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Poly(vinyl amine) as a matrix for a new class of polymers

<https://doi.org/10.1515/epoly-2018-0024>

Received February 3, 2018; accepted March 16, 2018; previously published online April 30, 2018

Abstract: Poly(vinyl amine) was utilized as a matrix for the synthesis of polymers bearing short polyamine chains (1–3 amine groups) grafted to the main macromolecular chain with long (eight atoms) spacers. The new polymers were characterized with nuclear magnetic resonance (NMR), Fourier transform infrared (FTIR) spectra, size exclusion chromatography and potentiometry. Poly(vinyl amine) was applied in the form of narrow molecular mass fractions and the modification proceeded without destruction of the main chain. Condensation of silicic acid in the presence of the polymeric amines gives rise to composite nanoparticles which are stable in aqueous medium. New polymers and composite nanoparticles effectively complex with DNA and RNA oligonucleotides and were found to display good internalization in cancer cells which indicates their promise towards gene delivery applications.

Keywords: acrylamide addition; gene delivery; nucleic acid; polyamine; poly(vinyl amine).

1 Introduction

Polymers are widely used in various areas of technology and in everyday life. The properties of polymers depend not only on their chemical structure but also on their chain length (1, 2). The other important parameter is polydispersity which is a measure of the distribution of the chain length in a given polymer sample. Polymers with

low polydispersity are highly desirable because of their optimal physical properties. Polydispersity is an important parameter for designing the biomedical applications of such polymers. The physiological activity of water-soluble polymers has been found to depend on the length of the polymer chain (2, 3) and there exists an optimal molecular weight for each polymeric system which corresponds to the highest activity. The presence of other polymer fractions, which are not equally active, decreases the activity of the whole sample and can cause undesirable side effects when the polymer is employed for therapeutic applications.

Decrease of the polymer polydispersity index (PDI) is possible during the synthesis stage by using living or pseudo-living polymerization (4, 5). These approaches result in polymers with a low PDI but the catalysts (initiators) employed are often expensive and the molecular weight of the samples cannot be regulated over a wide range. The other approach to obtaining polymers of low PDI includes the synthesis of a polydisperse sample, e.g. by radical polymerization, followed by fractionation. This strategy allows one to obtain a set of narrow polymeric fractions of various molecular weights. Modification of the existing polymers is a convenient way to obtain new polymeric structures and when the modification reaction is carried out under moderate conditions, the resulting polymer retains the initial polymerization degree and PDI.

The present work was aimed at utilization of poly(vinyl amine) (PVA) as a matrix for the synthesis of new polymers. PVA is easily produced from poly(vinyl formamide) (PVFA), which is available as narrow molecular weight fractions (6). The addition reaction of acrylamide double bond to the amine group was employed for PVA modification. We used acrylamide derivatives bearing short-chain polyamines as substituents (Scheme 1).

The remaining primary and secondary amine groups were converted to amide units by the reaction with acetic anhydride (Scheme 2).

The obtained polymers were characterized with ^1H and ^{13}C nuclear magnetic resonance (NMR), Fourier transform infrared (FTIR) spectroscopy, size exclusion chromatography and potentiometry in an aqueous solution.

Amine-containing polymers have immense potential as gene delivery systems due to their ability to complex anionic oligonucleotides through electrostatic interactions

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Scheme 1: Addition of acrylamide derivatives to PVA chain.

Scheme 2: Capping of primary and secondary amine groups with acetic anhydride.

(7–9). The conventionally employed gene delivery systems are limited by several drawbacks that have necessitated the search for novel systems to complex the therapeutic oligonucleotide. A promising way to design delivery systems with highly tunable complexing properties is the formation of composite nanoparticles based on polymeric amine and oligomeric silicates (10, 11). We have obtained soluble composite nanoparticles through silicic acid condensation in the presence of new polymers.

Any candidate for gene delivery application should demonstrate a high complication with the oligonucleotides as well as internalize rapidly into the target cells. The electrophoresis data obtained in the present work reveals that the new polymers and organo-silica nanocomposites are capable of interacting with DNA and RNA efficiently. Cell internalization studies were also performed using cancer cells to demonstrate the ability of RNA complexes to localize into cancer cells. The data suggest that these novel systems can be explored further for gene delivery applications.

2 Experimental

2.1 General

FTIR spectra were recorded with an Infracum FT-801 spectrometer (JSC Simex, Novosibirsk, Russian

Federation) using KBr pellets or films on a ZnSe plate. ^1H and ^{13}C NMR spectra were obtained using a Bruker DPX 400 spectrometer (Bruker Biospin Corporation, Billerica, MA, USA, 400.13 and 100.61 MHz, respectively) in deuterodimethylsulfoxide- d_6 (DMSO- d_6), CDCl_3 or CCl_4 (Merck KGaA, Darmstadt, Germany). Potentiometry measurements were performed on a “Multitest” ionometer (JSC Semico, Novosibirsk, Russian Federation) using a combined pH-electrode in a temperature controlled cell at $20 \pm 0.02^\circ\text{C}$. The molecular mass of the new polymers was estimated via size-exclusion chromatography (SEC) using a Milichrom A02 chromatograph (JSC Econova, Novosibirsk, Russian Federation) with 2×75 mm column filled with SRT SEC-100 5 μm phase (Sepax Technologies, Inc., Newark, DE, USA), operated at 35°C using 10:90 methanol:water solution of TFA, pH 2.5 (methanol and trifluoroacetic acid from Thermo Fisher Scientific, Waltham, MA, USA). The flow rate of the mobile phase was set at 0.03 ml min^{-1} (pressure 100 psi), whereas the injection volume for 1 g L^{-1} of the sample solution was $1 \mu\text{L}$. Fractionated samples of poly(vinyl formamide) (PVFA) (6) were applied as standards ($M_w/M_n < 1.5$). Zeta-potential (ζ) and particle size were measured with a Zetasizer Nano-ZS ZEN3600 (Malvern Instruments Ltd., Worcestershire, UK).

2.2 Reagents

PVA fractions were obtained by alkaline hydrolysis of fractionated samples of poly(vinyl formamide) (12). Molecular weight of the PVA samples was calculated from the molecular weight of the initial PVFA samples (6). Other chemicals were purchased from Sigma Aldrich, Fisher or Acros Chemicals and used as such without further treatment.

2.3 Conditions of mass spectrometric analysis

An Agilent 1200 chromatographic system controlled by Chemstation Version B.01.03 (Agilent Technologies, Inc.,

Santa Clara, CA, USA) and coupled to Agilent MSD-TOF 6200 with an electrospray ionization (ESI) source operated in the positive mode was used for mass spectrometric analysis of the samples. A chromatographic system high-performance liquid chromatography (HPLC) was carried out using a Zorbax 300 SB-C18 column (2.1×150 mm, 5 μ m, Agilent Technologies, Inc., Santa Clara, CA, USA) at 35°C, with a flow rate of 0.2 ml min⁻¹. The injection volume of sample solution was 20 μ l, water and acetonitrile with 0.1% (v/v) heptafluorobutyric acid (HFBA, Merck KGaA, Darmstadt, Germany) were used as eluting solvents A and B, respectively. The gradient conditions used was from 10% B to 100% B for 30 min, then to 100% B for 10 min. Other conditions were: capillary voltage 3500 V, nebulizer gas (N₂) at 30 psi, drying gas (N₂) at 5 l min⁻¹, desolvation temp 325°C, scan range m/z 70–2000.

2.4 Study of the copolymer composition by derivatization with trifluoroacetic acid (TFA)

The content of tertiary amine species introduced into the polymer structure was estimated using derivatization with TFA followed by ¹H NMR measurements (13). To a sample of polymer, typically ca. 30 mg, in a glass screw cap flat-bottom vial, 500 μ l of TFA was added. The vessel was heated at about 50°C and occasionally shaken for about 30 min to ensure the complete dissolution. Then the excessive TFA was removed to dryness at 50°C with argon flow. The cooled residue was mixed with 600 μ l of DMSO-d₆ and left overnight at room temperature. The resultant solution was filtered through a cotton pad in a 1 ml polypropylene pipette tip directly to a NMR tube. The molar ratio between -N(R)- species and N-vinyl acetamide units (NVA) was calculated from integral intensities of -N⁺H(R)- (9.2–11 ppm) and -C(O)-NH- (6.7–8.8 ppm) signals.

2.5 Synthesis of 2,6,10-trimethyl-2,6,10,14-tetraazapentadecane (N,N'-dimethyl-N-(3-dimethylaminopropyl)-N'-(3-methylaminopropyl)-1,3-propanediamine) (Scheme 3)

To a cooled (t < 10°C), stirred 15% solution of N,N'-dimethyl-N-(3-dimethylaminopropyl)-1,3-propanediamine [prepared according to earlier reported protocol (14)] in methanol, a 1.5-fold molar excess of methylacrylate (Merck KGaA, Darmstadt, Germany) was added drop-wise. The resultant mixture was refluxed for approximately 16 h. Then the volatile components were evaporated with a water pump and the residue warmed at 70°C in a water bath in vacuum for 90 min. After cooling to room temperature, the obtained oily ester was mixed with a two-fold molar excess of 8 M solution of methylamine in ethanol (Merck KGaA, Darmstadt, Germany). The mixture was maintained at room temperature until disappearance of 1740 cm⁻¹ C=O ester band in an FTIR spectrum of the vacuum-dried sample. Then the volatile components were removed as described earlier leaving the crude amide.

To a magnetically stirred solution of the amide (11.77 g, 43.2 mmol) in 112 ml of diethyl ether (Acros Organics, Thermo Fisher Scientific Inc., Waltham, MA, USA), 3.32 g of LiAlH₄ (Merck KGaA, Darmstadt, Germany) was added portion-wise in the course of an hour. The mixture was then refluxed for over 9 h. After cooling in an ice bath, the mixture was carefully quenched with the successive addition of 3 g of distilled water and 8 g of 50% (wt) solution of KOH in distilled water. The solid was filtered off using a Büchner funnel and rinsed with diethyl ether (3×40 ml). The combined filtrate was dried over potassium hydroxide powder, concentrated in a rotary evaporator and fractionally distilled (b.p. 130–132°C) in vacuum to yield 5.80 g of colorless liquid (52% yield). FTIR (film, cm⁻¹): ν_{NH} w 3300; $\nu_{\text{N-CH}_2}$ and $\nu_{\text{N-CH}_3}$ s 2785; $\nu_{\text{C-H}_2}$ and $\nu_{\text{C-H}_3}$ s 2945; δ_{CH_2}

Scheme 3: Synthesis of initial tetramine.

and δ_{CH_3} s 1460; ω_{NH} m 744. LC-MS: $[M+H]^+_{\text{calc.}}=259.286$
 $[M+H]^+_{\text{found}}=259.284$; $[M+HFBA+H]^+_{\text{calc.}}=473.272$, $[M+HFBA+H]^+_{\text{found}}=473.268$.

^1H NMR (CCl_4 , 400 MHz): δ_{H} 1.55–1.65 (6H, H-3,7), 2.17–2.22 (12H, H-1,5,6), 2.26 (8H, H-4), 2.41 (5H, H-2,9), 2.61 (2H, H-8). ^{13}C NMR (CCl_4 , 100 MHz): δ_{C} 25.49 (C-7), 27.4 (C-3), 36.42 (C-9), 41.95–42.23 (C-5,6), 45.35 (C-1), 50.74 (C-8), 55.68 (C-2), 56.15 (C-4).

2.6 Preparation of N-substituted N-methylacrylamides

N-substituted N-methylacrylamides ($n=1, 2, 3$) were prepared following method E from (9) (Scheme 4).

Novel acrylamide (N-(3-((3-(dimethylamino)propyl)(methylamino)propyl)(methylamino)propyl)-N-methylacrylamide) ($n=3$) was synthesized using a solution of 2,6,10-trimethyl-2,6,10,14-tetraazapentadecane (503.2 mg, 1.95 mmol) in 3.61 g of dry methylene chloride, a 3.16% (wt) solution of acryloyl chloride (Merck KGaA, Darmstadt, Germany) in methylene chloride (Acros Organics, Thermo Fisher Scientific Inc., Waltham, MA, USA, 6.19 g, corresponding to 2.16 mmol), 3 ml of a potassium carbonate water solution (50% w/w), anhydrous potassium carbonate (3 g). The product was purified by flash chromatography on 15–40 μm silica gel ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}:25\%$ wt. aqueous ammonia = 8:4.5:1, $R_f=0.84$) to obtain the target acrylamide as a slightly yellowish oily liquid (250 mg, 41%). FTIR (film, cm^{-1}): $\nu_{=\text{CH}_2}$ w 3096; $\nu_{\text{C-H}_2}$ and $\nu_{\text{C-H}_3}$ s 2941; $\nu_{\text{N-CH}_2}$ and $\nu_{\text{N-CH}_3}$ s 2760–2785; $\nu_{\text{C=C(N)=O}}$ vs 1613, 1649; δ_{CH_2} and δ_{CH_3} s 1456; $\delta_{\text{CH}_2 \text{ in } \text{CH=CH}_2}$ m 1402–1415; $\omega_{\text{C=C}}$ s 975. LC-MS: $[M+H]^+_{\text{calc.}}=313.297$, $[M+H]^+_{\text{found}}=313.300$.

Scheme 4: Synthesis of N-substituted N-methylacrylamides.

^1H NMR (CDCl_3 , 400 MHz): δ_{H} 1.56 (4H, m, H-10,12), 1.67 (2H, m, H-7), 2.07–2.17 (12H, s, H-9,11,14), 2.17–2.32 (10H, br. m, H-8,13), 2.93 and 3.01 (3H, s, H-5), 3.35 (2H, m, H-6), 5.60 (1H, t, H-1), 6.25 (1H, m, H-3), 6.57 (1H, m, H-2).

^{13}C NMR (CCl_4 , 100 MHz): δ_{C} 25.1 (C-7), 25.6 (C-10), 26.6 (C-12), 35.6 and 33.8 (C-5), 42.1 (C-9,11), 45.5 (C-14), 47.8 (C-6), 54.4 (C-13), 55.8 (C-8), 57.9 (C-8'), 127.4 ($\text{H}_2\text{C=}$), 127.8 ($=\text{CH}$), 166.4 (C-4).

2.7 Modification of PVA with N-substituted N-methylacrylamides (Scheme 1)

A solution of 0.286 g (6.65 mmol) of PVA, 2.9 mg of hydroquinone, and the required amount of corresponding acrylamide in 13.5 g of methanol was maintained in an argon atmosphere at 60°C over 39 h. The reaction solution was centrifuged at 12,500 g for 15 min. The obtained supernatant was concentrated in vacuum to leave about 3 g of the residue which was further triturated with cyclohexane (3 $\times$ 10 ml). The solution was discarded, whereas the solid was purified by dialysis in a cellulose tube (500–1000 MWCO) against distilled water, filtered through a 0.45 μm cellulose nitrate membrane and freeze-dried. Further the products were acetylated with acetic anhydride (Scheme 2).

Copolymers were stirred with acetic anhydride (Merck KGaA, Darmstadt, Germany, ca. 27 g per 1 g of copolymer) on an oil bath at 75°C over 2 h. Then the reaction mixture was evaporated in vacuum. The residue was triturated with diethyl ether (2 $\times$ 10 ml). The solution was discarded, whereas the solid was thrice reprecipitated from methanol with diethyl ether, or alternatively, dialyzed in a cellulose tube (Spectra/Por Dialysis Membrane, Biotech CE Tubing, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA, 500–1000 MWCO) against distilled water. Finally, a water solution of the product was filtered through a 0.45 μm cellulose nitrate membrane (Vladisart Ltd., Vladimir, Russian Federation) and freeze dried. The acrylamide loading and copolymer yields are presented in Table 1.

2.8 IR and NMR spectra of the new polymers

The prepared copolymers had a similar FTIR pattern. They contained bands of tertiary and secondary amide (amide I, vs. 1633–1643 cm^{-1} , $\nu \text{ C=O}$), secondary amide (amide II, s 1551 cm^{-1} , $\nu_{\text{C-N}} + \delta_{\text{N-H}}$; 3075 cm^{-1} , $2\nu_{\text{C-N}}$; very broad 3460 cm^{-1} , $\nu_{\text{N-H}}$; broad 3283 cm^{-1} , $\nu_{\text{N-H}}$), methyl and methylene groups (1350–1500 cm^{-1} , δ^a), methylene groups (2778 cm^{-1} , ν^s ; 2865 cm^{-1} , ν^s ; 2942 cm^{-1} , ν^a).

Table 1: New polymeric amines based on PVA.

Addition of polyamine substituted acrylamides on PVA

Entry	Acrylamide added			Yield (g)	Polymer composition (m, mol%) ^c	Acetic acid content (y, mol% from m)	M _n (kDa)	M _w (kDa)	PDI=M _w /M _n
	Z	g	mmol						
ZS-353-2 ^a	3	1.870	5.99	1.50	45 (39)	12	28.5	37.4	1.31
ZS-365-1 ^a	2	0.899	3.72	0.87	57 (57)	36	30.2	37.1	1.23
ZS-367-1 ^a	2	0.161	0.665	0.40	12 (11)	83	25.0	35.1	1.40
ZS-377-100 ^b	1	1.132	6.65	0.93	82 (75)	47	31.4	38.6	1.23
ZS-377-56 ^b	1	0.634	3.72	0.71	– (54)	22	34.0	42.7	1.25
ZS-377-10 ^b	1	0.113	0.665	0.32	13 (11)	44	26.5	35.6	1.34

^aPVA with M_n=12.8, M_w=17.6 kDa and PDI=1.38 was applied as a starting polymer. ^bPVA fraction with M_n=15.9, M_w=20.4 kDa and PDI=1.28 was applied as starting polymer. ^cCopolymer composition was calculated from ¹H NMR spectra after derivatization with TFA and from potentiometry data (values in brackets), in the case of ZS-377-56 the polymer-TFA adduct was not soluble in DMSO.

ZS-377-10, ZS-377-56, ZS-377-100 (a typical ¹³C NMR spectrum is presented on Figure 1).

¹H NMR (DMSO-d₆, 400 MHz): δ_H 1.47–1.67 (6H, H-1, H-6, H-14), 1.78 (3H, H-5), 1.88 (3H, CH₃ acetic acid),

Figure 1: A representative ¹³C NMR spectrum of ZS-377-10 in DMSO-d₆.

2.10 (9H, H-9, H-16), 2.15 (4H, H-11, H-15), 2.78 (5H, H-10, H-17), 2.94 (2H, H-13), 3.10–3.32 (4H, H-2, H-7), 6.6–8.3 (1H, H-3).

¹³C NMR (DMSO-d₆, 100 MHz): δ_C 21.8 (CH₃ acetic acid), 22.7 (C-5, 9), 24.8 (C-14), 31.8–40.4 (C-1,2,6,7,17), 45.1 (C-16), 46.9 (C-10), 55.9 (C-13), 56.7 (C-15), 168.8 (C-8,12), 169.9 (C-4).

ZS-353-2

¹H NMR (DMSO-d₆, 400 MHz): δ_H 1.42–1.62 (6H, H-1, H-6, H-14), 1.67–1.86 (3H, H-5), 1.88 (3H, CH₃ acetic acid), 2.11 (9H, H-9, H-17), 2.16–2.35 (12H, H-11, H-15, H-16), 2.78 (5H, H-10, H-19), 2.93 (2H, H-13), 3.17–3.33 (2H, H-2, H-7), 6.6–8.2 (1H, H-3).

¹³C NMR (DMSO-d₆, 100 MHz): δ_C 21.5 (CH₃ acetic acid), 22.8 (C-5, 9), 24.5 (C-14), 32–41.1 (C-1,2,6,7,19), 41.7 (C-18), 45 (C-17), 45.2 (C-10), 53.8–55.6 (C-13,15), 57.1 (C-16), 168.5 (C-8,12), 170.1 (C-4).

ZS-365-1 and ZS-367-1

^1H NMR ($\text{DMSO}-d_6$, 400 MHz): δ_{H} 1.19–1.66 (6H, H-1, H-6, H-14), 1.67–1.86 (3H, H-5), 1.88 (3H, CH_3 acetic acid), 2.11 (9H, H-9, H-17), 2.16–2.32 (8H, H-11, H-15, H-16), 2.78 (5H, H-10, H-19), 2.93 (2H, H-13), 3.10–3.38 (2H, H-2, H-7), 6.6–8.2 (1H, H-3).

^{13}C NMR ($\text{DMSO}-d_6$, 100 MHz): δ_{C} 21.8 (CH_3 acetic acid), 22.7 (C-5, 9), 24.8 (C-14), 31.8–44.1 (C-1,2,6,7,19), 41.6 (C-18), 45.1 (C-17), 46.9 (C-10), 53.8–55.4 (C-16), 168.8 (C-8,12), 170.1 (C-4).

2.9 Silicic acid condensation in the presence of new polymers and synthesis of soluble composites

Stock polymer (2 g L^{-1}), HEPES buffer (200 mM, pH=7.4) solutions and water were mixed in the desired proportions. $\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$ (100 mM) and HCl (0.5 M) were added rapidly to the solution under active stirring. The resulting solutions contained 0.4 g L^{-1} polymer, 5 or 7.5 mM silicic acid and 50 mM HEPES. The amount of HCl corresponds to full neutralization of sodium silicate. This procedure allowed us to prevent $\text{Si}(\text{OH})_4$ condensation at a pH higher than the desired value. The obtained solutions were stored at room temperature for 3 days which resulted in silicic acid condensation and formation of soluble composite nanoparticles similar to PVA based systems and polyampholytes (12, 15).

2.10 Study of oligonucleotide complexes with polymers and composites

The interaction between 21-mer DNA oligonucleotide GATCTCATCAGGGTACTCCTT-6-FAM (Evrogen JSC, Russia) 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 mM Tris acetate (adjusted to pH 7.4) and 1 mM EDTA. A glycerol gel loading buffer was used (0.5% sodium dodecyl sulfate, 0.1 M EDTA (pH=8), and 50% glycerol for 10× reagent). Electrophoresis experiments were performed at 90 V with a Mini-Sub ($7 \times 10 \text{ cm}$) 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 solution of 0.05% bromophenol blue was applied to visualize the “dye front” and to calculate the relative mobility (R_f).

Similar experiments were also performed with the small interfering RNA (si-RNA) against vascular endothelial growth factor (VEGF) (Eurofins Genomics, USA). The sequence of the VEGF si-RNA with dTdT overhangs at 3' in both strands used in the study are: sense – GGAGUACCCUGAUGAGAUC; anti-sense – CCUCAUGGGACUACUCUAG. Composite and siRNA complex were prepared by mixing aqueous solutions of composite and si-RNA in different ratios. The resulting mixture was vortexed and incubated for 30 min at ambient temperature. Then the polyplexes were mixed with a tracking dye (bromophenol blue, $2 \mu\text{l}$), loaded on 1% agarose gel and electrophoresed with Tris-acetate buffer (TAE) buffer at 80 V for 45 min. The gel was stained with ethidium bromide and visualized on a UV transilluminator using a Gel Documentation system (VILBER Lourmat, France).

2.11 Cell culture studies

A549 lung cancer cells were seeded in six well plates at a density of 5×10^4 cells well^{-1} and cultured on a glass coverslip in Dulbecco's Minimum Eagles Medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and incubated at 37°C and 5% CO_2 environment. After 24 h, when the cells attained confluency, the transfection mixtures were prepared by the complexation of polymers and Cy3 fluorescent probe-tagged si-RNA (Eurofins Genomics, USA) in the ratio of 4:1 (v/v) and the si-RNA concentration used was $10 \mu\text{M}$. The sequence of the sense and anti-sense strands of the fluorophore-tagged si-RNA employed for this study is as follows: sense – Cy3-GGAGUACCCUGAUGAGAUC; anti-sense: CCUCAUGGGACUACUCUAG-Cy3.

The cells were then washed with phosphate buffered saline (PBS) and the transfection mixtures were added to the appropriate wells in 2 ml of serum free DMEM and the final si-RNA concentration in each well was 5 nM. After 4 h

and 16 h of transfection, cells were washed with PBS and the coverslips were mounted over glass slides the images were analyzed using laser scanning confocal microscope (Olympus FV1000, Japan) at an excitation wavelength of 540 nm and at an emission wavelength 605 nm or using fluorescence microscopy (Nikon Eclipse TS100, Japan).

3 Results and discussion

Substituted acrylamides were obtained by the reaction of acryloyl chloride with corresponding amines according to the previously described procedure [Scheme 4 (14)]. Acrylamide containing three amine groups ($z=3$) was synthesized for the first time.

Interaction of narrow PVA fractions with substituted acrylamides resulted in new polymers which bear polyamine chains grafted to the main macromolecular chain with long (eight atoms) spacers (Table 1). The new amine containing copolymers are soluble in water, methanol and DMSO. Composition of the copolymers was calculated from ^1H NMR data after derivatization with trifluoroacetic acid (TFA) with the use of $-\text{N}^+\text{H}(\text{R})-$ (9.2–11 ppm) and $-\text{C}(\text{O})-\text{NH}-$ (6.7–8.8 ppm) signals. ^{13}C NMR data show the presence of acetic acid in the copolymers which forms a salt with amine groups.

Potentiometric titration of aqueous solutions of the copolymers from the alkali region ($\text{pH} \approx 11.4$) shows three inflections (Figure 2) which correspond to the titration of alkali excess, titration of amine groups and titration of sodium acetate. The potentiometry data allows for the

Figure 2: A representative potentiometric titration curve of ZS-367-1 sample in aqueous medium. Blue curve represents derivative from the titration curve. Numbers corresponds to titration of alkali excess (1), titration of amine groups (2), and titration of sodium acetate (3). The inflection between #1 and #2 corresponds to titration of one amine group in the side chain, this inflection was not visible in other copolymers. 0.1 M NaCl aqueous solution was applied as a solvent, sample mass was 0.0409 g, initial solution volume was 10 ml, pH was adjusted to 11.4 with 0.77 ml of 0.1 M NaOH, and 0.1 M HCl was applied as a titrant.

calculation of the copolymer composition which is in a good agreement with ^1H NMR data and to estimate the content of acetic acid in the copolymers (Table 1).

The molecular mass and PDI of the new copolymers were estimated from size exclusion chromatography (SEC) data. The obtained results show an increase of molecular weight after modification of PVA and the PDI is relatively low which confirms that the modification reactions proceed without the considerable destruction of the polymeric chains (Table 1). The observed increase of molecular weight after modification is the result of the addition of polyamine chains. We also must take into account that the introduction of ionizable amine groups can enlarge the size of macromolecular coils when compared with neutral PVFA, and it may contribute to the observed increase in the molecular weight from SEC.

Potentiometric titration allows the calculation of the ionization degree (α) and pK of acidic and basic compounds. We have calculated α according to the following equation (16):

$$\alpha = ([\text{NaOH}] + [\text{H}^+] - [\text{OH}^-]) / C_{\text{total}},$$

where $[\text{NaOH}]$ is the concentration of alkali added, C_{total} is the total concentration of the conjugated acid units. pK values were obtained from the Henderson-Hasselbach equation (17):

$$\text{pK} = \text{pH} - \log(\alpha / (1 - \alpha)).$$

In the case of polymeric amines, the calculated α and pK correspond to ionization of the conjugated acid $\equiv\text{NH}^+$. Dependence of pK vs. α is presented in Figure 3. It is found that the pK values are independent of α for low molecular compounds but polyelectrolytes show a decrease in pK

Figure 3: Dependence of pK vs. α for new polymeric amines.

Figure 4: Gel electrophoresis data for DNA (A, B), RNA (C) oligonucleotide complexes with new polymers and polyplex internalization (D). Gel electrophoresis data for DNA oligonucleotide complexes with new polymers (A, B). Lane 1: free oligonucleotide, 2 – ZS-377-10, 3 – ZS-377-56, 4 – ZS-377-100, 5 – ZS-367-1, 6 – ZS-353-2, and 7 – ZS-365-1. The concentration of the oligonucleotide was $10 \mu\text{M}$ and that of the polymers was 0.4 g L^{-1} . The polymer: oligonucleotide ratio (volume: volume) was 1:1 (A) and 4:1 (B). (C) Electrophoresis data for polyplexes with anti-VEGF-si-RNA complexed in the ratio of 4:2. Lane 1: control free si-RNA, lane 2: ZS-353-2(5), lane 3: ZS-365-1(7.5), lane 4: ZS-365-1(5), lane 5: ZS-367-1(7.5), and lane 6: ZS-367-1(5) composites (numbers in brackets represent silicic acid concentration in the composite solutions, mM). The concentration of si-RNA was $10 \mu\text{M}$. Internalization of polyplexes based on ZS-365-1(7.5) composite in A549 cancer cells (D): after 4 h observed using fluorescence microscopy and (E): 16 h after treatment observed with laser scanning confocal microscopy. Scale bar represents 100 (B) and 50 (C) μm .

with decrease of α (18) (Figure 3, PVA curve). Polymeric amines are not protonated at $\alpha=1$ (full dissociation of the conjugated acid) and protonation (α decrease) hampers the reaction with the next protons due to the electrostatic effect. The new polymers containing one amine group in the side chain do not exhibit a decrease in pK values with a corresponding decrease in α when the amine unit content is between 11 and 54% (ZS-377-10 and ZS-377-56). This effect is explainable due to the long distance of separation between isolated amine groups which prevents electrostatic interactions. An increase in the amine content in the copolymer (ZS-377-100) results in a considerable decrease of pK with a α value below 0.3 when 50% of the total polymeric units are protonated. Copolymers with two or three amine groups in the side chain show a more distinct decrease in pK with reduction in α values. This is on expected lines because protonation of one amine group in the side chain decreases basicity of the other groups in the same chain.

Due to their protonatable amine functionalities, polymeric amines are promising agents for gene delivery for therapeutic applications (19–21) that involves selective silencing of specific gene targets to curtail their expression or constitutively express under-expressed genes through the transfection of plasmids to target cells (21–23). In the present study, the new polymers with side chains

Table 2: Gel electrophoresis results for DNA oligonucleotide complex with new polymers.

Polymer	Polymer conc. ^a (g L^{-1})	Polymer: DNA ratio	R_f ^b
Free DNA oligonucleotide			1
ZS-377-10	4	4:1	–0.43
ZS-377-10	0.4	4:1	0; –0.2 to 0
ZS-377-10	0.4	1:1	0.49–1
ZS-377-56	4	4:1	0; –0.47 to 0
ZS-377-56	0.4	4:1	0
ZS-377-56	0.4	1:1	0
SZ-377-100	4	4:1	0; –0.57 to 0
SZ-377-100	0.4	4:1	0; –0.047 to 0
SZ-377-100	0.4	1:1	0
ZS-367-1	2	4:1	0; –0.44
ZS-367-1	0.4	4:1	0; –0.21 to 0
ZS-367-1	0.4	1:1	0–0.95
ZS-365-1	2	4:1	0; –0.44 to 0
ZS-365-1	0.4	4:1	0
ZS-365-1	0.4	1:1	0
SZ-353-2	2	4:1	0; –0.4 to 0
SZ-353-2	2	2:1	–0.35
SZ-353-2	0.4	4:1	0; –0.08 to 0
SZ-353-2	0.4	1:1	0; 0–0.87

^aDNA oligonucleotide was applied as $10 \mu\text{M}$ solution. ^bNegative R_f values correspond to substance movement towards the negative electrode. Interval of R_f values corresponds to the spreading electrophoretic blots.

of polyamine are capable of interacting with 21-mer DNA according to electrophoresis data (Figure 4, Table 2). The magnitude of the polymer-DNA interaction correlates with the content of the polyamine side chains. While ZS-377-10 and ZS-367-1 gave predominantly negative charged complexes and a considerable quantity of uncomplexed DNA, the most complete complexation was observed for SZ-377-100 and ZS-365-1 samples with the highest content of polyamine chains (Figure 4A). An increase in the polymer content in the complex with DNA (polyplex) (Figure 4B) results in the complete complexation for all samples. Polymers with the lowest polyamine content (ZS-377-10 and ZS-367-1) exhibited significant migration to the negative

electrode unlike the other polymer samples. Possibly, the high content of neutral hydrophilic units in these polymers facilitates the formation of more soluble complexes with higher electrophoretic mobility. In contrast, a high amine content in the polymer can promote the formation of large complexes that possess no net charge and do not exhibit electrophoretic mobility.

Condensation of silicic acid in the presence of new polymers results in diameter composite nanoparticles <100 nm (Figure 5, left) similar to composites based on poly(vinyl amine) (12). The addition of RNA to the solution does not change the particle size significantly. Transmission electron microscopy (TEM) images of

Figure 5: Dynamic light scattering (DLS) data (left) and transmission electron microscopy (TEM) images for composite particles and polyplexes. Dynamic light scattering (DLS) data (left) for aqueous solutions of composite particles (bottom black lines) obtained under silicic acid condensation in the presence of new polymers ($0.4 \text{ g} \cdot \text{L}^{-1}$) at pH 7.4, and composite – RNA complexes (top red lines). Silicic acid concentration (mM) is presented in brackets. Numbers at the left side – ζ -potential values. Solutions of free polymers contain mainly 1000–2000 nm aggregates. (Right) Transmission electron microscopy (TEM) images of composite particles obtained under silicic acid condensation in the presence of new polymers (A, C and E) and composite – DNA oligonucleotide complexes (B, D and F). A and B – SZ-353-2, C and D – ZS-365-1, E and F – ZS-367-1 polymers. Polymer concentration – $0.4 \text{ g} \cdot \text{L}^{-1}$, silicic acid concentration: 7.5 (A, B and E) and 5 (C, D and F) mM. Scale bar represents 100 (A and C), 200 (D, E and F) and 500 (B) μm .

Table 3: Gel electrophoresis results for DNA oligonucleotide complex with siliceous composites.

Polymer	Silicate conc. ^a (g L ⁻¹)	Polymer (composite): DNA ratio	R _f ^b
SZ-353-2	7.5	4:1	0; 0.36–0.38
SZ-353-2	5	4:1	0; –0.084 to 0
ZS-365-1	5	4:1	0; –0.028 to 0
ZS-367-1	7.5	4:1	0.4–0.54
ZS-367-1	5	4:1	0; –0.064 to 0

^aDNA oligonucleotide was applied as 10 μ M solution. Polymer concentration was 0.4 g L⁻¹. ^bNegative R_f values correspond to substance movement to the negative electrode. The interval of R_f values corresponds to spreading electrophoretic blots.

freeze-dried solutions of the composite nanoparticles (Figure 5, right) confirm the formation of the composite nanoparticles containing electron-dense siliceous inclusions. Interaction of the composite nanoparticles with oligonucleotide results in more dense particles. The electrophoresis data (Table 3) show that composites are able to interact with nucleic acid but the charges of the complexes are often negative due to the presence of polymeric silicic acid. The complexation ability of the composite nanoparticles with small interfering RNA (si-RNA) against vascular endothelial growth factor (VEGF) was confirmed with electrophoresis data (Figure 4C, lanes 2–4). Zeta-potential values are positive for nanocomposites and RNA complexes (Figure 5, left) which allows the expectation of penetration of these particles into living cells.

In order to investigate whether the polyplexes can internalize into the target cells where they are expected to deliver the therapeutic oligonucleotide, composites based on the ZS-365-1 polymer was complexed with fluorophore-tagged si-RNA. After 4 h, the internalization of the polyplex was observed in A549 cancer cells using fluorescence microscopy (Figure 4D). The appearance of red fluorescence in the cells indicate that the polyplexes have internalized into the cell. The confocal images also reveal that the localization of the si-RNA is in the cytosol (Figure 4E). Interestingly, the fluorescence in cells was observed even 16 h after administration suggesting that these polyplexes can be employed for persistent si-RNA-mediated gene silencing in cancer cells.

4 Conclusions

The addition reaction of polyamine modified acrylamides with poly(vinyl amine) gives rise to new polymers with

polyamine side chains. The reaction proceeds without visible destruction of the main polymeric chain and allows obtaining new polymeric amines with low polydispersity. The amine groups are placed far from the main polymeric chain which allows regulation of the acid-base properties of the polymer by changing the number of amine nitrogens in the side chain. Macromolecules with one amine unit in the side chain typically display behavior of low-molecular weight amines: pK of the conjugated acid insignificantly depend on ionization degree. Polymers with two or more amine groups in the side chain exhibit typical polyelectrolyte properties: basicity decreases with protonation of the polymer. The new polymeric amines and composite siliceous nanoparticles based on these polymers were investigated as potential gene delivery systems and were found to effectively complex DNA (RNA) oligonucleotides and internalize into the cancer cells. This opens up new vistas for this class of polymers in the realm of therapeutics.

Acknowledgments: We acknowledge financial support from a joint grant of the Russian Science Foundation (# 6-45-02001) and the Department of Science Technology of the Ministry of Science and Technology of the Republic of India (# INT/RUS/RSF/10). We are thankful to the Center of Ultramicroanalysis (Limnological Institute) for providing the equipment.

References

1. Carraher CE. Polymer chemistry. 6th ed. New York, Basel: Marcel Dekker Inc.; 2003. 960 p.
2. Samal SK, Dubrue P. Cationic polymers in regenerative medicine. Cambridge, UK: Royal Society of Chemistry; 2015. 618 p.
3. NPCS Board of Consultants & Engineers. The complete book on water soluble polymers. Delhi: Asia Pacific Business Press Inc.; 2009. 672 p.
4. Barner-Kowollik C, editor. Handbook of RAFT polymerization. Weinheim, Germany: Wiley-VCH; 2008. 544 p.
5. Hsieh HL, Quirk RP. Anionic polymerization: principles and practical applications. New York, Basel: CRC Press; 1996. 744 p.
6. Pavlov GM, Korneeva EV, Ebel C, GavriloVA II, Nesterova NA, Panarin EF. Hydrodynamic behavior, molecular mass and conformational parameters of poly(vinylformamide) molecules. Polym Sci Ser A 2004;46:1063–7.
7. Jagur-Grodzinski J. Biomedical applications of polymers 2001–2002. e-Polymers 2003;012:1–38.
8. Danilovtseva E, Krishnan UM, Pal'shin V, Annenkov V. Polymeric amines and ampholytes derived from poly(acryloyl chloride): synthesis, influence on silicic acid condensation and interaction with nucleic acid. Polymers 2017;9:624–41.

9. Zhang P, Wagner E. History of polymeric gene delivery systems. *Top Curr Chem. (Cham.)* 2017;375:1–39.
10. Bordianu-Antochi IE, Olaru M, Cotofana C. Novel hybrid formulations based on chitosan and a siloxane compound intended for biomedical applications. *React Funct Polym.* 2017;114:118–26.
11. Pandit V, Watson A, Ren L, Mixo A, Kotha SP. Multilayered nanoparticles for gene delivery used to reprogram human foreskin fibroblasts to neurospheres. *Tissue Eng Part C* 2015;21:786–94.
12. Annenkov VV, Danilovtseva EN, Pal'shin VA, Aseyev VO, Petrov AK, Kozlov AS, Patwardhan SV, Perry CC. Poly (vinyl amine) – silica composite nanoparticles: models of the silicic acid cytoplasmic pool and as a silica precursor for composite materials formation. *Biomacromolecules* 2011;12:1772–80.
13. Liu Q, Zhu M. Determination of molar ratio of primary secondary and tertiary amines in polymers by applying derivatization and NMR spectroscopy. *Polym Test.* 2016;56:174–9.
14. Annenkov VV, Zelinskiy SN, Danilovtseva EN, Perry CC. Synthesis of biomimetic polyamines. *ARKIVOC* 2009;xiii:116–30.
15. Annenkov VV, Pal'shin VA, Danilovtseva EN. Water-soluble copolymers of 2-methacryloyloxyethyl phosphate: synthesis and properties. *e-Polymers* 2012;024:1–9.
16. Strauss UP, Barbieri UP, Wong G. Analysis of ionization equilibria of polyacids in terms of species population distributions. Examination of a “two-state” conformational transition. *J Phys Chem.* 1979;83:2840–3.
17. Katchalsky A, Spitnik P. Potentiometric titrations of polymethacrylic acid. *J Polym Sci.* 1947;2:432–46.
18. Katchalsky A, Mazur J, Spitnik P. Polybase properties of polyvinylamine. *J Polym Sci.* 1957;23:513–32.
19. Grigsby CL, Leong KW. Balancing protection and release of DNA: tools to address a bottleneck of non-viral gene delivery. *J R Soc Interface* 2010;7:S67–82.
20. Dréan M, Debuigne A, Gonçalves C, Jérôme C, Midoux P, Rieger J, Guegan P. Use of primary and secondary polyvinylamines for efficient gene transfection. *Biomacromolecules* 2017;18:440–51.
21. Li L, Wei Y, Gong CJ. Polymeric nanocarriers for non-viral gene delivery. *Biomed Nanotechnol.* 2015;11:739–70.
22. Sparks SM, Waite CL, Harmon AM, Nusblat LM, Roth CM, Uhrich KE. Efficient intracellular siRNA delivery by ethyleneimine-modified amphiphilic macromolecules. *Macromol Biosci.* 2011;11:1192–200.
23. Vader P, van der Aa LJ, Storm G, Schiffelers RM, Engbersen JFJ. Polymeric carrier systems for siRNA delivery. *Curr Top Med Chem.* 2012;12:108–19.