Research Article

Quaternary Ammonium Polyethyleneimine: Antibacterial Activity

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Quaternary ammonium polyethyleneimine- (QA-PEI-) based nanoparticles were synthesized using two synthetic methods, reductive amination and N-alkylation. According to the first method, QA-PEI nanoparticles were synthesized by cross-linking with glutaraldehyde followed by reductive amination with octanal and further N-methylation with methyl iodide. The second method is based on crosslinking with dialkyl halide followed by N-alkylation with octyl halide and further N-methylation with methyl iodide. QA-PEI nanoparticles completely inhibited bacterial growth ($>10^6$ bacteria), including both Gram-positive, that is, *Staphylococcus aureus* at 80 $\mu\text{g/mL}$, and Gram-negative, that is, *Escherichia coli* at 320 $\mu\text{g/mL}$. Activity analysis revealed that the degree of alkylation and N-methylation of the QA-PEI nanoparticles plays a significant role in antibacterial activity of the reagent. The most potent compound was octyl alkylated QA-PEI alkylated at 1 : 1 mole ratio (primary amine of PEI monomer units/alkylating agent). Also, cytotoxicity studies on MAT-LyLu and MBT cell lines were performed with QA-PEI nanoparticles. These findings confirm previous reports that polycations bearing quaternary ammonium moieties inhibit bacterial growth *in vitro* and have a potential use as additives in medical devices which need antibacterial properties.

1. Introduction

During the last two decades continuous efforts have been made to develop polymers with antimicrobial properties. Positive charge and hydrophobic nature of the antibacterial reagent are important factors of cationic disinfectants [1]. Quaternary ammonium compounds have a broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria [2]. As compared with small-molecule antimicrobial agents, polymeric antimicrobials have advantages, such as that they are nonvolatile, chemically stable, have long-term antimicrobial activity, and are hard to permeate through the skin [3–5]. Distinctively, polycationic antimicrobials, bearing quaternary ammonium moieties, have high charge density exhibiting high antimicrobial activity [6].

Most of these compounds bearing quaternary ammonium groups appear to act by interacting with and disrupting negatively charged bacterial cell membrane followed by release of K^+ ions and other cytoplasmic constituents, resulting in immediate death of the bacterial cell. In contrast to the soluble polycations, insoluble quaternary ammonium macromolecules act on the surface of the microbial cell and display their antimicrobial activity only on contact without permeation [1]. An alternative mechanism of action was proposed by Kügler et al. for cationic surfaces which appear to induce an ion exchange between the positive charges and cations within the membrane [7]. Upon approaching a cationic surface, the structurally essential divalent cations of the membrane are relieved of their role in charge neutralization of the membrane components and are thus free to diffuse out of the membrane. The loss of these

structural cations results in a loss of membrane integrity [7].

A number of polycations possessing antimicrobial properties were developed for this purpose including ion exchange fibers [8], alkoxysilanes [9], soluble and insoluble pyridinium-type polymers [10, 11], polyionenes [12], polymer surfaces derivatized with poly(vinyl-N-pyridinium) [13, 14], and immobilized N-alkylated polyethyleneimine [15–17].

Polyethyleneimine (PEI) is a synthetic polymer prepared from aziridine by cationic polymerization. Its structure contains primary, secondary, and tertiary amino groups due to chain transfer reactions. The amino group ratio is 1:2:1 (i.e., 25% of primary amines, 50% of secondary amines, and 25% of tertiary amines). The amino groups of PEI are chemically reactive and consequently enable a wide variety of chemical modifications which provide PEI with appropriate physicochemical properties. Because of its positive charge and hydrophobicity, alkylated PEI has already attracted attention as a possible antimicrobial agent. For instance, a novel, nonrelease strategy for creating bactericidal surfaces which involves covalent coating with long hydrophobic polycationic chains is based on polyethyleneimine. Thus, coating with polycation was found effective against a variety of Gram-positive and Gram-negative bacteria [15–17]. In addition, Hilal et al. reported on strong antibacterial effect against *E. coli* achieved with polyvinylidene fluoride micro-filtration membranes which were modified with PEI using interfacial polymerisation method [18].

Prompted by these results, we examined the antibacterial activity of quaternary ammonium polyethyleneimine (QA-PEI) nanoparticles prepared via two synthetic methods including reductive amination and N-alkylation followed by N-methylation step. Of note, QA-PEI has been studied as antibacterial additive incorporated in clinically used restorative composite resins [19]. We have also shown that octyl alkylated QA-PEI based nanoparticles embedded in a restorative composite resin at 1% w/w demonstrated strong antibacterial effect against *S. mutans* which lasted for at least 3 months [20]. Moreover, incorporation of QA-PEI based nanoparticles in dental composite resin at 1% w/w was biocompatible [21].

Here we report on a series of QA-PEI nanoparticles which inhibit bacterial growth ($>10^6$ bacteria). The purpose of the present study was to evaluate the QA-PEI nanoparticle structure-activity relationship with respect to the degree of alkylation, N-methylation, PEGylation effect and counter ion, which can play a significant role in the antibacterial activity of the tested nanoparticles. The most potent compound was octyl alkylated QA-PEI alkylated at 1:1 mole ratio (primary amine of PEI monomer units/alkylating agent).

2. Materials and Methods

2.1. Materials. Polyethyleneimine (PEI) of molecular weight of 750-kDa (50% w/w aqueous solution) purchased from Fluka (Rehovot, Israel) was freeze-dried before use. Bromooctane, iodoctane, dibromopentane, diiodopentane,

iodomethane, sodium cyanoborohydride, octanal, polyethylene glycol monomethyl ether (MPEG₂₀₀₀), p-nitrophenyl chloroformate, and sodium bicarbonate were all purchased from Sigma-Aldrich (Rehovot, Israel) and used without further purification. Silver nitrate (AgNO₃) was purchased from Baker (Jerusalem, Israel). Aqueous solution of glutaraldehyde (25% w/w) was purchased from Frutarom (Haifa, Israel). All solvents and reagents were of analytical grade and were used as indicated.

IR spectra were recorded on a Perkin Elmer System 2000 FT-IR. ¹H-NMR spectra (DMSO-d₆ or CDCl₃) were obtained on a Varian 300-MHz spectrometer in 5 mm o.d. tubes. CDCl₃/DMSO-d₆ containing tetramethylsilane served as solvent, and shift reference particle size was determined by dynamic light scattering (DLS) at 25°C using the particle sizer ALV-NIBS/HPPS with ALV-5000/EPP multiple digital correlator (ALV-Laser Vertriebgesellschaft GmbH, Langen, Germany). The particle sizer used a 3 mW HeNe laser (wavelength 632.8 nm) and a scattering angle of 173°. The measurements were carried out in disposable plastic cuvettes (Sarstedt AG & Co., Nümbrecht, Germany), and samples were dispersed in double deionized water. The experimental data were analyzed using the ALV-5000/E software, taking the temperature correction of the viscosity into account. Degree of alkylation and methylation was estimated by elemental microanalysis of nitrogen (%N), carbon (%C), and iodine (%I) using a Perkin-Elmer 2400/II CHN analyzer. Zeta potential was measured using a Zetasizer 2000 (Malvern, UK). Measurements were done in DDW in triplicate. Optical density measurements were made using spectrophotometer (VERSAmax, Molecular Devices Corporation, CA, USA). Multiwell plates were purchased from Nunclon (Nunc, Copenhagen, Denmark). Tryptic Soy Broth (TSB) and Lambda Broth (LB) were purchased from Becton (Dickinson and Company).

2.1.1. Bacteria. *Staphylococcus aureus* (*S. aureus*) strain ATCC 29213 and *Escherichia coli* (*E. coli*) strain K12 MG1655 were selected for the bacterial inhibition study and grown in a tube containing Tryptic Soy Broth (TSB, Becton, Dickinson and Company), and Lambda Broth (LB, Becton, Dickinson and Company), respectively, at 37°C.

2.2. Methods

2.2.1. Synthesis of Quaternary Ammonium PEI-Based Nanoparticles via Reductive Amination. PEI (10 g, 0.23 mol of monomer units) was dissolved in 100 mL of absolute ethanol. Glutaraldehyde solution (230 µl) was added at 1:0.01 mole ratio (primary amine of PEI monomer units/glutaraldehyde). The crosslinking reaction was carried out at room temperature for 6 hours.

N-alkylation was conducted as follows: octanal was added at 1:1, 1:0.75, 1:0.5, and 1:0.25 mole ratio (primary amine of PEI monomer units/octanal, respectively; eventually leading to the 4 compounds no. A, no. B, no. C and no. D). The alkylation step was carried out at room temperature for 24 hours. The resulting yellow

suspension was reduced with sodium cyanoborohydride added at 1:0.33 mole ratio (octanal/ NaCNBH_3) for 24 hours at room temperature. Methylation was carried out with 43 mL of methyl iodide (0.69 mol) which was added at 1:3 mole ratio (monomer units of PEI /methyl iodide) at 42°C for 48 hours. During the methylation step, yellow precipitate was formed. An equivalent amount of sodium bicarbonate (0.23 mol, 19 g) was added to collect released HI during methylation step. Neutralization was continued at the same conditions for additional 24 hours. The precipitated crude was allowed to reach room temperature and washed with hexane and double deionized water (DDW) to remove traces of unreacted octanal, methyl iodide, sodium bicarbonate, and sodium iodide and then was freeze-dried. The purification step was repeated with additional amounts of hexane and DDW, and then freeze-dried. Average yield 75% (mol/mol).

FT-IR (alkylated PEI nanoparticles before reduction, KBr): 3400 cm^{-1} (N-H), 2927 cm^{-1} and 2852 cm^{-1} (C-H), 1617 cm^{-1} (N-H, small band), 1641 cm^{-1} (C=N), and 1464 cm^{-1} (C-H).

FT-IR (QA-PEI nanoparticles, KBr): 3440 cm^{-1} (N-H), 2953 cm^{-1} , 2924 cm^{-1} , and 2852 cm^{-1} (C-H), 1617 cm^{-1} (N-H, small band), 1465 cm^{-1} (C-H), and 952 cm^{-1} quaternary nitrogen.

$^1\text{H-NMR}$ (cross-linked PEI nanoparticles, CDCl_3): 1.4 ppm (m, 4H, glutaraldehyde protons), 1.5 ppm (m, 2H, glutaraldehyde protons), 2.3–3 ppm (m, 4H of PEI protons), and 7.5 ppm (m, 2H, glutaraldehyde imine protons).

$^1\text{H-NMR}$ (cross-linked PEI-octyl nanoparticles (reduced), CDCl_3): 0.8 ppm (t, 3H, CH_3 , octyl protons), 1.23 ppm (m, 10H, $-\text{CH}_2-$, octyl protons), 1.4 ppm (m, 2H, $-\text{CH}_2-$, octyl protons), and 2.4–2.7 ppm (m, 4H, $-\text{CH}_2-$, PEI protons and 2H, $-\text{CH}_2-$ of octyl protons).

$^1\text{H-NMR}$ (QA-PEI nanoparticles, $\text{DMSO}-d_6$): 0.85 ppm (t, 3H, CH_3 , octyl protons), 1.22 ppm (m, 10H, $-\text{CH}_2-$, octyl protons), 1.7 ppm (m, 2H, CH, octyl protons), and 2.6–4.4 ppm (m, methyl of quaternary amine, 4H of PEI and 2H of the octyl chain).

2.2.2. Synthesis of the Quaternary Ammonium Polyethyleneimine-Based (QA-PEI) Nanoparticles via N-Alkylation. PEI (10 g, 0.23 mol of monomer units) was dissolved in 100 mL of absolute ethanol. Dibromopentane was added at 1:0.04 mole ratio (monomer units of PEI/dibromopentane). The crosslinking reaction was carried out under reflux for 24 hours.

N-alkylation was conducted as follows: octyl halide was added at 1:1 mole ratio (primary amine of PEI monomer units/octyl halide) to the same flask. The alkylation was carried out under reflux conditions for 24 hours. Excess NaHCO_3 (1.25 equimolar of octyl halide) was slowly added to neutralize the released acid. Neutralization reaction was continued for 24 hours at the same conditions.

N-methylation was conducted as follows: 43 mL of methyl iodide (0.69 mol) was added to the same flask at 1:3 mole ratio (monomer units of PEI/methyl iodide). Methylation was continued at 42°C for 48 hours. An equivalent amount of sodium bicarbonate (0.23 mol, 19 g) was

slowly added to collect released HI during methylation step. Neutralization was continued under the same conditions for additional 24 hours. Formed NaI salt and excess of unreacted NaHCO_3 were discarded by decantation, and the liquid portion was precipitated in 300 mL of DDW, washed with hexane to remove traces of unreacted octyl halide, methyl iodide and with DDW to remove inorganic salts, and freeze-dried. The purification step was repeated with additional amounts of hexane and DDW, and freeze-dried. Average yield: 70% (mol/mol).

FT-IR (alkylated PEI nanoparticles, NaCl): 3480 cm^{-1} (N-H), 2950 cm^{-1} , 2930 cm^{-1} , and 2850 cm^{-1} (C-H), 1635 cm^{-1} (N-H, small band), and 1454 cm^{-1} (C-H).

FT-IR (QA-PEI nanoparticles, KBr): 3440 cm^{-1} (N-H), 2956 cm^{-1} , 2926 cm^{-1} , and 2853 cm^{-1} (C-H), 1617 cm^{-1} (N-H, small band), 1465 cm^{-1} (C-H), and 967 cm^{-1} quaternary nitrogen.

$^1\text{H-NMR}$ (cross-linked PEI nanoparticles, CDCl_3): 1.43 ppm (m, 2H, alkyl protons), 1.58 ppm (m, 4H, alkyl protons), and 2.1–3 ppm (m, 4H of PEI protons and 4H of alkyl protons).

$^1\text{H-NMR}$ (cross-linked PEI-octyl alkylated nanoparticles, CDCl_3): 0.86 ppm (t, 3H, CH_3 , octyl protons), 1.24 ppm (m, 10H, $-\text{CH}_2-$, octyl protons), 1.39 ppm (m, 2H, $-\text{CH}_2-$, octyl protons), and 2.36–2.7 ppm (m, 4H, $-\text{CH}_2-$, PEI protons and 2H of octyl protons).

$^1\text{H-NMR}$ (QA-PEI, $\text{DMSO}-d_6$): 0.845 ppm (t, 3H, CH_3 , octyl protons), 1.24 ppm (m, 10H, $-\text{CH}_2-$, octyl protons), 1.65 ppm (m, 2H, CH, octyl protons), and 3.2–3.6 ppm (m, methyl of quaternary amine, 4H of PEI and 2H of the octyl chain).

The primary amine content after alkylation step was determined by the trinitrobenzene sulfonic acid (TNBS) method [22].

2.2.3. Exchange Reaction of Iodide Form of Quaternary Ammonium Polyethyleneimine (QA-PEI) Nanoparticles to Nitrate Form. 1 g of the previously prepared QA-PEI nanoparticles ($M_{\text{w average}} = 223\text{ g/mol}$, 4.5 mmol) was dispersed in 100 mL of double deionized water (DDW). 50 mL of 1.8 M of AgNO_3 solution was added dropwise to QA-PEI dispersion. Exchange reaction was continued for 2 hours at 70°C. AgI salt was discarded by filtration. Filtrate was poured into a dialysis membrane (3500 cutoff, Membrane Filtration Products, Inc., San Antonio, TX) and dialyzed against DDW at 4°C for 3 days. The dialysate was lyophilized to dryness. Yield: 50% (w/w), elemental analysis of I (%) before exchange reaction: 54.26, elemental analysis of I (%) after exchange reaction: 4.65.

2.2.4. Exchange Reaction of Iodide Form of QA-PEI Nanoparticles to Acetate Form. 1 g of QA-PEI nanoparticles ($M_{\text{w average}} = 223\text{ g/mol}$, 4.5 mmol) was dispersed in 60 mL of 1 N acetic acid. The resulting dispersion was stirred for 3 hours at room temperature, then poured into a dialysis membrane (3500 cutoff cellulose tubing), and dialyzed against DDW at 4°C for 3 days. The dialysate was lyophilized to dryness. Yield: 50% (w/w), elemental analysis of I (%) before exchange reaction:

FIGURE 1: Kinetic measurements of bacterial outgrowth applying various concentrations of QA-PEI nanoparticles (i.e., no. I). The growth of the bacteria (a) *S. aureus* and (b) *E. coli* was measured every several hours during 24 hours. Each point on the curve is the average absorbance (A_{595}) measured simultaneously in three wells similarly prepared in the same microtiter plate.

TABLE 1: Characterization of QA-PEI nanoparticles at different degrees of alkylation prepared via reductive amination.

Code	Degree of alkylation ^a	Inhibition concentration ^b	Elemental analysis ^c			C/N ^d	Particlesize (nm) ^e	Zeta potential (mV) ^f
			%N	%C	%I			
A	1 : 1	40 µg/mL	7.03	32.74	54.00	3.61	24 ± 10	85 ± 10
B	1 : 0.75	40 µg/mL	9.05	33.63	49.25	3.16	116 ± 100	60 ± 31
C	1 : 0.5	80 µg/mL	8.85	30.94	54.25	2.81	56 ± 25	72 ± 31
D	1 : 0.25	160 µg/mL	9.26	29.60	53.70	2.45	108 ± 106	80 ± 47

Octanal was reacted with cross-linked PEI nanoparticles at various mole ratios via reductive amination followed by N-methylation under the same conditions as described in the experimental section.

^a Mole ratio of reactants (primary amine of PEI monomer units/alkylating agent).

^b Concentration of the tested compound used for a complete inhibition of $>10^5$ *S. aureus* growth. ^c Found Nitrogen, Carbon, and Iodine content of QA-PEI (elemental analysis). ^d C/N was determined by elemental analysis of the alkylated samples before methylation step, whereas C/N of the nonalkylated PEI was found to be 2.26. ^e Apparent particle size of QA-PEI nanoparticles (diameter). ^f Zeta potential of QA-PEI nanoparticles.

54.26, elemental analysis of I (%) after exchange reaction: 18.31.

2.2.5. QA-PEI-Methoxypoly (Ethylene Glycol) (MPEG) Conjugation. Activation of MPEG with p-nitrophenyl chloroformate was obtained as described elsewhere [23]. PEI (10 g, 0.23 mol of monomer units) was dissolved in 100 mL of absolute ethanol. Dibromopentane was added at 1:0.04 mole ratio (monomer units of PEI/dibromopentane). Crosslinking reaction was carried out under reflux conditions for 24 hours. MPEG₂₀₀₀-p-nitrophenyl carbonate (0.5% and 5% mol/mol to ϵ -NH₂) was added to the cross-linked PEI nanoparticles dispersion. The mixture was stirred under reflux conditions for 24 hours.

N-alkylation was conducted as follows: octyl halide was added at 1 : 1 mole ratio (primary amine of PEI monomer units/octyl halide) to the same flask. Alkylation step was carried out under reflux conditions for 24 hours. Excess NaHCO₃ (1.25 equimolar of octyl halide) was added to neutralize released acid. Neutralization reaction was continued from 3 to 24 hours at the same conditions.

N-methylation was conducted as follows: methylation step was carried out with 43 mL of methyl iodide (0.69 mol) added at 1 : 3 mole ratio to the same flask (monomer units of PEI/methyl iodide). Methylation was continued at 42°C for 48 hours. An equivalent amount of sodium bicarbonate (0.23 mol, 19 g) was added to collect released HI during methylation step. Neutralization was continued at the same conditions for additional 24 hours. Formed NaI salt and

TABLE 2: Characterization of QA- PEI nanoparticles prepared via reductive amination and N-alkylation.

Code	Degree of alkylation ^a	Inhibition concentration ^b	Elemental analysis ^c			C/N ^d	Particle size (nm) ^e	Zeta potential (mV) ^f	Primary amine ^g
			%N	%C	%I				
A	1 : 1	80 $\mu\text{g/mL}$	7.03	32.74	54.00	3.61	24 $\pm$ 10	85 $\pm$ 10	3.21 $\times 10^{-4}$
I	1 : 1	80 $\mu\text{g/mL}$	7.24	35.38	43.16	3.56	40 $\pm$ 18	63 $\pm$ 27	3.37 $\times 10^{-4}$

^{a,c-f} See notes in Table 1.

^b Concentration of the tested compound needed for a complete inhibition of $>10^6$ *S. aureus* growth.

^g Amount of primary amine (mmol/g) in conjugates determined by the TNBS method following alkylation step, while prior alkylation primary amine of PEI was 5.8 mmol/g.

TABLE 3: Characterization of PEI-based nanoparticles at different degrees of N-methylation.

Code	Inhibition concentration ^a	Elemental analysis ^b			C/N ^c	Particle size (nm) ^d	Zeta potential (mV) ^e
		%N	%C	%I			
F1	NA*	6.52	29.90	51.12	3.86	36 $\pm$ 28	79 $\pm$ 25
F2	NA*	6.06	28.13	52.60	3.86	20 $\pm$ 20	79 $\pm$ 38
F3	80 $\mu\text{g/mL}$	7.22	36.39	46.62	3.86	34 $\pm$ 29	97 $\pm$ 20

Cross-linked PEI nanoparticles were reacted with octyl halide at 1 : 1 mole ratio (primary amine of PEI/alkylating agent) followed by N-methylation with various equivalents of methyl iodide, under the same conditions as described in experimental section.

* Not active at 80 $\mu\text{g/mL}$ or below.

^a Concentration of the tested compound used for a complete inhibition of $>10^6$ *S. aureus* growth. ^b Found Nitrogen, Carbon, and Iodine content of QA-PEI (elemental analysis). ^c C/N was determined by elemental analysis of the alkylated samples before methylation step whereas C/N of the nonalkylated PEI was found to be 2.26.

^d Apparent particle size of QA-PEI nanoparticles (diameter). ^e Zeta potential of QA-PEI nanoparticles.

FIGURE 2: Evaluation of cytotoxicity of QA-PEI nanoparticles (i.e., no. I) (determined by cell growth inhibition). The cytotoxicity test was performed by the $[^3\text{H}]$ -thymidine incorporation method using MBT and MAT-LyLu cells which were incubated with various concentrations of QA-PEI. Each experiment was performed in triplicate.

excess of unreacted NaHCO_3 were discarded by decantation, and the residue was precipitated in 300 mL of DDW, washed with hexane and DDW to remove traces of the unreacted

octyl halide, methyl iodide, unreacted MPEG, released p-nitrophenol, and inorganic salts, respectively, and freeze-dried. The purification step was repeated with additional amounts of hexane and DDW, and the product was freeze-dried. Average yield: 49% (mol/mol).

The degree of PEGylation was found to be about 90% ($\sim$ to starting feed) as determined by spectrophotometric measurement of the released p-nitrophenol (UV, $\lambda = 410$ nm) [23].

$^1\text{H-NMR}$ (PEG-QA-PEI nanoparticles, DMSO-d_6): 0.853 ppm (t, 3H, CH_3 , octyl protons), 1.245 ppm (m, 10H, $-\text{CH}_2-$, octyl protons), 1.7 ppm (m, 2H, $-\text{CH}_2-$, octyl protons), 3.0–3.4 ppm (m, $-\text{CH}_2-$, MPEG protons), 3.4–4.0 ppm (m, methyl of quaternary amine, 4H of PEI and 2H of the octyl chain), and 4.2 ppm (m, 2H, $\text{CH}_3(\text{OCH}_2\text{CH}_2)_n\text{OCH}_2\text{CH}_2\text{OCONH}-$).

2.2.6. Antibacterial Activity of Quaternary Ammonium Polyethyleneimine (QA-PEI) Nanoparticles. Bacterial suspensions of *S. aureus* and *E. coli* were prepared by dilution in tryptic soy broth (TSB) and lambda broth (LB), respectively. The suspension was adjusted to an optical density of 0.15 at 595 nm. Working suspensions were then prepared by further dilution with the appropriate medium in order to reach 10^6 colony-forming units (CFU/250 μL /well within 96-well flat bottom plate) of bacteria. The microtiter plate was incubated at 37°C for 24 hours. During the incubation period, bacterial growth was estimated by following the changes in optical density (OD) measured at 595 nm using a spectrophotometer (VERSAmax, Molecular Devices Corporation, CA, USA). The minimal inhibitory concentration (MIC) of the tested compound was defined as the lowest concentration that

(a) Reductive amination method (#A)

(b) N-alkylation method (#I)

SCHEME 1: Continued.

(c) Modification with methoxypoly (ethylene glycol) (#E)

SCHEME 1: Synthesis of quaternary ammonium polyethyleneimine nanoparticles.

completely inhibits bacterial growth compared to the QA-PEI-free growth control. All the experiments were performed in triplicate and the mean values were calculated.

2.2.7. In Vitro Toxicity of Quaternary Ammonium Polyethyleneimine (QA-PEI) Nanoparticles. Serial dilutions of QA-PEI were prepared in RPMI 1640 growth medium. The cytotoxicity of QA-PEI was evaluated in Murine Bladder Tumor (MBT) and MAT-LYLU cells. Growth inhibition was estimated by [^3H]-thymidine incorporation method. Cells were cultured in flat-bottom flasks at 37°C. Before each experiment, the cells were washed and removed by trypsin treatment or scraped from the flask bottom, and an appropriate volume was centrifuged, resuspended, and diluted in growth medium to the desired cell concentration (4000 cells/200 μL). The growth medium consisted of RPMI 1640 and 10% fetal calf serum (FCS). By use of an automated dispenser, 200 μL of cell suspension was added to each well of a microtiter plate. After incubation overnight, the appropriate QA-PEI concentration, in triplicate, was added to test wells. QA-PEI-free medium was used as control. [^3H]-Thymidine (0.5 μCi) in 20 μL of medium was added the next day, and the plate was harvested and read by liquid scintillation counter (LKB, Finland) after additional 24 hours. The percent growth inhibition of the cells by QA-PEI nanoparticles was calculated as $[100 - (\text{count with}$

QA-PEI/control count) $\times 100$]. The effective dose 50 (ED_{50}) is calculated as QA-PEI concentration that reduces [^3H]-thymidine incorporation by 50%, relative to the control.

3. Results and Discussion

Quaternary ammonium polyethyleneimine (QA-PEI) nanoparticles were prepared using two synthetic methods, that is, reductive amination and N-alkylation. According to the first method, QA-PEI nanoparticles were synthesized by crosslinking of polyethyleneimine with glutaraldehyde followed by reductive amination with octanal and further N-methylation with methyl iodide as illustrated in Scheme 1(a). The second method is based on crosslinking with dibromopentane followed by N-alkylation with octyl halide and further N-methylation with methyl iodide resulting in nanoparticles of spherical shape [20] (Scheme 1(b)).

The most effective QA-PEI derivative was QA-PEI alkylated with octyl halide at 1:1 mole ratio (primary amine of PEI monomer units/alkylating agent), which inhibited $>10^6$ *S. aureus* growth at a concentration of 80 $\mu\text{g/mL}$.

There was a strict correlation between the activity of the compounds and their overall octyl content. Although several structural parameters influenced the antibacterial potency of the QA-PEI nanoparticles, the most important features appear to be the degree of alkylation and degree of

TABLE 4: Characterization of QA-PEI nanoparticles with different counter anions.

Code	Counter ion	Inhibition concentration ^a	Elemental analysis of (%) I ^b	C/N ^c	Particle size (nm) ^d	Zeta potential (mV) ^e
G	Nitrate	40 µg/mL	4.65	3.64	96 ± 80	64 ± 59
H	Acetate	40 µg/mL	18.31	3.64	154 ± 100	105 ± 30
I	Iodide	80 µg/mL	43.16	3.56	40 ± 18	63 ± 27

^{a-c} See notes in Table 3.

TABLE 5: Characterization of QA-PEI nanoparticles with different degrees of PEGylation.

Code	Degree of PEGylation	Inhibition concentration ^a	Elemental analysis ^b			C/N ^c	Particle size (nm) ^d	Zeta potential (mV) ^e
			%N	%C	%I			
I	0 %	80 µg/mL	7.24	35.38	43.16	3.56	40 ± 18	63 ± 27
J	0.5 %	40 µg/mL	7.03	32.74	54.00	3.79	24 ± 15	85 ± 10
E	5 %	40 µg/mL	8.85	30.94	54.25	3.84	56 ± 50	73 ± 31

^{a-c} See notes in Table 3.

methylation, while other parameters played a less significant role.

3.1. Determination of Minimal Inhibitory Concentration of QA-PEI Nanoparticles. The goal of this study was to evaluate antibacterial properties of QA-PEI against Gram-negative and Gram-positive bacteria including *E. coli* and *S. aureus*, respectively. Bacteria were exposed to various concentrations (40–320 µg/mL) of the QA-PEI for 24 hours at 37°C. Figure 1 summarizes the change in the bacterial growth after applying QA-PEI nanoparticles. A culture containing bacteria without nanoparticles was used as a negative control. Antibacterial analysis indicated that QA-PEI completely inhibited *S. aureus* and *E. coli* growth at 80 µg/mL (Figure 1(a)) and at 320 µg/mL (Figure 1(b)), respectively.

3.2. Effect of the Alkylation Degree on Antibacterial Activity. QA-PEI nanoparticles were prepared via reductive amination method followed by further N-methylation, which raises their positive charge by converting PEI's primary, secondary, and tertiary amino groups into cationic quaternary amino groups. Cross-linked PEI nanoparticles were allowed to react under similar conditions with increasing amounts of octanal ranging from 1:0.25 to 1:1 mole ratios (primary amine of PEI monomer units/octanal). The resulting imine conjugates were reduced to amine bonds by sodium cyanoborohydride. Table 1 summarizes the chemical data obtained with QA-PEI substituted with various degrees of octanal. Increased amount of octanal resulted in more hydrophobized compound which was evidenced by elevated carbon/nitrogen (C/N) ratio. For instance, QA-PEI nanoparticles alkylated at 1:1 mole ratio (primary amine of PEI monomer units/octanal), no. A, resulted in C/N of 3.61 in comparison with C/N of 2.45 obtained with QA-PEI alkylated at 1:0.25 mole ratio, no. D. According to the antibacterial assay, the alkylation degree was in correlation with the bactericidal activity of the QA-PEI (Table 1). In particular, QA-PEI nanoparticles alkylated at 1:1 mole ratio (primary amine of PEI monomer units/octanal) (no. A), and even lower, for

example, 1:0.75 mole ratio (no. B) were most effective in *S. aureus* growth inhibition, while a compound with very low degree of alkylation (1:0.25 mole ratio, no. D) depicted reduced antibacterial activity. Thus, the degree of alkylation had a decisive influence on the antibacterial properties of the nanoparticles as evidenced by reduced antibacterial activity of the QA-PEI modified with low degree of alkylation. We surmise that this inactivity of the less substituted QA-PEI is probably attributed to insufficient hydrophobic nature of the nanoparticles. Increased hydrophobicity, achieved due to increased alkylation degree, might enhance QA-PEI nanoparticles propensity to penetrate the hydrophobic bacterial membrane which is expressed in enhanced antibacterial properties. Indeed, Lin et al. reported on antibacterial activity of the alkylated PEI coatings for glass slide, suggesting that bactericidal surfaces with immobilized long polymeric chains have to be hydrophobic to be highly effective in bacterial removal [16]. On the basis of these results, 1:1 mole ratio (primary amine of PEI monomer units/alkylating agent) was used as optimal mole ratio for QA-PEI synthesis.

3.3. Comparison of QA-PEI Antibacterial Activity Prepared via Different Synthetic Methods. The purpose of this issue is to evaluate the relationship between the biological activity and the chemical characterization of QA-PEI nanoparticles prepared via two different synthetic methods, that is, reductive amination and N-alkylation. According to the chemical analysis (Table 2), QA-PEI nanoparticles prepared via two methods exhibit similar nitrogen (%N), carbon (%C), and iodine (%I) content, apparent particle size (ALV, nm), zeta potential, and primary amine content determined following alkylation step. Although reductive amination and N-alkylation methods resulted in QA-PEI nanoparticles bearing different particle sizes and zeta potential, no distinct difference in antibacterial activity was observed applying both types of QA-PEI nanoparticles. Based on these results the N-alkylation method was selected for further studies, due to its simplicity in synthesis, work up, and use of nonexpensive reagents.

TABLE 6: Characterization of QA-PEI nanoparticles.

Code	Elemental analysis ^a			C/N ^b	Particle size (nm) ^c	Zeta potential (mV) ^d
	%N	%C	%I			
I	7.24	35.38	43.16	3.56	40 ± 18	63 ± 27
K	7.31	36.45	43.00	3.69	42 ± 20	69 ± 16
L	6.87	35.31	43.36	3.64	40 ± 19	74 ± 35
M	7.22	36.39	46.62	3.86	34 ± 30	97 ± 20
N	7.39	35.16	45.72	3.60	16 ± 12	51 ± 6
P	6.60	34.31	49.98	3.65	12 ± 10	82 ± 40
O	7.07	34.43	48.71	3.71	16 ± 12	70 ± 43
Q	6.87	34.63	47.87	3.68	16 ± 5	79 ± 15

^{a-c} See notes in Table 3.

3.4. N-Methylation Effect on Antibacterial Activity. The purpose of this part of the study was to evaluate the N-methylation effect and its influence on the antibacterial activity of QA-PEI nanoparticles. Lin et al. have already shown that N-methylation of N-alkylated immobilized PEI enhanced its overall antibacterial activity [16]. Therefore, we tested the antibacterial properties of QA-PEI as a function of added equivalents of methyl iodide. QA-PEI nanoparticles were similarly prepared starting from cross-linked PEI nanoparticles and octyl halide added at 1 : 1 mole ratio (primary amine of PEI monomer units/octyl halide). Different equivalents of methyl iodide ranging from 1 : 1 to 1 : 3 mole ratios (monomer units of PEI/methyl iodide) were used during the methylation step. Table 3 summarizes the chemical characterization of QA-PEI nanoparticles, including nitrogen (%N), carbon (%C), and iodine (%I) content determined prior to the neutralization step, apparent particle size (ALV, nm), zeta potential and structure (FT-IR and ¹H-NMR are shown in Experimental Section). *S. aureus* was incubated with various concentrations (0–80 µg/mL) of QA-PEI for 24 hours. As seen in the Table 3, when 1 or 2 equivalents of methyl iodide were used (e.g., no. F1 and no. F2, resp.), no bacterial inhibition was observed with QA-PEI up to 80 µg/mL suggesting an insufficient N-methylation degree. However, 3 equivalents of methyl iodide yielded highly effective QA-PEI nanoparticles which abolished *S. aureus* growth at 80 µg/mL (e.g., no. F3). Thus, methylation following initial alkylation elevates bactericidal efficiency of the QA-PEI nanoparticles.

3.5. Antibacterial Activity of QA-PEI Nanoparticles as a Function of Counter Ion. To examine the effect of counter ion on bacterial growth, QA-PEI nanoparticles with various counter ions were applied. For this reason iodide counter anion of QA-PEI nanoparticles was exchanged with nitrate ion using precipitation method with AgNO₃ and with acetate using 1 N acetic acid solution. QA-PEI nanoparticles were characterized prior and following exchange reaction for their iodine (%I) content, apparent particle size (ALV, nm), and zeta potential as shown in Table 4. *S. aureus* was incubated with various concentrations (0–80 µg/mL) of QA-PEI for 24 hours. Although iodide counter ion was partly substituted with nitrate and acetate anions, antibacterial analysis

revealed that QA-PEI iodide, nitrate, and acetate form nanoparticles demonstrated similar efficiency in bacterial growth inhibition, indicating a minor effect on antibacterial activity. Previous studies have shown similar results, for example, Chen et al. revealed that counter ions including chloride and bromide showed no effect on the antibacterial activity of the quaternary ammonium biocides [24]. In addition, Panarin et al. reported on analogous findings obtained with various counter ions for quaternary ammonium groups of homopolymers based on vinylamine and methyl methacrylate [25]. However, Kanazawa et al. found that antibacterial properties of the poly(tributyl 4-vinyl benzyl phosphonium halide) were in the order of chloride > boron tetrafluoride > perchlorate > hexafluorophosphate indicating a counter ion dependence. Counter ion effect is widely an argued point; there is still no clear answer why counter ion should affect antibacterial properties except where it alters the solubility of the biocides [20, 26–28].

3.6. PEGylation Effect. The purpose of this part of the study was to evaluate the wettability effect of methoxy-poly(ethylene glycol) (MPEG) and its influence on antibacterial activity of QA-PEI. Previous studies have shown that hydrophilic coating may increase the surface energy of the material due to enhanced hydrophilicity following PEGylation and consequently increase bacterial interaction with the bactericide surface. For instance, Sellenet et al. have reported on quaternized polyvinyl pyridine incorporated with hydroxyethyl methacrylate and poly(ethylene glycol) methyl ether methacrylate that elevated antibacterial activity 20 times greater than the pure quaternized poly(vinyl pyridine) [29]. For this reason QA-PEI was modified with increasing amounts of MPEG (Scheme 1(c)). MPEG (M_w = 2 kDa) was activated with p-nitrophenyl chloroformate to obtain p-nitrophenyl carbonate derivative. Substitution of QA-PEI was carried out at reflux conditions in ethanol as described in Experimental Section. The amount of MPEG was fixed at 0.5% and 5% (mol/mol) to primary amines of polyethyleneimine. QA-PEI nanoparticles were characterized for their nitrogen (%N), carbon (%C), and iodine (%I) content, apparent particle size (ALV, nm), and zeta potential as shown in Table 5. *S. aureus* was incubated with various concentrations (80–320 µg/mL) of pegylated QA-PEI for

24 hours. No significant difference was observed in the antibacterial activity of MPEG derivatives (e.g., no. J and no. E) in comparison with unPEGylated QA-PEI (e.g., no. I). These findings indicate that MPEG modification did not significantly improve the bactericidal efficiency of the QA-PEI nanoparticles, suggesting a moderate effect on interaction between bacterial membrane and pegylated QA-PEI nanoparticles.

3.7. Cytotoxicity Evaluation of QA-PEI Nanoparticles. An important issue for the usefulness of antibacterial QA-PEI nanoparticles is whether these nanoparticles are harmful to mammalian cells. Lethal activity against bacteria with minimal effect on cell viability is required for clinical applications such as medical devices with antimicrobial properties. [³H]-thymidine incorporation is a well-known technique for testing cell proliferation. QA-PEI nanoparticles (i.e., no. I) which toxicity might be attributed to strong positive charge of the nanoparticles, were incubated with MAT-LyLy and MBT cell lines for 48 hours. The results presented in Figure 2 lead to several conclusions. First, no effect on MAT-LyLy cultures was observed using up to 37 $\mu\text{g/mL}$ of QA-PEI. The calculated ED_{50} was 75 $\mu\text{g/mL}$. Second, exposure of MBT cultures to QA-PEI resulted in ED_{50} of 30 $\mu\text{g/mL}$. Thymidine incorporation was completely abolished in both cultures after exposure to 111 $\mu\text{g/mL}$ of QA-PEI nanoparticles. The inhibitory effect of QA-PEI on the proliferation of the MAT-LYLU cells was in a much lesser extent than on MBT cell line.

3.8. Reproducibility. In a typical experiment, 8 QA-PEI samples were prepared in different batches starting from cross-linked PEI nanoparticles and octyl halide added at 1 : 1 mole ratio (primary amine of PEI monomer units/octyl halide) followed by methylation step. The purpose of this experiment was to evaluate the reproducibility of the synthesis and antibacterial activities of the various batches. Table 6 summarizes chemical analysis of these compounds.

According to the elemental analysis, averages of %N, %C, and %I in these batches were 7.07 ± 0.47 , 35.25 ± 1.2 , and 46.05 ± 3.9 , respectively. The apparent particle size and zeta potential of these nanoparticles were $25 \text{ nm} \pm 15 \text{ nm}$ and $72.98 \text{ mV} \pm 23.9 \text{ mV}$, respectively. The antibacterial effect of these QA-PEI nanoparticles was evaluated against *S. aureus*, and the average inhibition concentration was 80 $\mu\text{g/mL}$ indicating good reproducibility in antibacterial activity.

4. Conclusion

A series of cross-linked quaternary ammonium polyethyleneimine- (QA-PEI-) based nanoparticles were prepared using two synthetic methods including reductive amination between cross-linked PEI and octanal and N-alkylation with octyl halide followed by N-methylation with methyl iodide. Although the majority of the tested compounds were found to be effective in inhibition of bacterial growth, QA-PEI nanoparticles alkylated at 1 : 1 mole ratio (primary amine of PEI monomer units/alkylating agent) showed the

highest antibacterial activity. This work emphasizes that the structure of the polycationic nanoparticles has a significant role in the antibacterial activity. The presented results suggest that QA-PEI nanoparticles might be used as antibacterial additives for biomedical devices.

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