Effect of glycounits on the antimicrobial properties and toxicity behavior of polymers based on quaternized DMAEMA

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S *Supporting Information*

ABSTRACT: Polymers with quaternary ammonium groups such as quaternized poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMAQ) have been used as antimicrobial agents because of their demonstrated good antimicrobial activities against a huge number and types of microbes, although their cytotoxicity is also well-known. In this work block copolymers based on PDMAEMAQ were synthesized containing hydrophobic segments of poly(butyl methacrylate) to improve the antimicrobial activity and glycomonomer units with the aim of decreasing the cytotoxicity of the polymers. Hydrophobic butyl methacrylate (BMA) blocks were chain extended by statistical and block copolymers of DMAEMA and 2-{[(D-glucosamin-2-N-yl)carbonylethyl methacrylate (HEMAGl) glycomonomer of different compositions. In order to find the balance between antimicrobial activity and cytotoxicity, the selectivity index of each polymer was obtained from minimum inhibitory concentrations (MIC) and white and red blood cells toxicity measurements.
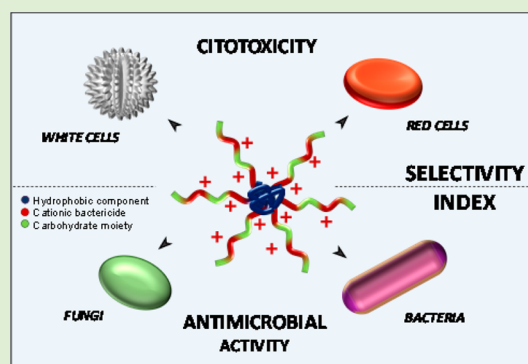
INTRODUCTION

Microbes form an integral part of our daily lives. Whereas many of these are present in our bodies and are actually beneficial, such as the intestinal bacteria *Escherichia coli*, or form part of natural processes, as in bread fermentation by *Saccharomyces cerevisiae* yeast, many global health problems are caused by microbial infections. Unfortunately, not all of these infections can be easily controlled by common antibiotics, and under certain conditions or high pathogenicity of microorganisms, infections can become uncontrolled. In fact, according to a report of the World Health Organization,[1] around 17 million of worldwide deaths annually are caused by infectious diseases (mainly by bacteria). Although new antibiotics are continuously being discovered, infections still remain in the top of rankings of mortality from disease. The use of broad-spectrum antibiotics, abuses in their consumption, open the way for antimicrobial resistance. Nowadays, common hospital and community bacteria already present resistance against first, second, and even third lines of antibiotics, rendering current treatments almost ineffective. For instance, *Klebsiella pneumonie* have become resistant against cephalosporins and carbapenems, *Escherichia coli* against cephalosporins and fluoroquinolones,

and *Staphylococcus aureus* adapts rapidly to acquire resistance against penicillin, methicillin, tetracycline, and erythromycin.[2]

It is clear from the above that there is an ongoing need in developing new antimicrobial agents. In this sense, antimicrobial polymers offer a high versatility for avoiding microbial contaminations or infections. Surface coatings, food packaging, biomedical devices, or water purification systems are some of the ways in which antimicrobial polymers can be incorporated, providing antimicrobial properties to the material.[3−6] Possible design parameters for the polymers include the functional active groups, comonomer composition, molecular weight,[7] the hydrophobic/hydrophilic balance,[8] and the polymer architecture,[9] all of which are known to affect the antimicrobial activity.[4]

In terms of functional antimicrobial groups, phosphonium or sulfonium salts, phenol and benzoic acid derivatives, or fluorine-containing molecules, among others,[5,6] can be found. However, structures carrying quaternary ammonium groups are most widely reported.[10,11] Polymers containing quaternized 2-

(dimethylamino)ethyl methacrylate (DMAEMAQ) units are among those most widely studied due to their demonstrated good antimicrobial activity,[12] despite their demonstrated cytotoxicity. In addition, unquaternized poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) displays high transfection efficiencies and pH response around 40 °C; thus, it is useful not only for antimicrobial purposes, but also for gene transfection[13] or drug delivery.[14]

As is well-known, bacterial membranes are negatively charged due to the high content in phosphatidylethanolamine on the outside.[15] Then cationic biocides are able to adsorb onto the bacterial cell surface through electrostatic interactions, increasing accessibility into the cell. Once the polymer is attached on the bacteria, it should pass through the cell wall and cross the hydrophobic cytoplasmic membrane. Many studies changing the hydrophobic nature of the polymer have demonstrated that a certain amount of hydrophobic content improves its antimicrobial activity.[16] Kuroda et al.[17] studied the antimicrobial behavior of random amphiphilic copolymers based on DMAEMA and hydrophobic methacrylate monomers with side chains from methyl to hexyl and found that mole fractions of the hydrophobic component up to 0.4 decreases the minimum inhibitory concentrations (MICs) at least 10-fold, irrespective of the hydrophobic chain type. The type of hydrophobic chain, however, does play an important role in determining the cytotoxicity of the polymer. The longer the hydrophobic chain, the lower the hemolytic concentration (HC_{50}), and similar results have been described in the work of Dutta et al.[18] for octyl and dodecyl alkyl moieties.

In summary, a good balance between antimicrobial activity and cytotoxicity should be found and, in this sense, many attempts have been carried out to decrease toxicity while maintaining antimicrobial activity. For instance, Locock et al.[19] used guanidine moieties instead of amines as cationic biocidal group to obtain low hemotoxicity. In this work we incorporate carbohydrates into the copolymers based on DMAEMA with the expectation that the toxicity of these antimicrobial polymers will decrease due to the high biocompatibility of carbohydrate units. Some sugars are specifically recognized by membrane-proteins in many human and bacterial membranes acting as sequestering agents. Also, glycopolymers have been previously synthesized as nontoxic vehicles in delivery systems.[20] With the aim of finding this balance between antimicrobial activity and cytotoxicity, and taking into account the considerations as above, a library of polymers was prepared containing these three main components in different compositions and sequences (statistical and block): the antimicrobial agent (DMAEMAQ), the hydrophobic compound (butyl methacrylate, BMA), and the glycomonomer (HEMAGl). In order to address common current problems, the antimicrobial activity was assessed against common pathogenic bacteria and fungi, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or *Candida parapsilosis* by the microbroth dilution method,[21] which is a well-established method for routine studies in microbiology departments in hospitals. Additionally, the cytotoxicity and selectivity of these polymers were determined to evaluate potential compositions that may be used in medical treatments against bacteria and fungi.

■ EXPERIMENTAL SECTION

Materials. *n*-Butyl methacrylate (BMA, 99%, Sigma-Aldrich) was passed through a basic alumina column in order to remove inhibitor, and 2-hydroxyethyl methacrylate (HEMA, 99%, Fluka) and

N,N,N',N'',N''-pentamethyldiethylenetriamine (PMDETA, 99%, Sigma-Aldrich) were distilled prior to use. The reagents D-(+)-glucosamine hydrochloride (Fluka, 99%), *p*-nitrophenyl chloroformate (Fluka, 97%), ethyl 2-bromoisobutyrate (EBrIB, 98%), copper(I) bromide (CuBr, 99.999%), copper(I) chloride (CuCl, 99.995%), 2-(dimethylamino)ethyl methacrylate (DMAEMA, 98%) and lithium bromide salt (LiBr, >99%) were purchased from Sigma-Aldrich, and the quaternizing agent iodomethane (MeI, >99%) was obtained from Riedel-de-Haën, all of them used as received. Regenerated cellulose dialysis tubing (CelluSep T1, M_{WCO} 3500−5000 Da) was purchased from Membrane Filtration Products, Inc. The AR grade solvents, triethylamine (TEA, Scharlau, 99.8%), tetrahydrofuran (THF, Scharlau, 99.8%), toluene (Merck), chloroform (Merck), N,N-dimethylformamide (DMF, Scharlau), and dimethyl sulfoxide (DMSO, Scharlau), were utilized without further purification.

For microbiological and hemolytic studies, sodium chloride solution (NaCl 0.9%, BioXtra, suitable for cell culture), phosphate buffered saline powder (PBS, pH 7.4), and Triton X-114 solution (BioUltra, for molecular biology, ~10% in H_2O) were purchased from Aldrich and were used as received. Sheep blood (5%) Columbia agar plates were purchased from bioMérieux and cation adjusted BBL Mueller Hinton broth used as microbial growth media was purchased from Becton, Dickinson and Company. For cytotoxicity assays, Trypan Blue solution used for cell staining (0.4%, Sigma-Aldrich), sterile RPMI-1640 culture medium with 20 mM HEPES and L-glutamine (Sigma-Aldrich), and the cell viability reagent AlamarBlue (AbD Serotec) were used as received. American Type Culture Collection (ATCC): *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 27853), *Escherichia coli* (*E. coli*, ATCC 25922), *Staphylococcus aureus* (*S. aureus*, ATCC 29213), and a staphylococcus coagulase-negative *Staphylococcus epidermidis* (*S. epidermidis*, ATCC 12221), were used as bacterial strains, and the yeast *Candida parapsilosis* (*C. parapsilosis*, ATCC 22109) was used as fungal strain and purchased from Oxoid. Human blood was taken from healthy donors (Hospital Universitario de Móstoles, Madrid, Spain).

Measurements. Copolymer compositions were determined using ^{1}H NMR spectroscopy. NMR spectra of PBMA macroinitiators and PBMA-*b*-PDMAEMA copolymer samples in CDCl$_3$ (10 mg/mL) were recorded on Bruker AV 300 MHz spectrometer at 25 °C. Copolymers containing glycomonomer were analyzed with a Varian Inova 400 MHz spectrometer at 70 °C in DMF-d_7 (10 mg/mL). The number-average molecular weights (M_n) and polydispersity indexes (M_w/M_n) of PBMA macroinitiators were measured by size exclusion chromatography (SEC) which was carried out on a Waters system (Waters Division Millipore) equipped with a Waters model 410 refractive-index detector and tetrahydrofuran as eluent at a flow rate of 1 mL/min. Separation module operating at 35 °C was composed of Styragel packed columns HR1, HR3, HR4E, and HR5E (Waters Division Millipore). SEC was calibrated with poly(methyl methacrylate) (PMMA) standards (Polymer Laboratories, Ltd.) ranging between 2.4×10^6 and 9.7×10^2 g/mol. The Fourier Transform Infrared (FTIR) spectra of KBr pellets were recorded using a PerkinElmer Spectrum One.

Materials for biological experiments were already sterile or were sterilized in an autoclave before use. The optical density of the microorganism suspensions was measured in McFarland units by a DensiCHEKTM Plus (VITEK, bioMérieux) and incubations were performed at 37 °C in a Jouan IQ050 incubator. Absorbances of 96-well plates were read with a Triturus microplate reader (Grifols).

Preparation of PBMA Macroinitiators. Poly(butyl methacrylate) macroinitiators were obtained by atom transfer radical polymerization (ATRP) in toluene at 100 °C and in the presence of CuBr/PMDETA as catalyst using similar conditions as reported in the literature for methacrylate monomers.[22−24] The purities of the resulting polymers, PBMA$_{70}$-Br (M_n = 9900 g/mol; M_w/M_n = 1.24) and PBMA$_{132}$-Br (M_n = 18770 g/mol, M_w/M_n = 1.18), were confirmed from ^{1}H NMR spectra and their molecular weights and polydispersity indexes determined by SEC, relative to PMMA standards.

Preparation of Block Copolymers Having Statistical Hydrophilic Copolymer Segment: PBMA -*b*-P(DMAEMA-*co*-HEMAGl).

Scheme 1. Schematic Representation of the Diverse Copolymers' Synthesis
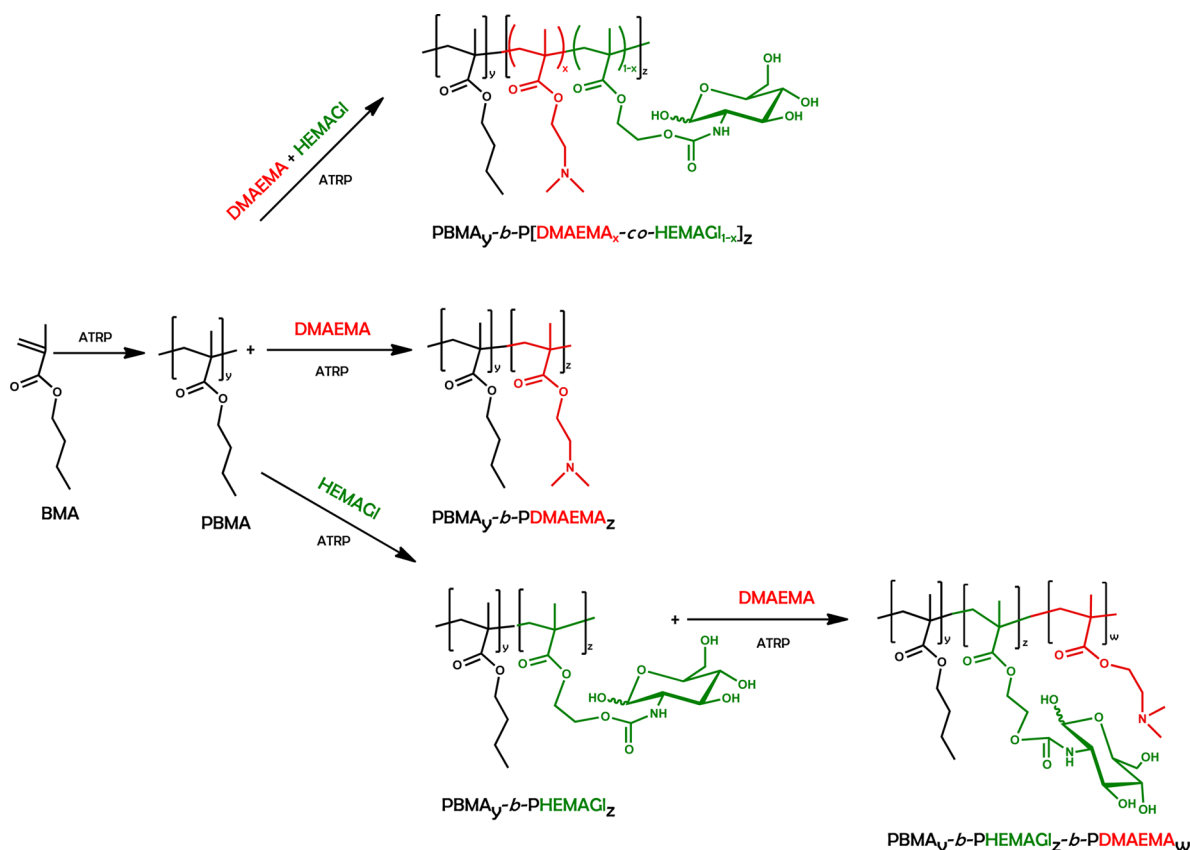
Statistical copolymerizations of DMAEMA and 2-{[(D-glucosamin-2-N-yl)carbonylethyl methacrylate (HEMAGl, synthesized following a procedure described previously by our group[25]) monomers were carried out by ATRP copolymerization in a Schlenk tube from both PBMA-Br macroinitiators. CuCl/PMDETA was used as catalyst in DMF at 80% w/w (80% of total weight is the solvent). Initial DMAEMA mole fractions in the monomer feed for the second block were 0.25, 0.5, and 0.75. The solution was purged with argon during at least 20 min before adding CuCl and PMDETA sequentially. The tube was immediately submerged in a thermostated bath at 90 °C for 4 h, after which the reaction was stopped by cooling (up to 20 °C), exposing catalyst to air and diluting with DMF. The final products were isolated by dialysis against distilled water for 72 h and lyophilized. Final compositions of block copolymers were determined via ^{1}H NMR spectroscopy.

In addition to the PBMA-b-P(DMAEMA-co-HEMAGl) copolymers, PBMA-b-PDMAEMA, PBMA-b-PHEMAGl and PBMA-b-PHEMAGl-b-PDMAEMA diblock and triblock copolymers were also synthesized from PBMA macroinitiators using the same procedure, as described above. PDMAEMA homopolymer (M_n = 12100 g/mol; M_w/M_n = 1.16) was also synthesized for comparative purposes as described in the literature.[26] The compositions were determined by ^{1}H NMR.

Quaternization of Synthesized Polymers. All synthesized polymers containing DMAEMA were quaternized to quaternary amine trimethylaminoethyl methacrylate (DMAEMAQ). Polymers were dissolved in DMF and methylation reaction was carried out with two equivalents of methyl iodide per amine group at room temperature. Full quaternatization was achieved after 24 h of reaction as confirmed by FTIR and NMR spectroscopies of resultant polymers. Solvent and methyl iodide excess were eliminated by dialysis and lyophilization, after which a washing step with hexane was performed to ensure the complete elimination of methyl iodide.

Evaluation of Antimicrobial Activity. Antimicrobial activities of the synthesized polymers were evaluated by determining their minimum inhibitory concentrations (MIC) against the bacterial strains S. aureus, S. epidermidis, and P. aeruginosa and the fungal strain C. parapsilosis. Starting from stock solutions containing 1 mg/mL of polymer the standard broth microdilution method[27] was applied to obtain the MIC. The noncomplete solubility of polymers in aqueous media necessitated an additional first step in which the polymers are dissolved in DMSO. This solution was then diluted with sterile Mueller-Hinton broth to obtain a final concentration of 1 mg/mL (the maximum final DMSO content should be below 6%). Bacterial and fungal strains were grown in 5% sheep blood Columbia agar plates, dispersed, and adjusted with saline solution to a turbidity equivalent of 0.5–1 McFarland turbidity standard ($1-3 \times 10^8$ CFU/mL). A 1:100 dilution with broth provides the bacteria suspension working solution (10^6 CFU/mL). For the broth microdilution method, as a general procedure, 100 μL from each polymer stock solution were placed in a 96-well round-bottom microplate. From these ones, 50 μL were half diluted with 50 μL broth and then, subsequent 1:2 serial dilutions were made across the plate. A well without antimicrobial agent, containing only bacteria or fungi, was used as positive growth control. After 24 or 48 h of incubation at 37 °C for bacteria and fungi, respectively, the MIC was visually determined from the lowest concentration of antimicrobial agent at which no bacterial growth was observed.

Hemolytic Activity (HC$_{50}$). The capability of the synthesized polymers to induce hemolysis of erythrocytes was studied on fresh human blood obtained from healthy donors. Whole blood was collected in blood collecting tubes containing EDTA as anticoagulant and centrifuged immediately after extraction at 3500 rpm for 20 min. Supernatant (plasma) was removed, buffy coat at the middle (containing white blood cells) was isolated and kept for a later white blood cells assay, while the red blood cells (RBC) at the bottom were subsequently washed with cool sterile PBS and centrifuged three times. Once the red blood cells were isolated, a 5% v/v suspension in PBS of red blood cells was prepared and used as working solution. Polymer stock solutions were prepared by dissolving the polymers first in DMSO and subsequent 2-fold dilution with PBS (20 or 40 mg/mL). For the hemolytic assay, 100 μL from each polymer stock

Table 1. Composition of Statistical Copolymers PBMA-b-P(DMAEMA-co-HEMAGl)^{a}

macroinitiator	polymer	f_{DMAEMA}/f_{HEMAGl}	F_{DMAEMA}/F_{HEMAGl}	F_{BMA}	M_{n} (Da)
PBMA$_{70}$	**S1** PBMA$_{70}$-b-P(DMAEMA$_{0.18}$-co-HEMAGl$_{0.82}$)$_{89}$	0.25:0.75	0.18:0.82	0.44	27100
	S2 PBMA$_{70}$-b-P(DMAEMA$_{0.53}$-co-HEMAGl$_{0.47}$)$_{234}$	0.50:0.50	0.53:0.47	0.23	56200
	S3 PBMA$_{70}$-b-P(DMAEMA$_{0.68}$-co-HEMAGl$_{0.32}$)$_{263}$	0.75:0.25	0.68:0.32	0.21	56100
PBMA$_{132}$	**S4** PBMA$_{132}$-b-P(DMAEMA$_{0.38}$-co-HEMAGl$_{0.62}$)$_{117}$	0.25:0.75	0.38:0.62	0.53	31230
	S5 PBMA$_{132}$-b-P(DMAEMA$_{0.63}$-co-HEMAGl$_{0.37}$)$_{224}$	0.50:0.50	0.63:0.37	0.37	49830
	S6 PBMA$_{132}$-b-P(DMAEMAQ$_{0.88}$-co-HEMAGl$_{0.12}$)$_{127}$	0.75:0.25	0.88:0.12	0.51	22630

$^{a}f_{DMAEMA}$ and F_{DMAEMA}, are the molar fraction of DMAEMA in the feed and in the copolymer, respectively; F_{BMA} is the molar fraction of BMA in the copolymer; and M_{n}, is the number average molecular weight estimated by NMR of statistical copolymers (hydrophilic segment).
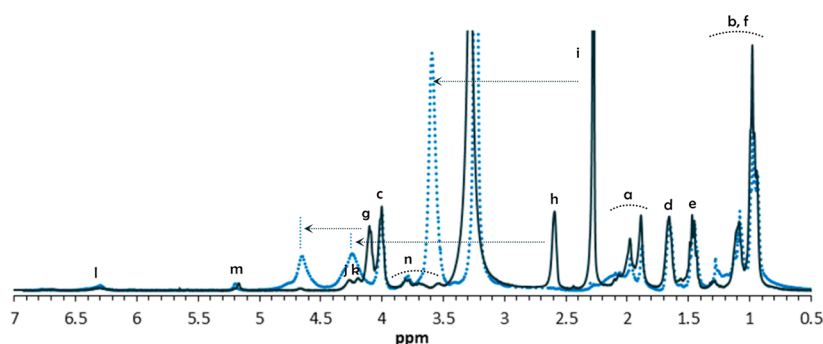
Figure 1. Scheme of PBMA$_{y}$-b-P(DMAEMA$_{x}$-co-HEMAGl$_{1-x}$)$_{z}$ block copolymer structure and ^{1}H NMR spectrum of copolymer S6 (solid line) and its quaternized derivative S6Q (dotted line).

solution were placed in a 96-well round-bottom microplate and 2-fold serial dilutions were performed with PBS.[16,19,28] Then, 150 μL of 5% red blood cell suspension were added to each well. Triton X-114 (50 μL of a 1% v/v solution in PBS) was used as positive control for 100% of hemolysis. Also, a negative control consisting of 150 μL of red blood cell suspension mixed with 50 μL of PBS was placed in order to obtain 0% hemolysis data and correct absorbance values. The microplate was incubated for 1h at 37 °C, centrifuged at 1000 rpm during 10 min and the supernatant was then transferred to an empty 96-well microplate to measure hemoglobin absorbance at 550 nm using a Triturus (Grifols) microplate reader. DMSO was demonstrated to be nontoxic at the experimental concentrations used in this study.

Cytotoxicity Assay with White Blood Cells. Cytotoxicity against white blood cells (WBC) was evaluated using the alamar blue assay. Through this colorimetric assay the reduction of white blood cells viability when exposed to antimicrobial polymers relative to untreated cells was determined. Once white blood cells were isolated from whole blood as described above it is necessary to clean them completely from red blood cells to avoid interactions with polymers and possible colorimetric interferences. Red blood cell traces were eliminated by lysis of erythrocytes with ammonium chloride lysing solution.[29] This isolation method ensures a good viability of the cells after the treatment. Cleaned white blood cells were suspended in RPMI-1640 medium at the desired concentration. After optimization, an optimum cell concentration of 8.4×10^{4} cells/mL and incubation time of 20 h were chosen as experimental conditions to be used in the alamar blue assay. The number of white blood cells in the final suspension was assessed by trypan blue dye exclusion and subsequent counting in a Neubauer chamber. The polymer stock solutions were the same as those utilized for hemolytic assays.

In a same way, and following manufacturer instructions for executing alamar blue assays, polymer solutions (50 μL) were placed in a 96-well round-bottom microplate, followed by 2-fold serial dilutions with RPMI-1640 medium and addition of 100 μL of white blood cells suspension to each well. After 1 h of incubation at 37 °C, 15 μL of alamar blue were added to each well, and subsequently, the microplate was placed again into an incubator for 24 h. Absorbances were measured at 550 and 620 nm (corresponding to the reduced and oxidized forms of alamar blue, respectively) in a microplate reader and cytotoxicity was determined by comparison against the positive growth control (only white blood cells in RPMI-1640 medium). Blanks of RPMI medium and colored polymers were measured before the alamar blue addition to correct the final absorbance values.

Reproducibility and Statistical Analysis of Results. All experiments were done in triplicate at the same moment of the study and, in addition, MIC determination was repeated twice on different days. The data are expressed as mean values of replicated measurements and error bars correspond to standard deviations of the results.

■ RESULTS AND DISCUSSION

Synthesis of Copolymers. New amphiphilic copolymers were synthesized by atom transfer radical polymerization (ATRP) (see Scheme 1). The hydrophobic PBMA as macroinitiator was obtained by polymerization of butyl methacrylate in toluene at 100 °C using 1 equiv of CuBr as catalyst, 1 equiv of PMDETA as ligand, and 1 equiv of ethyl 2-bromoisobutyrate as initiator, [BMA]/[CuBr]/[PMDETA]/[EBrIB] = 100 or 200:1:1:1.[30] Two macroinitiators with different lengths were obtained. Their characterization by ^{1}H NMR and SEC confirmed the structure and controllability of the reaction. The number-average molecular weights, M_{n}, were 9900 and 18770 g/mol corresponding to polymerization degrees of 70 and 132 units, respectively, (PBMA$_{70}$ and PBMA$_{132}$) and their polydispersity indexes were relatively low, 1.24 for PBMA$_{70}$ and 1.18 for PBMA$_{132}$. The obtained PBMAs were later used as macroinitiators to polymerize hydrophilic blocks composed of DMAEMA and HEMAGl in statistical distributions using a halogen exchange catalyst system, CuCl/PMDETA, for better reaction control.[31] Molar ratios for catalyst, ligand and initiator were maintained at one equivalent while DMAEMA mole fractions were changed to obtain three different compositions: 0.25, 0.5, and 0.75; the relationship is [DMEMA + HEMAGl]/[CuCl]/[PMDETA]/[EBrIB] = 100 or 200:1:1:1. Additionally, diblock and triblock copolymers of DMAEMA and HEMAGl were prepared by ATRP using the same conditions as for the statistical copolymers. In the case of

triblock copolymers, these were obtained polymerizing first the HEMAGl block and then the DMAEMA block to obtain PBMA-*b*-PHEMAGl-*b*-PDMAEMA. These copolymers could not be analyzed by SEC due to the excessive adhesion of DMAEMA in the separation columns. Final compositions of copolymers were then calculated from ^{1}H NMR spectra in DMF-d_7 at 70 °C; these results are summarized in Table 1. The M_n of the hydrophilic segment was then obtained from the composition, which is determined by NMR, subtracting the molecular weight of macroinitiator previously determined by SEC.

A typical ^{1}H NMR spectrum and the general chemical structure of the synthesized copolymers are shown in Figure 1. The quadruplet (e) at about 1.5 ppm corresponding to CH$_2$CH$_3$ of the BMA unit, the peak at 2.6 ppm from the methylene protons adjacent to the amine group of DMAEMA, and the peak at 5.2 ppm from the anomeric proton of HEMAGl were used as reference signals for calculating the copolymer composition. It is well-known that quaternary amines show better antimicrobial activities than tertiary, secondary, and primary ones.[5,32] For this reason, DMAEMA was quaternized to its quaternary ammonium salt DMAEMAQ by reaction with an excess of methyl iodide at room temperature.[33] Complete methylation was reached after 24 h, as revealed by the shift of peaks at 2.3, 2.6, and 4.1 ppm to 3.6, 4.2, and 4.7 ppm, respectively; all of these are associated with the nearest protons to the nitrogen of DMAEMA (see Figure 1). Quantitative quaternization was also confirmed by FTIR spectroscopy (see Supporting Information, Figure S1) with the disappearance of the 2760 and 2820 cm^{-1} bands attributed to the stretching vibration of carbon−hydrogen bonds in amines.

Similarly, the characterization of the di- and triblock copolymers was performed by NMR and the results are summarized in Table 2. Subsequently, the quaternization was performed for selected polymers and in all the cases the reaction was quantitative.

Table 2. Composition of Block Copolymers[a]

	polymer	F_{BMA}	F_{DMAEMA}	F_{HEMAGl}	M_n (Da)
homopolymer	**PDMAEMA**	0.00	1.00	0.00	12100
diblock copolymer	**BD** PBMA$_{70}$-*b*-PDMAEMA$_{65}$	0.52	0.48	0.00	10300
	BH PBMA$_{132}$-*b*-PDMAEMA$_{440}$	0.23	0.00	0.77	147430
triblock copolymer	**T1** PBMA$_{70}$-*b*-PHEMAGl$_6$-*b*-PDMAEMA$_{45}$	0.58	0.37	0.05	9100
	T2 PBMA$_{132}$-*b*-PHEMAGl$_{25}$-*b*-PDMAEMA$_{73}$	0.57	0.32	0.11	19830

[a]F_{BMA}, F_{DMAEMA}, and F_{HEMAGl} are the molar fractions of BMA, DMAEMA, and HEMAGl in the block copolymer, respectively, and M_n is the number average molecular weight estimated by NMR (hydrophilic segment).

Antimicrobial Studies. There are different parameters that influence the antimicrobial activity of polymers. It does not only depend on the antimicrobial moiety employed, but also on the physicochemical characteristics of the macromolecule as a whole, including the hydrophilic/hydrophobic balance, charge density, monomer nature, and its distribution within the polymer backbone among other things.[6] For this reason, results can vary from those expected based on the literature for similar

polymers. To carry out a complete evaluation of the antimicrobial capability of these positively charged statistical, diblock and triblock copolymers, minimum inhibitory concentrations (MICs) against Gram-positive bacteria (*S. aureus* and *S. epidermidis*), Gram-negative bacteria (*P. aeruginosa*), and fungi (*C. parapsilosis*) were determined by applying the broth microdilution method.[21] In addition, quaternized PDMAEMA homopolymer and some of nonquaternized polymers were studied to compare the results. Polymer stock solutions were prepared at a concentration of 1 mg/mL in Mueller−Hinton broth with DMSO content up to 6% (higher DMSO concentrations were demonstrated to be toxic for bacteria).[34] For all the studied microbes, 100 μL of each stock solution were placed in the first column of a 96-well plate and several half-dilutions were done across the plate maintaining the last column as positive growing control. Then, 50 μL of corresponding 10^6 CFU/mL bacteria solution were added on each well and plates were incubated. By doing this, polymer concentrations tested varied from 500 to 0.5 μg/mL. The measurements were done at least in triplicate and the results summarized in Table 3. As can be seen, the polymers behave differently depending on the microorganism strain, being more effective, with low MIC values, against Gram-positive *S. epidermidis* and *S. aureus* bacteria and against *C. parapsilosis* fungus and almost ineffective against Gram-negative *P. aeruginosa* and *E. coli* bacteria (*E. coli* data not shown). The double cellular membrane of Gram-negative bacteria complicates polymer permeation inside the bacteria, the inner membrane disruption, and finally, the bacterial death.[35] It is important to remark that the quaternized polymers show smaller MIC values than their corresponding pristine ones. The best results obtained for statistical copolymers were for S6Q with a MIC equal to 8 μg/mL against *S. epidermidis*, while for block copolymers the lowest MIC value was found for BDQ (4 μg/mL).

As mentioned above, PDMAEMAQ homopolymer was studied because it is considered to be an excellent antimicrobial polymer but at the same time, depending on the cell types and the concentration used, can be cytotoxic.[36−38] It is clear that copolymers with low or no DMAEMA content such as S1Q and BH do not show any antimicrobial activity. In contrast, almost similar antimicrobial abilities are found in the cases of S6Q, BDQ, and T1Q polymers, which contain the highest mole fractions of quaternized DMAEMA, confirming that the DMAEMA content is crucial for the antimicrobial activity (Figure 2a). A minimum DMAEMA content, $F_{DMAEMA} > 0.3$, is needed to achieve comparable values to BDQ or PDMAE-MAQ. It has been demonstrated that polymers with DMAEMA contents lower than 0.1 are not able to kill bacteria or fungi.[16]

Considering the hydrophobic BMA block in the copolymer, it can be seen in Figure 2 that an increase in the global composition of BMA produces a reduction in MIC values to reach a minimum at intermediate composition, $F_{BMA} \sim 0.5$. Higher BMA contents will result in higher hydrophobicity and then, an imbalance between hydrophobic and hydrophilic segments. The later could produce an increase of the MIC values[17] besides favoring the polymer insolubility.

Hemolytic Assays. Absorbance measurements of released hemoglobin due to erythrocytes hemolysis produced by action of the copolymers against red blood cells were used to evaluate their toxicity. The positive control to produce 100% of hemolysis (maximum absorbance) was 1%Triton X-114 in PBS, which acts as a detergent for cell membranes disrupting

Table 3. Minimum Inhibitory Concentrations (μg/mL) Obtained for Quaternized (Q) and Some Native Polymers against Bacteria and Fungi Strains[a]

	F_{BMA}	F_{DMAEMA}	F_{HEMAGl}	S. aureus	S. epidermidis	P. aeruginosa	C. parapsilosis
S1Q	0.44	0.10	0.46	>500	>500	>500	>500
S2	0.23	0.41	0.36	>500	125		
S2Q				125	32	>500	32
S3Q	0.21	0.54	0.25	32	8	500	32
S4Q	0.53	0.18	0.29	125	62	>500	>500
S5	0.37	0.40	0.23	125	62		
S5Q				125	32	500	>500
S6	0.51	0.43	0.06	32	16		
S6Q				16	8	250	8
PDMAEMAQ	0.00	1.00	0.00	16	4	250	16
BDQ	0.52	0.48	0.00	32	4	>500	8
BH	0.23	0.00	0.77	>500	>500	>500	>500
T1Q	0.58	0.37	0.05	16	16	250	8
T2Q	0.57	0.32	0.11	64	32	250	16

[a]F_{BMA}, F_{DMAEMA}, and F_{HEMAGl} are the molar fractions in the copolymer of BMA, DMAEMA, and HEMAGl, respectively.
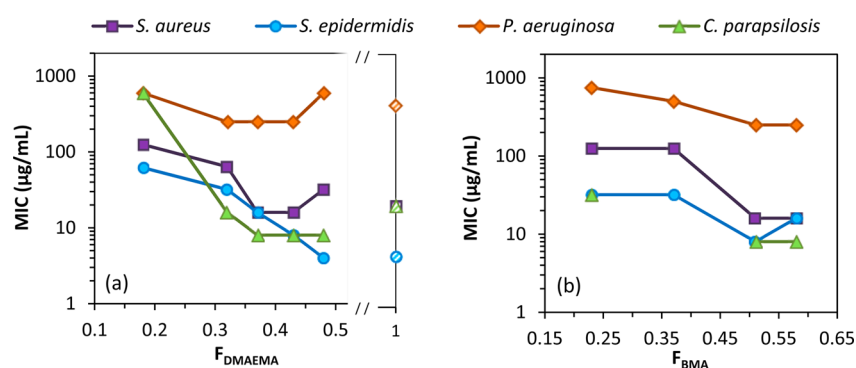
Figure 2. MIC dependence on BMA and DMAEMA content in the copolymers. (a) Polymers S4Q, S6Q, T1Q, and T2Q with similar BMA content and values for PDMAEMAQ homopolymer (streaked fill) and (b) polymers S2Q, S5Q, S6Q, and T1Q with similar DMAEMA content.
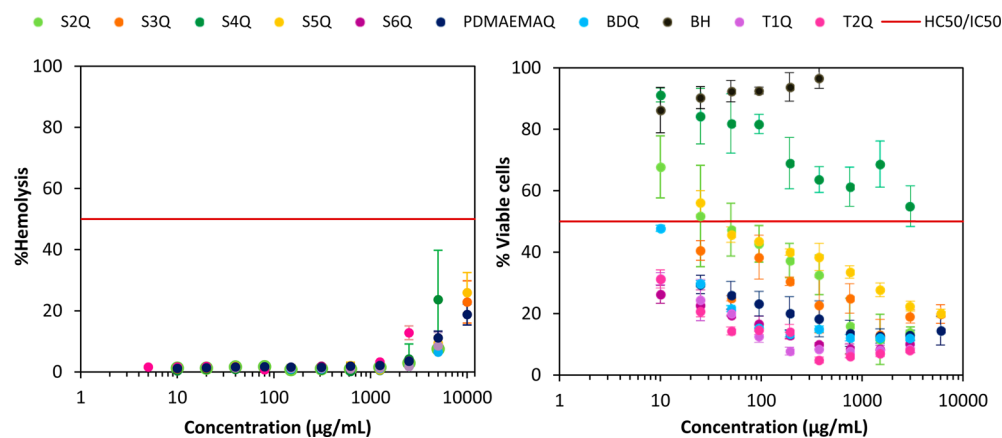
Figure 3. Toxicity of quaternized polymers for red blood cells represented as percentage of hemolyzed erythrocytes (left) and as percentage of viable white blood cells (right) against concentration. Red line situated at 50% represents theoretical HC_{50} and IC_{50} (half maximal inhibitory concentration) for red and white blood cells, respectively.[39]

them completely. The recorded absorbance for each polymer relative to the absorbance corresponding to 100% hemolysis determines the polymer toxicity (see Supporting Information for data analysis details). Hemolysis produced by quaternized polymers was studied at concentrations limited by the solubility of the polymers. The concentration used was 5 mg/mL for all of copolymers except for T2Q (2.5 mg/mL), S3Q, S5Q, and PDMAEMAQ (10 mg/mL).

Hemolytic activity is generally defined by the HC_{50} parameter, which represents the concentration of the toxic agent at which 50% of red blood cells are hemolyzed. In Figure 3 (left) is represented %hemolysis against polymer concentration. The toxicity of S1Q was not determined because of its inactivity against any of the microbes studied, and although BH is inactive, its toxicity was estimated in this case to prove that the DMAEMA content is responsible for the polymer toxicity.

Table 4. HC$_{50}$ and IC$_{50}$ and Their Selectivity Index Values of Quaternized Copolymers against *S. aureus*, *S. epidermidis*, *P. aeruginosa*, and *C. parapsilosis*

	HC$_{50}$	IC$_{50}$	*S. aureus*		*S. epidermidis*		*P. aeruginosa*		*C. parapsilosis*	
			SI$_{RBC}$	SI$_{WBC}$	SI$_{RBC}$	SI$_{WBC}$	SI$_{RBC}$	SI$_{WBC}$	SI$_{RBC}$	SI$_{WBC}$
S1Q	n/a	n/a								
S2Q	>5000	37	>40	0.3	>150	1.2			>150	1.2
S3Q	>10000	<25	>300	<0.8	>1250	<3	>20	<0.1	>300	<0.8
S4Q	>5000	>3000	>40	>24	>75	>45				
S5Q	>10000	33	>80	0.3	>300	1	>20	0.1		
S6Q	>5000	<10	>300	<0.6	>625	<1.2	>20	<0.04	>625	<1.2
PTMAEMAQ	>10000	<25	>625	<1.6	>2500	<2.5	>40	<0.1	>625	<1.6
BDQ	>5000	10	>150	0.3	>1250	2.5		<0.02	>625	1.2
BH	>5000	>3000								
T1Q	>5000	<10	>300	<0.6	>300	<0.6	>20	<0.04	>625	<1.2
T2Q	>2500	<10	>40	<0.2	>75	<0.3	>10	<0.04	>150	<0.6

As can be seen, the maximum %hemolysis reached is under 30% in all the cases and no significant differences can be found between the different polymers. In fact, up to 1 mg/mL (which is twice the value of the highest MIC found) the survival of red blood cells was above of 95%. This fact confirms that these polymers are not toxic for human erythrocytes. Moreover, the selectivity index (SI), which is defined as HC$_{50}$/MIC, is very satisfactory in all the cases (see Table 4). These values indicate that polymers are potentially clinically effective antimicrobial agents exhibiting selective toxicity toward the microorganism rather than the host.

White Blood Cell Viability. As mentioned above, the main goal of this study is to maintain the antimicrobial activity of PDMAEMA while decreasing its cytotoxicity. Incorporation of HEMAGl units into the copolymers should help to achieve the objective, higher activity with lower toxicity. Since white blood cells are essential in fighting infections and are more sensitive than red blood cells, we also studied the toxicity against these cells. It is worth mentioning that only a few studies have been published to date that investigate toxicities against white blood cells. Most related studies have been done with leukemic cells using a MTT assay test for cell viability.[40−42] To the best of our knowledge, only a few studies used the alamar blue assay to measure the viability of entire peripheral white blood cells.[43−45] This colorimetric assay determines cell viability under action of a toxic agent compared to untreated cells based on monitoring the redox process produced for cell metabolism on this reagent by measuring the absorbance at determined wavelengths with time. At the beginning, alamar blue is in its 100% oxidized form (blue and nonfluorescent) and during the experiment, the cell growth transforms it into its reduced form (red and fluorescent). The redox process can be monitored following the changes in absorbance or fluorescence values. A reduced medium is indicative of cell proliferation while an oxidized medium means growth inhibition. In this way, cell viability can be directly related to toxicity. Absorbances at 550 and 620 nm were monitored for this assay. Cell viability is obtained by comparing the reduction of alamar blue by the sample in the presence of toxic agents (polymers) with respect to the reduction produced by only white blood cells (see Supporting Information). First, cell number and time of reduction were optimized. A linear response between 1.6×10^4 and 2.6×10^5 cells per well in 24 h yields the working range (see Figure S2). The alamar blue assay was performed at 8.4×10^4 WBC/well, and the results were determined at 20 h. The test was carried out at a final polymer concentration of 6 mg/mL for S3Q, S5Q,

and PDMAEMAQ and 3 mg/mL for all the others (first well discarded due to DMSO toxicity). Figure 3 (right) displays the viability of white blood cells at different polymer concentrations. It is clear that most of the polymers are highly toxic for white blood cells even at low concentrations. However, polymers with a considerable proportion of HEMAGl and low DMAEMA content, that is, S4Q and BH, are innocuous in the entire concentration range studied although they are very ineffective against microbes. Of particular interest are the S2Q and S5Q copolymers, which have similar DMAEMA content, approximately 0.40, but HEMAGl molar fractions in the copolymer of 0.36 and 0.23, respectively. These polymers have IC$_{50}$ values of 37 and 33 μg/mL, respectively; indicating that they are not toxic at these concentrations. The two most toxic polymers are S6Q and T1Q, with only 0.05 and 0.11 mole fractions of HEMAGl, respectively. From these results it may be concluded that maintaining the DMAEMA content constant, a certain amount of HEMAGl in the copolymer can decrease the polymer toxicity. This is in line with what was previously found for cationic polymers in gene transfer applications: the incorporation of glycounits in the structure decreases their toxicity.[20,46] Moreover, the copolymers with statistical hydrophilic block showed greater cell viability than the triblock copolymer containing comparable HEMAGl and DMAEMAQ content. To the best of our knowledge, this is the first study, which has evaluated the role of glycopolymers on antimicrobial cationic polymers.

■ CONCLUSIONS

In summary, novel amphiphilic block copolymers containing DMAEMA and HEMAGl units well-arranged in a statistical or block manner in the hydrophilic segment have been synthesized by atom transfer radical polymerization. It has been demonstrated the necessity to have a perfect balance between hydrophilicity and hydrophobicity or positive charge density, as well as a distribution of the active sites along the structure, to achieve a nontoxic and potent antimicrobial polymer. Remarkably, it was established that the incorporation of certain amount of carbohydrate pendants groups increases the cell viability while maintaining the antimicrobial activity. Therefore, the appropriate antimicrobial polymer will depend on the final application the polymer. For instance, S6Q (i.e., PBMA$_{132}$-*b*-P(DMAEMAQ$_{0.88}$-*co*-HEMAGl$_{0.12}$)$_{127}$) and T1 (i.e., PBMA$_{132}$-*b*-PDMAEMAQ$_{45}$-*b*-PHEMAGl$_6$) would be very promising materials against Gram-positive bacteria and fungi in coatings and paints because their demonstrated high

antimicrobial activity, whereas S4Q (i.e., PBMA$_{132}$-b-P-(DMAEMAQ$_{0.38}$-co-HEMAGl$_{0.62}$)$_{117}$), which is less active but also less harmful, could potentially be used in vivo treatments. Obviously, it is always dangerous to extrapolate in vitro results to real clinical applications and further in vivo studies will be required.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

FTIR spectra of PBMA macroinitiator as well as S5 and S5Q statistical block copolymers. The data analysis details corresponding to the hemotoxicity and alamar tests. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
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