Bioinspired Water-soluble Polymers with Grafted Polyamine Chains: Synthesis and Complexation with Oligonucleotides

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Abstract The siliceous frustules of diatom algae contain complex proteins known as silaffins, which consist of a peptide chain with grafted polyamine chains. These polyamines contain twenty or more nitrogen atoms with trimethylene groups between the nitrogens. We synthesized a set of polymers containing grafted long-chain polyamine fragments by using acryloyl chloride (ACh) polymers and activated acrylic acid copolymers as the starting materials. The new polymers contained 0.05 mol%−3.2 mol% of polyamine chains, which corresponded to 0.06−3.56 mmol·g^{-1} amine groups. The new amine-containing polymers formed complexes with short (19-21-mer) deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) strands, and these complexes penetrated into model yeast cells and A549 lung cancer cell. This study demonstrates the potential of these species based on long-chain polyamines to serve as novel gene delivery systems.

Keywords Polyamine; Polymeric amine; Gene delivery; Acryloyl chloride

INTRODUCTION

Synthetic water-soluble polymers have been intensively studied as physiologically active substances and drug delivery agents[1−3]. The activity of the polymers depends on several factors, *i.e.*, the presence of functional groups, chemical nature, length, and branching of the main chain. Various combinations of these factors provide infinite possibilities for synthesizing targeted polymeric structures. The study of polymers in living organisms is a valuable source of inspiration for the design of new artificial polymers. At the end of the past century, interesting polymers were discovered in biogenic silica produced by diatom algae[4]. These polymers are known as silaffins and they consist of a peptide chain with grafted polyamine chains. The polyamines are derivatives of spermidine and contain over twenty nitrogen atoms with trimethylene groups between the nitrogens. Some of the nitrogens in the polyamine chains are methylated or quaternized depending on the diatom species[5]. The discovery of silaffins stimulated the synthesis and study of polymeric amines as model biomacromolecules with the objective to understand the physiological role of silaffins and to obtain new siliceous materials.

Synthetic polymeric amines are promising molecules for gene delivery applications as transfection vehicles[6−8]. These polymeric amines include polyethyleneimine and dendrimers such as poly(amidoamine) and poly(propyleneimine), polyspermine, poly-L-lysine, poly-L-ornithine, poly-L-arginine, poly(*N*-alkyl-4-vinylpyridinium) bromide, and poly(vinyl amine). The chains of the polymeric amines interact with nucleic acids and the obtained positively charged complexes are captured by living cells *via* endocytosis. A polymeric structure must satisfy several requirements to be effective for transfection applications. Specifically, the polymer must show low toxicity, which is unusual in the case of polymeric amines because positively charged macromolecules can interact with the negatively charged cell membrane and destroy it. Alternately, increased electrostatic interactions between the polymer chains and cell surface can cause non-specific internalization of the polymer in non-target cells, leading to undesirable effects. The polymer must form a stable complex with nucleic acids, but this complex must bedestabilized in the target cell, *e.g.*, the nucleic acid cargo must be released into the cytosol when the pH of the endosomes decreases. If the carrier fails to escape or release the oligonucleotide cargo into the cytosol, it will

remain trapped within the endosomal vesicle; the vesicle will be progressively acidified and will fuse with the lysosomes. The carrier with the oligonucleotides will then be degraded by the lysosomal enzymes, making the therapy ineffective. When transfection is used for therapeutic purposes, the polymer must protect the nucleic acid during transportation of the complex from the site of injection to the targeted cells. Short chain ribonucleic (RNA) oligomers (si-RNA, about 20 units) have been extensively investigated for anti-sense cancer therapy[9–11]. After penetration into the cancer cell, these oligonucleotides interact with a specific target messenger-RNA, which interferes with the translation process, thereby preventing protein synthesis by this RNA. This in turn can stop further division of the cancer cell. With the aim to satisfy the requirements of polymeric carriers for si-RNA delivery, various complex polymeric systems containing amine and neutral sequences, including stimuli-responsive polymeric assemblies have been investigated[12, 13].

Silaffins belong to a class of proteins, found in diatoms, that regulate silica deposition. It is presumed that silaffins act as carriers of siliceous nanoparticles in biosilicification[14]. Inspired by the structure of silaffin, we set out to design similar polymers as potential agents for gene delivery. Herein, we report the synthesis of long-chain oligo-propylamines (LCPAs) that simulate the side polyamine chains of silaffins[15]. These LCPAs contain two terminal secondary amine groups that can be used to attach LCPA fragments to the main polymer chain, whereas the other amine groups are fully methylated. The main chain of the new polymers consists of 1-vinylpyrrolidone or *N*,*N*-dimethylacrylamide units. Length of LCPAs chains is comparable with oligonucleotides and we can expect formation of aggregates containing oligonucleotides coordinated with side chains and hydrophilic main chain. Polymers containing amine groups in the main chain are usually applied as transfection agents but it was found that grafting of polyethyleneimine short chains on a neutral main chain resulted in very promising systems[16].

The structure of the obtained polymers is studied using Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy, as well as size exclusion chromatography. The interaction of the polymers with a model oligonucleotide is investigated using electrophoresis. Yeast cells (*Saccharomyces cerevisiae*) are employed as models to estimate the cytotoxicity of these new polymers and to assess the ability of the polymer-oligonucleotide complexes to be internalized in living cells.

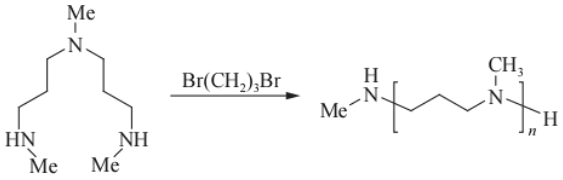
EXPERIMENTAL

Reagents

Diethyl ether, dichloromethane, dimethylamine (40 wt% in H_2O), triethylamine, *N*-hydroxysuccinimide, *N*-hydroxy-phthalimide, 1,4-dioxane, potassium hydroxide, potassium carbonate, dimethyl formamide (DMF), azobisiso-butyronitrile (AIBN), acryloyl chloride (ACh), 1-vinylpyrrolidone (VP), ethidium bromide, and fluorescein diacetate were of reagent grade (Sigma-Aldrich, Fisher, or Acros Chemicals). Dimethyl sulfoxide-d_6 (DMSO-d_6, 99.8 atom D%) and trifluoroacetic acid (TFA) (99 wt% purity) were purchased from Sigma-Aldrich. ACh and VP were distilled before polymerization. DMF was dried with anhydrous $CuSO_4$ and 3Å molecular sieves, followed by distillation. AIBN was recrystallized from ethanol.

A solution of dimethylamine in 1,4-dioxane (9.01 wt%) was prepared *via* saturation of dry 1,4-dioxane with gaseous dimethylamine. The amine concentration was determined from the resultant weight gain, as well as by using potentiometric titration. To obtain gaseous dimethylamine, a 40 wt% solution of $HN(CH_3)_2$ in water was added drop-wise to a large excess of potassium hydroxide flakes. The evolved gas was dried by passing through a glass tube filled with KOH flakes.

The LCPAs were obtained according to the reported method[15] by condensation of *N*,*N*-bis[3-(methylamino) propyl]methylamine with 1,3-dibromopropane, as shown in Scheme 1.



Scheme 1 Synthesis of LCPAs

The average number of nitrogen atoms in the LCPAs was 15.5 based on ^{1}H-NMR analysis, and the LCPA chains contained 4−30 nitrogen atoms based on mass spectral (MS) analysis.

The yeast *Saccharomyces cerevisiae*, parent-type strain W303-1B (*MATα ade2-1 his3-11, 15 trp1-1 leu2-3112 ura3-1 [rho$^+$]*) was procured from the collection of the Siberian Institute of Plant Physiology and Biochemistry (SIPPB SB RAS). Fluorescein 3′-tagged DNA oligonucleotide GATCTCATCAGGGTACTCCTT was purchased from Evrogen JSC (Russia).

A549 lung cancer cell line was procured from the National Center for Cell Sciences (NCCS), Pune. Dulbecco's modified eagles medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) and trypsin were purchased from Gibco, USA. The small interfering RNA (si-RNA) against vascular endothelial growth factor (VEGF) and Cyanine-3 fluorescent tagged si-RNA were purchased from Eurofins Genomics, USA. The sequences of the VEGF si-RNA with dTdT overhangs at 3′ in both strands used in the study are sense GGAGUACCCUGAUGAGAUC; anti-sense − CCUCAUGGGACUACUCUAG.

Equipment

^{1}H- and ^{13}C-NMR spectra were recorded on a DPX 400 Bruker instrument in $CDCl_3$ or DMSO-d_6 at 400 MHz for ^{1}H- and 100 MHz for ^{13}C-NMR. Infrared spectra were recorded on an Infralum FT-801 spectrophotometer (SIMEX, Russia) using KBr pellets. Potentiometry measurements were performed on a “Multitest” ionometer with a combined pH

electrode in a temperature-controlled cell at (20 ± 0.02) °C. The molecular mass of the new polymers based on poly(acryloyl chloride) (PACh) was estimated *via* size-exclusion chromatography (SEC) using a Milichrom A02 chromatograph (JSC Econova) with 2 × 75 mm column filled with SRT SEC-100 5 μm phase (Sepax Technologies, Inc.), operated at 35 °C using 150 mmol·L^{-1} phosphate buffer at pH = 7. The flow rate of the mobile phase was set at 0.03 mL·min^{-1} (pressure 0.69 MPa), whereas the injection volume for 1 g·L^{-1} of the sample solution was 2 μL. Fractionated samples of poly(vinyl formamide)[17] were applied as standards (M_w/M_n < 1.5). Electrophoresis experiments were performed with a Mini-Sub (7 × 10 cm) Cell GT System (Bio-Rad Laboratories, Inc.) with an ELF-4 power supply (DNA-Technology LLC) and TCP-20.LC transilluminator (Vilber Lourmat), operated at 254 nm. A Motic AE-31T microscope equipped with a fluorescence attachment and Moticam Pro 205A camera was used for visual observation of theyeast cells.

Synthesis of Initial Activated Polymers

Poly(acryloyl chloride) (PACh) was synthesized by a method similar to that of earlier reports[18] *via* radical polymerization of the monomer. Briefly, AIBN (0.2325 g, 1.416 mmol) was dissolved in 9.30 g (0.103 mol) of acryloyl chloride. The solution was diluted with 39.09 g of dry 1,4-dioxane and portioned out into six pre-weighed screw-cap glass bottles, followed by purging with argon, hermetization, and incubation at 60 °C for 72 h. The resulting solutions were stored at −20 °C. To estimate the yield and degree of polymerization of PACh, the reaction mixture was poured into water (50 mL) and dialyzed against water. After freeze drying, poly(acrylic acid) was obtained in 89% yield. Based on viscometry data[19], the degree of polymerization of the poly(acrylic acid) and correspondingly, of PACh, was 220.

Poly(*N*,*N*-dimethylacrylamide-*co*-*N*-acryloxysuccinimide) (ZS-243) was obtained by using PACh precipitated from 1,4-dioxane with cyclohexane.

Three initial solutions were prepared:

Solution A. All of the polymer prepared from 17.73 mmol of acryloyl chloride was dissolved in 26.46 g of dry DMF.

Solution B. Triethylamine (1.26 g, 12.4 mmol), 9.01 wt% dimethylamine solution in 1,4-dioxane (6.21 g, corresponding to 12.41 mmol of $HN(CH_3)_2$), and 4.90 g of DMF were combined.

Solution C. *N*-hydroxysuccinimide (0.816 g, 7.09 mmol) and triethylamine (0.77 g, 7.6 mmol) were dissolved in 5.24 g of DMF.

Solution A was magnetically stirred and cooled under flowing tap water (14 °C) and solution B was then added. Ten minutes after the cooling process was stopped, the mixture was left at room temperature for 140 min. Solution C was then added to the mixture in one batch. The resulting mixture was left at room temperature overnight. The following day, the formed precipitate was filtered off and the filtrate was concentrated under vacuum. The addition of 50 mL of dry ether precipitated a viscous mass, which was washed with 20 mL of ether, dissolved in 10 mL of dichloromethane, and precipitated by adding 60 mL of ether. The obtained solid was redissolved in 10 mL of dichloromethane. The solution was centrifuged (4000 g, 10 min), followed by vacuum evaporation of the supernatant to obtain 1.774 g oflight-brown solid (ZS-243).

Poly(*N*,*N*-dimethylacrylamide-*co*-*N*-acryloxyphthalimide) (ZS-267-1, ZS-244-1) was obtained using PACh precipitated from 1,4-dioxane with cyclohexane.

Three initial solutions were prepared:

Solution A. All of the polymer obtained from 31.13 mmol of acryloyl chloride was dissolved in 43 g of dry DMF.

Solution B. Triethylamine (2.18 g, 21.5 mmol), 9.01 wt% dimethylamine solution in 1,4-dioxane (10.76 g, corresponding to 21.5 mmol of $HN(CH_3)_2$), and 4.47 g of DMF were combined.

Solution C. *N*-hydroxyphthalimide (2.04 g, 12.5 mmol) and triethylamine (1.62 g, 16.0 mmol) were dissolved in 8.76 g of DMF.

Solution A was magnetically stirred and cooled under flowing tap water (14 °C); solution B was then added. Ten minutes after the cooling process was stopped, the mixture was left at room temperature for 140 min, followed by the addition of solution C. The resulting mixture was left at room temperature overnight. The formed precipitate was then filtered off, and the filtrate was evaporated to ~14 g under vacuum. The addition of 100 mL of dry ether precipitated a viscous mass, which was washed with ether (2 × 35 mL) andreprecipitated twice from dichloromethane with ether. Vacuum drying at room temperature gave 2.760 g of light-brown solid (ZS-267-1). Copolymer ZS-244-1 was prepared analogously to ZS-267-1.

Poly(1-vinylpyrrolidone-*co*-acryloyl chloride) (VP-ACh) samples were obtained by a method similar to that of earlier reports[20] by radical polymerization indioxane (12 g of 25% solution) in the presence of AIBN (2% relative to the monomer mass) at 60 °C under argon atmosphere. The reaction was carried out in hermetically sealed 30 mL vials for 18 h. The resulting solutions were stored at −20 °C. The composition of the copolymers was estimated *via* FTIR spectroscopy after hydrolysis of 1 mL of the reaction mixture in water (20 mL), dialysis against water through a cellophane membrane (4 kDa cutoff), and freeze-drying. The obtained samples were dissolved in water (2 g·L^{-1}), the pH was adjusted to 10, and the solutions were freeze-dried. Calibration mixtures were obtained by mixing and freeze-drying 20 g·L^{-1} solutions of poly(1-vinylpyrrolidone) and the sodium salt of poly(acrylic acid) prepared by hydrolysis of PACh. The characteristic $\nu_{C=O}$ (−COO^-, 1570 cm^{-1}) and ν_{CN} (1288 cm^{-1}) FTIR absorptions were used to confirm product formation. The monomer loading and copolymer composition used herein are presented in Table 1. The yield of the copolymers was 35%−40%.

Table 1 Synthesis of VP-ACh copolymers

Copolymer	Monomer mixture (g)		Copolymer composition (mol%)	
	VP	ACh	VP	ACh
D-22p	2.9610	0.0297	95.2	4.8
D-20p	2.8973	0.0503	89.2	10.8
D-21p	2.8760	0.1067	88.9	11.1

Synthesis of Polymers with LCPA Chains

Reaction ofLCPAs with PACh

After ACh polymerization (described above), the reaction mixture was precipitated into cyclohexane (20 mL), centrifuged (30 min, 4000 g), and washed with cyclohexane. The obtained PACh was dissolved in dry DMF. The LCPAs were added to 9.01 wt% dimethylamine solution indioxane and both solutions (PACh and LCPAs) were cooled to 0 °C. The LCPA solution was then promptly added to the PACh solution and shaken at −4 °C to 0 °C for 1 h. In the case of the PV16PO-1 and PV16PO-2 samples, the second portion of LCPA solution was added 30 min after commencement of the reaction. The mixture was poured into water (85 mL) and purified by dialysis (4 kDa cutoff). The obtained aqueous solution was filtered off (0.45 μm) and freeze-dried. The reagent loading and copolymer yields are presented in Table 2.

Table 2 Amounts of the key reagents and yields in the reaction of LCPAs with PACh

Sample	Composition of the reaction mixture (g)			Yield (g)
	PACh	LCPAs	$(CH_3)_2NH$	
PV16PO-0	1.175	–	0.7220	0.9764
PV16PO-1	1.264	0.6406	0.5048 0.2508	1.3078
PV16PO-2	1.264	0.6380	0.3612 0.3852	1.3459
PV16PO-3	1.344	0.6820	0.7993	1.3361
PV16PO-5	1.246	0.9462	0.7378	0.9695
PV16PO-6	1.540	0.3901	0.9138	1.1556

Grafting ofoligo(N-methyltrimethylene imine) onto poly(N,N-dimethylacrylamide-co-N-acryloxyphthalimide) and poly(N,N-dimethylacrylamide-co-N-acryloxysuccinimide)

A solution of 0.80414 g of oligo(*N*-methyltrimethylene imine) (ZS-188-1) in 11.93 g of dry 1,4-dioxane was mixed with 2.76 g of poly(*N*,*N*-dimethylacrylamide-*co*-*N*-acryloxy-phthalimide) (ZS-267-1) in 18.70 g of DMF under argon. The reaction vessel was left at room temperature for about 15 h, followed by heating (60 °C) and stirring with a magnetic stirrer for 8.5 h. Subsequently, 2.53 g of 9.01 wt% dimethylamine solution in 1,4-dioxane was added to the mixture, which was then cooled to room temperature. After about 15 h, the vessel was heated to 60 °C. After 6 h, a new portion of the dimethylamine solution (2.02 g) was added to the mixture and heating was continued for 2.5 h. The mixture was then diluted with ~15−20 mL of distilled water and dialyzed against distilled water using a cellulose bag until the conductivity of the wastewater fell to 5.5 $\mu S \cdot cm^{-1}$. The resultant solution was concentrated by rotary evaporation, and separated from the suspended matter by centrifugation (15000 g, 40 min) and membrane syringe filtration (0.45 μm pores); the solution was then freeze-dried to obtain 1.756 g of an off-white to pale yellow fluffy material (ZS-268).

Copolymers ZS-247 and ZS-248 were prepared analogously to ZS-268 under the conditions listed in Table 3.

Table 3 Amounts of the key reagents and yields in synthesis of poly[*N*,*N*-dimethylacrylamide-*co*-*α*-acryloyl oligo(*N*-methyltri-methylene imine)] (ZS-247 and ZS-248)

	Loading of the reagents (g)	
	ZS-247	ZS-248
LCPAs	0.4084	0.4238
Poly(*N*,*N*-dimethylacrylamide-*co*-*N*-acryloxysuccinimide) (ZS-243-1)	1.3884	–
Poly(*N*,*N*-dimethylacrylamide-*co*-*N*-acryloxyphthalimide) (ZS-244-1)	–	1.4593
Yield of grafted copolymer	0.7063	0.9841

Grafting ofLCPAs onto VP-ACh copolymers

The previously obtained solutions of VP-ACh copolymer in dioxane were precipitated in cyclohexane (35 mL) in a 50-mL centrifuge tube. The polymer was separated by centrifugation (5 min, 4500 g) and washed with a fresh portion of cyclohexane. The copolymer was dissolved in DMF (10 mL), cooled in an ice/water bath, and the LCPAs in 10 mL of a DMF-CH_2Cl_2 (1:1, *V*:*V*) mixture were added. The solution was shaken for 30 min at room temperature and a solution of diethylamine in DMF (0.3 mL) was added. After 30 min of shaking, the solution was poured into water (60 mL) and purified by dialysis (4 kDa cutoff). The obtained aqueous solution was filtered off (0.45 μm) and freeze-dried. The reagent loading and copolymer yields are presented in Table 4.

Determination ofthe copolymer composition

The percentage of grafted LCPA chains was determined from the ^{13}C-NMR spectra by using the signals at 55.1 ppm (−$\underline{C}H_2$−N(Me) −$\underline{C}H_2$−) and 174.7 ppm (C=O) by taking into account the average length of the LCPA chains (15.5). In the case of a low content of LCPAs (< 1 mol%), the sensitivity of ^{13}C-NMR was not sufficient for detection of the polyamine chains and we used the polymer derivatization with trifluoroacetic acid (TFA) according to the method reported earlier[21], followed by ^{1}H-NMR determination of the terminal −N(Me)H_2^+ groups. Typically, 500 μL ofTFA was added to *ca.* 30 mg of polymer in a glass screw-cap flat-bottom vial. The vessel was heated to about 50 °C and occasionally shaken for about 30 min to ensure complete dissolution. The excess TFA was then removed at 50 °C by purging with argon. The cooled residue was mixed with 600 μL of DMSO-d_6 and left overnight at room temperature. The resultant solution was filtered through a cotton pad into a

Table 4 Grafting of LCPAs onto VP-ACh copolymers

Copolymer	Initial copolymer	Loading ofLCPAs (g)	Loading of diethylamine (g)	Yield (g)
D-22p-2-O	D-22p	0.1155	0.0436	1.74
D-22p-3-O	D-22p	0.5340	0.0481	1.85
D-20p-4-O	D-20p	0.0936	0.0840	1.42
D-20p-5-O	D-20p	0.4328	0.0780	1.77
D-20p-6-O	D-20p	0.2212	0.0492	1.58
D-21p-8-O	D-21p	0.1790	0.1632	0.56

1 mL of polypropylene pipette tip and directly into a NMR ampoule. Details of the measurements are described in the electronic supplementary information (ESI). The TFA-based method was compared for three samples using ^{13}C-NMR and the deviation in the content of the LCPAs was found to be about 10%.

Synthesis and electrophoresis of oligonucleotide complexes with polymers and composite nanoparticles

The interaction between 21-mer DNA oligonucleotide GATCTCATCAGGGTACTCCTT-6-FAM and the synthesized polymers was investigated by electrophoresis on agarose gel. Complexes were prepared by mixing solutions of the polymer and oligonucleotide. The samples were incubated at room temperature for 30 min and placed into the wells of the 1% agarose gel. Free oligonucleotide, as a control, was also loaded onto the gel. The gel running buffer was 40 mmol·L^{-1} Tris acetate (adjusted to pH 7.4) and 1 mmol·L^{-1} EDTA. A glycerol gel loading buffer was used (0.5% sodium dodecyl sulfate, 0.1 mol·L^{-1} EDTA (pH = 8), and 50% glycerol for 10× reagent). The gel was run at 90 V and the fluorescein-tagged oligonucleotide was visualized on a UV transilluminator.

Study of cytotoxicity and gene delivery with model yeast culture

The cells of *S. cerevisiae* were maintained at 30 °C on YEPD medium (0.5% yeast extract, 1% peptone, 2% glucose). The cells were grown at 30 °C in 10 mL plastic vials with 2 mL of liquid YEPD. Cells in the logarithmic or stationary growth phase were used in the experiments. The cytotoxicity of the polymers and polymer-oligonucleotide complexes was studied after 24 h of cultivation of the yeast cells in the presence of the polymer sample. Acetyl fluorescein and ethidium bromide were used to visualize vital and dead cells, respectively, according to the method described elsewhere[22, 23]. The dyes were added to the culture 20 min before observation; 50 mg·L^{-1} of acetyl fluorescein and 25 mg·L^{-1} of ethidium bromide were used for staining. Penetration of the model DNA oligonucleotide (fluorescein 3'-tagged GATCTCATCAGGGTACTCCTT)-polymer complex into the yeast cells was monitored by following the emission of fluorescein at 470 nm for 5 h after addition of the complex to the cell culture. The experiments performed with 24 and 48 h cultures did not show any considerable differences relative to the 5 hdata.

Study of cell viability and polyplex internalization with A549 lung cancer cells

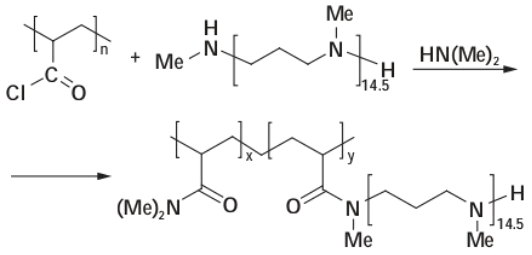
Polymer and si-RNA complexes were prepared by mixing aqueous solutions of polymer and si-RNA in different ratios. The resulting mixture was vortexed and incubated for 30 min at ambient temperature. Toxicity of the polymer and polyplex was evaluated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay (Cell Titer 96 Aqueous one solution, Promega, USA). Four thousand A549 cells per well were seeded in a 96-well plate and incubated at 37 °C in 5% CO_2. After the cells achieved confluency, the cells were washed with PBS to remove the non-adherent cells and the medium was replaced with serum-free medium. The polyplexes were then added to the serum-free medium and the final si-RNA concentration in each well was 0.1 μmol·L^{-1}. After 4 h, the medium was replaced with the fresh medium and incubated for 24 h or 48 h. The medium was then removed followed by the addition of MTS reagent (20 μL) and 200 μL of serum-free media to each sample well and incubated at 37 °C for 2 h. The reaction was stopped by addition of 25 μL of 10% sodium dodecyl sulfate (SDS) solution. The absorbance was measured at 490 nm using multimode reader (Biotek, Epoch 2, USA).

Internalization of the polyplex in A549 cells was evaluated using fluorescent si-RNA[24]. A549 cells were cultured on a cover slip in a 6-well plate with a seeding density 10^5 cells/well. After the cells attained confluency, the medium was removed and washed with PBS to remove the non-adherent cells. The polyplexes were added to the cells (final si-RNA concentration was 0.1 μmol·L^{-1}) in serum-free conditions and the cells were incubated for 4 h or 12 h. After the incubation time, the serum-free medium was removed and replaced with fresh medium. The cells were stained with the nuclear stain Hoechst (λ_{ex} = 405 nm and λ_{em} = 430−505 nm) and the images were captured using laser scanning confocal microscopy (FV1000, Olympus, Tokyo, Japan). The fluorescent si-RNA was imaged at λ_{ex} = 550 nm and λ_{em} = 560−650 nm.

RESULTS AND DISCUSSION

New polymers were obtained by three strategies:

i. Reaction of PACh with LCPAs in the presence of dimethylamine, which prevents cross-linking of the polymer by the bifunctional LCPAs molecules and introduces non-ionizable *N*,*N*-dimethylacrylamide units into the polymeric chain (Scheme 2).

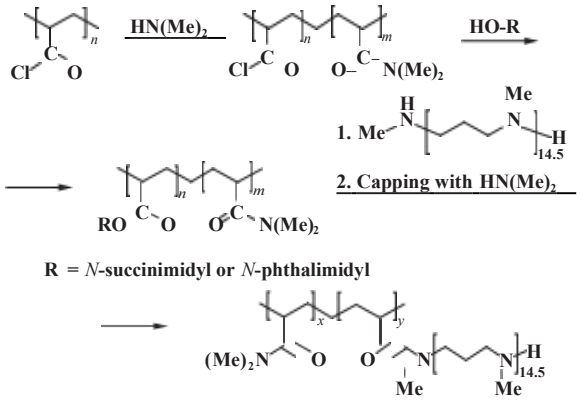


Scheme 2 LCPAs grafting to PACh

This reaction proceeds readily at moderate temperature and allows the engrafting of various structures into the polymeric chain. However, PACh is easily hydrolysable, which complicates manipulation of this polymer, including purification from the monomer or fractionation.

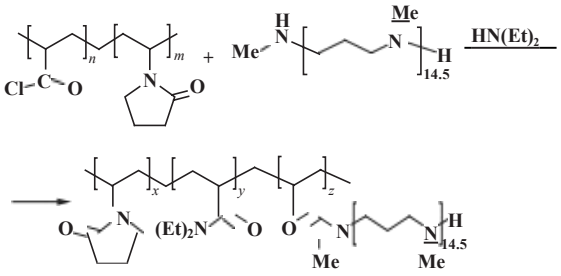
ii. Preliminary reaction of PACh with dimethylamine and *N*-hydroxysuccinimide or *N*-hydroxyphthalimide, which results in a polymer bearing activated units of acrylic acid. However, these units are not as sensitive as acryloyl chloride. The activated polymer can be processed in the presence of atmospheric moisture, which allows various manipulations with this intermediate, such as fractionation. The next stage of modification involves the reaction of the activated polymer with the LCPAs and capping of the

remaining activated units with dimethylamine (Scheme 3).



Scheme 3 LCPAs grafting to activated poly(acrylic acid)

iii. Copolymerization of ACh with 1-vinylpyrrolidone followed by grafting with the LCPAs is another route for generating the LCPA-containing copolymers (Scheme 4).



Scheme 4 Copolymerization of ACh with 1-vinylpyrrolidone

The structure of the polymers was confirmed by NMR analyses (Figs. 1 and 2, Figs. S1−S4 in ESI) and infrared (IR) (Fig. 3). The composition of the new polymers is presented in Table 5. The content of LCPA units varied from 0.05 mol% to 3.2 mol%, corresponding to a concentration of 0.06−3.56 mmol·g^{-1} of amine groups. The degree of polymerization of the initial PACh was 220 based on the viscometry data obtained after PACh hydrolysis. Grafting of the LCPA chains onto the PACh chain or activated esters of poly(acrylic acid) could be complicated by inter- or intra-molecular cross-linking because the LCPA molecules have two active —NH groups. The position and shape of the SEC profiles of the obtained polymers (Fig. 4) were similar to those of the PV16PO-0 sample, which was obtained by the reaction of PACh with excess dimethylamine. Some samples showed a shoulder in the region corresponding to a low degree of polymerization, which can be attributed to the presence of an admixture of a fraction of intra-molecularly cross-linked macromolecules. The molecular mass of the polymers based on 1-vinylpyrrolidone was estimated by viscometry using the parameters for poly(1-vinylpyrrolidone)[25]. The obtained value of 500 kDa (degree of polymerization: 4500) is close to the data obtained for poly(1-vinylpyrrolidone) under the same conditions.

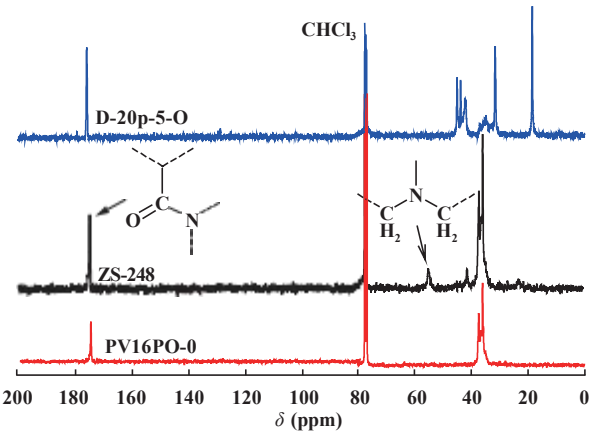
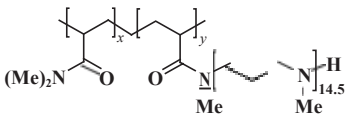
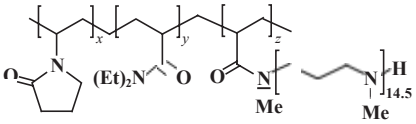


Fig. 1 ^{13}C-NMR spectra of PV16PO-0, ZS-248, and D-20p-5-O

The ability of the new polymers to form complexes with a model 21-mer DNA oligonucleotide was studied using electrophoresis (Fig. 5). Increasing the content of LCPAs in the polymer resulted in a decrease in the amount of uncomplexed DNA, and in the case of the PV16PO-1, PV16PO-5, and ZS-268 samples, a positively charged

Table 5 Structure and composition of the new polymers with grafted polyamine chains

Sample	Structure	Composition (mol%)	
		y	*z*
PV16PO-0		0	–
PV16PO-1		1.85	–
PV16PO-2		1.59	–
PV16PO-3		1.75	–
PV16PO-5		2.36	–
PV16PO-6		1.46	–
ZS-247		1.59	–
ZS-248		3.05	–
ZS-268		3.24	–
D-22p-2-O		4.80	0.19
D-22p-3-O		4.80	0.06
D-20p-4-O		10.80	0.12
D-20p-5-O		10.80	0.26
D-20p-6-O		10.80	0.22
D-21p-8-O		11.10	0.05

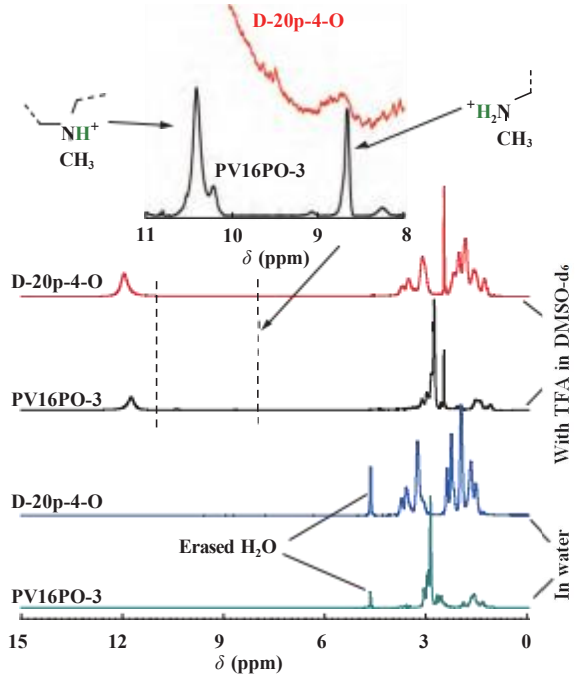


Fig. 2 ^{1}H-NMR spectra of polymer samples in D_2O (pure) and DMSO-d_6 (derivatized with TFA)

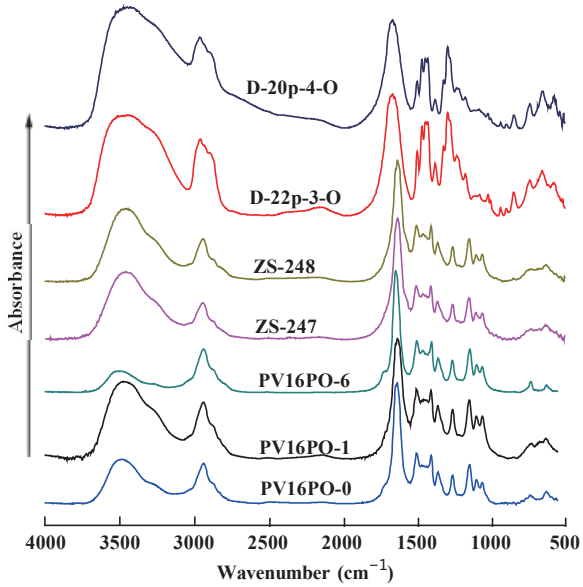


Fig. 3 Representative FTIR spectra of the new polymers. The spectra contain bands of amine groups (1150, 1100, 1050 cm^{-1}, $\nu_{C=N}$), amide (1635−1660 cm^{-1}, $\nu_{C=O}$), methyl, and methylene groups (1460 cm^{-1}, δ_a; 2790−3000 cm^{-1}, ν)[26, 27]

polymer-DNA complex was formed. The D-20p-4-O polymer contained 0.12 mol% of LCPA units only and neutral complexes were formed at high polymer concentrations (Fig. 5, lanes 11 and 12).

S. cerevisiae yeast cells were applied to estimate toxicity and ability of the new polymers to help oligonucleotide penetration into cells. Incubation of the yeast cells with the polymer-DNA complexes indicated DNA penetration into the cells in the presence of the LCPA-containing polymers (Figs. S5A−S5H in ESI). The cytotoxicity of the polymers

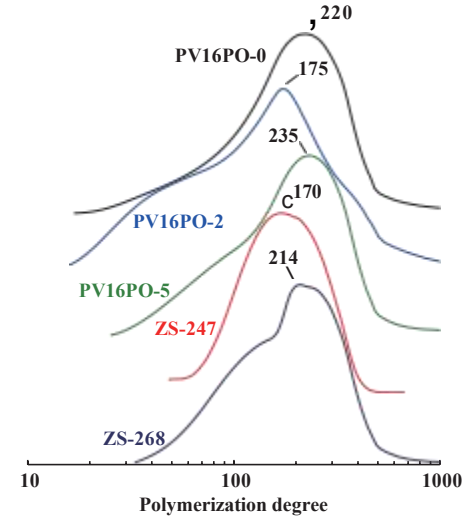


Fig. 4 Representative SEC data for new polymers obtained on the basis of PACh

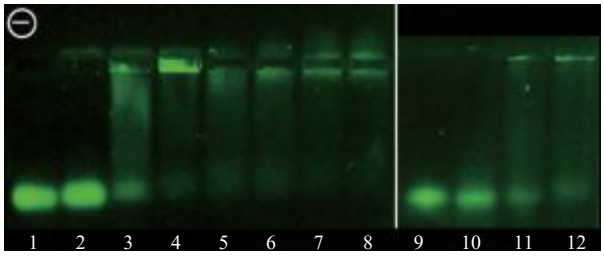


Fig. 5 Gel electrophoresis data for DNA oligonucleotide complex with new polymers. Lane 1: free oligonucleotide, 2: D-21p-8-O, 3 and 9−12: D-20p-4-O, 4: D-20p-5-O, 5: PV16PO-6, 6: PV16PO-1, 7: PV16PO-5, 8: ZS-268. The concentration of the oligonucleotide was 10 μmol·L^{-1} and that of the polymers was 4 g·L^{-1} (lanes 2−8 and 12), 0.5, 1, and 2 g·L^{-1} for lanes 9−11, correspondingly. The polymer:oligonucleotide ratio (*V*:*V*) was 2:1. The gel was run for 20 min.

and polymer-oligonucleotide complexes was studied with acetyl fluorescein and ethidium bromide according to the protocols reported earlier[22, 23]. Non-fluorescent acetyl fluorescein is hydrolyzed by the action of enzymes in vital cells, giving rise to fluorescein, which shows a characteristic emission (Figs. S5I and S5J in ESI). Ethidium bromide penetrates through the membranes of dead cells and stains the DNA orange (Figs. S5K and S5L in ESI). The polymers with a high LCPA content showed signs of cytotoxicity after 24 hof incubation (Figs. S5M and S5N inESI),whereas the corresponding complexes with DNA were found to be non-toxic (Fig. S5P in ESI) and the cell death was found to be comparable to that of the control. The change in the charge upon complexation may be the main factor for mitigation of the cytotoxicity of the cationic polymer.

Experiments with mammalian cells were carried out using A549 lung cancer cells. Cytotoxicity was tested using MTS cell viability test and the toxicity of the polymer and polyplex was evaluated using MTS assay for 24 and 48 h, respectively (Fig. 6). The results show that the synthesized polymer and polyplex are non-toxic *in vitro* such that no significant reduction in cell viability was observed. The

polyplexes formed by ZS-247, ZS-248 and PV16PO-1 show about 20% reduction in cell viability after 48 hof treatment while the polyplexes formed by D-20P-40 polymer did not exhibit any cytotoxicity.

The cell internalization of si-RNA complexed with the different polymers was investigated for a period of 4 and 12 h after treatment A549 cells using fluorescent si-RNA (Figs. 7 and 8). The images captured using laser scanning confocal microscopy are shown in Figs. 7 and 8.

Among the polyplexes investigated, ZS-247 si-RNA shows good internalization when compared to free si-RNA and other polyplexes after 4 h of treatment. PV16PO-1 and D-20P-40 also showed internalization in to A549 cells but no internalization was discernible after 4 h in cells treated with ZS-248 polyplexes. All the polyplexes as well as free si-RNA showed internalization in the cytosolic compartment and not in the nucleus.

Fig. 8 shows the internalization of the polyplexes after 12 h. The free si-RNA treated cells showed faint intensity while the ZS-247 polyplex showed the strongest intensity after 12 h. The polyplex was localized in the cytosol which augers well for gene therapy as the site of action of si-RNA

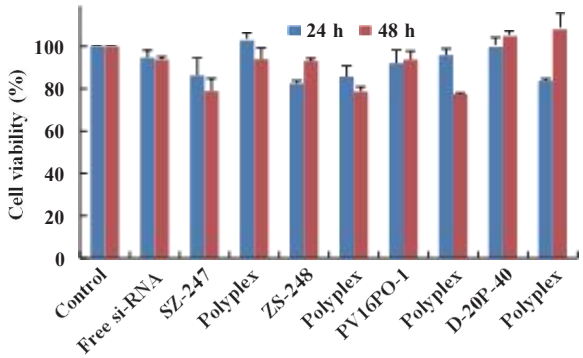


Fig. 6 Cell viability of A549 cancer cells after treatment with different carrier systems both independently as well as after complexation with anti-VEGF si-RNA

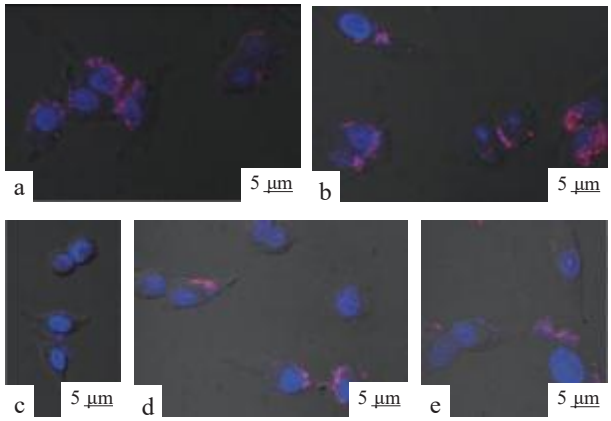


Fig. 7 Laser scanning confocal images of A549 cells 4 h after treatment with polyplexes containing fluorescent si-RNA: (a) free si-RNA, (b–e) polyplexes: (b) ZS-247, (c) ZS-248, (d) PV16PO-1, and (e) D-20P-40. Polymer concentration: (b–d) 4 mg·mL^{-1}, (e) 8 mg·mL^{-1}, si-RNA concentration is 10 μmol·L^{-1}. Polymer:si-RNA ratio: (b–d) 4:1, and (e) 8:1. Blue fluorescence: Hoechst stained DNA in cancer cells; red fluorescence: cyanine-3 tagged si-RNA

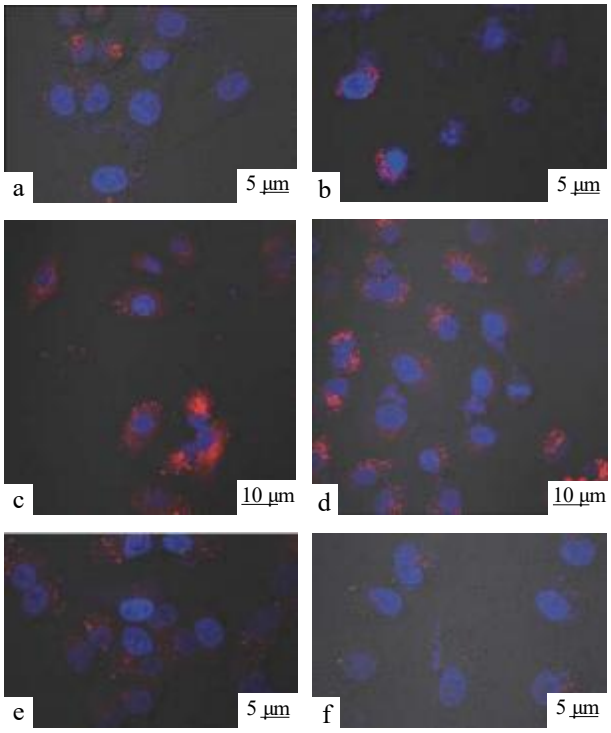


Fig. 8 Laser scanning confocal images of A549 12 h after treatment with polyplexes containing fluorescent si-RNA: (a) free-siRNA, (b–f) polyplexes: (b) ZS-248, (c) ZS-247, (d) ZS-248, (e) PV16PO-1, and (f) D-20P-40. Polymer concentration: (b–e) 4 mg·mL^{-1}, and (f) 8 mg·mL^{-1}; si-RNA concentration is 10 μmol·L^{-1}. Polymer: si-RNA ratio: (b–e) 4:1, and (f) 8:1. Blue fluorescence: Hoechst stained DNA in cancer cells, red fluorescence: Cyanine-3 tagged si-RNA

is the cytosol. The polyplex formed from D-20P-40 showed only a faint intensity which is much lower than the intensity observed after 4 h, indicating that this polyplex is degraded or eliminated from the cells with 12 h. This may explain the lack of cytotoxicity for the polyplex formed by this polymer after 48 h of treatment. The polyplex ZS-248 shows better internalization in the ratio of 8:1 when compared to the complex at 4:1 ratio, indicating that higher polymer concentration resulted in better encapsulation efficiency. The polyplex formed by PV16PO-1 did not show very high fluorescence, indicating its limited lifetime in the intracellular milieu. Considering together the results from the cell viability and cell uptake experiments, the polyplex formed by ZS-247 appears to show promise for gene therapy applications. Its anti-cancer efficacy may further be improved when used in combination with chemotherapy.

CONCLUSIONS

A set of polymers containing grafted long-chain polyamine fragments on the acryloyl chloride polymer and acryloyl chloride copolymers with 1-vinylpyrrolidone were synthesized successfully. The new polymers contained 0.05 mol%–3.2 mol% of polyamine chains, corresponding to 0.06–3.56 mmol·g^{-1} of amine groups. The polymers based on poly(acryloyl chloride) retained the same degree of polymerization as the initial polymer (near 170–240). The

molecular mass of the 1-vinylpyrrolidone-based polymers was 500 kDa (polymerization degree: 4500). The new amine-containing polymers can form complexes with short (19-21-mer) DNA and RNA oligonucleotides. These complexes were internalized in model yeast cells and A549 lung cancer cells, suggesting their suitability for gene delivery applications.

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