

VILNIUS UNIVERSITY
CENTER FOR PHYSICAL SCIENCES AND TECHNOLOGY

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**POLYALKYLENEIMINES, THEIR DERIVATIVES AND
POLYAMIDOAMINES: SYNTHESIS, CHARACTERIZATION AND
APPLICATION FOR GENE DELIVERY**

Summary of doctoral dissertation

Physical Sciences, Chemistry (03P)

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The scientific work was carried out in 2011-2015 at Vilnius University, Faculty of Chemistry, Department of Polymer Chemistry.

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VILNIAUS UNIVERSITETAS
FIZINIŲ IR TECHNOLOGIJOS MOKSLŲ CENTRAS

ALMA BOČKUVIENĖ

**POLIALKILENIMINŲ, JŲ DARINIŲ IR POLIAMIDOAMINŲ
SINTEZĖ, TYRIMAS IR PRITAIKYMAS GENŲ PERNAŠAI**

Daktaro disertacijos santrauka

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1. INTRODUCTION

Relevance of the work. Gene therapy, as a potential method in medical diagnostics and treatment of genetic disorders such as immunodeficiency, cystic fibrosis, neurodegenerating diseases or cancer, has gained essential attention over the past decade. For gene therapy (genetic engineering) DNA or siRNA are generally inserted into cells using variety of methods and carriers. Among them, polymeric vectors were considered as the most suitable and promising due to their low immunogenicity and toxicity, possibility of repeat administration, and low production cost. Consequently, polymeric vectors for transfection of DNA and/or siRNA to cells belong to hot topics in polymer science. The ideal polymeric vector must have proper molecular weight, structure and chemical composition for achieving long circulation time, low immunogenicity, good biocompatibility, selective targeting and efficient penetration of physiological barriers.

Poly(2-hydroxyalkylene imines) possessing both imine and hydroxyl groups in their repeating units have been synthesized recently and shown to be efficient gene transfection reagents. More detailed examination of poly(2-hydroxypropylene imine) (PHPI) revealed that this polymer had no significant effect on tested cell viability and was very efficient gene delivery reagent, comparable or even better than commercially available transfection reagents Exgen500, Lipofectamine, and others. Unfortunately, at higher concentrations PHPI showed substantial cytotoxicity which seriously restricted *in vivo* application of PHPI. Presumably, toxicity of PHPI is associated with low biocompatibility of this polymer and could be substantially reduced, if polymer would be more hydrophilic or biodegradable. Hydrophilicity of PHPI could be increased by introducing highly hydrophilic segments (like poly(ethylene glycol)) into the structure of PHPI. On the other hand, biodegradable properties may be provided to PHPI by introducing easily reducible (cleavable) linkages into the main chain of PHPI.

Polyamidoamines is a very interesting group of biodegradable polymers. They can be used for delivery of a great variety of therapeutic molecules including peptides, plasmid DNA and siRNA. However, until now no attempts were made to synthesize polyamidoamine-type delivery vehicles containing hydroxyl and ethereal groups in the main chain. We expect that such polar groups could assist improving biocompatibility of

polyamidoamines and providing additional complexation ability to the polymer in respect to the deliverable material.

In this work, poly(2-hydroxypropylene imine) derivatives containing cystamine (CT) or metoxy-poly(ethylene glycol) (MPEG) segments, and polyamidoamines containing hydroxyl or ethereal groups in the main chain have been synthesized and investigated. They are expected to be non-cytotoxic and efficient reagents for *in vitro* and *in vivo* transfection of DNA and(or) siRNA.

The aim of the present work is to synthesize new cationic polymers polyalkyleneimines, their derivatives and polyamidoamines that should be non-toxic to cells, biodegradable and suitable for DNA or siRNA delivery.

The objectives of the research are the following:

- ✓ To synthesize poly(ethyleneiminehexamethyleneimines), poly(2-hydroxypropyleneimines) and derivatives of poly(2-hydroxypropyleneimine) containing disulfide linkages or metoxy-poly(ethylene glycol) chains.
- ✓ To synthesize polyamidoamines containing hydroxyl and ethereal groups.
- ✓ To study the effect of the reaction conditions of polycondensation and polyaddition on the yield, molecular characteristics and cationicity of the newly synthesized polyalkyleneimines.
- ✓ To evaluate polycondensation reaction rate and extent of the reaction in polar protic and aprotic solvents.
- ✓ To evaluate cytotoxicity of the newly synthesized cationic polymers and their efficiency in DNA/siRNA transfection.
- ✓ To evaluate biodegradability of poly(2-hydroxypropylene imines) containing cystamine units and of polyamidoamines containing hydroxyl groups.

Scientific novelty and practical value of the dissertation.

High molecular weight polyalkyleneimines were synthesized for the first time by polycondensation of aliphatic diamines and dibromides at appropriate reaction conditions. Isothermal reaction calorimetry was used to study kinetics of the polycondensation reaction.

PHPI derivatives containing disulfide linkages in the main chain or attached MPEG chains were synthesized for the first time. Polyamidoamines containing hydroxyl and ethereal groups in the main chain were prepared for the first time as well.

Newly synthesized polymers were tested as DNA or siRNA delivery agents. Transfection results showed that PHPI derivatives containing disulfide linkages in the main chain or MPEG segments could be great candidates for *in vitro* and *in vivo* transfection.

Defensive statements:

- ✓ Polar aprotic solvents are the most suitable for polycondensation of 1,6-diaminohexane with 1,2-dibromoethane and 1,3-diamino-2-propanol with 1,3-dibromo-2-propanol.
- ✓ Degree of branching of polycondensational polyalkyleneimines depends on basicity of a diamine monomer, the distance between amino groups in this monomer, and the solvent.
- ✓ Poly(2-hydroxypropyleneimine) derivatives containing disulfide linkages in the main chain can be synthesized by polycondensation of 1,3-dibromo-2-propanol with either cystamine or a mixture of cystamine and 1,3-diamino-2-propanol.
- ✓ Poly(2-hydroxypropyleneimine) derivatives containing disulfide linkages in the main chain are biodegradable, less toxic to cells than PHPI, and are suitable for a double transfection.
- ✓ Poly(2-hydroxypropyleneimine) derivatives containing methoxy-poly(ethyleneglycol) chains can be synthesized by polycondensation of 1,3-dibromo-2-propanol with 1,3-dibromo-2-propanol and methoxy-poly(ethyleneglycol) iodides. These derivatives have high molecular weight, they are suitable for siRNA transfection.
- ✓ Polyamidoamines obtained by polyaddition of 1,3-diamino-2-propanol and *N,N'*-methylenebisacrylamide are biodegradable polymers.

Approbation of the research results. Results of the research were presented in 3 scientific papers in the journals included into the Thomson Reuters Web of Science database (WOS). Results were also reported in 6 international and 1 national conferences.

Structure of the doctoral dissertation. The doctoral dissertation is written in Lithuanian and contains the following chapters: Introduction with the motivation of the research objectives, Literature Survey, Experimental Part, Results and Discussion, Conclusions, List of References (257 entries) and List of Scientific Publications. Material of the dissertation is laid out in 203 pages, including 23 schemes, 42 figures and 16 tables.

2. MATERIALS AND METHODS

Main materials. Methoxy-poly(ethylene glycols) (MPEG^o1000, MPEG^o2000, and MPEG^o5000), 1,3-diamino-2-propanol (DAP, 98%), 1,3-dibromo-2-propanol (DBP, 95%), 1,6-diaminohexane (DAH, 98%), 1,2-dibromomethane (DBE, 98%), *N,N'*-methylenebisacrylamide (MBAA, 99%), cystamine dihydrochloride, 2,2'-(ethylenedioxy)diethylamine (EDODA, 98%), *N,N*-dimethylacetamide (DMAC, 99%), *N,N*-dimethylformamide (DMF, 99%), methanol (MeOH, 99.5%), 2-propanol (IPOH, 99.7%), and dichloromethane (CH₂Cl₂, 99%). Prior to use, the solvents were purified according to standard procedures and stored over molecular sieves (3Å). Other reagents used were of analytical grade. **Reagents for transfection:** Dubelcco's Modified Eagle's Medium (DMEM), RPMI-1640 medium, Trypsin-EDTA, L-glutamine, gentamicin sulfate and doxycycline were purchased from Sigma Aldrich, Fetal bovine serum (FBS) – from GE Healthcare Life Sciences, *HeLa* cells – from ATCC (LGS Standards, UK), HEK293iGFP cells were kindly provided by Thermo Fisher Scientific, Lafayette, US. GFP-specific and control siRNAs were purchased from Ambion (Life Technologies), eGFP encoding plasmid was synthesized at GenScript USA Inc. All other reagents were of analytical reagent grade.

Synthesis of polyalkyleneimines, their derivatives and polyamidoamines. Polycondensation of DAH and DBE as well as of DAP and DBP were carried out using equimolar ratio of the monomers in DMF, DMAC, MeOH, IPOH and water. Poly(ethyleneiminehexamethyleneimines) (PEIHI) were synthesized at 50 °C using 50% total concentration of monomers during 5-168 hours. PHPI were synthesized at 60 and 80 °C, total monomer concentration 40%, reaction time up to 168 hours. PHPI containing segments of MPEG (M_n=1000, 2000, 5000) or cystamine (CT) were

synthesized in DMAC using DAP, DBP, MPEG iodide and cystamine (CT) as starting materials. Trying to find conditions favourable for attachment of MPEG chains, MPEG iodide was added to the initial mixture of the monomers (method A) or was introduced later, at the end of the reaction (method B). In all cases were used 10 mmol of DAP, 9.5 mmol of DBP and 1 mmol of MPEGI. Variation of the ratio CT to DAP enabled to synthesize PHPI-SS with different amount of disulfide bonds. Total concentration of the monomers was 40%, reaction temperature was set to 80 °C, and duration of the reaction was 7 days. Synthesis of polyamidoamines (PAMAM) was carried out using equimolar amounts of MBAA and DAP, and small amounts of EDODA in water as well as in water-methanol mixtures (10 and 50 vol% of water). Total concentration of the monomers was 20%, reaction temperature was set to 25 °C, duration of the reaction 7 days.

PEIHI, PHPI, MPEG'ylated PHPI, PHPI-SS and PAMAM were ultrafiltrated through a *Pellicon* membrane (*Millipore*) with nominal cut-off 10 kDa against water, and the polymers as solids were isolated by freeze-drying.

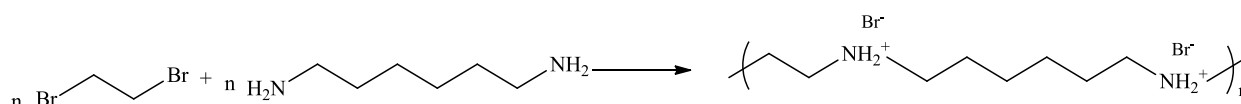
Characterization of PEIHI, PHPI, derivatives of PHPI and PAMAM. Synthesized polymers were characterized by FTIR and NMR spectroscopy, SEC, DLS, DSC, XPS and potentiometric titration, and were tested for DNA and siRNA delivery *in vitro*.

Cell culture and transfection. For DNA transfections, one day before the experiment *HeLa* cells were seeded in a 24-well tissue culture plate at the density of $6 \cdot 10^4$ cells per well in the total volume of 1 mL DMEM culture medium supplemented with 10% FBS. The cells were incubated at 37 °C in a CO₂ incubator until they reached 70-80% confluency (usually within 24 h). For siRNA delivery, *HEK293iGFP* cells were seeded in a 48 well tissue culture plate at the density of $2 \cdot 10^4$ cells per well in the total volume of 250 µl of RPMI culture medium supplemented with 10% FBS. GFP expression was induced with doxycycline (final concentration in cell culture – 1 µg/ml). The cells were incubated in a CO₂ incubator for 24 h at 37 °C. The transfection efficiency was evaluated 48 h later by flow cytometry using Guava EasyCyte8HT system and *Guava CytoSoft 2.2.3* cell acquisition/analysis software (Millipore). Also was evaluated double DNA transfection and RNA extraction after transfection.

3. RESULTS AND DISCUSSION

3.1 Synthesis and study of poly(ethyleneiminehexamethyleneimines)

Polycondensation reaction between 1,6-diaminohexane (DAH) and 1,2-dibromoethane (DBE) (Scheme 1) was studied in order to elucidate the effect of the reaction conditions on the molecular weight of poly(ethyleneiminehexamethyleneimine) (PEIHI).



Scheme 1. Synthesis of poly(ethyleneiminehexamethyleneimine)

Kinetics of polycondensation between DAH and DBE in various solvents was studied using isothermal reaction calorimeter. The heat flow profiles confirm that the reaction in all solvents starts immediately, but the rate of the reaction differs, being the highest in aprotic solvents.

Molecular characteristics of PEIHI were determined by size exclusion chromatography (SEC) with triple detection system using LALS/RALS, viscosity and RI detection (Table 1). As determined by SEC, yield (η) of the purified PEIHI is in the range of 0.2-7%. Weight average molecular weight (M_w) of PEIHI was from 21 to 81 kDa, and dispersity (M_w/M_n) from 2 to 5. Polymers with the highest molecular weight were obtained in DMF, DMAC and water.

^{13}C NMR spectrum of PEIHI revealed that this polymer is branched. Chemical shift at 40 ppm is associated with the carbon next to the primary amino group ($-\text{H}_2\text{N}-\underline{\text{C}}\text{H}_2-\text{CH}_2-$); the shift at 43-47 ppm is attributed to the carbon attached to differently protonated secondary amino groups ($-\text{NH}-\underline{\text{C}}\text{H}_2-\text{CH}_2-$ and $-\text{NH}_2^+-\underline{\text{C}}\text{H}_2-$); the shift at 48-52 ppm is related to the carbon next to tertiary amino group ($>\text{N}-\underline{\text{C}}\text{H}_2-\text{CH}_2-\text{NH}-$ and $>\text{N}-\underline{\text{C}}\text{H}_2-\text{CH}_2-\text{N}<$). Since tertiary amino group represents the branching point on PEIHI, the degree of branching was calculated as the molar ratio of secondary to tertiary amino groups. This ratio shows average number of secondary amino groups existing between

two branched points. This number is between 6 and 7. PEIHI with the highest degree of branching were obtained in polar aprotic solvents. Presumptive mechanism of branching of polyalkyleneimines (PEIHI and PHPI) see section 3.2.

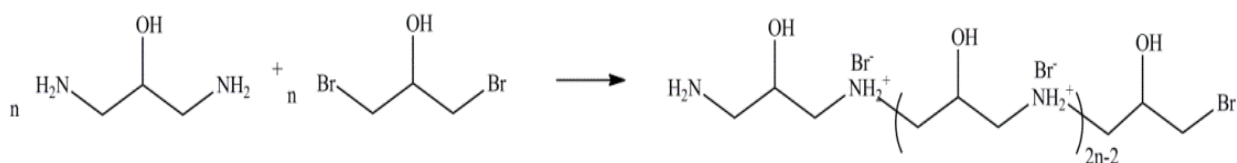
Table 1. Molecular characteristics of PEIHI, synthesized in various solvents

Solvent	t, hrs	η , %	M_w , kDa	M_w/M_n
MeOH	168	5.4	22.1	2.4
IPOH		0.2	21.1	3.2
Water		2.8	68.6	4.1
DMF	168	8.6	70.5	4.0
DMAC	168	7.3	81.4	5.2
PC	48	1.0	29.8	5.1

Acid-base potentiometric titrations and DLS investigation revealed that PEIHI were typical polyelectrolytes. pKa values of PEIHI irrespective of the synthesis conditions were close and equal to 7.8-8.1). Slope values ($n=1.82-2.25$) of the dependence in the coordinates of the Henderson-Hasselbalch equation) show that ionization of amino groups is strongly affected by ionization state of the adjacent groups. Buffering capacity of PEIHI is in the range of pH 6.7-9.1. Such values indicate that PEIHI may well perform as delivery vehicle. DLS investigation revealed that hydrodynamic radius of PEIHI depends upon ionic strength and pH of solution.

3.2 Synthesis and characterization of poly(2-hydroxypropylene imines)

Polycondensation of 1,3-diamino-2-propanol (DAP) and 1,3-dibromo-2-propanol (DBP) was studied in five different solvents, namely, methanol (MeOH), 2-propanol (IPOH), water, *N,N*-dimethylacetamide (DMAC) and *N,N*-dimethylformamide (DMF) (Scheme 2). Reactions were carried at 60 °C (for MeOH) and at 80 °C (for all remaining solvents).



Scheme 2. Synthesis of poly(2-hydroxypropylene imines)

Kinetics of this reaction was followed according to bromide concentration in the reaction mixture. The highest reaction rate and the highest extent of the reaction were characteristic for polycondensation in DMAC. In parallel, kinetics of polycondensation between DAP and DBP in various solvents was studied using isothermal reaction calorimeter. The heat flow profiles confirm that the reaction starts immediately, but the rate of the reaction differs, being the highest in aprotic solvents. The same tendency was observed during polycondensation of DAH and DBE (see section 3.1).

Purification of PHPI synthesized in alcohols, water and aprotic solvents was done by ultrafiltration using membrane with nominal cut-off 10 kDa. Obviously, the yield of the ultrafiltrated PHPI was low, ranging between 14 and 20% (Table 2). Molecular weight (M_w) of the purified PHPI was medium (8-17 kDa), and dispersity index about 2 for the samples synthesized in methanol and water, and nearly 5 for those synthesized in aprotic solvents. High dispersity of PHPI synthesized in DMAC and DMF are predetermined by high-molecular fraction present in these samples (M_w approaching and even exceeding 100 kDa) (Figure 1).

Table 2. Characteristics of PHPI synthesized in various solvents

Solvent	η^a , %	M_w , kDa	M_w/M_n	α^b	T_g^c
MeOH	14.2	12.7	2.04	0.50	-21.8
IPOH	5.7	9.70	2.71	0.52	-20.9
Water	17.4	8.06	1.94	0.50	-26.0
DMF	15.7	16.4	4.72	0.44	35.7
DMAC	19.5	17.6	4.84	0.43	32.0

^a determined by gravimetric method; ^b slope of Mark-Houwink plot; ^c glass transition temperature determined by DSC.

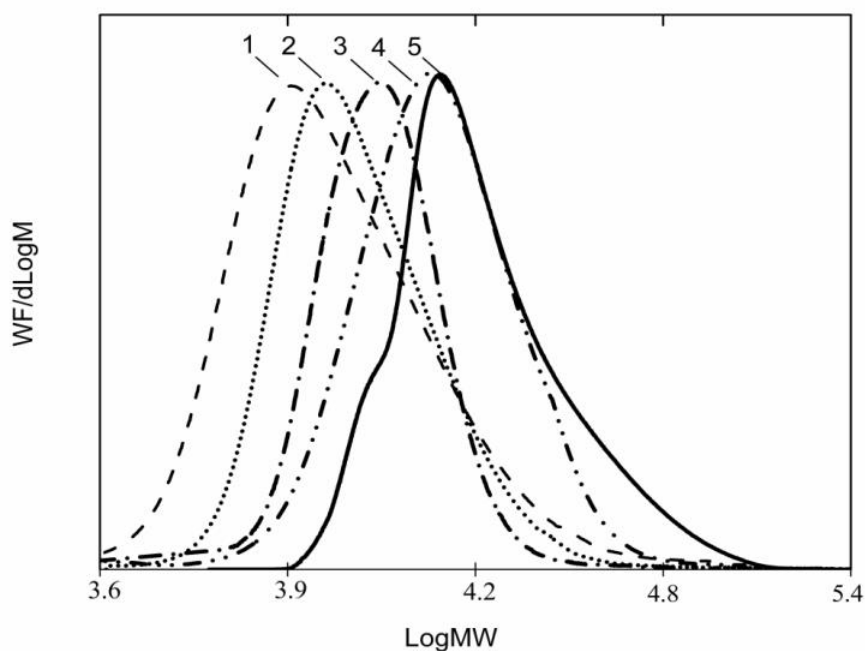


Fig. 1. Molecular weight distribution curves of ultrafiltrated PHPI synthesized in IPOH (1), water (2), MeOH (3), DMF (4), and DMAC (5).

^1H NMR spectrum of the deprotonated PHPI contains broad signal at 2.4 ppm attributed to $-\text{NH}-\underline{\text{C}}\text{H}_2-\text{NH}-\underline{\text{C}}\text{H}_2-\text{CH}(\text{OH})-\underline{\text{C}}\text{H}_2-$, and two signals at 3.5 ppm and 3.6 ppm attributed to $-\text{C}\underline{\text{H}}_2-\underline{\text{C}}\text{H}(\text{OH})-\text{C}\underline{\text{H}}_2-$. More information about structure of PHPI gives ^{13}C -NMR spectrum of this compound (Fig. 2). Chemical shift at 43.3 ppm belongs to the carbon associated with primary amino group ($\text{NH}_2-\underline{\text{C}}\text{H}_2-$), while the shift at 52.8 ppm is related to the carbon directly attached to the secondary amino group ($-\underline{\text{C}}\text{H}_2-\text{NH}-\underline{\text{C}}\text{H}_2-\text{CH}(\text{OH})-\text{C}\underline{\text{H}}_2-$). The shift at 59.1 ppm is related to the carbon next to tertiary amino group ($\text{N}(\underline{\text{C}}\text{H}_2-\text{CH}(\text{OH})-\text{C}\underline{\text{H}}_2-)_3$), and that at 64.2-63.8 ppm belongs to the carbon associated with hydroxyl group $-\text{C}\underline{\text{H}}_2-\underline{\text{C}}\text{H}(\text{OH})-\text{C}\underline{\text{H}}_2-$.

Since tertiary amino group represents the branching point on PHPI, the degree of branching can be calculated by the molar ratio of secondary to tertiary amino groups. This ratio shows how many repeating units of PHPI are between two branched points. Irrespective of the solvent, all synthesized PHPI are branched polymers. The highest degree of branching was obtained in aprotic solvents DMF and DMAC (the branching points were separated by approximately 2 repeating units). Synthesis in water and

alcohols lead to less branched products, branching points being separated by approximately 3 repeating units.

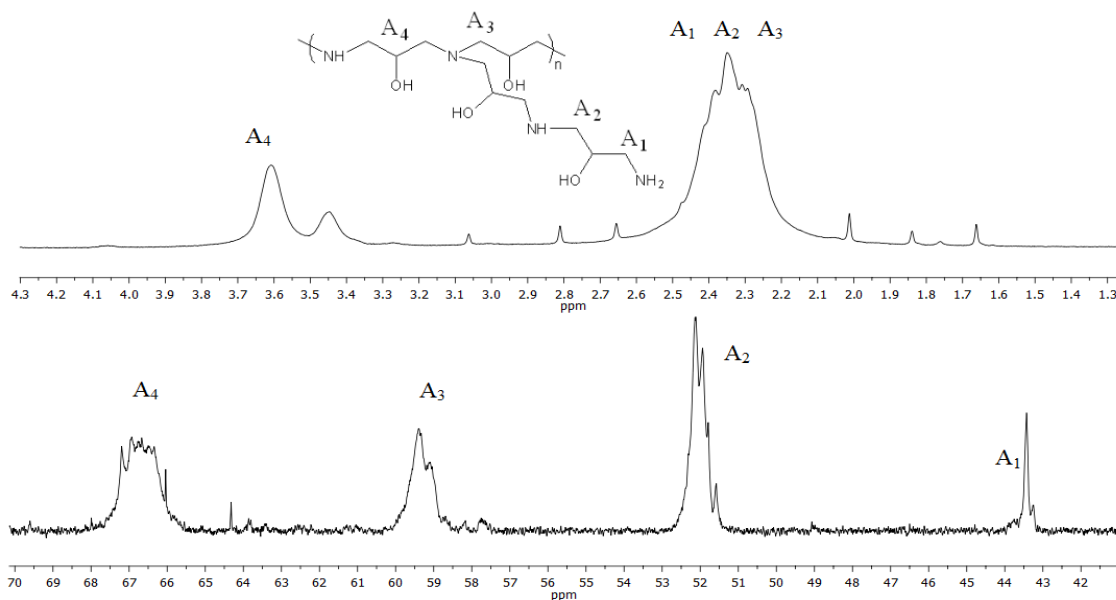
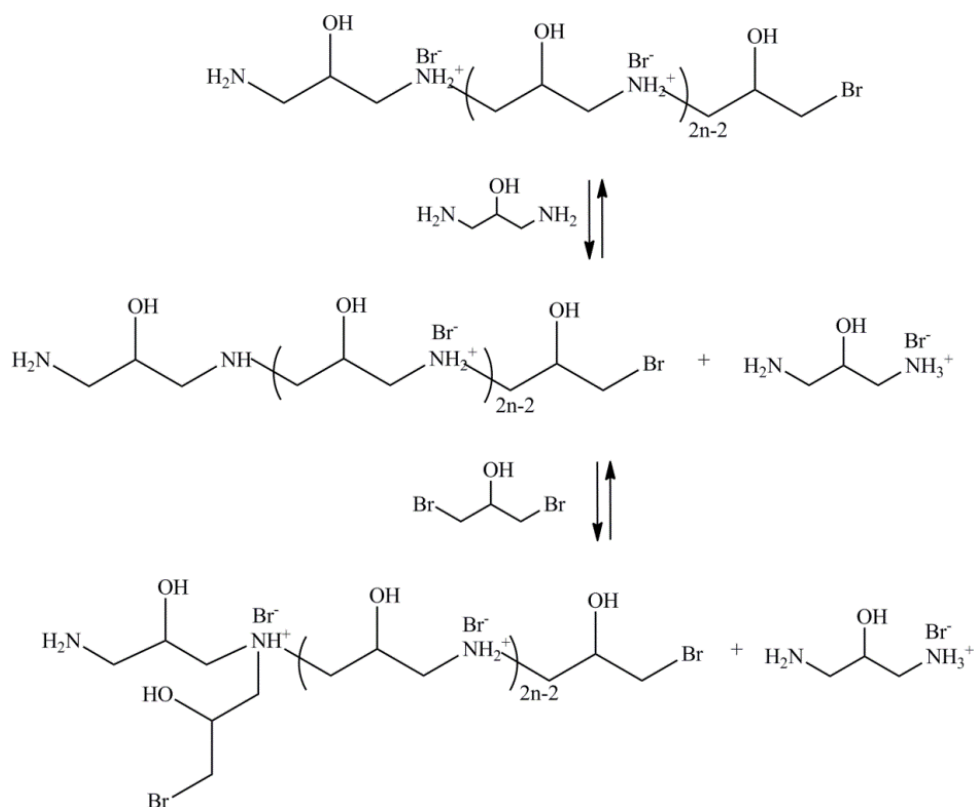


Fig. 2. ^1H NMR (top) and ^{13}C NMR (bottom) spectra of ultrafiltrated PHPI synthesized in DMAC. Spectra were recorded in D_2O containing several drops of NaOD, pH 13-14 (samples in a deprotonated form).

Presumptive mechanism of branching during polycondensation of DAP and DBP is presented in Scheme 3. Branching, possibly, proceeds because of deprotonation of secondary amino groups which is related to proton migration from the protonated secondary amino groups of the polymer to non-protonated amino groups of DAP or terminal amino groups of the polymer (oligomer). Likely, the effect of solvents on degree of branching of PHPI is predetermined by H-bonding which changes nucleophilicity of amino groups. In protic solvents, H-bonded amino groups partly lose their nucleophilicity and capability to deprotonate secondary amino groups, which lower possibility to branching. Contrarily, aprotic polar solvents act as proton acceptors increasing nucleophilicity (basicity) of amino groups and, the same, tendency to branching. This facilitates ion-exchange reactions between protonated secondary amino groups and non-protonated primary or even secondary amino groups leading to higher degree of branching.



Scheme 3. Presumptive mechanism of branching during polycondensation of DAP and DBP

Complementary information about chain conformation and branching of PHPI was abstracted from the data obtained using SEC with triple detection (Table 2). Downward curvature in Mark-Houwink plots characteristic for branched polymers was not evident (perhaps, because of relatively low molecular weight), but the slope values below 0.5 are associated with compact, usually branched, structures. Consequently, both NMR and SEC data confirmed formation of highly branched PHPI under polycondensation of DAP and DBP in aprotic solvents DMF and DMAC.

Subject to the degree of branching, glass transition temperature (T_g) of PHPI differ (Table 2). DSC analysis revealed that PHPI are amorphous polymers possessing T_g at negative ($-20 - -26$ °C, synthesis in alcohols and water) or positive ($32-36$ °C, synthesis in aprotic solvents) temperatures.

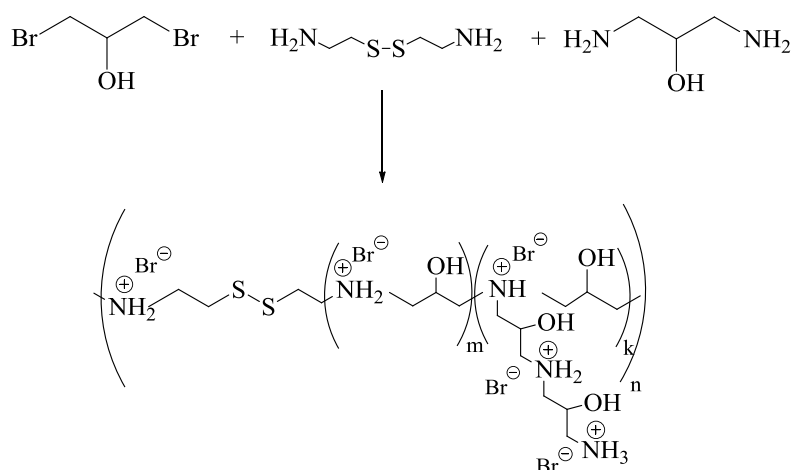
Acid-base titration studies gave pK_a values of PHPI at 7.2-7.8. The slope values (n) of the dependence in the coordinates of the Henderson-Hasselbalch equation are 2.1-2.6.

DLS investigation revealed that hydrodynamic radius of PHPI depends upon ionic strength and pH of solution. These results show that PHPI are typical polyelectrolytes.

3.3 Synthesis and characterization of PHPI derivatives containing cystamine units

PHPI containing multiple biodegradable disulfide linkages in the main chain (PHPI-SS) were synthesized by polycondensation of 1,3-dibromo-2-propanol (DBP) with either cystamine (CT) or a mixture of CT and 1,3-diamino-2-propanol (DAP). Variation of the ratio CT to DAP enabled to synthesize PHPI-SS with different amount of disulfide bonds (Scheme 4). The polymers in descending order of CT units were named as PHPI-SS1, PHPI-SS2, PHPI-SS3 and PHPI-SS4 (Table 3).

In order to remove low molecular weight fractions, PHPI-SS were ultrafiltrated through 10 kDa cut-off membrane. Unfortunately, together with oligomeric fractions, part of the polymer was lost, which resulted in low yields 2-7% of the ultrafiltrated PHPI-SS. Weight average molecular weights (M_w) of PHPI-SS after ultrafiltration ranged from ~4 to 8 kDa with dispersity index 1.5-2 (Table 3). Molecular weight of PHPI-SS1 and PHPI-SS2 are close while that of PHPI-SS3 and PHPI-SS4 containing the smallest amount of cystamine units is about twice higher. Molecular weight distribution (MWD) curves of the polymers (Fig. 3) have slightly expressed bimodality and high-molecular “tails”.



Scheme 4. Synthesis of PHPI derivatives containing cystamine units

Table 3. Yield (η) and molecular characteristics of PHPI-SS after ultrafiltration

Polymer	Ratio of the			η^a , %	M_w^b	M_w/M_n^b	N_2/N_3^c
	monomers						
	DBP	CT	DAP				
PHPI-SS1	1	1	-	4.5	4.2	1.98	1.76
PHPI-SS2	1	0.7	0.3	1.4	3.9	1.52	1.65
PHPI-SS3	1	0.5	0.5	4.8	7.9	1.56	2.01
PHPI-SS4	1	0.25	0.75	7.1	6.9	1.43	1.94
PHPI	1	-	1	19.5	17.6	4.84	1.74

Determined by: ^a gravimetric method; ^b SEC; ^c ¹³C NMR spectroscopy

Structure of the polymers was proved by ¹H and ¹³C NMR spectroscopy. It was determined, that approximately every fourth nitrogen atom of PHPI-SS had a branching point, i.e. these polymers were highly branched.

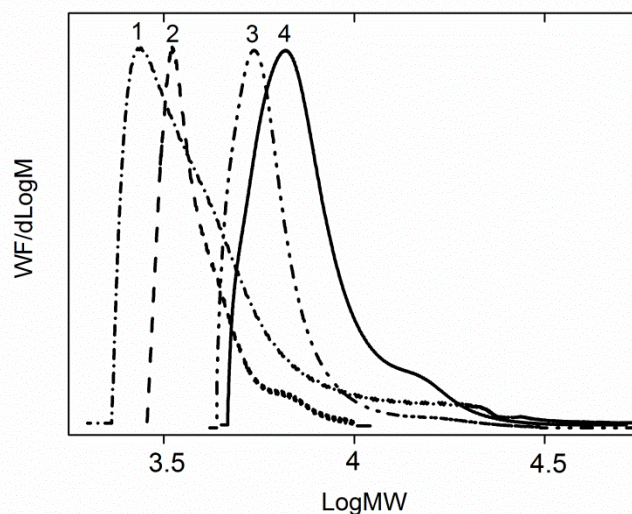


Fig. 3. MWD curves of ultrafiltrated PHPI-SS1 (1), PHPI-SS2 (2), PHPI-SS3 (4) and PHPI-SS4 (3).

The presence of cystamine moieties and disulfide linkages in the polymers were proven by NMR spectroscopy, elemental analysis and chemical determination. Experimental molar ratio between 2-hydroxypropylene imine and ethylenedithioethylene

imine segments in PHPI-SS (HPI/EDEI) determined by different methods (Table 4) is in good agreement with the theoretical ratio HPI/EDEI calculated according to the initial feed of the monomers (Table 4). The most reliable results gave ^1H -NMR spectroscopy. The ratio HPI/EDEI calculated from the experimentally determined content of the disulfide groups close to theoretical value means that PHPI-SS do have disulfide linkages.

Table 4. Ratio of HPI/EDEI in various PHPI-SS

Polymer	HPI/EDEI ^a	HPI/EDEI calculated from:			
		^1H NMR	^{13}C NMR	EA ^b	-SS- ^c
PHPI-SS1	1	0.98	1.19	0.59	0.87
PHPI-SS2	1.85	1.93	2.29	1.76	2.16
PHPI-SS3	3	2.98	3.20	2.98	2.63
PHPI-SS4	7	6.42	7.47	6.62	6.31

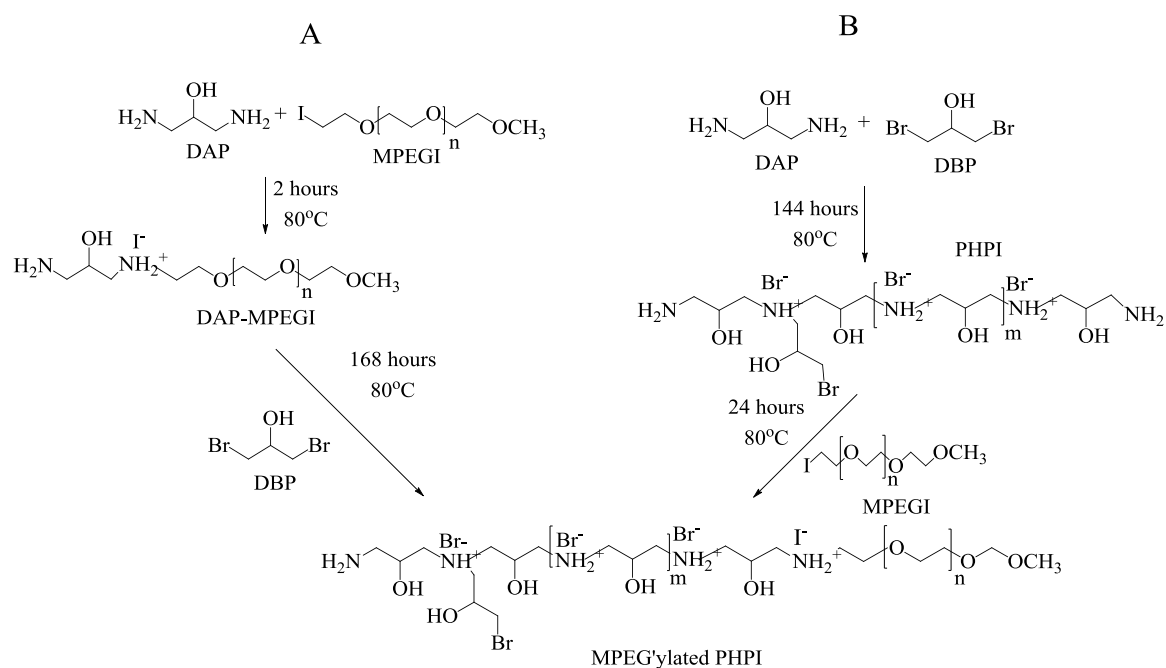
^a ratio in the initial feed; ^b from elemental analysis of sulfur; ^c from chemical analysis

3.4 Synthesis and characterization of MPEG'ylated derivatives of PHPI

MPEG'ylated derivatives of PHPI were synthesized by polycondensation of DAP and DBP in the presence of MPEG iodides (MPEGI) (Scheme 5). In all cases molar proportions between DAP:DBP:MPEGI = 10:9.5:1 mmol. Trying to find conditions suitable for the synthesis of PHPI derivatives with different content of MPEG chains, MPEGI was added to the initial mixture of the monomers (method A), or it was introduced later, at the end of the reaction (method B). Moreover, MPEGI with different molecular weight (M_n 1000, 2000, and 5000) was used, which expanded possibilities to synthesize PHPI-MPEG derivatives with different structure, i.e. length and density of MPEG chains and molecular weight. PHPI derivatives were purified by extracting unreacted MPEGI along with the residual MPEG with CH_2Cl_2 , and low molecular weight fractions from polymer were eliminated by ultrafiltration through 10 kDa membrane.

Molecular characteristics of MPEG'ylated derivatives of PHPI are summarized in Table 5. Yield of MPEG'ylated PHPI was low, usually less than 30%. Especially low

yield was characteristic for PHPI derivatives containing segments of MPEG 5000. Molecular characteristics of MPEG'ylated PHPI depend also on the method of the synthesis. High content of oxyethylene (OE) segments exceeding 60 mol% was characteristic for PHPI derivatives synthesized by the method A. In this case, 1 to 1.25 MPEG chains were attached per one macromolecule of PHPI (value k in Table 5). Contrarily, this number for MPEG'ylated PHPI obtained by the method B was considerably less.



Scheme 5. Synthesis of MPEG'ylated derivatives of PHPI

Fig. 5 presents molecular weight distributions (MWD) curves of MPEG'ylated PHPI. Obviously, molecular weight and MWD of the polymers depend on molecular weight of MPEG and, especially, on the method of the synthesis. Weight-average molecular weight of MPEG'ylated derivatives of PHPI synthesized by the method A was rather low, from 8 to 53 kDa. The polymers synthesized by the method B (MPEGI was introduced at the end of the reaction) were characterized by considerably higher weight-average molecular weight (138-162 kDa) and very high dispersity (M_w/M_n up to 13) (Table 5). MWD curves of these polymers were bimodal showing the presence of the fractions with molecular weight up to several millions.

Table 5. Molecular characteristics of MPEG'ylated PHPI

Method	Sample	MPEG	η , %	OE, mol%	k	M_w , kDa	M_w/M_n	α	N_2/N_3
A	1	1000	28.1	59	1.25	7.7	2.84	0.38	2.5
	2	2000	23.2	67	1.06	15.6	4.03	0.33	2.4
	3	5000	5.97	72	1.20	52.6	5.29	0.29	2.3
B	4	1000	20.9	3.3	0.22	138	11.7	0.35	2.7
	5	2000	17.1	2.4	0.09	145	12.1	0.33	2.8
	6	5000	6.04	9.3	0.15	162	13.6	0.32	2.6

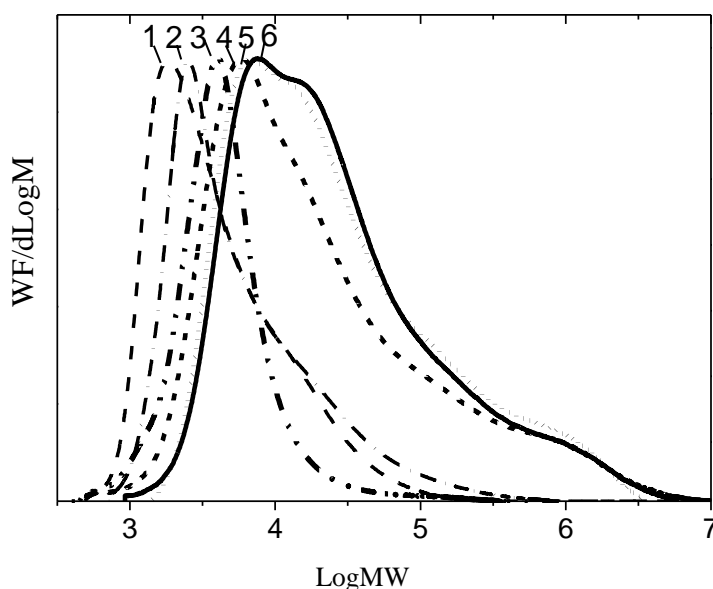


Fig. 5. MWD of ultrafiltrated MPEG'ylated PHPI. Numbers on the curves correspond to the number of the samples in Table 5.

Structure of MPEG'ylated derivatives of PHPI was established using ^1H and ^{13}C NMR spectra. ^1H NMR spectrum of PHPI modified by MPEG°2000 (Figure 6) contains the signals attributed to MPEG ($-\text{CH}_2\text{CH}_2\text{O}-$, at 3.6 ppm) and to PHPI (see section 3.2). ^{13}C NMR spectra of MPEG'ylated PHPI indicate the presence of MPEG chains since they have the signal attributed to $-\text{O}-\text{CH}_2-\text{CH}_2-$ (69,5 ppm) groups (Fig. 6). The signals attributed to PHPI segments evidence branched structure of the polymers (Section 3.2). It was determined that every second or third repeating unit of PHPI chain had a branching point. The polymers synthesized by the method B appeared to be slightly

more branched than those synthesized by the method A (Table 5). SEC analysis confirmed branched structure of the PHPI derivatives: the values of the constant α in Mark-Houwink equation were very low varying from 0.29 to 0.38 (Table 5).

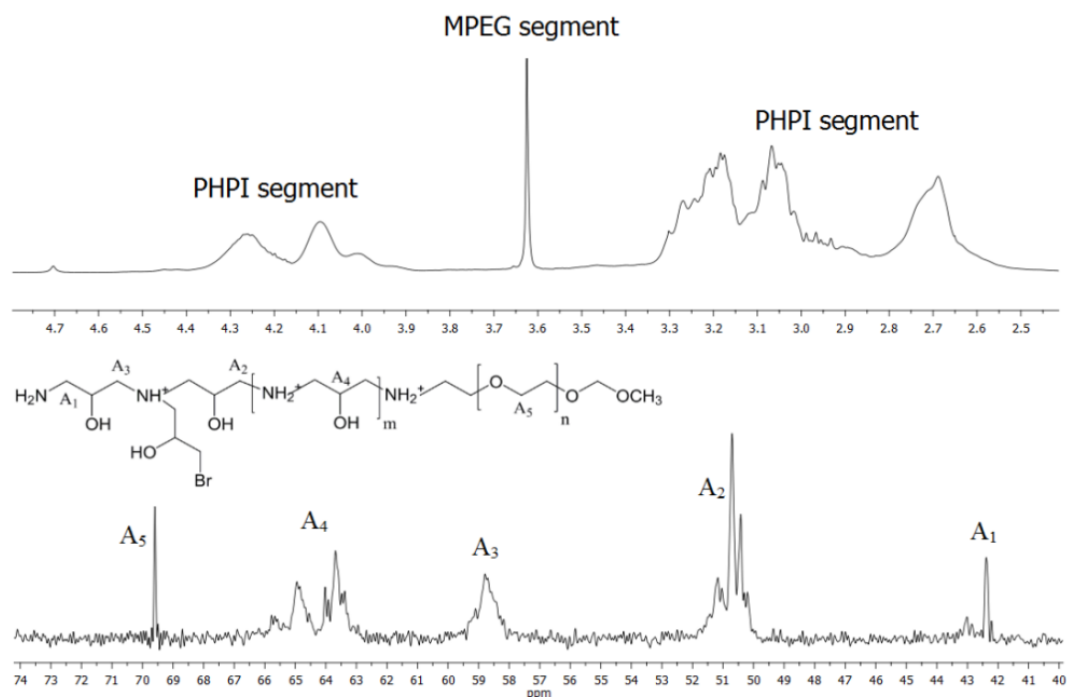


Fig. 6. ^1H NMR (top) and ^{13}C NMR (bottom) spectra of PHPI derivative with MPEG°2000 in D_2O (sample 5 in Table 5).

The presence of MPEG segments in PHPI derivatives was confirmed also by FT-IR spectra and DSC measurements. FT-IR spectra showed the absorption bands at 1100 and 1470 cm^{-1} corresponding to the stretching vibrations of C-O-C in $(-\text{O}-\text{CH}_2-\text{CH}_2-)$, and to deformation vibrations of $-\text{OCH}_3$. DSC thermograms of PHPI derivatives with high content of MPEG showed melting at 31, 48 and 51 $^\circ\text{C}$ for the polymers no 1, 2 and 3 (Table 5), respectively. DSC thermograms of PHPI derivatives with low content of MPEG didn't show melting behavior. Glass transition temperature (T_g) of MPEG'ylated derivatives of PHPI was at negative temperatures and ranged from -2 to -7 $^\circ\text{C}$.

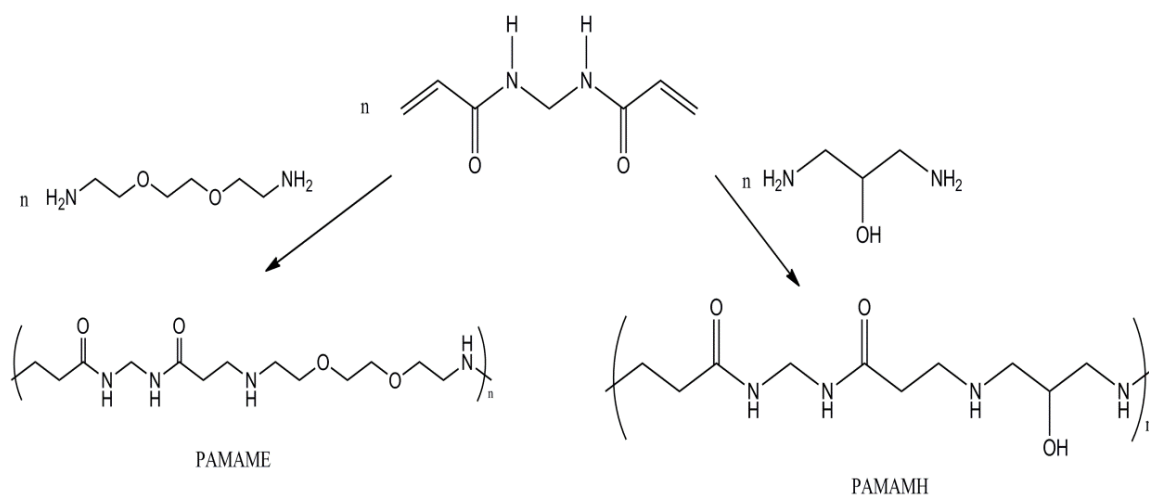
For cationic gene delivery vehicle, its buffering capacity and degree of unprotonation β at physiological pH (pH 7.3) are very important characteristics with

respect to endosomal escape of polyplexes and high transfection efficiency. Acid-base potentiometric titrations of MPEG'ylated PHPI gave titration curves very similar to those of PHPI. PHPI and MPEG'ylated derivatives of PHPI are characterized by high slope values (n) in the coordinates of the Henderson-Hasselbalch equation. This suggests that ionization of amino groups of these polymers is seriously affected by neighbouring already protonized imine groups. All the polymers studied exhibited very close pK_a values (7.4-7.8). According to pK_a , polymers synthesized by the method A are more similar to PHPI than those obtained by the method B. Overall buffering capacity of PHPI derivatives covers pH range from 6.4 to 8.8, and the degree of unprotonation of amino groups at the pH of the cytosol (pH 7.3) is at about 39-48%. These numbers indicate that MPEG'ylated derivatives of PHPI should be considered as good cationic gene delivery vehicles.

DLS investigation revealed that macromolecules of MPEG'ylated PHPI are coiled to a smaller diameter both in neutral and acidic environment, compared to non-MPEG'ylated counterpart.

3.5 Synthesis and characterization of polyamidoamines

Novel type of hydroxyl (PAMAMH) and ethereal (PAMAME) groups containing polyamidoamines (PAMAM) were synthesized by polyaddition of 1,3-diamino-2-propanol (DAP) and 2,2'-(ethylenedioxy)diethylamine (EDODA) to *N,N'*-methylenebisacrylamide (MBAA) (Scheme 6). Using equimolar amounts of MBAA and DAP (or EDODA), the addition reaction was carried out in methanol, water and water-methanol mixtures (10 and 50 vol% of water). It was found that the yield (η) of the purified PAMAM was monomer-dependent: 11-20% for PAMAMH and 2.5-4.6% for PAMAME. Molecular weight of PAMAM was determined by SEC and was in the range of 8-76 kDa (PAMAMH) or 17.4-31.8 kDa (PAMAME). Molecular weight and dispersity of the polymers were dependent on the solvent as well: PAMAM synthesized in water had the highest molecular weight and dispersity (Table 6).



Scheme 6. Synthesis of polyamidoamines

Table 6. The results of PAMAM synthesis

PAMAMH			
Solvent	η , %	M_w , kDa	M_w/M_n
H ₂ O	12.4	76.3	4.19
MeOH/H ₂ O (50/50)	21.3	10.7	1.28
MeOH/H ₂ O (90/10)	11.8	8.29	1.52
MeOH	19.9	12.2	1.86
PAMAME			
Solvent	η , %	M_w , kDa	M_w/M_n
H ₂ O	4.6	17.4	4.97
MeOH/H ₂ O (50/50)	5.8	24.0	1.22
MeOH/H ₂ O (90/10)	2.5	25.3	1.49
MeOH	3.5	31.8	2.18

PAMAMH and PAMAME were characterized by ¹H and ¹³C NMR spectroscopy. ¹³C NMR spectroscopy revealed that in PAMAMH, approximately every second imine group is a branching point, and in PAMAE every fourth imine group is a branching point, i.e. these polymers are highly branched.

Acid-base titration showed that imine groups of PAMAM at physiological pH 7.4 are partly protonated only, therefore in the cells they may act as a good “proton sponge”. Along with that, being presumably biodegradable and containing additional hydroxyl and ethereal group functionalities, which should impart some biocompatibility and

ability to bind DNA through additional hydrogen-bonding, PAMAM may be promising gene delivery agent.

3.6 Gene delivery mediated by cationic polymers

Transfection experiments were performed to characterize the newly synthesized biodegradable polymers and to determine an optimal polymer concentration for further experiments. GFP expression analysis (Fig. 7) showed that efficiency of PHPI-SS1, PHPI-SS2 and PHPI-SS3 was comparable to that of PHPI, and the use of these polymers gave similar results with GFP⁺ cell percent reaching 80-90%. Cytotoxicity levels for all tested PHPI-SS (especially, considering that much higher polymer concentrations were used) were similar or lower than that of PHPI suggesting that polymer ability to degrade within the cell contributes to the better health and survival of the cells. DLS analysis revealed that PHPI containing cystamine units formed complexes of similar size as that of PHPI (~150 nm).

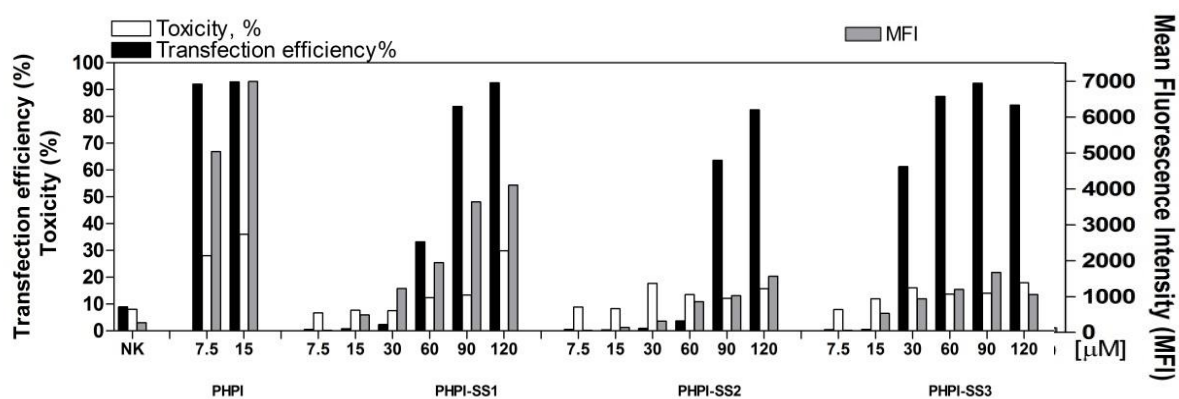


Fig. 7. DNA transfection efficiency of PHPI-SS. Concentration of the polymers [μ M] is indicated in the X-axis. Transfection efficiency (GFP⁺ cell, %) and toxicity are plotted on the left Y-axis, MFI – on the right Y-axis.

In order to better understand the differences observed in transfection results, we further analysed the ability of PHPI-SS to disintegrate and release DNA following the reducing agent DTT treatment. Transfection results showed that the most promising polymers are PHPI-SS1 and PHPI-SS3. These two polymers were further analysed by

gel filtration to better understand their degradation process and was revealed that PHPI did not degrade upon DTT treatment while PHPI-SS1 and PHPI-SS3 were broken down to oligomers. RNA recovery from PHPI-SS transfected cells was not affected even after the double transfection suggesting that PHPI-SS1 and PHPI-SS2 could be great candidates for *in vitro* and *in vivo* transfection.

Characteristics of DNA or siRNA complexes with PHPI and its PEG'ylated derivatives were examined using DLS technique. Complexes of MPEG'ylated derivatives of PHPI with DNA are larger (100-170 nm) compared to those with siRNA (40-70 nm). High molecular weight polymers (Table 5, no. 4-6) transfected DNA better than their counterparts with low molecular weight, and the length of MPEG chains attached to the polymers had no effect over the transfection efficiency. Comparing with PHPI, MPEG'ylated derivatives demonstrated reduced DNA-transfection efficiency. The difference was especially apparent (3-4 fold) at the level of GFP fluorescence (Fig. 8).

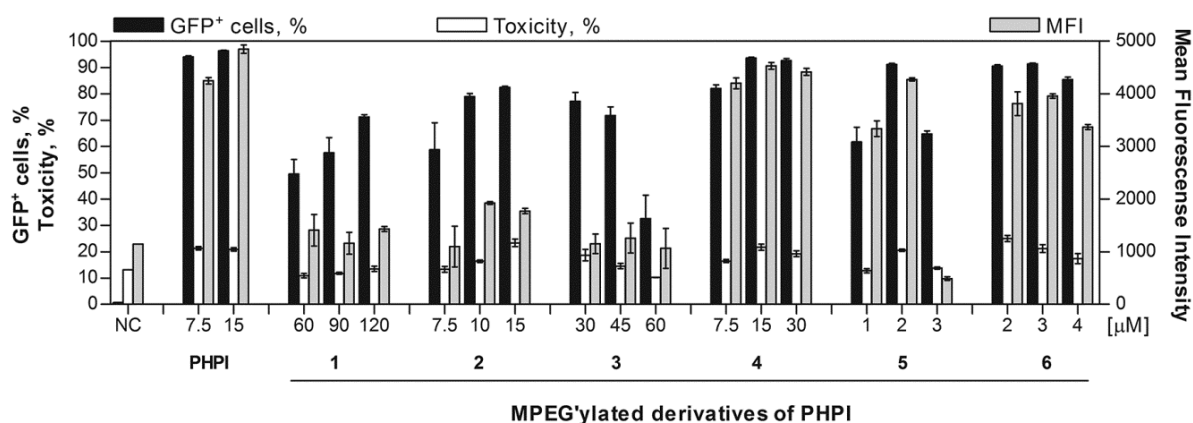


Fig. 8. DNA transfection efficiency of MPEG'ylated PHPI. Concentration of the polymers is shown on X-axis; the numbers below correspond to the samples in the Table 5. Graph shows mean values \pm SD ($n=3$).

The GFP knockdown efficiency was calculated based on the reduction of mean fluorescence intensity comparing samples transfected with non-targeting control vs. GFP-specific siRNA (Figure 9).

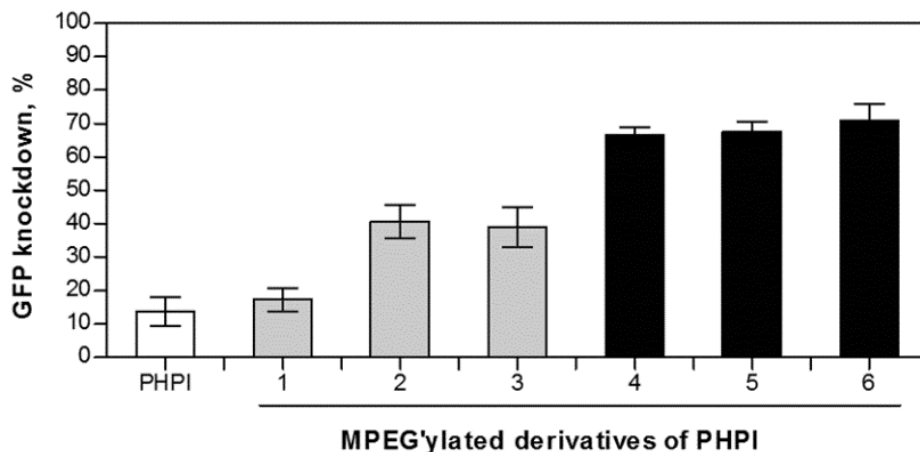


Fig. 9. siRNA-mediated gene knockdown using PHPI and its MPEG'ylated derivatives. The numbers on X-axis correspond to the samples in Table 5. Graph shows mean values \pm SD ($n=3$).

Similarly as with DNA transfection, high molecular weight polymers performed better transfecting siRNA, for which GFP suppression levels reached 70% (black histograms), while for low molecular weight polymers – only 40% (grey histograms). Thus, MPEG'ylation of PHPI facilitates improved siRNA mediated gene suppression effect.

Transfection experiments were also performed to characterize the newly synthesized PAMAMH polymers and to determine these polymers biodegradability. GFP expression analysis showed that efficiency of PAMAH was lower to that of PHPI, but cytotoxicity levels for all tested PAMAM in lower concentrations were similar or lower than that of PHPI. Biodegradability test was obtained using phosphate-buffered saline (PBS) solution (pH 7.3) and it was revealed that hydroxyl groups containing polyamidoamines are susceptible to biodegradation.

CONCLUSIONS

1. Polar aprotic solvents are the mostly suitable for the synthesis of poly(alkylene imines) by polycondensation of 1,3-diamino-2-propanol and 1,3-dibromo-2-propanol or 1,6-diaminohexane and 1,2-dibromoethane. These solvents facilitate achieving high polycondensation reaction rate, high reaction extent and preparing polymers with reasonable high molecular weight.
2. Poly(alkylene imines), their derivatives and polyamidoamines are branched but soluble polymers. Degree of branching of poly(alkylene imines) is increasing by the use of a diamine monomer with higher basicity and shorter distance between primary amino groups, and carrying polycondensation in polar aprotic solvents.
3. Low molecular weight fractions of poly(alkylene imines), their derivatives and polyamidoamines are removed by ultrafiltration thus reducing dispersity of the polymers.
4. Poly(2-hydroxypropylene imines) (PHPI) containing multiple disulfide linkages in the main chain (PHPI-SS) were synthesized via polycondensation of 1,3-dibromo-2-propanol with either cystamine or a mixture of cystamine and 1,3-diamino-2-propanol. Amount of disulfide linkages in these polymers depend on the initial ratio of the monomers. M_w of the ultrafiltrated PHPI-SS is rather low (3.9–7.9 kDa).
5. MPEG'ylated derivatives of poly(2-hydroxypropylene imines) can be synthesized by polycondensation of 1,3-diamino-2-propanol and 1,3-dibromo-2-propanol in the presence of MPEG iodides whose molecular weight is 1000, 2000, and 5000 Da. Yield of MPEG'ylated derivatives, their molecular weight and degree of MPEG'ylation depend on the reaction time when MPEG iodide is introduced in the reaction mixture. Introduction at the earlier stages results in polymers with relatively low molecular weight (M_w 8-53 kDa) having high content of oxyethylene groups (over 60 mol%) and from 1 to 1.25 MPEG chains per macromolecule. Introduction at late stages results in polymers with high molecular weight (M_w 140-160 kDa) having high polydispersity, low content of oxyethylene groups (less than 9 mol%) and from 0.09 to 0.22 MPEG chain per macromolecule. Yield of MPEG'ylated poly(2-hydroxypropyleneimines) is less than 30% and does not much depends on the method of synthesis.

6. Cytotoxicity levels for PHPI-SS are lower than that of PHPI. PHPI-SS are able to disintegrate within the cells and therefore are suitable for double transfection.
7. For siRNA delivery high molecular weight MPEG'ylated poly(2-hydroxypropyleneimines) having low content of MPEG chains perform significantly better than low-molecular-weight polymers having high content of MPEG chains or non-MPEG'ylated poly(2-hydroxy propyleneimine). Using high molecular weight MPEG'ylated poly(2-hydroxypropyleneimines) GFP suppression levels reached 70%.
8. Novel type of hydroxyl and ethereal groups containing polyamidoamines (PAMAM) were synthesized by polyaddition of 1,3-diamino-2-propanol and 2,2'-(ethylenedioxy)diethylamine to *N,N'*-methylenebisacrylamide (MBAA) Although hydroxyl groups containing polyamidoamines are susceptible to biodegradation, their transfection efficiency is lower than that of PHPI.

LIST OF SCIENTIFIC PUBLICATIONS ON THE THEME OF DISSERTATION

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1. Bočkuvienė A., Vareikis A., Makuška R. Polycondensation of hydroxyl-group containing aliphatic diamines and dibromides yielding highly branched poly(2-hydroxypropylene imines), *Chemija*, 2015, 26 (1), P.51-59.
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Patents

Filled application for US Patent: Zigmantas Š., Žaliauskienė L., Makuška R., Bočkuvienė A., Vareikis A., Biodegradable cationic polymers and uses thereof.

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2014 04 21 – 2015 04 21 Participation in the project „Mokinių jaunujų tyrėjų atskleidimo ir ugdymo sistemos sukūrimas – II etapas“. This work was funded by the European Structural Fund, Nr. VP1-3.2.-ŠMM-02-V-03-001.

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POLIALKILENIMINŲ, JŲ DARINIŲ IR POLIAMIDOAMINŲ SINTEZĖ, TYRIMAS IR PRITAIKYMAS GENŲ PERNAŠAI

SANTRAUKA

Genų terapija – nauja sritis, kurioje plačiai pritaikomi ir sintetiniai katijoniniai polimerai. Tai perspektyvus metodas gydyti genetines ligas, vėžį ar AIDS. Pastaruoju metu domimasi ne tik DNR, bet ir specifinių RNR, dalyvaujančių genų raiškoje, panaudojimu genų terapijoje. Genų terapijos sėkmė priklauso nuo genų pernašos technologijos, todėl labai svarbu parinkti tinkamas ir geromis savybėmis pasižyminčias pernašos sistemas. Katijoninis polimeras turi atitikti tam tikrus reikalavimus, iš jų svarbiausi yra tinkama polimero molekulinė masė, makromolekulių krūvio tankis, polimero biosuderinamumas bei bioskalumas. Reguluojant polimero molekulinę masę bei krūvio tankį, galima keisti ne tik polimero toksiškumą ląstelėms, bet ir polimero-DNR/siRNR komplekso savybes. Didinant polimero hidrofiliškumą, jo biosuderinamumas dažnai padidėja, todėl genų pernašos vektoriai, turintys hidrofiliųjų hidroksi- ar oksi- grupių, yra itin perspektyvus. Kita vertus, bioskalus vektorius tuo pačiu bus ir biosuderinamas. Polimerinis vektorius bus bioskalus tuomet, jei jo pagrindinėje grandinėje bus bioskalių ryšių, tokių kaip esterinis, amidinis ar disulfidinis.

Pagrindinis šio darbo tikslas – susintetinti naujus polialkileniminų ir poliamidoaminų darinius – DNR ar siRNR pernašai tinkamus katijoninius polimerus, kurie būtų mažai toksiški ląstelėms ir bioskalūs ląstelių citoplazmoje.

Svarbiausi šio darbo rezultatai, atspindintys naujumą, originalumą ir svarbą:

Keičiant sintezės sąlygas, pirmą kartą polikondensacijos būdu susintetinti didelės molekulinės masės poli(etileniminheksametileniminai) ir poli(2-hidroksipropileniminai), turintys pirminių, antrinių ir tretinių aminogrupių. Atrinktos optimalios polialkileniminų sintezės sąlygos bei įvertintos produktų savybės.

Pirmą kartą išsamiai ištirta diaminoalkanų ir dibromalkanų polikondensacijos kinetika, naudojant izotermišką reakcijų kalorimetrą.

Pirmą kartą polikondensacijos būdu susintetinti modifikuoti poli(2-hidroksipropileniminai) su šoninėmis metoksipolietilenglikolio grandinėmis, o taip pat su disulfidiniais ryšiais pagrindinėje grandinėje.

Pirmą kartą susintetinti hidrofiliniai poliamidoaminai iš hidroksi- ir oksigrupių turinčių diaminų ir *N,N'*-metilenbisakrilamido. Atrinktos optimalios tokių poliamidoaminų sintezės sąlygos, gaunant transfekcijai tinkamos molekulinės masės ir savybių polimerus.

Nustatyta naujai susintetintų polimerų geba transfekuoti DNR/siRNR bei šių polimerų toksiškumas ir bioskalumas.

Poliniai aprotoniniai tirpikliai yra tinkamiausia terpė 1,6-heksametilendiamino ir 1,2-dibrometano, o taip pat 1,3-diamino-2-propanolio (DAP) ir 1,3-dibrom-2-propanolio (DBP) polikondensacijai. Tokiuose tirpikliuose pasiekiamas didžiausias polikondensacijos reakcijos greitis, reakcijos baigties laipsnis, susidaro didžiausios molekulinės masės polimerai.

Polikondensacijos būdu sintetinant polialkileniminius ir jų darinius, o taip pat poliadicijos būdu sintetinant poliamidoaminus su oksi- ir hidroksigrupėmis susidaro šakoti, bet tirpūs katijoniniai polimerai. Labiau šakoti polimerai susidaro polikondensaciją vykdant poliniuose aprotoniniuose tirpikliuose, naudojant baziškesnį diamino monomerą ir tais atvejais, kai monomerų reaguojančios funkcinės grupės mažiau nutolusios viena nuo kitos.

Ultrafiltravimo būdu gryninant polialkileniminius, jų darinius ir poliamidoaminus, iš jų pašalinamos mažamolekulės frakcijos, taip išskiriant didesnės molekulinės masės bei mažiau polidispersinius polimerus.

Metoksi-poli(etilenglikolio) (MPEG) grandinių turintys PHPI dariniai (PHPI-MPEG) susintetinti vykdant DAP polikondensaciją su DBP ir MPEG jodidais, kurių molekulinė masė 1000, 2000 ir 5000 Da. Parenkant MPEG jodido dozavimo į reakcijos mišinį laiką, galima reguliuoti MPEG kiekį PHPI darinyje, tokio darinio molekulinę masę bei išeigą. MPEG jodidą dedant reakcijos pradžioje, susidaro nedidelės molekulinės masės (8-53 kDa) polimerai, turintys virš 60 mol% oksietileno grandžių ir po 1-1,25 MPEG grandinę makromolekulėje. MPEG jodidą dedant baigiantis reakcijai, susidaro didelės molekulinės masės (140-160 kDa) polidispersiniai polimerai, turintys mažiau nei 9 mol% oksietileno grandžių ir po 0,09-0,22 MPEG grandinių makromolekulėje. PHPI-MPEG išeiga mažesnė nei 30 % ir mažai priklauso nuo MPEG molekulinės masės. MPEG grandinių ilgis PHPI-MPEG dariniuose neturi įtakos šių darinių efektyvumui transfekuoti DNR. Mažos molekulinės masės ir daug MPEG

grandinių turinčių PHPI darinių efektyvumas DNR transfekcijoje nusileidžia PHPI efektyvumui. Didelės molekulinės masės ir mažai MPEG grandinių turinčių PHPI darinių efektyvumas DNR transfekcijoje artimas PHPI efektyvumui. Didelės molekulinės masės PHPI-MPEG darinių efektyvumas siRNR transfekcijoje siekia 70 % ir gerokai viršija PHPI ir mažos molekulinės masės darinių efektyvumą. .

Disulfidinių ryšių pagrindinėje grandinėje turintys PHPI dariniai geba suirti ląstelių citozolyje, todėl jie mažiau toksiški už PHPI ir yra tinkami pakartotinei transfekcijai.

Didelės molekulinės masės ir mažai MPEG grandinių turintys PHPI-MPEG siRNR transfekuoja geriau už mažos molekulinės masės PHPI-MPEG darinius ar PHPI. Didelės molekulinės masės PHPI-MPEG efektyvumas siRNR transfekcijoje siekia 70 %.

Poliadicijos būdu iš *N,N'*-metilenbisakrilamido ir DAP bei 2,2'-(etilendioksi)diethylamino susintetinti oksid- ir hidroksigrupių turintys poliamidoaminai. Hidroksigrupių turintys poliamidoaminai yra bioskalūs, tačiau GFP geną į *HeLa* ląsteles transfekuoja ne taip efektyviai, kaip PHPI.