.
4. Koplín, S. A.; Lin, S.; Domanski, T., Evaluation of the antimicrobial activity of cationic polyethylenimines on dry surfaces. *Biotechnol Prog* **2008**, 24, (5), 1160-5.
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., Surface characterization and antibacterial activity of chitosan-grafted poly(ethylene terephthalate) prepared by plasma glow discharge. *J Appl Polym Sci* **2001**, 81, (11), 2769-2778.
6. Makal, U.; Wood, L.; Ohman, D. E.; Wynne, K. J., Polyurethane biocidal polymeric surface modifiers. *Biomaterials* **2006**, 27, (8), 1316-26.
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., Surface modification of orthodontic wires with photocatalytic titanium oxide for its antiadherent and antibacterial properties. *Angle Orthod* **2007**, 77, (3), 483-8.
8. Pasquier, N.; Keul, H.; Heine, E.; Moeller, M., From multifunctionalized poly(ethylene imine)s toward antimicrobial coatings. *Biomacromolecules* **2007**, 8, (9), 2874-82.
9. Imazato, S.; Torii, M.; Tsuchitani, Y.; McCabe, J. F.; Russell, R. R., Incorporation of bacterial inhibitor into resin composite. *J Dent Res* **1994**, 73, (8), 1437-43.
10. Leung, W. K.; Lau, A. P.; Yeung, K. L., Bactericidal and sporicidal performance of a polymer-encapsulated chlorine dioxide-coated surface. *J Appl Microbiol* **2009**.
11. Shinsako, K.; Okui, Y.; Matsuda, Y.; Kunimasa, J.; Otsuka, M., Effects of bead size and polymerization in PMMA bone cement on vancomycin release. *Biomed Mater Eng* **2008**, 18, (6), 377-85.
12. Moon, J. S.; Kim, H. K.; Koo, H. C.; Joo, Y. S.; Nam, H. M.; Park, Y. H.; Kang, M. I., The antibacterial and immunostimulative effect of chitosan-oligosaccharides against infection by *Staphylococcus aureus* isolated from bovine mastitis. *Appl Microbiol Biotechnol* **2007**, 75, (5), 989-98.
13. Butun, V.; Armes, S. P.; Billingham, N. C., Synthesis and aqueous solution properties of near-monodisperse tertiary amine methacrylate homopolymers and diblock copolymers. *Polymer* **2001**, 42, (14), 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., Fluorescently tagged star polymers by living radical polymerisation for mucoadhesion and bioadhesion. *Functional Polymers* **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., Atom transfer polymerization of methyl methacrylate

mediated by alkylpyridylmethanimine type ligands, copper(I) bromide, and alkyl halides in hydrocarbon solution. *Macromolecules* **1999**, 32, 2110-2119.

16. Zhang, X.; Matyjaszewski, K., Synthesis of Well-Defined Amphiphilic Block Copolymers with 2-(Dimethylamino)ethyl Methacrylate by Controlled Radical Polymerization. *Macromolecules* **1999**, 32, (6), 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., Living free-radical polymerization by reversible addition - fragmentation chain transfer: The RAFT process. *Macromolecules* **1998**, 31, (16), 5559-5562.
18. Jana, S.; Rannard, S. P.; Cooper, A. I., Structure-LCST relationships for end-functionalized water-soluble polymers: an "accelerated" approach to phase behaviour studies. *Chem Commun (Camb)* **2007**, (28), 2962-4.
19. Liu, G.; Wu, D.; Ma, C.; Zhang, G.; Wang, H.; Yang, S., Insight into the origin of the thermosensitivity of poly[2-(dimethylamino)ethyl methacrylate]. *Chemphyschem* **2007**, 8, (15), 2254-9.
20. van de Wetering, P.; Cherng, J. Y.; Talsma, H.; Hennink, W. E., Relation between transfection efficiency and cytotoxicity of poly(2-(dimethylamino)ethyl methacrylate)/plasmid complexes. *J Control Release* **1997**, 49, (1), 59-69.
21. Gu, Z.; Yuan, Y.; He, J.; Zhang, M.; Ni, P., Facile approach for DNA encapsulation in functional polyion complex for triggered intracellular gene delivery: design, synthesis, and mechanism. *Langmuir* **2009**, 25, (9), 5199-208.
22. Hoogeveen, N. G.; Cohen Stuart, M. A.; Fleer, G. J., Can charged (block co)polymers act as stabilisers and flocculants of oxides? *Colloids Surf A Physicochem Eng Asp* **1996**, 117, (1-2), 77-88.
23. Zhu, S.; Yang, N.; Zhang, D., Poly(*N,N*-dimethylaminoethyl methacrylate) modification of activated carbon for copper ions removal. *Materials Chemistry and Physics* **2009**, 113, (2-3), 784-789.
24. Keely, S.; Ryan, S. M.; Haddleton, D. M.; Limer, A.; Mantovani, G.; Murphy, E. P.; Colgan, S. P.; Brayden, D. J., Dexamethasone-pDMAEMA polymeric conjugates reduce inflammatory biomarkers in human intestinal epithelial monolayers. *J Control Release* **2009**, 135, (1), 35-43.
25. Lowe, A. B.; Vamvakaki, M.; Wassall, M. A.; Wong, L.; Billingham, N. C.; Armes, S. P.; Lloyd, A. W., Well-defined sulfobetaine-based statistical copolymers as potential antibioadherent coatings. *J Biomed Mater Res* **2000**, 52, (1), 88-94.
26. Kusumo, A.; Bombalski, L.; Lin, Q.; Matyjaszewski, K.; Schneider, J. W.; Tilton, R. D., High capacity, charge-selective protein uptake by polyelectrolyte brushes. *Langmuir* **2007**, 23, (8), 4448-54.
27. Zhang, Q.; Xia, F.; Sun, T.; Song, W.; Zhao, T.; Liu, M.; Jiang, L., Wettability switching between high hydrophilicity at low pH and high hydrophobicity at high pH on surface based on pH-responsive polymer. *Chem Commun (Camb)* **2008**, (10), 1199-201.
28. Lee, S. B.; Koepsel, R. R.; Morley, S. W.; Matyjaszewski, K.; Sun, Y.; Russell, A. J., Permanent, nonleaching antibacterial surfaces. 1. Synthesis by atom transfer radical polymerization. *Biomacromolecules* **2004**, 5, (3), 877-82.
29. Huang, J.; Koepsel, R. R.; Murata, H.; Wu, W.; Lee, S. B.; Kowalewski, T.; Russell, A. J.; Matyjaszewski, K., Nonleaching antibacterial glass surfaces via "Grafting

- Onto": the effect of the number of quaternary ammonium groups on biocidal activity. *Langmuir* **2008**, 24, (13), 6785-95.
30. Roy, D.; Knapp, J. S.; Guthrie, J. T.; Perrier, S., Antibacterial cellulose fiber via RAFT surface graft polymerization. *Biomacromolecules* **2008**, 9, (1), 91-9.
  31. Lenoir, S.; Pagnouille, C.; Detrembleur, C.; Galleni, M.; Jerome, R., Antimicrobial activity of polystyrene particles coated by photo-crosslinked block copolymers containing a biocidal polymethacrylate block. *e-Polymers* **2005**, 074, 1-11.
  32. Huang, J.; Murata, H.; Koepsel, R. R.; Russell, A. J.; Matyjaszewski, K., Antibacterial polypropylene via surface-initiated atom transfer radical polymerization. *Biomacromolecules* **2007**, 8, (5), 1396-9.
  33. Yancheva, E.; Paneva, D.; Maximova, V.; Mespouille, L.; Dubois, P.; Manolova, N.; Rashkov, I., Polyelectrolyte Complexes between (Cross-linked) N-Carboxyethylchitosan and (Quaternized) Poly[2-(dimethylamino)ethyl methacrylate]: Preparation, Characterization, and Antibacterial Properties. *Biomacromolecules* **2007**, 8, (3), 976-984.
  34. Ward, M.; Sanchez, M.; Elasri, M. O.; Lowe, A. B., Antimicrobial activity of statistical polymethacrylic sulfopropylbetaines against Gram-positive and Gram-negative bacteria. *J Appl Polym Sci* **2006**, 101, (2), 1036-1041.
  35. Yousefi Rad, A.; Ayhan, H.; Kisa, U.; Piskin, E., Adhesion of different bacterial strains to low-temperature plasma treated biomedical PVC catheter surfaces. *J Biomater Sci Polym Ed* **1998**, 9, (9), 915-29.
  36. Keely, S.; Rawlinson, L. A.; Haddleton, D. M.; Brayden, D. J., A tertiary amino-containing polymethacrylate polymer protects mucus-covered intestinal epithelial monolayers against pathogenic challenge. *Pharm Res* **2008**, 25, (5), 1193-201.
  37. Ikeda, T.; Yamaguchi, H.; Tazuke, S., New polymeric biocides: synthesis and antibacterial activities of polycations with pendant biguanide groups. *Antimicrob Agents Chemother* **1984**, 26, (2), 139-44.
  38. Keller, R. N.; Wycoff, H. D., Copper (I) chloride. *Inorganic syntheses* **1946**, II, 1-4.
  39. You, Y. Z.; Manickam, D. S.; Zhou, Q. H.; Oupicky, D., Reducible poly(2-dimethylaminoethyl methacrylate): synthesis, cytotoxicity, and gene delivery activity. *J Control Release* **2007**, 122, (3), 217-25.
  40. Shah, R. R.; Merreceyes, D.; Husemann, M.; Rees, I.; Abbott, N. L.; Hawker, C. J.; Hedrick, J. L., Using atom transfer radical polymerisation to amplify monolayers of initiators patterned by microcontact printing into polymer brushes for pattern transfer. *Macromolecules* **2000**, 33, (2), 597-605.
  41. O'Mahony, R.; Quinn, T.; Drudy, D.; Walsh, C.; Whyte, P.; Mattar, S.; Fanning, S., Antimicrobial resistance in nontyphoidal *Salmonella* from food sources in Colombia: evidence for an unusual plasmid-localized class 1 integron in serotypes Typhimurium and Anatum. *Microb Drug Resist* **2006**, 12, (4), 269-77.
  42. Mack, D.; Siemssen, N.; Laufs, R., Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for functional relation to intercellular adhesion. *Infect Immun* **1992**, 60, (5), 2048-57.

43. CLSI, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard - Seventh Edition. M7-A7. In CLSI, Wayne, PA, USA: 2006.
44. NCCLS, Methods for antimicrobial susceptibility testing of anaerobic bacteria; Approved Standard - Fifth Edition. M11-A5. In NCCLS, Wayne, PA, USA: 2001.
45. NCCLS, Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved Standard - Second Edition. M27-A2. In NCCLS, Wayne, PA, USA: 2002.
46. NCCLS, Methods for determining bactericidal activity of antimicrobial agents; Approved Guideline. M26-A. In NCCLS, Wayne, PA, USA: 1999.
47. Mattiuzzo, M.; Bandiera, A.; Gennaro, R.; Benincasa, M.; Pacor, S.; Antcheva, N.; Scocchi, M., Role of the *Escherichia coli* SbmA in the antimicrobial activity of proline-rich peptides. *Mol Microbiol* **2007**, *66*, (1), 151-63.
48. Je, J. Y.; Kim, S. K., Chitosan derivatives killed bacteria by disrupting the outer and inner membrane. *J Agric Food Chem* **2006**, *54*, (18), 6629-33.
49. Ibrahim, H. R.; Sugimoto, Y.; Aoki, T., Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism. *Biochim Biophys Acta* **2000**, *1523*, (2-3), 196-205.
50. Essodaigui, M.; Broxterman, H. J.; Garnier-Suillerot, A., Kinetic analysis of calcein and calcein-acetoxymethylester efflux mediated by the multidrug resistance protein and P-glycoprotein. *Biochemistry* **1998**, *37*, (8), 2243-50.
51. Edgerton, M.; Koshlukova, S. E.; Lo, T. E.; Chrzan, B. G.; Straubinger, R. M.; Raj, P. A., Candidacidal activity of salivary histatins. Identification of a histatin 5-binding protein on *Candida albicans*. *J Biol Chem* **1998**, *273*, (32), 20438-47.
52. Hollo, Z.; Homolya, L.; Davis, C. W.; Sarkadi, B., Calcein accumulation as a fluorometric functional assay of the multidrug transporter. *Biochim Biophys Acta* **1994**, *1191*, (2), 384-8.
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., Antibacterial, antitumor and hemolytic activities of alpha-helical antibiotic peptide, P18 and its analogs. *J Pept Res* **2001**, *58*, (6), 504-14.
54. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **1983**, *65*, (1-2), 55-63.
55. Maher, S.; McClean, S., Investigation of the cytotoxicity of eukaryotic and prokaryotic antimicrobial peptides in intestinal epithelial cells in vitro. *Biochem Pharmacol* **2006**, *71*, (9), 1289-98.
56. van de Wetering, P.; Moret, E. E.; Schuurmans-Nieuwenbroek, N. M.; van Steenberg, M. J.; Hennink, W. E., Structure-activity relationships of water-soluble cationic methacrylate/methacrylamide polymers for nonviral gene delivery. *Bioconjug Chem* **1999**, *10*, (4), 589-97.
57. Muller, S., Modes of cytometric bacterial DNA pattern: a tool for pursuing growth. *Cell Prolif* **2007**, *40*, (5), 621-39.
58. Lienkamp, K.; Madkour, A. E.; Musante, A.; Nelson, C. F.; Nusslein, K.; Tew, G. N., Antimicrobial polymers prepared by ROMP with unprecedented selectivity: a molecular construction kit approach. *J Am Chem Soc* **2008**, *130*, (30), 9836-43.



59. Endo, Y.; Tani, T.; Kodama, M., Antimicrobial activity of tertiary amine covalently bonded to a polystyrene fiber. *Appl Environ Microbiol* **1987**, 53, (9), 2050-5.
60. Cho, S. H.; Jhon, M. S.; Yuk, S. H.; Lee, H. B., Temperature-induced phase transition of poly(N,N-dimethylaminoethyl methacrylate-co-acrylamide). *J Polym Sci B: Polymer Physics* **1997**, 35, (4), 595-598.
61. Chung, Y. C.; Su, Y. P.; Chen, C. C.; Jia, G.; Wang, H. L.; Wu, J. C.; Lin, J. G., Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. *Acta Pharmacol Sin* **2004**, 25, (7), 932-6.
62. Saha, S.; Savage, P. B.; Bal, M., Enhancement of the efficacy of erythromycin in multiple antibiotic-resistant Gram-negative bacterial pathogens. *J Appl Microbiol* **2008**, 105, (3), 822-8.
63. Champney, W. S.; Tober, C. L., Specific inhibition of 50S ribosomal subunit formation in *Staphylococcus aureus* cells by 16-membered macrolide, lincosamide, and streptogramin B antibiotics. *Curr Microbiol* **2000**, 41, (2), 126-35.
64. Vaara, M., Outer membrane permeability barrier to azithromycin, clarithromycin, and roxithromycin in Gram-negative enteric bacteria. *Antimicrob Agents Chemother* **1993**, 37, (2), 354-6.
65. Moreau, E.; Domurado, M.; Chapon, P.; Vert, M.; Domurad, D., Biocompatibility of polycations: *in vitro* agglutination and lysis of red blood cells and *in vivo* toxicity. *J Drug Target* **2002**, 10, (2), 161-73.
66. Layman, J. M.; Ramirez, S. M.; Green, M. D.; Long, T. E., Influence of polycation molecular weight on poly(2-dimethylaminoethyl methacrylate)-mediated DNA delivery *in vitro*. *Biomacromolecules* **2009**, 10, (5), 1244-52.
67. Jones, R. A.; Poniris, M. H.; Wilson, M. R., pDMAEMA is internalised by endocytosis but does not physically disrupt endosomes. *J Control Release* **2004**, 96, (3), 379-91.
68. Yancheva, E.; Paneva, D.; Danchev, D.; Mespouille, L.; Dubois, P.; Manolova, N.; Rashkov, I., Polyelectrolyte complexes based on (quaternized) poly[(2-dimethylamino)ethyl methacrylate]: behavior in contact with blood. *Macromol Biosci* **2007**, 7, (7), 940-54.
69. Carreño-Gómez, B.; Duncan, R., Evaluation of the biological properties of soluble chitosan and chitosan microspheres. *Int J Pharm* **1997**, 148, 231-240.