Synthesis and antibacterial properties of quaternary ammonium derivative of polyethylenimine

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This publication is dedicated to the memory of the scientist Prof. Andrzej Duda

Abstract: The quaternary derivative of branched polyethylenimine (bPEI-met) was synthesized and its antibacterial activity against Gram-positive bacterium (*Staphylococcus aureus*) and Gram-negative bacterium (*Escherichia coli*) was studied. The values of minimum inhibitory concentration (*MIC*) and minimum bactericidal concentration (*MBC*) of bPEI-met against mentioned bacteria were determined. These indicated that bPEI-met could be considered as an effective alternative to antibiotics for the treatment of selected bacterial strains. The results obtained can be useful in search for drugs allowing treatment of antibiotic resistant bacterial strains.

Keywords: polyethylenimine, polycation, liposomes, minimum inhibitory concentration, minimum bactericidal concentration.

Synteza i właściwości antybakteryjne czwartorzędowej amoniowej pochodnej polietylenoiminy

Streszczenie: Zsyntetyzowano czwartorzędową amoniową pochodną rozgałęzionej polietylenoiminy (bPEI-met) i badano jej działanie antybakteryjne przeciwko bakteriom Gram-dodatnim na przykładzie gronkowca złocistego (*Staphylococcus aureus*) i Gram-ujemnym na przykładzie pałeczki okrężnicy (*Escherichia coli*). Wyznaczono wartości minimalnego stężenia hamującego (*MIC*) i minimalnego stężenia bakteriobójczego (*MBC*) bPEI-met w stosunku do obu wymienionych szczepów bakterii. Wyniki tych badań sugerują, że bPEI-met może być rozważana jako alternatywa dla antybiotyków w zwalczaniu wybranych szczepów bakterii, szczególnie tych, które charakteryzują się antybiotykoopornością.

Słowa kluczowe: polietylenoimina, polikation, liposomy, minimalne stężenie hamujące, minimalne stężenie bakteriobójcze.

Polyethylenimines (PEI) are weak polycations that have found various applications in industry and medicinal chemistry. PEI exist in linear (lPEI) and branched (bPEI) architectures [1]. The linear chain has only secondary amine nitrogen atoms ($[-CH_2CH_2NH-]_y$), whereas the branched chain consists of primary, secondary, and tertiary amine nitrogen atoms ($[-CH_2CH_2N<]_x[-CH_2CH_2NH-]_y[-CH_2CH_2NH_2]_z$). The conventional applications of PEI include the use as a reagent for pulp dehydration in the paper industry and in fiber board production, as electrolyte extractors for zinc and cadmium plating, and as flocculants in coal production [2]. PEI has also been used in water purification for the removal from water of heavy metal ions by their complexation [3]. In addition, PEI is among the most versatile and frequently used nonviral vectors for DNA complexation and transfection into several cell lines and tissues [4]. These polycations are characterized by an excellent gene complexing ability (formation of polyplexes) and exceptional transfectant properties.

Due to the application of PEI in biotechnology the effect of this polymer and its derivatives on phospholipid bilayers or cell membranes was studied using various experimental techniques and computer simulations. Sikor *et al.* [5] studied the impact of ionic strength on the stability of zwitterionic phosphatidylcholine (PC) vesicles in the presence of bPEI. They have shown that the introduction of polymer in excess to the lipid content at high ionic strength resulted in the stabilization of liposomes and bPEI was able to penetrate the bilayer. Fur-

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ther research on the stability of various PEI-decorated liposomes was carried out by Sabin *et al.* [6]. The results demonstrated a remarkable dependence of the stability of zwitterionic and anionic liposomes on the polymer concentration, pH, temperature, and on the initial size of the liposomes. Molecular dynamics (MD) simulations provided insight into PEI-bilayer interactions at the molecular level [7, 8].

Herein, we present the results of our study on the synthesis and biocidal properties of quaternary ammonium polyethylenimine. The studies are in line with the search for novel compounds which could be used to combat pathogens, especially the growing number of antibiotic resistant bacteria. We investigated its interaction with lipid membranes of both the zwitterionic and anionic nature that were used as models for bacterial membrane. Next, the antibacterial activity of bPEI-met was evaluated against *Staphylococcus aureus* (Gram-positive bacterium), and *Escherichia coli* (Gram-negative bacterium).

EXPERIMENTAL PART

Materials

Branched polyethylenimine (bPEI) with an average molecular weight of ~10 000 was purchased from Sigma-Aldrich. Lipids: 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (POPC, ≥99 %) and 1,2-dioleoyl-*sn*-glycero-3-phosphoric acid monosodium salt (DOPA, ≥98 %) were obtained from Sigma. *N*-methyl-2-pyrrolidone (NMP, spectrophotometric grade), iodomethane (≥99 %), DMSO-d_6 (99.9 atom % D), and D_2O (99.9 atom % D) were delivered by Aldrich and used as received. NaI (≥99 %) was received from POCh (Gliwice, Poland). Benzoylated dialysis tubing (2 000 molecular weight cutoff) was purchased from Sigma-Aldrich. Millipore-quality water was used for all solution preparations. Tryptic soy broth (TSB), Luria-Bertani broth (LB), and tryptic soy agar (TSA) were supplied by Fluka.

Synthesis of quaternary ammonium derivative of bPEI (bPEI-met)

bPEI (0.901 g, 20.92 mmol of the amino groups) was dissolved in water (6 cm^3) and the solution was mixed with 25 cm^3 of NMP. The mixture was stirred for 1 h at room temperature. Next, 15 % NaOH solution (9.7 cm^3), iodomethane (13.3 cm^3, 10-fold excess to the amino groups), and NaI (1.6 g) were added. Reaction was carried out with stirring for 3 days at 50 °C. To exchange the iodide counterions for chloride ones, the reaction mixture was placed in dialyzing tube and dialyzed in the following sequence (2 days in each environment): against deionized water, 0.1 M KCl solution, and again deionized water. Finally, the product was recovered by lyophilization as fine white hygroscopic crystals in amount of 0.914 g. The yield of the synthesis was 60.1 %.

Preparation of liposomes

Small unilamellar phospholipid vesicles (SUV) were prepared by an extrusion technique as described previously [9]. POPC was weighed into a glass flask and dissolved in chloroform (0.2 cm^3). To obtain the anionic SUV, a chloroform solution of DOPA was added to reach a 9:1 molar ratio of POPC to DOPA. The solvent was evaporated under a gentle stream of nitrogen to complete dryness. A 1 mM NaCl solution of pH adjusted to 7.4 was added until the desired lipid concentration was attained (usually 0.5 or 1.0 mg/cm^3), and the sample was vortex mixed for 5 min. The resulting multilamellar vesicle dispersion was subjected to five freeze-thaw cycles from liquid nitrogen temperature to 60 °C and then extruded ten times through the membrane filters with 100 nm pores using a gas-pressurized extruder.

Characterization of bPEI-met

^{1}H NMR (300 MHz) measurements were performed on a Bruker Avance II 300 spectrometer. The NMR spectra were taken at 80 °C in the mixture of D_2O/DMSO-d_6 (1:8, v/v) using DMSO-d_6 residual peaks as internal standards. Elemental analysis was performed on a EuroEA 3000 Elemental Analyzer. A Malvern Nano ZS light-scattering apparatus (Malvern Instrument, Worcestershire, UK) was used for dynamic light scattering (DLS) and zeta potential measurements. The samples were illuminated with a 633 nm laser, and the intensities of scattered light at an angle of 173° were measured using an avalanche photodiode. The *z*-average diameter (d_z), dispersity index ($Đ$), and distribution profiles of the samples were automatically calculated using the software provided by Malvern. The zeta potential was measured using the technique of laser Doppler velocimetry. Samples of polymers (1 mg/cm^3) in water were filtered through a 0.22-μm pore size filter.

Test of antibacterial properties

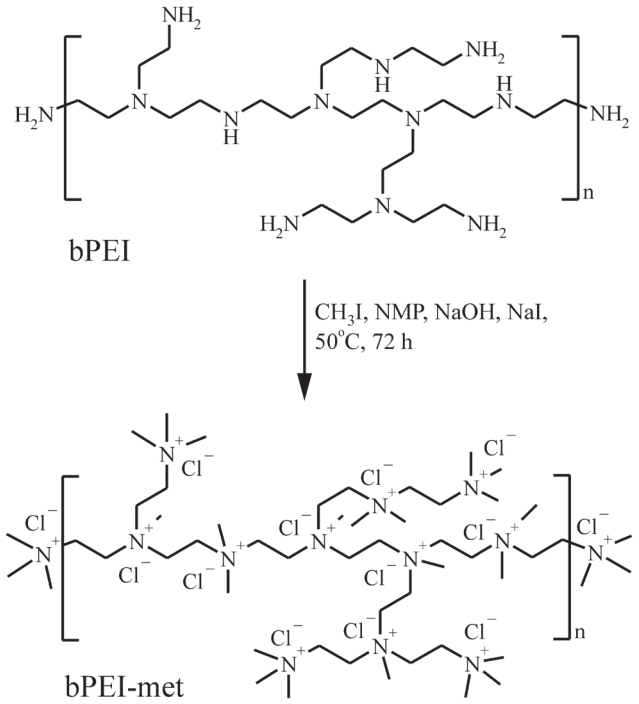
The minimum inhibitory concentration (*MIC*) and minimum bactericidal concentration (*MBC*) were determined using a broth microdilution method [10]. Overnight cultures (37 °C, shaking) of *Staphylococcus aureus* ATCC 25923 in TSB and *Escherichia coli* ATCC 25922 in LB were diluted 10-fold and incubated (37 °C, shaking) until they reached the exponential growth phase. A series of dilutions of the polymer solution (from 2.5 to 0.00976 mg/cm^3) in TSB or LB was prepared in a 96-well plate (0.2 cm^3 per well). Wells with no polymer added were used as positive growth controls. The diluted bacterial suspension was added to each well to give the final concentration of $1 \cdot 10^6$ colony-forming units per cm^3 (CFU/cm^3), confirmed by viable counts. Wells without bacteria added were used as negative growth controls. The plate was incubated for 20 h at 37 °C and the growth of the bacteria was assessed turbidimetrically (optical density at 600 nm). *MIC* was determined as

the lowest polymer concentration without visible growth. From all wells not showing visible growth, 0.01 cm^3 of the broth was plated on TSA and the number of colonies was counted following overnight incubation at 37 °C. *MBC* was determined as the lowest concentration reducing the initial inoculum by ≥99.9 %. The test was performed at least three times for each strain and the modal value was taken.

RESULTS AND DISCUSSION

Synthesis of bPEI-met

bPEI was transformed into a strong polycation by direct alkylation of amino groups. The synthesis and the chemical structure of bPEI-met, which is the quaternized derivatives of bPEI, are presented in Scheme A.



Scheme A

^{1}H NMR spectra of the unmodified bPEI and its quaternized derivative (bPEI-met) are shown in Fig. 1. One can observe that unmodified PEI displayed only the $–CH_2–$ proton signals at 2.4–2.6 ppm corresponding to different ethylene groups in the polymer backbone ($[–CH_2CH_2N<]_x[–CH_2CH_2NH–]_y[–CH_2CH_2NH_2]_z$). The obtained spectrum is similar to that of bPEI dissolved in D_2O reported in the literature [11]. The spectra of the product confirmed the occurrence of methylation of the amino groups. The appearance of the new peaks at chemical shift (δ) in the range of 3.1–3.4 ppm in the ^{1}H NMR spectrum of bPEI-met was attributed to the quaternized ammonium groups. At δ = 2.06 ppm one can observe another peak that can be ascribed to dimethylated amino groups, which indicates that the methylation of the bPEI was not complete. The degree of substitution by the methyl group was determined from the ^{1}H NMR spectrum. It was roughly estimated that bPEI-met contains about 88 % of the quaternary ammonium groups. Elemental analysis of bPEI-met gave results: % C 41.58, % H 9.89, and % N 12.98. The more exact calculations carried out based on results of the elemental analysis for bPEI-met revealed the presence of 86.9 % of quaternary ammonium groups in the structure of bPEI-met.

The quaternization of bPEI by the reaction with iodomethane was previously reported by Thomas and Klibanov [12]. bPEI was reacted with a 12-fold excess of iodomethane in methanol or ethanol. In spite of a higher excess of iodomethane, the level of quaternization was found to be the same as in our experiment (about 87 %). NMP was previously used as an effective reaction medium for methylation of chitosan and poly(allylamine) (PAH) with iodomethane [13, 14]. Therefore, we believe that value *ca.* 87 % is the maximum level of quaternization, which can be achieved for branched PEI.

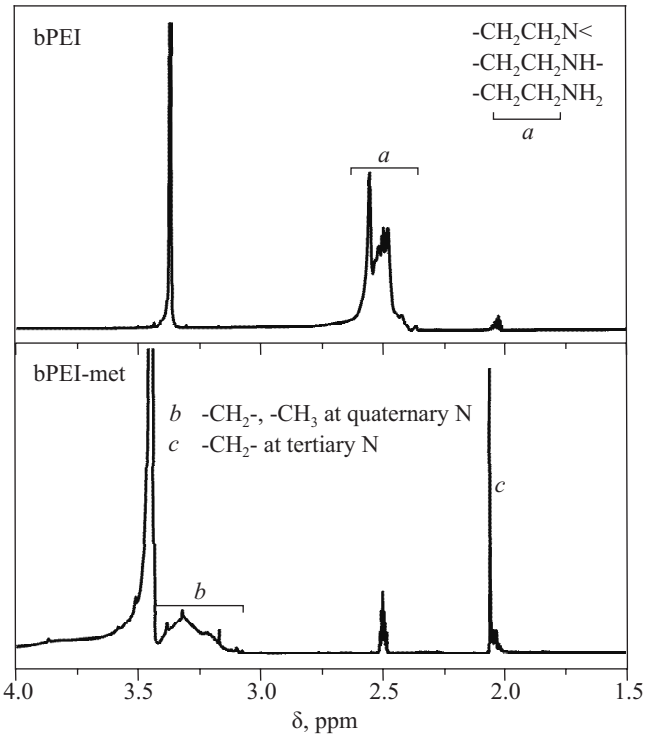


Fig. 1. ^{1}H NMR spectra of bPEI and bPEI-met in the D_2O/DMSO-d_6 mixture (1:8, v/v)

Interaction of bPEI-met with liposomes

Polycations exhibit antibacterial properties due to the interaction and disruption of the bacterial cell membranes [15]. Zwitterionic membranes are often used as models of mammalian cell membranes, while the negatively charged membranes are used as models for the bacterial cell membrane [16]. To evaluate the ability of bPEI-met to interact with the cell membrane we have carried out studies in which liposomes were used as models of cellular membranes. Small unilamellar vesicles/liposomes (SUV) were prepared from zwitterionic POPC or from a mix-

T a b l e 1. Values of the mean hydrodynamic diameter (d_z) and dispersity ($Đ$) of POPC and POPC/DOPA SUV dispersed in a 1 mM NaCl solution of pH = 7.4 and treated with bPEI-met*)

System	bPEI-met concentration μg/cm^3	bPEI-met content wt %	d_z (n = 5) nm	$Đ$ (n = 5)
POPC SUV	0	0	111.3 ± 1.0	0.04 ± 0.02
POPC SUV with bPEI-met	10	1.0	>10^3	0.31 ± 0.15
	20	2.0	>10^4	0.85 ± 0.18
	30	3.1	>10^4	1.00 ± 0.00
	50	5.3	>10^4	1.00 ± 0.00
	100	11.1	>10^4	1.00 ± 0.00
	150	17.6	>10^4	1.00 ± 0.00
POPC/DOPA SUV	0	0	110.5 ± 0.9	0.05 ± 0.02
POPC/DOPA SUV with bPEI-met	10	1.0	193.5 ± 3.5	0.24 ± 0.01
	20	2.0	>10^5	1.00 ± 0.00
	30	3.1	>10^3	1.00 ± 0.00
	40	4.2	168.8 ± 2.9	0.25 ± 0.03
	50	5.3	139.6 ± 1.4	0.15 ± 0.01
	60	6.4	132.5 ± 0.2	0.12 ± 0.02
	100	11.1	143.5 ± 1.1	0.18 ± 0.01

*) Values are given as the mean ± standard deviation.

ture of POPC and anionic DOPA (9:1). A series of samples containing SUV and various weight fractions of polycations with regard to the lipid content (0.5 mg/cm^3) were prepared. The measurements of the vesicle size using dynamic light scattering (DLS) were performed to assess the possibility of vesicle aggregation/stabilization upon the polymer addition. The results of the measurements of the hydrodynamic diameter (d_z) and the dispersity index ($Đ$) of vesicles exposed to various amounts of the polymer are collected in Table 1.

The size of the extruded SUV prepared from pure POPC or from the mixture POPC and DOPA was around 110––112 nm, and $Đ$ value was less than 0.06 indicating that the population of liposomes had a narrow size distribution. The zeta potential of POPC liposomes was about -9 mV and decreased to -33 mV after incorporation of DOPA (an anionic lipid). As shown in Table 1, the effect of bPEI-met on liposomes was strongly dependent on the type of liposomes. Values of d_z and $Đ$ of the POPC liposomes substantially increased after the introduction of even the smallest mass fraction of bPEI-met. Thus, the presence of the polymer caused a strong aggregation of the zwitterionic vesicles in the whole range of polymer concentrations studied. In the case of the anionic liposomes, the addition of bPEI-met in amount lower then 3.2 wt % resulted in a significant increase in d_z of the liposomes and in a higher dispersity $Đ$ value of the sample, indicating vesicle aggregation. Further increasing the polycation concentration caused a gradual reduction of d_z and $Đ$. After the addition of more than 5.3 wt %, d_z reached a constant value of ~140 nm and a $Đ$ of ~0.15. The size distributions were stable and invariable for several days.

The zeta potential of the liposomes was determined to confirm the adsorption of the polymers on the liposome surface. The changes in zeta potential with the increasing content of bPEI-met in the sample are shown in Fig. 2. In the case of the POPC liposomes, the introduction of bPEI-met caused only small increase in the zeta potential. For example, the addition of 12 wt % of bPEI-met compared to the lipid content changed the zeta potential to the slightly positive value. On the contrary, the zeta potential of the anionic SUV became positive after the treatment with the polymer mass fraction higher than 2 wt %. With increasing bPEI-met content, zeta potential increased and reached constant values of 28–32 mV at a content of about 6 wt %. That observation confirmed the adsorption of the polymers on the bilayer surface. The positively charged groups of bPEI-met were exposed to the bulk solution, thus increasing the surface potential of the liposomes.

Dispersions of microparticles characterized by zeta potential values above +30 mV or below -30 mV are generally considered to be well stabilized by the strong electrostatic repulsion forces operating between particles and preventing their aggregation [9]. Thus, the bPEI-met at content of about 6 wt % is sufficient to obtain the stable isolated polyelectrolyte-coated liposomes, as confirmed by the DLS measurements. A similar aggregation-dissociation process was previously observed using direct transmission cryo--electron microscopy (cryo-TEM) for the same liposomes composed of the POPC/DOPA mixture and incubated with various strong polycations at various molar ratios [17].

Antibacterial activities of bPEI-met in solution

Biocidal properties (ability to destroy microorganism – *MIC*) of bPEI-met were studied using a dilution method against Gram-negative *Escherichia coli* ATCC 25922 and

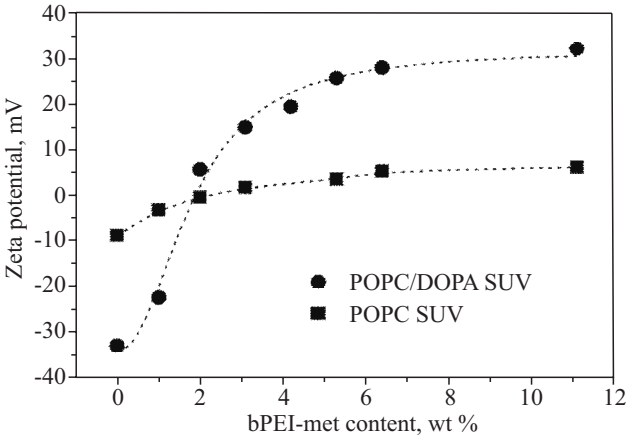


Fig. 2. The zeta potential of POPC and POPC/DOPA liposomes treated with various content of bPEI-met

Gram-positive *Staphylococcus aureus* ATCC 25923 strains. The results of the *in vitro* antibacterial activity of bPEI--met are given in Table 2. The values of *MIC* indicate that bPEI-met can inhibit the growth of *Staphylococcus aureus*. The antimicrobial activity of the quaternized derivative of bPEI can be compared to the unmodified bPEI. The *MIC* value of bPEI with the molecular weight of 60 000 against *Staphylococcus aureus* was previously determined to be 0.195 mg/cm^3 [18]. This indicates that the modification of PEI by methylation with iodomethane improved considerably the antibacterial activity of the polymer against *Staphylococcus aureus*. The value of *MIC* for bPEI--met was 2.5 times lower compared to that characteristic for the parent polymer.

T a b l e 2. Bacteriostatic (ability to inhibit the growth or reproduction of bacteria – *MIC*) and bactericidal (ability to destroy bacteria – *MBC*) activity of bPEI-met against *Staphylococcus aureus* and *Escherichia coli*

	MIC, mg/cm^3	*MBC*, mg/cm^3
Escherichia coli in LB (Gram -)	>2.5	>2.5
Staphylococcus aureus in TSB (Gram +)	0.078	0.078

Polymers with the quaternized ammonium groups have been previously shown as biocides against various microorganisms [13, 19–24]. Holappa *et al.* [19] studied the derivatives of chitosan. They concluded that the attachment of the quaternary ammonium moiety to a polymer chain is not sufficient prerequisite to induce antimicrobial action. The key issue is the optimal position of the positive charges in relation to the polymer backbone. Recently, we have studied the quaternary ammonium derivatives of PAH, poly(allyltrimethylammonium chloride) (PATA) and poly[(3-allylamino-2-hydroxypropyl)trimethylammonium chloride] (PAHT) [13]. Both polycations had the $-N^+(CH_3)_3$ groups in their chemical structure, but their distances from the polymer backbone were different. In PATA these groups were closely linked to the polymer chain by a methylene linker, whereas in PAHT they were separated from the chain by a methylamino-2-propanol spacer. We observed that this difference in the polymer structures significantly influenced the antimicrobial activity against *Staphylococcus aureus*. The *MIC* values were 0.625 and 2.5 mg/cm^3 for PATA and PAHT, respectively. In the case of bPEI-met, the quaternary ammonium groups are incorporated directly into the polymer backbone and its *MIC* value for *Staphylococcus aureus* was 8 times lower compared to PATA. That supports our earlier observation that the localization of the cationic groups with respect to the polymer backbone is essential for the antibacterial activity of the polymers.

Bactericidal activity (*MBC*) was also determined. bPEI-met is less bactericidal against Gram-negative bacteria (*Escherichia coli*) than against Gram-positive ones (*Staphylococcus aureus*). Similar result has been previously observed for PATA and PAHT [13]. This difference in antibacterial activity is most likely due to a different construction of the cell wall of the *Escherichia coli* and *Staphylococcus aureus* bacteria. Gram-negative bacteria have an additional outer membrane, which is stabilized by the lipopolysaccharides layer. Such multilayered structure of the cell envelope seems to be more difficult for penetration by bactericidal agents.

CONCLUSIONS

This study shows that the strong polycation (bPEI-met) can be obtained by exhaustive methylation of bPEI with iodomethane in NMP. The maximum level of quaternization that can be achieved for that branched polymer is about 87 %. bPEI-met can interact strongly with the negatively charged lipid membranes, the model of bacterial cell membranes, changing their properties. bPEI-met displays the strong bacteriostatic effect against Gram--positive bacteria, while the antibacterial activity against Gram-negative bacteria was lower.

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