Peer-reviewed version available at Polymers 2018, 10, 1297; doi:10.3390/polym10121297

Article

Design of the oligonucleotide carriers: importance of polyamine chain length

Vadim V. Annenkov [1,*], Uma Maheswari Krishnan [2], Viktor A. Pal'shin [1], Stanislav N. Zelinskiy [1], Gayathri Kandasamy [2] and Elena N. Danilovtseva [1]

[1] Limnological Institute of the Siberian Branch of the Russian Academy of Sciences, 3, Ulan-Batorskaya St., P.O. Box 278, Irkutsk 664033, Russia; danilovtseva@yahoo.com (E.N.D.); acrom@mail.ru (V.A.P.)
[2] Centre for Nanotechnology & Advanced Biomaterials (CeNTAB), School of Chemical and Biotechnology, SASTRA University, Thanjavur 613401, Tamil Nadu, India; umakrishnan@sastra.edu
* Correspondence: annenkov@lin.irk.ru; Tel.: +7-914-8982577

Abstract: Amine containing polymers are extensively studied as special carriers for short-chain RNA (13–25 nucleotides) which are applied as gene silencing agent in gene therapy of various diseases including cancer. Elaboration of the oligonucleotide carriers requires knowledge about peculiarities of oligonucleotide - polymeric amine interaction. Critical length of the interacting chains is the important parameter which allows to design sophisticated constructions containing oligonucleotide binding segments, solubilizing, protective and aiming parts. We studied interaction of (TCAG)n, n=1-6 DNA oligonucleotides with polyethylenimine and poly(N-(3-((3-(dimethyl-amino)propyl)(methyl)amino)propyl)-N-methylacrylamide). Critical length for oligonucleotides in interaction with polymeric amines is 8-12 units and complexation at these length can be accompanied by "all-or-nothing" effects. New dimethylacrylamide based polymers with grafted polyamine chains were obtained and studied in complexation with DNA and RNA oligonucleotides. The most effective interaction and transfection activity into A549 cancer cells was found for a sample with average number of nitrogens in polyamine chain equal to 27, i.e. for a sample in which all grafted chains are longer the critical length for polymeric amine - oligonucleotide complexation.

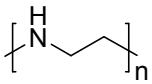
Keywords: polymeric amines; oligonucleotides; critical length; grafted polyamines; gene delivery

1. Introduction

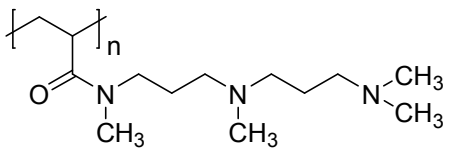
Gene silencing is a promising approach to combat various diseases including cancer [1-4]. The corresponding techniques include delivery of short nucleic acids (SNA, 13–25 nucleotides) into the cell cytoplasm. Special carriers are employed to protect SNA in circulation, to facilitate SNA penetration into cell and to escape destruction of SNA in lysosomes [5]. Amine containing polymers are extensively studied in this area and the most well-known polymer in this category is polyethylenimine (PEI) [6]. High buffer capacity of PEI at pH 6-7.5 causes escape of the polymer-SNA complex from endosomes by the "proton pump" mechanism. The other requirement for SNA carriers is low cytotoxicity which is a limitation of polymeric amines. Dedicated design of the oligonucleotide carriers requires knowledge about peculiarities of SNA - polymeric amine interaction. This interaction is a typical interpolymeric reaction [7-8] and one of the important parameters of these reactions is the critical length (L_c) of the interacting chains. Sub-optimal length results in weak interactions while higher L_c results in relatively irreversible reactions. The importance of L_c value in SNA - polymer interactions was addressed in several articles [9-12]. Synthetic polymeric amines of various length and SNA of a fixed length have been explored in several earlier reports [9,10] but the set of amine chains investigated was restricted. A recent work had investigated a set of SNA with varying chain length [11] and the minimal length of the SNA chain was determined as 14 nucleotides in reaction with virus particle of unknown chemical structure. A study of polylysine interaction with plasmid DNA [12] revealed that complexation

occurred at 8 lysine units and an absence of interaction was observed with samples with 3 lysine units. The lack of information about L_c in SNA - polymeric amine interactions stems from the fact that the main objective of studies in the field of gene therapy has focused on achieving complexation using SNA with definite length, usually near 20 nucleotides. However, polymeric constructs for SNA delivery often contain combinations of short amine, neutral and sometimes acidic sequences and hence information about L_c values is required for the design of these constructs and understanding their properties.

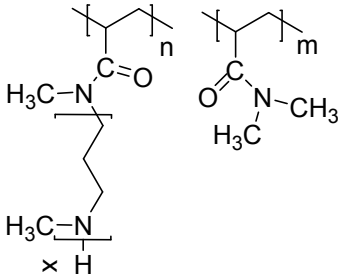
Our work has two objectives with the first being estimation of L_c for DNA oligonucleotides in the reaction with PEI and new polymeric amine poly(N-(3-((3-(dimethylamino)propyl)(methyl)amino)propyl)Nmethylacrylamide) (AKX-182, Scheme 1) [13]. We have used the DNA oligonucleotides $(TCAG)_n$, where n = 1-6, which contain equal amount of the each nucleotide and cannot form stable hairpins or self-dimers. The second objective of our work is to synthesize and study new polymers as gene delivery agents. These polymers originate from bioinspired polymers with grafted polyamine chains [14]. Polyamines containing 3-4 nitrogen atoms, including spermine and spermidine, play an important role in cell physiology: protein and nucleic acid synthesis, gene expression, protection from oxidative damage and etc. [15]. The so-called long-chain polyamines (LCPAs), more exactly oligomeric polyamines, have been found in bacteria [16], diatom algae [17], in the siliceous sponge [18] and in haptophyte [19]. In the case of diatoms, LCPAs are present as post-translationally grafted side chains in specific proteins namely, silaffins [20]. These LCPAs contain up to two tens of partially methylated nitrogen atoms separated by trimethylene or tetramethylene fragments [21]. Some polymeric amines [22,23] capable of interacting with oligomers of silicic acid and forming stable composite nanoparticles were considered as models of cytoplasmic silicon containing vesicles. We have elaborated [24] a method to synthesize LCPAs as an oligomeric mixture containing polyamines with 6-30 nitrogen atoms. Grafting of the LCPA chains on to poly(acrylic acid) resulted in polyampholytes which can control condensation of silicic acid giving rise to silica, the structure of which was similar to biogenic silica [25]. Neutral polymers with pendant LCPAs can interact with SNA and the obtained complexes are promising particles for gene therapy [14]. Medical applications of polymers require homogeneous macromolecules and in this work we synthesized poly(N,N-dimethylacrylamide) (PDMAAm) containing grafted LCPA chains starting from narrow polymer and LCPA fractions (Scheme 1). Influence of the length of pendant LCPA chains on the capability to interact with SNA was investigated taking in to account L_c values for SNA.



PEI



AKX-182



ZS-371-1(2,3,5)

Scheme 1. Structures of the studied polymers.

2. Materials and Methods

2.1 Materials

2,2'-Azobis(2-methylpropionitrile) (AIBN) (Sigma-Aldrich, St. Louis, MO, USA) was recrystallized from ethanol prior to use. Diethyl ether (Acros, Geel, Belgium) and 1,4-dioxane (Sigma-Aldrich) were distilled under sodium. Dimethylformamide (DMF) (Sigma-Aldrich) was dried with CuSO4 (30 min) and distilled at 5 mm Hg. Acryloyl chloride (Sigma-Aldrich) was distilled before polymerization. Dichloromethane (Acros, Geel, Belgium) was refluxed over phosphorus pentoxide and distilled under argon. Dimethylamine 40 wt. % aqueous solution,

triethylamine, 1,3-dibromopropane, N-hydroxysuccinimide, N-hydroxyphthalimide, potassium hydroxide, potassium carbonate of reagent grade (Sigma–Aldrich, Fisher, or Acros Chemicals) were used in the study. Dimethyl sulfoxide-d6 (DMSO-d6, 99.8 atom D %), deuterochloroform (99.8 atom D %), heptafluorobutyric acid (HFBA, ≥ 99.0 % (GC)), acetonitrile (HPLC Far UV / gradient grade, Avantor Performance Materials B.V., Deventer, The Netherlands) and trifluoroacetic acid (TFA, 99 wt. % purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fisher (Hampton, NH, USA), or Acros (Geel, Belgium) chemicals and used without further treatment. A 9.01 wt. % solution of dimethylamine in 1,4-dioxane was prepared via saturation of dry 1,4-dioxane with gaseous dimethylamine. The amine concentration was determined from the resulted weight gain as well as with potentiometric titration. To obtain gaseous dimethylamine, its 40 wt. % aqueous solution was added dropwise to a large excess of potassium hydroxide flakes. The evolved gas was passed through a drying column packed with KOH flakes.

N,N-bis[3-(methylamino)propyl]methylamine was prepared following the technique from our earlier work [26]. Oligo(N-methylazetidine) (LCPA, ZS-309 sample) was prepared from 1,3-dibromopropane and N,N-bis[3-(methylamino)propyl]methylamine according to earlier work from our group [24]. AKX-182 polymer was prepared according to the protocol described earlier [13]. PEI (M_w = 30000-40000) was obtained from SERVA Fine Biochemica (Heidelberg).

FAM 3'-tagged DNA oligonucleotides were purchased from Evrogen JSC, Russia. Small interfering RNA (si-RNA) against vascular endothelial growth factor (VEGF) was obtained from Eurofins Genomics, USA. The sequence of the sense and anti-sense strands of the fluorophore-tagged si-RNA is as follows: sense - Cy3-GGAGUACCCUGAUGAGAUC and antisense: CCUCAUGGGACUACUCUAG-Cy3..

2.2. Instrumentation

^{1}H nuclear magnetic resonance (NMR) spectra were obtained on a DPX 400 Bruker instrument (400.13 MHz, Billerica, MA, USA) in $CDCl_3$ and DMSO-d_6. Spectra of LCPA were recorded for $CDCl_3$ solutions, whereas those of poly(N,N-dimethylacrylamide)s grafted with LCPA were obtained for DMSO-d_6 solutions of samples derivatized with trifluoroacetic acid as follows: to a sample of polymer, typically of ca. 30 mg, in a glass screw cap flat-bottom vial, 500 μL of TFA was added. The vessel was heated at about 50 °C and occasionally shaken for about 30 min to ensure complete dissolution. Then, the excessive TFA was removed to dryness at 50 °C with argon flow. The cooled residue was mixed with 600 μL 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 ampoule.

Mass spectrometric analysis was performed on an Agilent 6210 TOF LC/MS System. The samples were dissolved in acetonitrile. Water and acetonitrile with 0.1% (v/v) HFBA were used as eluting solvents A and B, respectively (Solvent A – 90%, B – 10%). The flow rate of the mobile phase was set at 0.2 mL/min, while the injection volume of sample solution was 20 μL. The conditions for TOF MS were as follows: the mass range was m/z 100-1000 and scan time was 1 s with an interscan delay of 0.1 s; mass spectra were recorded under ESI+, V mode, centroid, normal dynamic range, capillary voltage 3500 V, desolvation temp 350°C, nitrogen flow 5 L/min.

The molecular masses of the new polymers were estimated via size-exclusion chromatography (SEC) using a Milichrom A02 chromatograph (JSC Econova, Novosibirsk, Russia) with 2 mm × 75 mm column filled with SRT SEC-100 5 μm phase (Sepax Technologies, Inc., Newark, NJ, USA), operated at 35 °C using phosphate buffer solution 0.15 M, pH 6.86. 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 μL. Fractionated samples of poly(vinyl formamide) [27] were applied as standards (M_w/M_n < 1.3).

Atomic force microscopy (AFM) was performed using Scanning Probe Microscope CMM-2000 (PROTON-MIET, ZAVOD, JSC, Russia) operated in contact mode in air at room temperature using silicon probes (nominal probe curvature radius of 10 nm). Height mode images (512x512 pixels) were collected with a scan speed between 1 and 2 Hz. The samples were placed on mica slips, water

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 23 October 2018 doi:10.20944/preprints201810.0515.v1

Peer-reviewed version available at Polymers 2018, 10, 1297; doi:10.3390/polym10121297

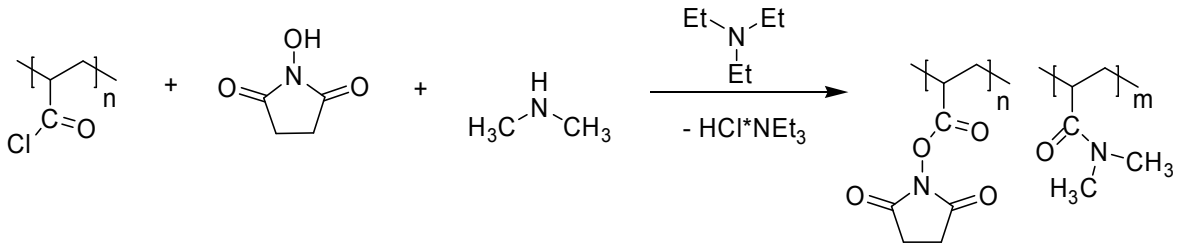
was removed with a filter paper after 30 min and slips were air dried. The software package Gwyddion was used for AFM image processing.

2.3. Synthesis of poly(N,N-dimethylacrylamide) with grafted LCPA chains (ZS-371-n)

2.3.1. Synthesis of Poly(acryloyl chloride) (PAC)

PAC was synthesized similar to the protocol described earlier [28] by polymerization of acryloyl chloride (6.336 g, 0.070 mol) in 25 mL of dioxane with the addition of 0.1267 g AIBN in argon atmosphere at 60 °C for 48 h. PAC was precipitated from the reaction solution using 138 mL of cyclohexane. The supernatant was decanted and the residue was dissolved in DMF (Solution A, see below) for further use. With the objective to estimate yield and polymerization degree of the PAC, the reaction mixture was poured into water (50 mL) and dialyzed against water. After freeze drying, poly(acrylic acid) was obtained with 90% yield. According to viscometry data, [29] the polymerization degree of the poly(acrylic acid) and, correspondingly of PAC, was found to be 220.

2.3.2. Preparation of poly(N,N-dimethylacrylamide-co-N-acryloxysuccinimide) (ZS-358)



Three initial solutions namely Solution A (The prepared PAC in 104.4 g of DMF), Solution B (N-hydroxysuccinimide (1.61 g, 14.0 mmol) and triethylamine (1.70 g, 16.8 mmol) dissolved in 11 g of DMF), and Solution C (Triethylamine (7.22 g, 71.4 mmole), 10.1 wt. % dimethylamine solution in 1,4-dioxane (26.55 g, corresponds to 59.48 mmol of HN(CH3)2) and 22.9 g of DMF) were combined.

Solution B was added to magnetically stirred solution A (cooling on an ice bath) for eight minutes. The mixture was kept stirred with cooling for 31 minutes followed by dropwise addition of solution C for 30 minutes. Stirring was continued with cooling for 30 minutes and then at room temperature for two hours. The reaction vessel was left in a refrigerator for about 15 hours. The white precipitate was filtered off using a filter funnel with sintered glass disc. The light yellowish filtrate was concentrated to a volume of 10-15 mL under vacuum at room temperature. Addition of 110 mL of toluene precipitated a yellow sticky mass which was washed with toluene (50 mL × 1), THF (25 mL × 2) and dried using an oil vacuum pump for four hours to yield 7.496 g of ZS-358.

2.3.2. Fractional precipitation of ZS-358

A solution of ZS-358 (6.434 g) in 26 mL of CH_2Cl_2 was filtered through a glass disc (10-15 μm) and diluted to a total volume of 215 mL with CH_2Cl_2. The first fraction (1F) was precipitated with careful addition of 19.64 g of n-hexane to the stirred solution. The supernatant was decanted in about 90 minutes for the successive precipitations (Table 1).

2.3.3. Preparative exclusion chromatography (SEC) fractionation of ZS-309

The fractionation was performed on a glass jacketed chromatography column (0.7 × 85 cm) packed with Sephadex G-25 (coarse, 100-300 μm). A solution of 100 mg of ZS-309 in 200 μL of 0.1 M HCl was loaded on the wet sorbent surface followed by gravity elution at 35°C with a 25 mM acetate buffer solution containing 0.2 M NaCl. Collected fractions were analyzed by silica gel TLC (CH_2Cl_2:CH_3OH: 25% aq. NH_3 = 2:2:1). The spots were visualized in red color by Dragendorff's reagent [30]. Chosen fractions were combined and analyzed with LC-MS (Figure S1) with the objective to estimate effectiveness of the fractionation. Then the solutions were evaporated to dryness under vacuum, mixed with a solution of K_2CO_3 (50%) in water and extracted with CH_2Cl_2.

Peer-reviewed version available at Polymers 2018, 10, 1297; doi:10.3390/polym10121297

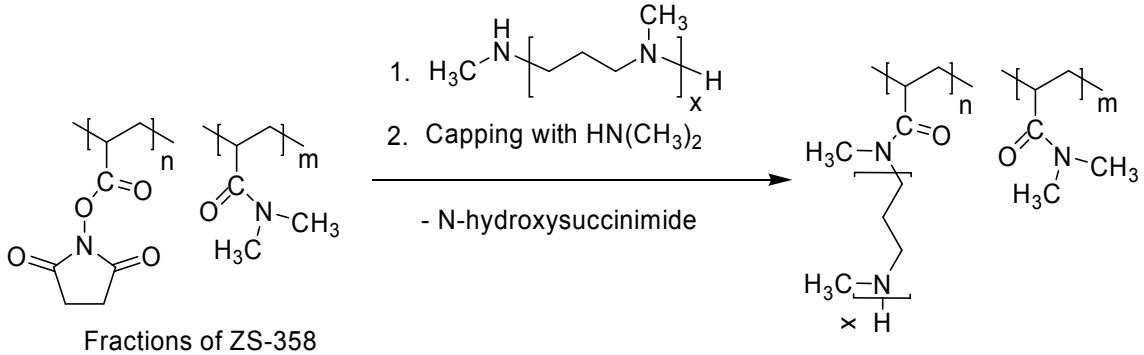
The extracts were dried over anhydrous potassium carbonate, evaporated and finally kept under vacuum of an oil pump for two days. The obtained LCPA fractions contain 27.6 (ZS-309-1), 16.3 (ZS-309-2), 13.4 (ZS-309-3) nitrogen atoms in molecule as determined with ^{1}H NMR (Figure S2) with the use signals at 2.40 ppm (terminal methyl groups) and 2.16 ppm (methyl groups in the polyamine chain).

Table 1. Fractionation of ZS-358.

Fraction #	Portion of n-hexane, g	Precipitation time	Weight of dried fraction, g
1F	19.64	1.5 hour	-*
2F	24.38	0.5 hour	0.6449
3F	23.79	over night	1.2384
4F	15.36	over night	0.7422
5F	18.93	over night	0.7243
6F	21.52	over night	0.8777
7F	34.39	over night	0.5444

*1F was unsuitable for the further usage because of poor solubility in organic solvents. Fractions 3-7 were precipitated from solutions kept in a refrigerator.

2.3.4. Grafting of oligo(N-methylazetidine) onto poly(N,N-dimethylacrylamide-co-N-acryloxysuccinimide) (ZS-371-n)



Preparation of ZS-371-1. ZS-309-3 (0.06602 g), a fraction of oligo(N-methylazetidine), was dissolved in a mixture of 0.70 g of DMF and 1.02 g of 10.8% $HN(CH_3)_2$ in 1,4-dioxane (1-st portion) followed by addition of 0.24958 g of 3F (a ZS-358 fraction) solution in 1.83 g of DMF. The solution was purged with argon, sealed in a glass vessel and kept at 60 °C for 24 hours. After that the 2-nd portion of the dimethylamine solution (0.38 g) was added and heating continued for additional 24 hours. Then the mixture was rotary evaporated and further kept under vacuum of an oil pump at room temperature for 3 hours. The sticky residue was thoroughly triturated with diethyl ether multiple times, the ether solutions discarded and the product reprecipitated three times from methylene chloride/methanol to ether. After vacuum drying, the polymer was dissolved in deionized water, filtered through a 0.45 μm cellulose acetate membrane and freeze-dried to give 0.222 g of ZS-371-1.

The other polymers ZS-371-(2-7) were prepared similarly, see the Table 2.

The content of grafted units was calculated from ^{1}H NMR spectra (Figure S3) of TFA-derivatized samples dissolved in DMSO-d_6 [31]. The integral intensities of the 1H NMR signal at 10.4 ppm (protons at tertiary nitrogen atoms) was compared with the integral intensities of the all C-H protons within 0.8-4.6 ppm except for those of DMSO, keeping in mind the average length of the grafted oligoamines.

Peer-reviewed version available at Polymers 2018, 10, 1297; doi:10.3390/polym10121297

208 **Table 2.** Grafting of oligo(N-methylazetidine) onto fractions of ZS-309.

Item	ZS-371-1	ZS-371-2	ZS-371-3	ZS-371-5
Fraction of ZS-358	3F	3F	3F	4F
Amount, g	0.24958	0.51380	0.19950	0.31191
DMF for dissolving ZS-358, g	1.83	3.37	3.37	2.95
Oligo(N-methylazetidine) fraction	ZS-309-3	ZS-309-2	ZS-309	ZS-309-1
Length of the Oligo(N-methylazetidine)	13.4	16.3	15.5	27.6
Amount, g	0.06602	0.25028	0.07295	0.26000
1-st portion of 10.8% $HN(CH_3)_2$ in 1,4-dioxane, g	1.02	2.10	0.81	1.27
2-nd portion of 10.8% $HN(CH_3)_2$ in 1,4-dioxane, g	0.38	0.45	0.49	0.43
Yield of copolymer, g	0.22189	0.42537	0.16356	0.32097
M_n, kDa	11.0	11.4	11.9	8.6
M_w/M_n	1.24	1.22	1.36	1.16
Grafting degree, %	1.8	3.0	2.3	3.4

209 *2.4. Study of polymer - oligonucleotide interactions and in vitro activity of the polyplexes*

210 2.4.1. Interaction with DNA oligonucleotides

211 The interaction between DNA oligonucleotides and polymers was investigated by
212 electrophoresis on agarose gel. Complexes were prepared by mixing solutions of the polymer and
213 oligonucleotide. The samples were incubated at room temperature for 30 min and placed into the
214 wells of the 1% agarose gel. Free oligonucleotide, as a control, was also loaded onto the gel. The gel
215 running buffer was 40 mM Tris acetate (adjusted to pH 7.4) and 1 mM EDTA. A glycerol gel loading
216 buffer was used (0.5% sodium dodecyl sulfate, 0.1 M EDTA (pH = 8), and 50% glycerol for 10×
217 reagent). The gel was run at 90 V and the fluorescein-tagged oligonucleotide was visualized on a UV
218 transilluminator.

219 2.4.2. RiboGreen assay

220 Polyplexes were prepared in the ratio of 4:1 and diluted to 100 µL with deionized water in a
221 96–well plate. 100 µL of 1:1000 diluted ribogreen dye (Invitrogen, USA) was added to each well and
222 the fluorescence intensity was measured using a multimode reader (Synergy H1, Biotek, USA)
223 following incubation for 5 min in dark. The sample was excited at 490 nm and the emission was
224 measured at 525 nm.

225 2.4.3. Study of the polymer and polyplex toxicity

226 Toxicity of the polymer and polyplex was evaluated using (3-(4,
227 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) MTS assay
228 (Cell Titer 96 Aqueous one solution, Promega, USA). Four thousand A549 cells per well were seeded
229 in a 96-well plate and incubated at 37 ºC in 5 % CO_2. After the cells achieved confluency, the medium
230 was removed and washed with PBS (pH 7.4) to remove the non-adherent cells. Polyplexes were
231 prepared by mixing 10 µM siRNA (Eurofins) with 4 mg/mL polymer solution in 1:4 ratio. Then, 5 µL
232 of the polyplex or 2 mg/mL polymer solution were mixed with 100 µL of serum-free media and
233 added to the cells. After 4 h, the medium was replaced with the fresh medium and incubated for 24 h
234 or 48 h. MTS reagent (Promega, USA, 10 µL) and 100 µL of serum-free media were added to each
235 sample well and incubated at 37 ºC for 2 h. The reaction was stopped by addition of 25 µL of 10%
236 sodium dodecyl sulfate (SDS) solution. The absorbance was measured at 490 nm using multimode
237 reader (Synergy H1, Biotek, USA).

2.4.4. Study of the polyplex internalization

These studies were carried out with a polymer concentration of 4 mg/mL and a siRNA concentration of 10 μM with a polymer to siRNA ratio of 4:1. The internalisation of the polyplex in A549 cells was evaluated using fluorescent siRNA (Eurofins) that is excited at 538 nm and emits at 640 nm. A549 cells were cultured in DMEM (Gibco, USA) 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 phosphate buffered saline (PBS, pH 7.4) to remove the non-adherent cells. Then, 5 μL of polyplexes were added to 200 μL of serum-free media and incubated for specific time points. The medium was then replaced with fresh medium containing fetal bovine serum (Gibco, USA). The cells were stained with Hoechst 33342 (Invitrogen, USA) and the images were captured using laser scanning confocal microscopy (FV1000, Olympus, Japan).

3. Results and Discussion

3.1. Determination of critical length of DNA sequence in reaction with polymeric amines

The influence of length of single-strand DNA oligonucleotide on the ability to interact with PEI and AKX-182 was studied at N/P ratio (ratio of amine groups to nucleotides) equal to 20. The high excess of polymeric amine minimizes stacking and other possible side effects. Gel electrophoresis data (Figure 1) shows formation of slightly positive complex with 12-24-mer DNA for PEI and 16-24 mer DNA in the case of AKX-182. Decrease the DNA length to 8-12 units results in a diffuse spot which corresponds to some negative-charged complexes. 4-mer DNA does not interact with PEI and AKX-182. The formation of negative-charged products is unexpected, poor interaction of short oligonucleotides with polymeric amines could result in the presence of free DNA but the observed diffuse spots are far from free DNA position. We see here two kinds of DNA-containing particles: neutral (or slightly positive) and negative charged. These complexes were observed at various N/P ratios and DNA length (Table 3). Increase of the DNA length and N/P ratio results in disappearance of negative charged complex. Nucleic acids can interact with amines by the means of ionic interactions through phosphate groups or by hydrogen bonds through nucleobases [32,33 and references in this review]. The latter mechanism suggests that the negatively charged free phosphate groups compensates the positive charge of the free amine units. We can hypothesize formation of negatively charged coordinated regions surrounded with positive charged parts of the polymer (Scheme 2(a)). Increase of the polymeric amine content results in presence of positive charged complex only. The formation of just only negative particles with short DNA chains is possibly rises according to "all-or-nothing" scheme (Scheme 2(b)) which is often realized with weak associative interactions [34,35]. Experiments with double stranded DNA (Figure 2) show similar behavior of the DNA-polymer complexes.

Thus, 8-12 nucleotides represent a critical length of DNA chains at which interaction with polymeric amines proceeds at high excess of the amine and is accompanied by "all-or-nothing" effects.

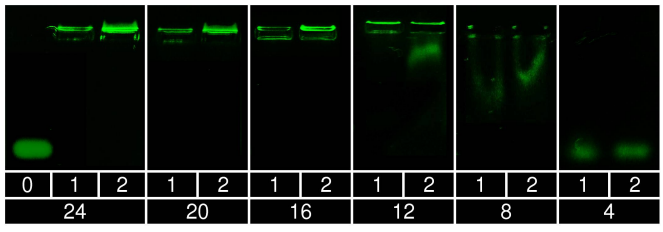


Figure 1. Gel electrophoresis data for PEI (1) and AKX-182 (2) complexes with (TCAG)n DNA (last row - length of the DNA), "0" - free DNA (similar blots were observed irrespective of DNA length). DNA concentration was 2.5 μM (calculating on DNA chains), N/P ratio = 20.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 23 October 2018 doi:10.20944/preprints201810.0515.v1

Peer-reviewed version available at Polymers 2018, 10, 1297; doi:10.3390/polym10121297

Table 3. Types of complexes observed in DNA - polymer interactions (pH = 7.4) [1]

DNA length	N/P ratio									
	PEI					AKX-182				
	5	10	20	40	80	5	10	20	40	80
4	N	N	N	N	N	N	N	N	N	N
8	N	-	-, +	-, +	+	N	-	-, +	-, +	+
12	-, +	-, +	+	+	+	-	-	-, +	+	+
16	-, +	-, +	+	+	+	-, +	-, +	+	+	+
20	-, +	-, +	+	+	+	-, +	-, +	+	+	+
24	-, +	-, +	+	+	+	-, +	-, +	+	+	+

[1] "N" - no interaction; "-" - a diffuse spot in negative area; "+" - neutral or slightly positive band.

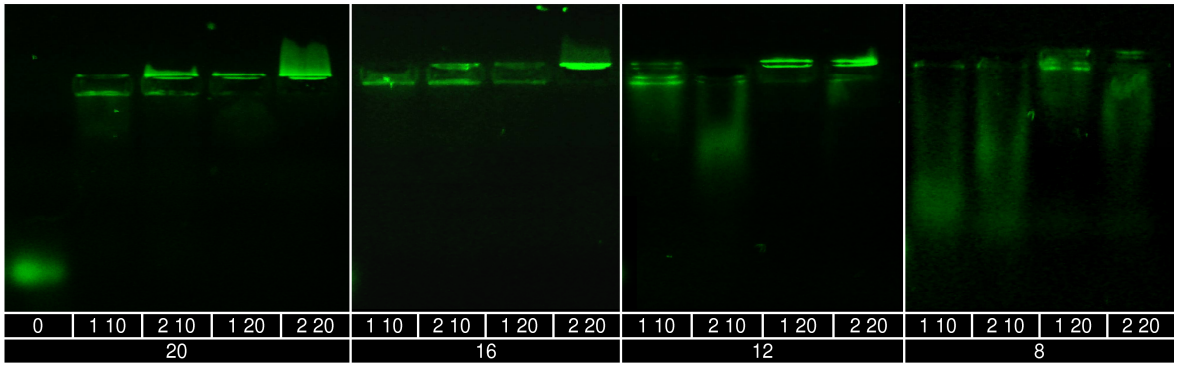


Figure 2. Gel electrophoresis data for PEI (1) and AKX-182 (2) complexes with duplex DNA (last row - length of the DNA), "0" - free DNA (similar blots were observed irrespective of DNA length). DNA concentration was 2.5 μM (calculating on DNA chains), N/P ratio presented as second number in first legend row.

3.2. Synthesis of poly(N,N-dimethylacrylamide) with grafted LCPA chains

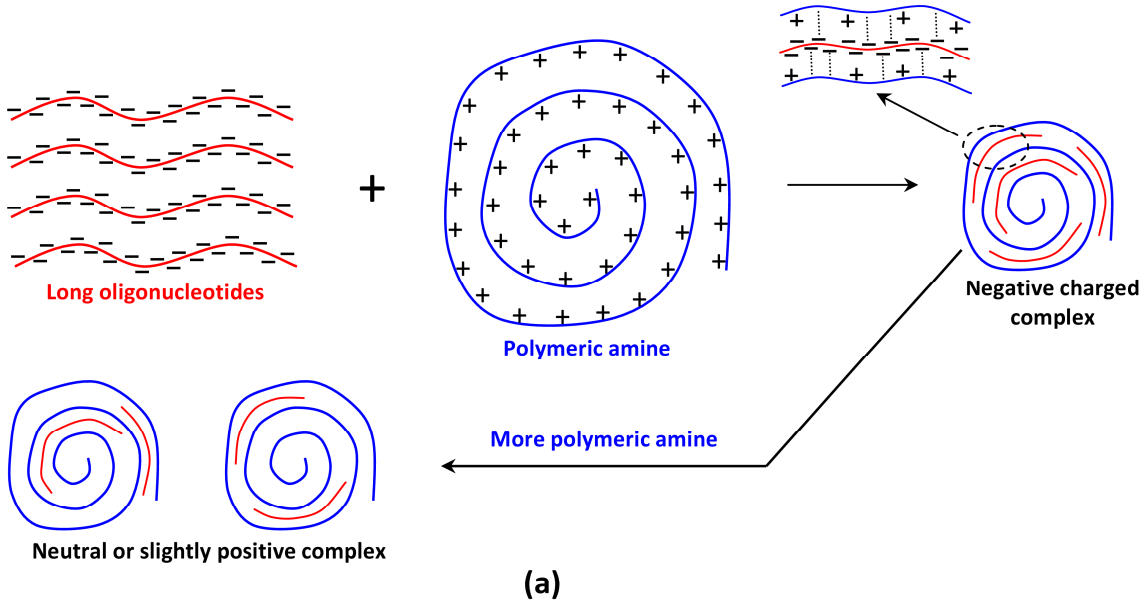
The desired PDMAAm with grafted LCPA chains was synthesized starting from poly(acryloyl chloride) which was converted into PDMAAm containing activated acrylic ester (oxysuccinimide) units. This copolymer was fractionated by precipitation from CH_2Cl_2 with n-hexane. The mixture of LCPA oligomers was fractionated with flash-SEC and three fractions containing oligomers with average 13, 16 and 27 nitrogen atoms were obtained. These oligomers as well as non-fractioned sample were involved in the reaction with activated polymer giving rise to the target polymers (Scheme 3, Table 2). Grafting degree of the LCPA chains is 1.8-3.4, M_w/M_n ratio is 1.16-1.24 for polymers with fractionated LCPA chains which is significantly lower than the values for polymers based on poly(acryloyl chloride) without fractionation (1.37-1.58 [13]).

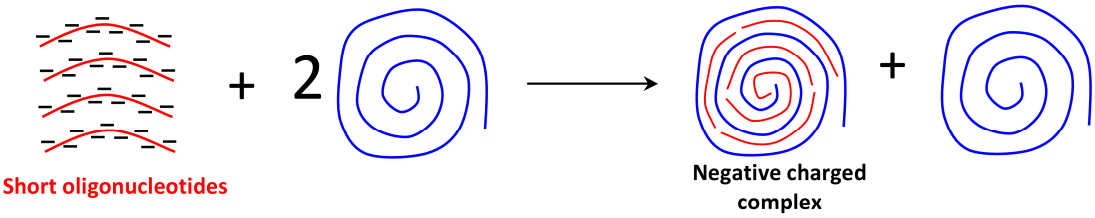
3.3. Interaction of LCPA containing polymers with oligonucleotides

Complexation between PDMAAm with grafted LCPA and oligonucleotides was studied with gel electrophoresis (Figure 4). AKX-371-5 sample only interacts with 16-24-mer DNA N/P = 20. The other polymers give complexes at N/P > 40. This behaviour corresponds to the data with PEI and AKX-182 samples: AKX-371-5 contains long (>16 units) side polyamine chains and all these chains exceed the critical length for interaction with DNA. AKX-371-1 (LCPA chain length - 13.4) is not active in the complexation because LCPA chain is not enough for the reaction with DNA and a stacking structures from several LCPA chains are not possible due to low grafting degree. AKX-371-2 and AKX-371-3 contain a certain fraction of LCPA longer than 20 units and increase of the polymer concentration results in approximately full complexation at N/P = 150.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 23 October 2018 doi:10.20944/preprints201810.0515.v1

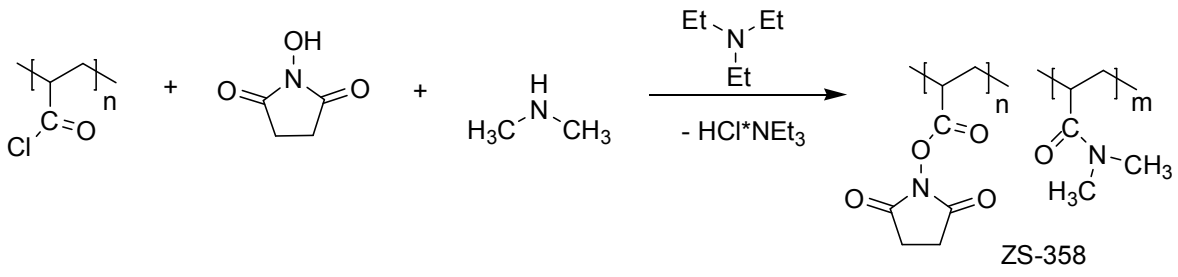
Peer-reviewed version available at Polymers 2018, 10, 1297; doi:10.3390/polym10121297



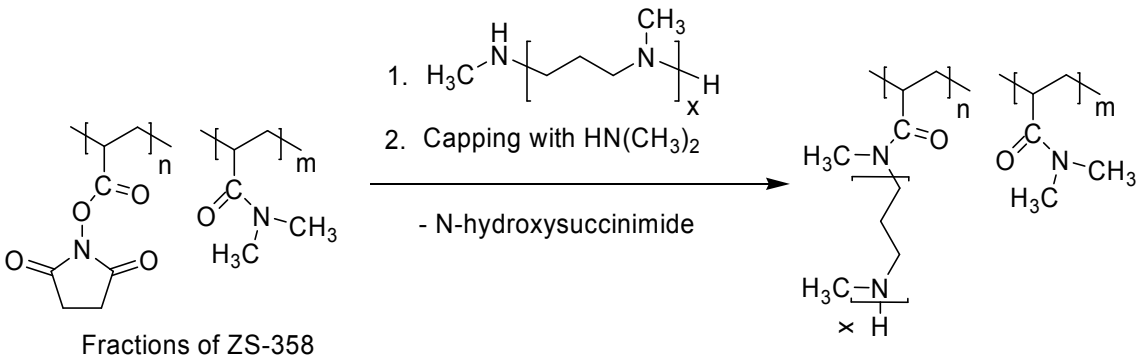


307

308 **Scheme 2.** Interaction of long (a) and short (b) oligonucleotides with polymeric amine.



309



310

311 **Scheme 3.** Synthesis of poly(N,N-dimethylacrylamide) with grafted LCPA chains

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 23 October 2018 doi:10.20944/preprints201810.0515.v1

Peer-reviewed version available at Polymers 2018, 10, 1297; doi:10.3390/polym10121297

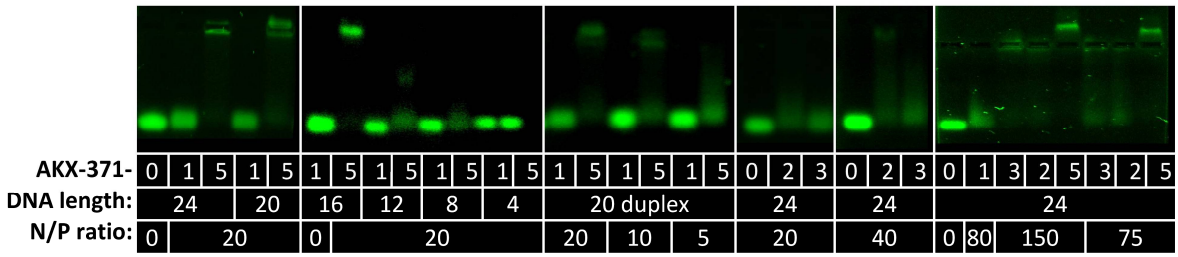


Figure 4. Gel electrophoresis data for AKX-371-N (N = 1,2,3,5) complexes with DNA. "0" - free DNA (similar blots were observed irrespective of DNA length). DNA concentration was 2.5 μM (calculating on DNA chains).

Interaction of the polymers with 19-mer double stranded si-RNA against vascular endothelial growth factor (VEGF) was studied with RiboGreen assay [36] which allows to measure concentration of free, non-complexed RNA (Table 4). The most stable complexes were obtained with ZS-371-5 polymer and the complexation proceeds to a large degree at N/P ratio 20 which corresponds to formation of partially negative charged complexes. Complexes of the LCPA containing polymers with single and double strand 20-mer DNA exist as 100-200 nm particles (Figure 5) which is appropriate for internalization into living cells [37-39].

Table 4. Interaction of si-RNA with LCPA containing polymers

Polymer	N/P ratio	Free RNA, %
ZS-371-1	20	6.4
ZS-371-2	20	5.6
ZS-371-3	20	11.9
ZS-371-5	20	3.8
ZS-371-1	80	8.9
ZS-371-2	150	5.8
ZS-371-3	155	7.4
ZS-371-5	180	5.3

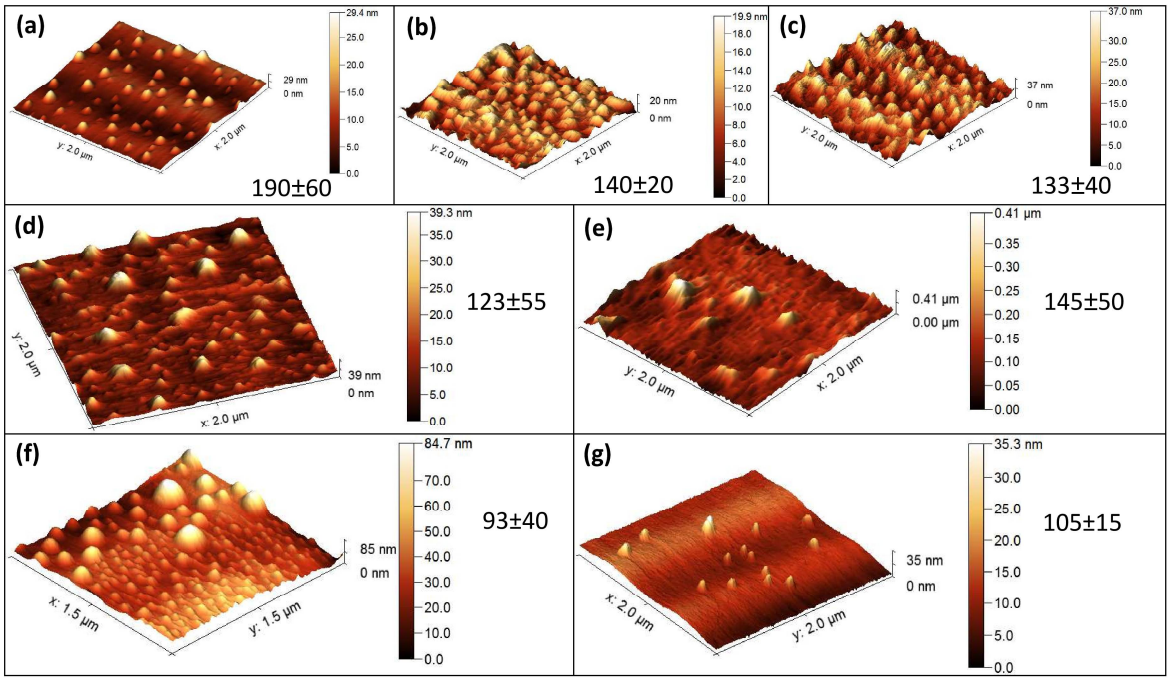


Figure 5. AFM images of AKX-371-5 polymer (a), complexes of polymers AKX-371-5 (b and c), AKX-371-2 (d and e) and AKX-371-3 (f and g) with single-strand (b, d and f) and double strand (c, e and g) 20-mer DNA. N/P ratios are 180 (b and c), 150 (d and e) and 155 (f and g). AKX-371-1 did not

Peer-reviewed version available at Polymers 2018, 10, 1297; doi:10.3390/polym10121297

give particles appropriate for AFM study. Numbers near pictures present average size of the particles, nm.

3.4. In vitro study of transfection activity of si-RNA polyplexes based on LCPA containing polymers

The new polymeric amines were studied as transfection agents with the use of 19-mer si-RNA which can silence vascular endothelial growth factor (VEGF) [40]. Viability (Figure 6) and internalization assays (Figure 7) were performed to estimate the transfection potential of the new polymers.

The free siRNA does not show significant reduction in viability after 24 h as well as 48 h. The free ZS-371-1 shows a slight but insignificant decrease in the viability of the A-549 cells after 48 h but its complex with anti-VEGF siRNA does not show any toxicity after both time points. This suggests that the surface charges on the uncomplexed polymer may interfere with the viability of the proliferating cells. Complexation with the siRNA neutralizes the surface charges that is manifested in the absence of any marked decrease in the cell viability after 48 h. The ZS-371-2 polymer does not alter the viability at both time points indicating its lack of toxicity. However, its polyplex with siRNA shows a slight reduction in the viability after 48 h. This suggests an effect of the VEGF silencing on different signalling pathways in the polyplex treated cells. In comparison, the ZS-371-3 did not alter the cell viability after 24 h or 48 h both in the uncomplexed as well as complexed forms. This may arise due to poor internalization in to the cells or due to poor complexation or extremely high affinity complexation that restricts the release of the siRNA or due to poor ability to escape from the endosome. But, the internalization studies (Figure 6a) show that ZS-371-3 polyplex localizes rapidly within 4 h in the cells thereby ruling out the possibility of poor internalization. Further, ribogreen assay reveals that the complexation efficiency is about 92% and hence poor siRNA could not explain the observed cell viability. Therefore, it could be possible that the siRNA release or endosomal escape may be the factors that could have influenced the lack of toxicity for this formulation. The viability of cells treated with ZS-371-5 shows a small but insignificant reduction in the viability after 48 h but its complex with the anti-VEGF siRNA shows significant decrease in the cell viability after 48 h indicating the effect of VEGF silencing in the cells. Several reports [41,42] have indicated that VEGF silencing can mediate cell death and decreased proliferation by modulating the PI3K/Akt and Notch signalling pathways respectively. Internalization studies show (Figure 6b) significant internalization of si-RNA after 6h. si-RNA localizes mostly in cytoplasm (not in nucleus) which is necessary for the effective silencing. The ribogreen assay revealed that ZS-371-5 shows the maximum complexation efficiency followed by the ZS-371-2 which is explainable with the length of grafted LCPA chains. This could be one of the factors that could have influenced the cell viability assay. Additional factors such as endosomal escape and release of siRNA could also have influenced the viability that needs further validation.

Conclusions

We have found that critical length for oligonucleotides in interaction with polymeric amines is 8-12 units and complexation at these length can be accompanied by "all-or-nothing" effects. New polymers with grafted polyamine chains were obtained and studied in complexation with DNA and RNA oligonucleotides. The most effective interaction and transfection activity was found for a sample with average number of nitrogens in polyamine chain equal to 27, i.e. for a sample which all grafted chains are longer the critical length for polymeric amine - oligonucleotide complexation.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 23 October 2018 doi:10.20944/preprints201810.0515.v1

Peer-reviewed version available at Polymers 2018, 10, 1297; doi:10.3390/polym10121297

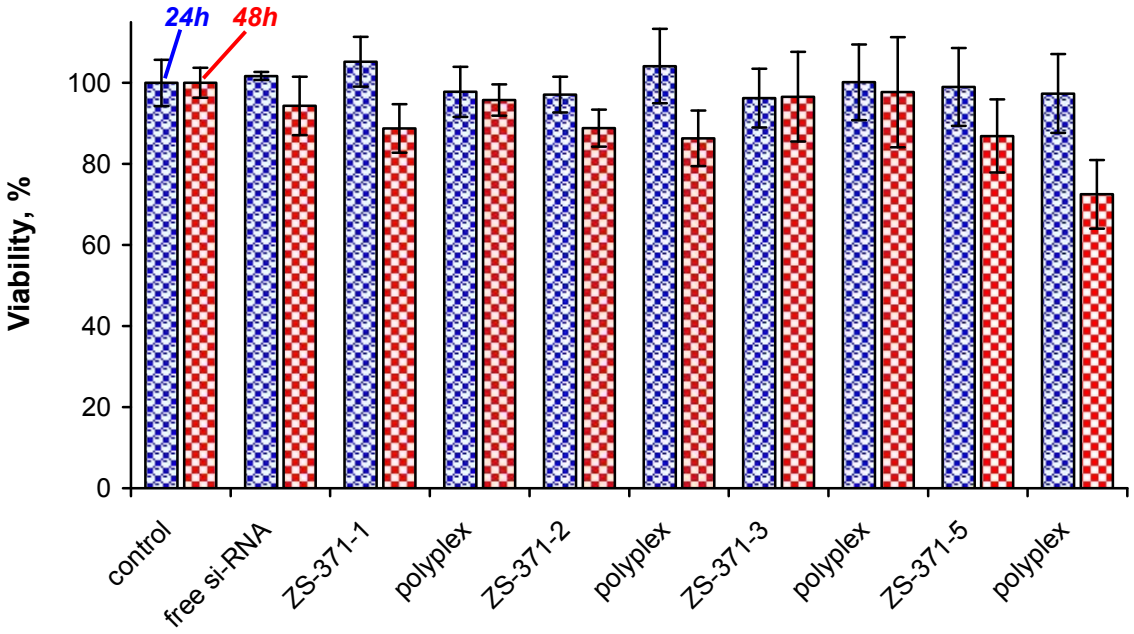


Figure 6. Cell viability of A549 cancer cells after treatment with different polymers both independently as well as after complexation with anti-VEGF si-RNA.

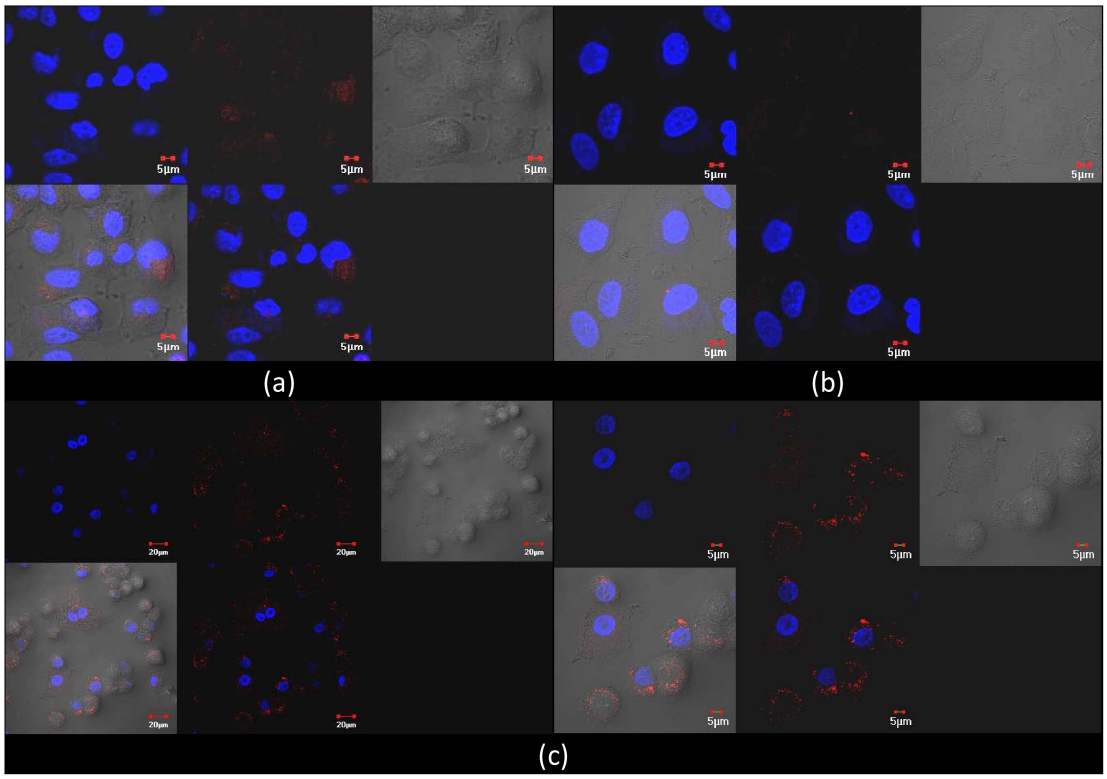


Figure 7. Internalization of polyplexes based on ZS-371-3 (a) and ZS-371-5 (b and c) polymers in A549 cancer cells after 4 (a and b) and 6 (c) h observed using laser scanning confocal microscopy. Scale bar represents 5 (a, b and c, right) and 25 (c, left) μm.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1. Liquid chromatography-mass spectrometry (LC-MS) analysis of ZS-309 fractions; Figure S2. 1H NMR spectra of LCPA (ZS-309) fractions.; Figure S3 1H NMR spectra of copolymers (derivatized with TFA) in DMSO-d6.

Author Contributions: Vadim V. Annenkov conceived and designed the experiments on polymer synthesis and study of polymer - DNA interactions; Uma M. Krishnan conceived and designed the experiments on polymer - si-RNA interaction and *in vitro* study of the polyplexes; Elena N. Danilovtseva performed experiments on study of polymer - DNA interactions and analyzed the data; Viktor A. Pal'shin and Stanislav N. Zelinskiy performed synthesis of new polymers; Gayathri Kandasamy performed the experiments on polymer - si-RNA interaction and in vitro study of the polyplexes; and Vadim V. Annenkov and Uma Maheswari Krishnan wrote the paper.

Funding: We acknowledge financial support from a joint grant of the Russian Science Foundation (16-45-02001) and the Department of Science Technology of the Ministry of Science and Technology of the Republic of India (INT/RUS/RSF/10).

Acknowledgments: We are thankful to the Center of Ultramicroanalysis (Limnological Institute) and the Baikal Analytical Center (A. E. Favorsky Irkutsk Institute of Chemistry) for providing equipment.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Innus, M. Antisense cancer therapy: do antisense oligonucleotides hold promise as a cure for cancer? Chem. Sci. J. 2017, 8, e117, DOI: 10.4172/2150-3494.1000e117.
2. Zaimy, M.; Saffarzadeh, N.; Mohammadi, A.; Pourghadamyari, H.; Izadi, P.; Sarli, A.; Moghaddam, L.; Paschepari, S.; Azizi, H.; Torkamandi, S.; Tavakkoly-Bazzaz, J. New methods in the diagnosis of cancer and gene therapy of cancer based on nanoparticles. Cancer Gene Ther. 2017, 24, 233–243, DOI: 10.1038/cgt.2017.16 3.
3. Gurav, B.; Srinivasan, G. Antisense oligonucleotides as therapeutics and their delivery. Curr. Sci. 2017, 1, 490-498, DOI: 10.18520/cs/v112/i03/490-498.
4. Bhavsar, D.; Subramanian, K.; Sethuraman, S.; Krishnan, U.M.; Nano-in-nano' hybrid liposomes increase target specificity and gene silencing efficiency in breast cancer induced SCID mice. Eur. J. Pharm. Biopharm. 2017, 119, 96-106, DOI: 10.1016/j.ejpb.2017.06.006.
5. Behr, J.P. The proton sponge: a trick to enter cells the viruses did not exploit. Chimia 1997, 51, 34-36
6. Akinc, A.; Thomas, M.; Klibanov, A.M.; Langer, R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J. Gene Med. 2005, 7, 657-663, DOI: 10.1002/jgm.696.
7. Tsuchida, E.; Abe, K. Interactions between macromolecules in solution and intermacromolecular complexes. Adv. Polym. Sci., 1982, 45, 1-119, DOI: 10.1007/BFb0017549.
8. Antipina, A.D.; Baranovskii, V.Yu.; Papisov, I.M.; Kabanov, V.A. Equilibrium peculiarities in the complexing of polymeric acids with poly (ethylene glycols). Polym. Sci. U.S.S.R., 1972, 14, 1047-1057, DOI: 10.1016/0032-3950(72)90108-6.
9. Dufresne, M.H.; Elsabahy, M.; Leroux, J.C. Characterization of polyion complex micelles designed to address the challenges of oligonucleotide delivery. Pharm. Res. 2008, 25, 2083-2093, DOI: 10.1007/s11095-008-9591-6.
10. Sparks, S.M.; Waite, C.L.; Harmon, A.M.; Nusblat, L.M.; Roth, C.M.; Uhrich, K.E. Efficient intracellular siRNA delivery by ethyleneimine-modified amphiphilic macromolecules. Macromol. Biosci. 2011, 11, 1192–1200, DOI: 10.1002/mabi.201100064.
11. Maassen, S.J.; de Ruiter, M.V.; Lindhoud, S.; Cornelissen, J.J.L.M. Oligonucleotide length dependent formation of virus-like particles. Chem. Eur. J. 2018, 24, 7456-7463, DOI: 10.1002/chem.201800285.
12. Wadhwa, M.S.; Collard, W.T.; Adami, R.C.; McKenzie, D.L.; Rice, K.G. Peptide-mediated gene delivery: influence of peptide structure on gene expression. Bioconjugate Chem. 1997, 8, 81-88, DOI: 10.1021/bc960079q.
13. Danilovtseva, E.; Maheswari Krishnan, U.; 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, DOI: 10.3390/polym9110624.

14. Annenkov, V.V.; Krishnan, U.M.; Pal'shin, V.A.; Zelinskiy, S.N.; Kandasamy, M.; Danilovtseva, E.N. Bioinspired water-soluble polymers with grafted polyamine chains: synthesis and complexation with oligonucleotides. Chinese J. Polym. Sci. 2018, 36, 1114-1122, DOI: 10.1007/s10118-018-2133-8.
15. Pegg, A.E. The function of spermine. IUBMB Life 2014, 66, 8-18, DOI: 10.1002/iub.1237.
16. Knott, J.M. Biosynthesis of long-chain polyamines by crenarchaeal polyamine synthases from Hyperthermus butylicus and Pyrobaculum aerophilum. FEBS Lett. 2009, 583, 3519-3524, DOI: 10.1016/j.febslet.2009.10.014.
17. Kröger, N.; Deutzmann, R.; Bergsdorf, C.; Sumper, M. Species-specific polyamines from diatoms control silica morphology. Proc. Natl. Acad. Sci. U. S. A. 2000, 97, 14133–14138, DOI: 10.1073/pnas.260496497.
18. Matsunaga, S.; Sakai, R.; Jimbo, M.; Kamiya, H. Long-chain polyamines (LCPAs) from marine sponge: possible implication in spicule formation. ChemBioChem 2007, 8, 1729–1735, DOI: 10.1002/cbic.200700305.
19. Durak, G.M.; Taylor, A.R.; Walker, C.E.; Probert, I.; de Vargas, C.; Audic, S.; Schroeder, D.; Brownlee, C.; Wheeler, G.L. A role for diatom-like silicon transporters in calcifying coccolithophores. Nat. Commun. 2016, 7, 10543, DOI: 10.1038/ncomms10543.
20. Kroger, N.; Deutzmann, R.; Sumper, M. Polycationic peptides from diatom biosilica that direct silica nanosphere formation. Science 1999, 286, 1129-1132, DOI: 10.1126/science.286.5442.1129.
21. Annenkov, V.V.; Shishlyannikova, T.A.; Zelinskiy, S.N.; Bridoux, M.C.; Danilovtseva, E.N. Unusual polyamines from Baikalian diatoms. ChemistrySelect 2018, 3, 9708-9713, DOI:10.1002/slct.201802032.
22. Brunner, E.; Lutz, K.; Sumper, M. Biomimetic synthesis of silica nanospheres depends on the aggregation and phase separation of polyamines in aqueous solution. Phys. Chem. Chem. Phys. 2004, 6, 854–857, DOI: 10.1039/b313261g.
23. Annenkov, V.V.; Danilovtseva, E.N.; Pal'shin, V.A.; Aseyev, V.O.; Petrov, A.K.; Kozlov, A.S.; Patwardhan, S.V.; Perry, C.C. 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, 1772–1780, DOI: 10.1021/bm2001457.
24. Annenkov, V.V.; Zelinskiy, S.N.; Danilovtseva, E.N.; Perry, C.C. Synthesis of biomimetic polyamines. ARKIVOC, 2009 xiii 116-130, DOI: 10.3998/ark.5550190.0010.d10.
25. Annenkov, V.V.; Pal'shin, V.A.; Verkhozina, O.N.; Larina, L.I.; Danilovtseva, E.N. Composite nanoparticles: a new way to siliceous materials and a model of biosilica synthesis. Mater. Chem. Phys. 2015, 165, 227-234., DOI: 10.1016/j.matchemphys.2015.09.022.
26. Annenkov, V.V.; Patwardhan, S.V.; Belton, D.; Danilovtseva, E.N.; Perry, C.C. A new stepwise synthesis of a family of propylamines derived from diatom silaffins and their activity in silicification. Chem. Commun. 2006, 1521–1523, DOI: 10.1039/b515967a.
27. Pavlov, G.M.; Korneeva, E.V.; Ebel, C.; Gavrilova, I.I.; Nesterova, N.A.; Panarin, E.F. Hydrodynamic behavior, molecular mass and conformational parameters of poly(vinylformamide) molecules. Polym. Sci. A 2004, 46, 1063–1067.
28. Buruiana, E.C.; Buruiana, T.; Hahui, L. Preparation and characterization of new optically active poly(N-acryloyl chloride) functionalized with (S)-phenylalanine and pendant pyrene. J. Photochem. Photobiol. A 2007, 189, 65–72, DOI: 10.1016/j.jphotochem.2007.01.008.
29. Newman, S.; Krigbaum, W.R.; Laugier, C.; Flory, P.J. Molecular dimensions in relation to intrinsic viscositie. J. Polym. Sci. 1954, 14, 451–462, DOI: 10.1002/pol.1954.120147704.
30. Farnsworth, N.R. Biological and phytochemical screening of plants. J. Pharm. Sci. 1966, 55, 225-276, DOI: 10.1002/jps.2600550302.
31. 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, 174-179, DOI: 10.1016/j.polymertesting.2016.10.013.
32. Kondinskaia, D.A.; Gurtovenko, A.A. Supramolecular complexes of DNA with cationic polymers: the effect of polymer concentration. Polymer 2018, 142, 277-284, DOI: 10.1016/j.polymer.2018.03.048.
33. Shen, Z.L.; Xia, Y.Q.; Yang, Q.S.; Tian, W.D.; Chen, K.; Ma, Y.Q. Polymer-nucleic acid interactions. Top. Curr. Chem. 2017, 375, 44, DOI: 10.1007/s41061-017-0131-x.
34. Lopez-Fontal, E.; Milanesi, L.; Tomas, S. Multivalence cooperativity leading to "all-or-nothing" assembly: the case of nucleation-growth in supramolecular polymers. Chem. Sci. 2016, 7, 4468-4475, DOI: 10.1039/c6sc00520a.

35. Li, Y.; Zhao, T.; Wang, C.; Lin, Z.; Huang, G.; Sumer, B.D.; Gao, J. Molecular basis of cooperativity in pH-triggered supramolecular self-assembly. Nat. Commun. 2016, 7, 13214, DOI: 10.1038/ncomms13214.
36. Michel, T.; Luft, D.; Abraham, M.K.; Reinhardt, S.; Medina, M.L.S.; Kurz, J.; Schaller, M.; Avci-Adali, M.; Schlensak, C.; Peter, K.; Wendel, H.P.; Wang, X.; Krajewski, S.; Cationic nanoliposomes meet mRNA: efficient delivery of modified mRNA using hemocompatible and stable vectors for therapeutic applications. Mol. Ther. Nucleic Acids 2017, 8, 459–468, DOI: 10.1016/j.omtn.2017.07.013.
37. Wang, W.; Naolou, T.; Ma, N.; Deng, Z.; Xu, X.; Mansfeld, U.; Wischke, C.; Gossen, M.; Neffe, A.T.; Lendlein, A. Polydepsipeptide block-stabilized polyplexes for efficient transfection of primary human cells. Biomacromolecules 2017, 18, 3819−3833, DOI: 10.1021/acs.biomac.7b01034.
38. Kasper, J.C.; Schaffert, D.; Ogris, M.; Wagner, E.; Friess, W. Development of a lyophilized plasmid/LPEI polyplex formulation with long-term stability—a step closer from promising technology to application. J. Controlled Release 2011, 151, 246–255, DOI:10.1016/j.jconrel.2011.01.003.
39. Pezzoli, D.; Giupponi, E.; Mantovani, D.; Candiani, G. Size matters for in vitro gene delivery: investigating the relationships among complexation protocol, transfection medium, size and sedimentation. Sci. Rep. 2017, 7, 44134, DOI: 10.1038/srep44134.
40. Espana-Serrano, L.; Chougule, M.B. Enhanced anticancer activity of PF-04691502, a dual PI3K/mTOR inhibitor, in combination with VEGF siRNA against non-small-cell lung cancer. Mol. Ther. Nucleic Acids 2016; 5, e384, DOI: 10.1038/mtna.2016.90.
41. Peng, N.; Gao, S.; Guo, X.; Wang, G.; Cheng, C.; Li, M.; Liu, K. Silencing of VEGF inhibits human osteosarcoma angiogenesis and promotes cell apoptosis via VEGF/PI3K/AKT signaling pathway. Am. J. Transl. Res. 2016, 8, 1005-1015.
42. Li, G.J.; Yang, Y.; Yang, G.K.; Wan, J.; Cui, D.L.; Ma, Z.H.; Du, L.J.; Zhang, G.M. Slit2 suppresses endothelial cell proliferation and migration by inhibiting the VEGF-Notch signaling pathway. Mol Med Rep. 2017, 15, 1981-1988, DOI: 10.3892/mmr.2017.6240.