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Water-soluble N-[(2-hydroxy-3-trimethylammonium)propyl]chitosan chloride as a nucleic acids vector for cell transfection

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ABSTRACT

To endow the cationic polysaccharides with solubility in the whole pH-range without loss of functionality of the amino groups, different chitosan samples were treated with glycidyltrimethylammonium chloride. Each modified unit of the exhaustively alkylated quaternized chitosan (QCht) contained both quaternary and secondary amino groups. The intercalated dye displacement assay and ζ-potential measurements implied stability of QCht polyplexes at physiological conditions and protonation of the secondary amino groups in slightly acidic media which is favorable for transfection according to proton sponge mechanism. The cytotoxicity and transfection efficacy increased with the chain lengthening. Nevertheless, the longest chains of QCht, 250 kDa were less toxic than PEI for COS-1 cells and revealed comparable and even significantly higher transfection activity of siRNA and plasmid DNA, respectively. Thus, highly polymerized QCht (250 kDa) provided the highest level of the plasmid DNA transfection being 5 and 80 times more active than QCht (100 kDa) and QCht (50 kDa), respectively, and 4-fold more effective than PEI, 25 kDa. The established influence of QCht molecular weight on toxicity and transfection efficacy allows elaborating polysaccharide vectors that possess rational balance of these characteristics.

1. Introduction

In recent years the using of biocompatible and biodegradable cationic polysaccharide chitosan as a vector for delivering of nucleic acids in the cells (transfection) have gained increasing interest (He, Tabata, & Gao, 2010; Jayakumar et al., 2010; Mao, Sun, & Kissel, 2010; Mintzer & Simanek, 2009; Muzzarelli, 2010; Rudzinski & Aminabhavi, 2010). However, in the overwhelming majority of the investigations, the level of the transfection was relatively low. The factors that hinder the development and implementation of polyelectrolyte complexes of nucleic acids (so-called polyplexes) on the base of chitosan are poor solubility of the cationic polysaccharide in neutral media, insufficient stability of the polyplexes at physiological conditions and moderate selectivity with respect to different cell lines.

There are different ways to enhance chitosan solubility, specifically methylation (i.e. exhausted alkylation) of the primary amino groups. The presence of quaternary amino groups in the chains endows the methylated chitosan with solubility in the whole pH range and the ability to form stable polyplexes (Germershaus, Mao, Sitterberg, Bakowsky, & Kissel, 2008). The toxicity of polyplexes based on the methylated chitosan and the efficacy of the transfection depend on molecular characteristics of the vector (Kean, Roth, & Thanou, 2005; Thanou, Florea, Geldorf, Junginger, & Borchard, 2002). In the experiments with MCF-7 and COS-7 cell lines, an increase in the methylation degree virtually did not influence on toxicity of oligomeric polysaccharides but resulted in a growth in toxicity which remained lower as compared with polyethyleneimine (PEI). As to transfection, the maximal efficacy was provided by cationic oligomers and polymers containing a half of the modified repeat units in the chains as well as by exhaustively alkylated highly polymerized chitosans. The drawback to the methylation, apart from the other disadvantages, is a significant lowering of the polysaccharide molecular mass late in the modification. The grafting of the methylated chitosan with polyethylene glycol (PEG) (Germershaus et al., 2008) was attended with lowering of the toxicity and facilitated accumulation of the polyplexes in the cell. The efficacy of the transfection increased in the order:

Abbreviations: QCht, quaternized chitosan; PEI, polyethylenimine; GTMAC, glycidyltrimethylammonium chloride; SG, SYBR Green I; duDNA, short-chained DNA duplex (19 base pairs).

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chitosan < trimethylated chitosan < PEG-grafted trimethylated chitosan, but did not attain the activity of PEI-based polyplexes.

An alternative method of introducing the quaternary amino groups in chitosan chain is the treatment with betaine (Gao, Zhang, Chen, Gu, & Li, 2009). The toxicity and transfection efficacy of the betaine derivatives increased with the modification degree but did not exceed the toxicity and activity of PEI-based polyplexes.

The presence in the alkylated chitosans of a large part of partly substituted derivatives that consist of secondary and tertiary amino groups as well as O-substituted fragments is the challenge for their application. By modification of chitosan with betaine in the Holappa approach (Runarsson, Holappa, Jonsdottir, Steinsson, & Masson, 2008), one can prepare the polycation of definite structure. However this way is labor consuming since it consists of several successive stages of repeated treatments including numerous protection–deprotection procedures of the functional (hydroxylic) groups.

The exhausted alkylation of chitosan primary amino groups with glycidyltrimethylammonium chloride (GTMAC) appears to be promising way of synthesis highly charged cationic polysaccharide of homogeneous structure (Gorshkova et al., 2011; Lim & Hudson, 2004; Loubaki, Ourevitch, & Sicsic, 1991). The alkylation results in formation of the modified units, each containing quaternary amino group and secondary amino group that provide solubility of the quaternized chitosan (QCht) in the whole pH range. The protonation of the secondary amino groups in slightly acidic media could be important for transfection. According to proton sponge mechanism (Boussif et al., 1995), the protonation provides the timely escape to cytosol of the polyplexes that were captured by endosomes or lysosomes.

In a number of works on synthesis and properties of QCht, the structure and molecular characteristics of the polysaccharide have been confirmed (Gorshkova et al., 2011; Yevlampieva et al., 2011), and complex formation of QCht with either synthetic polyanions (Izumrudov, Volkova, Grigoryan, & Gorshkova, 2011) or DNA (Kostina, Gorshkova, & Izumrudov, 2011) have been studied. The information accumulated to date allows going from the model polyelectrolyte systems to QCht-based polyplexes as vectors for gene and drug delivery.

Herein we report the results of studying QCht polyplexes with either plasmid DNA or oligonucleotides. The established factors that control both toxicity and transfection efficacy of the polyplexes can form the basis for development of efficient chitosan vectors.

2. Materials and methods

2.1. Materials

Acetone, methanol, ethanol, hydrochloric acid, GTMAC, sodium hydroxide were purchased from Aldrich (USA). All solutions were prepared with the use of bidistilled water that was additionally purified with a Milli-Q system (Millipore, USA). RPMI 1640 and DMEM medium, fetal bovine serum (FBS), gentamicin, trypsin/EDTA solution, HEPES buffer were purchased from Paneco (Russia).

2.2. Polysaccharides

The sample of chitosan (degree of chitin deacetylation 90%, sol fraction 5% by mass) was purchased from ZAO Bioprogress (Russia). Before using, the chitosan was purified by precipitating from the acidic solution with alkaline solution, washed with distilled water to neutral pH value, and lyophilized. Polysaccharides of different molecular weights were prepared by partial depolymerization of the purified chitosan as described elsewhere (Huang, Khor, & Lim, 2004).

QCht samples were synthesized by alkylation of amino groups of the corresponding chitosan with glycidyltrimethylammonium chloride as described in Gorshkova et al. (2011). In brief: three portions of 3 mL of GTMAC (15 mmol) were added with 2 h intervals to dispersion of 0.8 g chitosan (5 mmol) in water at 80 °C under permanent stirring. After 10 h the product was precipitated in acetone (30 mL), stored for 12 h at 4 °C, dissolved in 25 mL of water:methanol (2:1) mixture, precipitated in 75 mL of acetone:ethanol (4:1) mixture. The precipitate was extracted for 2 days with ethanol in a Soxlet apparatus, dissolved in water, dialyzed for 48 h, and lyophilized. The quaternization degree of QCht samples was determined by NMR spectroscopy and element analysis on chlorine content (Gorshkova et al., 2011). The molecular mass of the chitosans and QCht samples was determined by light-scattering (Yevlampieva et al., 2011).

QCht samples with degree of alkylation 90 ± 3% and molecular weights 250, 100, and 50 kDa denoted as QCht(250), QCht(100), and QCht(50), respectively were prepared and used. The chitosan sample, 100 kDa was used for comparison.

2.3. Nucleic acids

Oligonucleotide sequences of siRNA against firefly luciferase siRNA (all sequences are written from 5′ to 3′) were UUUCCGUCAUCGUCUUUCC-dTdT and GGAAGACGAUGACGGAAA-dTdT. As a control, “scrambled siRNA” against phosphoprotein gene of respiratory-syncytial virus (strain “Long”) that consisted of UCUUGCAGUUAUAUUAUCG-dTdT and CGAUAAUAUAACUGCAAGA-dTdT oligoribonucleotides (Bitko, Musiyenko, Shulyayeva, & Barik, 2005) was used. siRNA samples were prepared by mixing of equimolar amounts of the self-complementary oligoribonucleotides, heating for 2 min at 50 °C, and cooling to room temperature. The dilution of siRNA to final concentration 0.2 μg/μL of the duplex was conducted by RNAase-free water.

DNA duplex (duDNA) comprising oligonucleotide sequences TTTCCGTCATCGTCTTTCCTT and GGAAAGACGATGACGGAAATT with 19 base pairs was prepared in a similar way and used on the fluorescence intercalator displacement assay.

All oligonucleotides were purchased from ZAO Sintol (Russia).

The plasmid “pGL3-Promoter Vector” (Promega, USA) containing SV-40 promoter-driven firefly luciferase gene was used for gene transfer experiments.

The “highly polymerized” calf thymus DNA was purchased from Sigma (USA).

2.4. Cell culture

CHO (Chinese hamster ovary cells) and COS-1 (African green monkey kidney cells) were maintained in RPMI 1640 and DMEM medium, respectively, and supplemented with 5% fetal bovine serum (FBS) and gentamicin (40 μg/mL) at 37 °C and 5% CO_2. The cells were subcultured regularly using trypsin/EDTA. For siRNA delivery experiments, CHO cells stably transfected with firefly luciferase gene (CHO-luc) were used as described elsewhere (Iijima et al., 2007).

2.5. Measurements of ζ-potential and size of the polyplexes

ζ-Potential and size of the particles were determined at 25 °C using Zetasizer Nano_ZD (Malvern Instruments). ζ-Potential measurements were performed with polyplexes formed by “highly polymerized” calf thymus DNA and QCht in 0.01 M solutions of Tris, HEPES, and MES buffers at pH 9.0, 7.2, and 5.5, respectively.

Concentration of DNA phosphate groups [P] in the solution before the titration was 6.0×10^{-5} mol/l. The data were presented as the dependence of ζ-potential on $[N_q]$:[P] ratio, where $[N_q]$ is molar concentration of quaternized amino groups of QCht. The size of the particles formed in mixtures of solutions of "highly polymerized" calf thymus DNA solution and QCht(250) or QCht(50) was estimated at $[N_q]$:[P] = 16 in 0.01 M HEPES buffer, pH 7.2 at the same concentration of the phosphate groups.

2.6. Fluorescence, spectrophotometry and luminescence measurements

All measurements were performed with the use of Varioskan® Flash 2.4 spectral scanning multimode reader (Thermo Fisher Scientific, USA).

2.7. Fluorescence intercalator displacement assay

The efficiency of the polycations binding with nucleic acids was estimated by fluorescence of intercalating dye SYBR Green I (Sigma–Aldrich, USA) (SG) which was measured in black 96-well plates (NUNC, Denmark). In the experiments, 5010 base pairs plasmid pGL3-Promoter and short-chained DNA duplex (duDNA) were used as plasmid DNA and the model of siRNA, respectively. The series of a polycation 2-fold dilutions were prepared in 96-well plate in amount of 50 μL per well followed by addition to each well of 50 μL of SG mixtures with plasmid DNA or duDNA in 10 mM HEPES buffer, pH 7.2 (final dilution of SG was 1:20,000). After 10 min incubation at room temperature and on permanent stirring, fluorescence intensity (F) was measured at λ_{ex} = 480 nm and λ_{em} = 520 nm. Data were presented as a dependence of F on [N]:[P] ratio, where [N] is molar concentration of all amino groups of the polycation. Stability of the polyplexes in water–salt media was assessed analogously by fluorimetric titration with 4 M NaCl of mixtures comprising SG, polycation, and nucleic acid. Data were represented as the dependence of relative fluorescence intensity F/F_0 on concentration of the low-molecular-weight electrolyte, where F_0 is fluorescence intensity of DNA·SG or duDNA·SG complexes at the same ionic strength.

2.8. Cytotoxicity assay

CHO and COS-1 cell lines were used. Cells were seeded into 96-well cell culture plates (NUNC, Denmark) at 2×10^5 cells/mL with 5% FBS, allowed to attach and grow for 24 h, and treated with 2-fold dilutions of polycations in FBS-free culture media for 24 h. The cytotoxicity of different polymers was assessed using "CellTiter 96® Non-Radioactive Cell Proliferation Assay" kit (Promega, USA) in accordance with the manufacturer's instructions. The MTT assay is based on the intracellular conversion of a tetrazolium salt to a red formazan product that is easily detected using the 96-well plate reader. The absorbance was recorded at 570 nm wavelength (with reference wavelength 700 nm). The data were presented in term of IC_{50} value, which corresponds to concentration of the polycation that inhibits cell proliferation by 50%.

2.9. Transfection by plasmid DNA

In vitro gene transfer efficiency of the polymers was estimated in COS-1 cells using firefly luciferase reporter gene as a part of pGL3-Promoter plasmid. Cells were seeded at 2×10^5 cells per well in 12-well Plates 24 h before the transfection. Immediately prior to transfection, the medium was replaced with Opti-MEM (Invitrogene, USA). The plasmid polyplexes were prepared directly before the transfection by mixing of 2 μg per well of the plasmid with a polycation at different [N]:[P] ratio and 15 min incubation at room temperature. The complexes were added to the plate (100 μL per well) and incubated 4 h at 37 °C. Then the medium was replaced by fresh growth medium. The transfection efficiency was evaluated 24 h after the transfection by measurement of the luciferase activity in the cells that was performed in the black 96-well plates (NUNC, Denmark) using "Luciferase Assay System" (Promega, USA) according to the manufacturer's instructions. The data were presented as relative light units (RLU) per 10^5 cells. In the transfection experiments, branched polyethylenimine (PEI, 25 kDa) (Sigma–Aldrich, USA) was used as a control.

2.10. Gene silencing with siRNA

For in vitro gene silencing experiments, CHO-luc cells stably transfected with firefly luciferase gene were used. The protocol was virtually the same as described above for the plasmid transfection with the two exceptions, i.e. the amount of siRNA in polyplexes was 1 μg per well and Opti-MEM medium was used for polyplex preparation. The data were presented as a residual luciferase activity in the cells treated with specific anti-firefly luciferase siRNA which was normalized to the activity of the cells treated with a nonspecific "scrambled siRNA". Lipofectamine 2000 (Invitrogene, USA) was used as a control of siRNA delivery.

2.11. Statistical analysis

Results are expressed as mean ± SD. Statistical comparisons were performed with the use of Graphpad Prism 4 software (USA). Data were analyzed using unpaired t-test and were accepted as significantly different when $p < 0.05$.

3. Results and discussion

3.1. Binding of the modified chitosan with nucleic acids

The efficacy of the binding was estimated by fluorescence intensity of intercalated dye SYBR Green I. The intercalation of the dye between the base pairs and formation of DNA·SG complex is accompanied by increase in the intensity, whereas the following electrostatic binding of DNA·SG with the added cationic polymer is attended with the fluorescence quenching caused by the competitive displacement of SG (Zhiryakova & Izumrudov, 2007).

In the experiments, highly polymerized plasmid DNA pGL3-Promoter (5010 base pairs) and duDNA were used. The model duplex which was composed of two complementary chains each consisted of 19 base pairs played the role of siRNA.

All studied QCht samples displaced the intercalated dye from the plasmid (Fig. 1), though the efficacy of the binding was lower as compared with PEI. Thus, the addition of PEI was accompanied by a complete fluorescence quenching (curve 1) indicating the quantitative displacement of the intercalator from the plasmid. On the contrary, the titration of DNA·SG with QCht solution was characterized by a noticeable residual fluorescence even at a large excess of the polysaccharide (curves 2–4). Besides, the quenching by PEI was virtually completed at the equivalence of the charges, namely at [N]:[P] ≈ 1.2 (curve 1), whilst the maximal quenching by QCht occurred at more than 2-fold excess of all amino groups of the polysaccharide, [N]:[P] ≈ 2.4 (curves 2–4).

The shift of inflexion points of the titration curves to higher values of [N]:[P] implies that a large part of QCht amino groups do not participate in formation of ion pairs with DNA. This assumption is supported by ζ-potential measurements (Fig. 2).

The reversal of the polyplexes charge ($\zeta = 0$) in neutral (pH 7.2) or slightly alkaline media (pH 9.0) occurs at practically equimolar content of QCht(250) quaternary amino groups and DNA phosphate groups, $[N_q]$:[P] ≈ 1 (Fig. 2). In other words, the quantity of

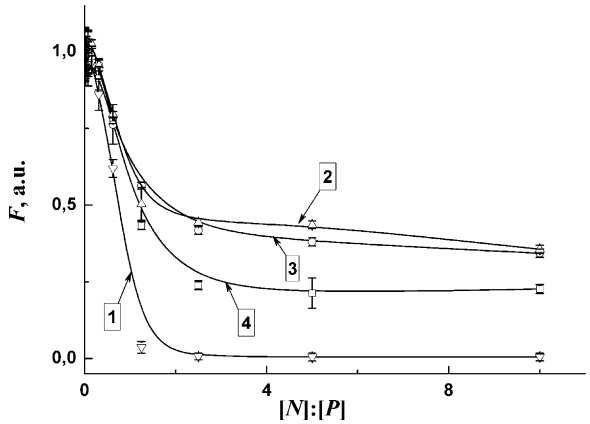

Fig. 1. Dependence of fluorescence intensity on [N]:[P] ratio upon titration of DNA·SG complex with PEI, 25 kDa (1) or QCht of different molecular mass, kDa: 250 (2), 100 (3), and 50 (4). pH 7.2, 25 °C, λ_{ex} = 480 nm, λ_{em} = 520 nm; [P] = 5×10^{-6} M.

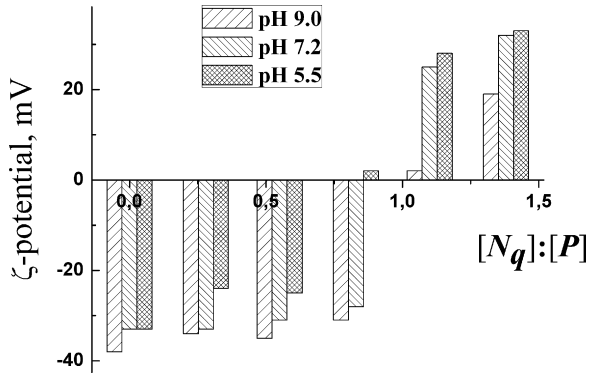

Fig. 2. Dependence of ζ-potential of polyplexes formed in DNA mixtures with QCht(250) at different pH on [N_q]:[P] ratio, where N_q are quaternary amino groups of the polysaccharide; [P] = 5.1×10^{-5} M, 25 °C.

these groups is quite enough for neutralization of the nucleic acid whereas the secondary amino groups do not participate in the electrostatic interaction. Their protonation and hence the contribution to the polyplex formation is accomplished only in slightly acidic media. This follows from the shift of the reversal charge region to [N_q]:[P] ≈ 0.75 at pH 5.5 (Fig. 2). The analogous shift was observed in the DNA mixtures with other QCht samples.

The marked residual fluorescence (up to 40%) at large excess of the polysaccharides suggests relatively low efficacy of the electrostatic interaction between the components. Note that similar residual fluorescence is typical for the methylated chitosans (Germershaus et al., 2008). In all likelihood, the binding of QCht molecules that are characterized by a pronounced stiffness (Yevlampieva et al., 2011) with the rigid double helix is controlled by steric restrictions which hinder the matching of the polyelectrolytes. Therefore QCht binding with DNA is less effective as compared with relatively flexible PEI (Fig. 1, curve 1). Argument in favor of the important role of the steric hindrances is the run of the curves of DNA·SG titration with QCht molecules of different molecular masses. Contrary to the expectations, curve 4 which corresponds to the shortest QCht chains is located well below curves 2 and 3 related to highly polymerized polysaccharides. This unusual growth in efficacy of the DNA binding with the shortening of QCht chain could stem from higher ability of the short chains to match with rigid double helix promoting formation of the ion pairs.

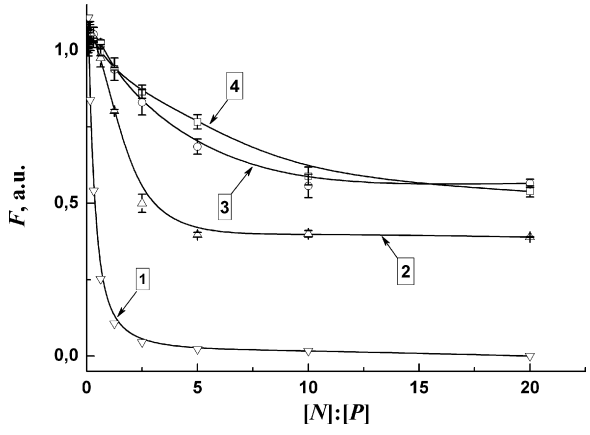

Fig. 3. Dependence of fluorescence intensity on [N]:[P] ratio upon titration of duDNA·SG complex with PEI, 25 kDa (1) or QCht of different molecular mass, kDa: 250 (2), 100 (3), and 50 (4). Other conditions are the same as in caption of Fig. 1.

The products of the cationic polymers interaction with short-chained nucleic acid behaved approximately in a like manner. Judging from the run of the titration curves (Fig. 3), in this case the replacing of PEI by the polysaccharides also halved the efficacy of the binding. In addition, the substitution of the short-chained duplex (Fig. 3) for plasmid DNA (Fig. 1) resulted in shift of all titration curves to higher value of [N]:[P] ratio which implies the weakening of the binding. This finding is in accordance with well-documented relatively low efficacy of interaction between the oppositely charged components when charged oligomers are used (Tsuchida and Abe, 1982). It is interesting that unlike plasmid DNA which binds more efficiently with short QCht chains (Fig. 1), the duplex interacts preferentially with highly polymerized polysaccharide (Fig. 3). Most likely, in this case the steric hindrances are not so pronounced and hence, the effect of local concentrating of the positive charges in the coil of highly polymerized QCht proved to be crucial for the interaction.

3.2. Stability of the polyplexes in water–salt media

Inasmuch as the transfection of eukaryotic cells is performed at physiological values of pH and ionic strength (pH 7.2, 0.14 M NaCl), stability of polyplexes in water–salt solutions is important characteristic. Using the same approach based on fluorescence intensity of the intercalated dye, one can study dissociation of the polyplexes by the added low-molecular-weight electrolyte. The dissociation of the ion pairs allows the dye to intercalate into getting free sites of the nucleic acid that is accompanied by growth in relative fluorescence intensity F/F_0, where F_0 is the fluorescence intensity of DNA·SG complex (or duDNA·SG complex) under the same conditions (Zhiryakova & Izumrudov, 2007).

The titration curves of mixtures of plasmid (Fig. 4A) or duplex (Fig. 4B), dye and the QCht sample (curves 2–4) differ from the PEI curve (curve 1). In QCht mixtures, the addition of salt is attended with regular increase in F/F_0 values, whereas in the PEI mixture, these values remain close to 0 in a wide range of the ionic strength with insignificant growth of F/F_0 at the end of the titration. This finding is in accordance with the influence of a nature of the polycation amino groups on stability of polyelectrolyte complexes in water–salt media. Specifically, the affinity of nucleic acid to polycation gradually decreased on replacement of chains with primary amino groups by the chains with secondary, tertiary, and quaternary amino groups, respectively (Izumrudov, Zhiryakova, & Kudaibergenov, 1999). PEI molecules contain primary, secondary, and tertiary amino groups in the ratio 1:2:1 (Lukovkin, Pshezhetsky, & Murtazaeva, 1973). The charge of QCht in neutral media is conditioned solely by quaternary amino groups that form the least stable ion pairs with phosphate groups. Thus QCht polyplexes dissociate noticeably even on addition of the insignificant quantity of the low-molecular-weight electrolyte.

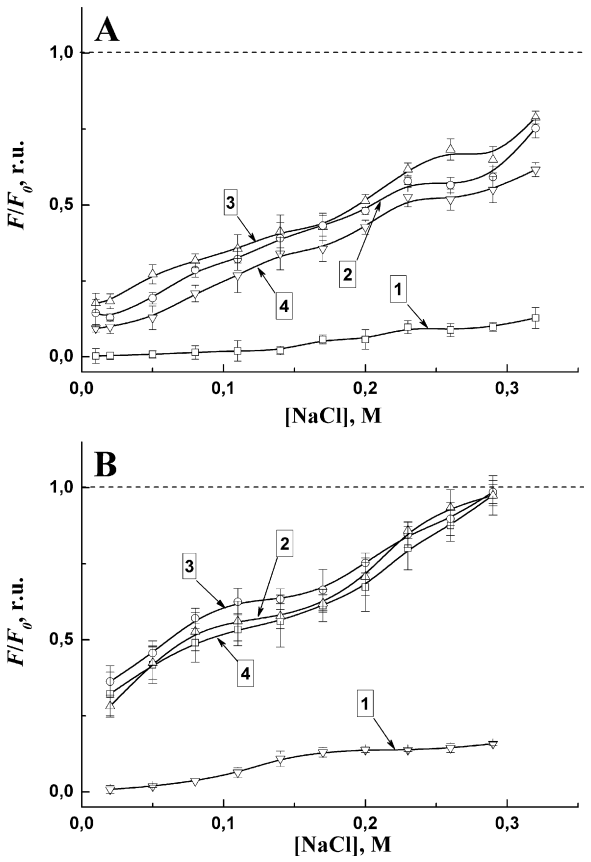

Fig. 4. Dependence on the salt concentration of relative fluorescence intensity in mixture of DNA·SG complex (A) or duDNA·SG complex (B) with PEI, 25 kDa (1) or QCht of different molecular mass, kDa: 250 (2), 100 (3), and 50 (4). [N]:[P] = 16, other conditions are the same as in caption of Fig. 1. Data were represented as the dependence of relative fluorescence intensity F/F_0 on concentration of the low-molecular-weight electrolyte, where F_0 is fluorescence intensity of DNA·SG or duDNA·SG complexes without polycation at the same ionic strength.

In all probability, the monotonic increase of the fluorescence intensity on the titration of plasmid mixture with QCht (Fig. 4A, curves 2–4) is conditioned by broad molecular mass distribution of QCht samples, i.e. the presence in the polysaccharides of a significant part of short chains that form with DNA of relatively unstable polyplexes. Slightly appeared jump on the curves with the inflection point at 0.2 M NaCl implies the cooperative destruction of a certain part of the ion pairs.

The polyplexes of short-chained DNA behave in a similar manner with the only difference that above effects are exhibited at lower salt concentration that is reasonably explained by decrease in the complex stability with the shortening of the chains (Fig. 4B). It is well known that the most pronounced destabilization occurs on replacing highly polymerized chain by the oligomers bearing relatively small amount of charges like the chains of duDNA.

So, at physiological ionic strength (0.14 M NaCl), the dissociation of the studied polyplexes is relatively high but not crucial (Fig. 4). At least a half of the ion pairs remain stable, i.e. the use of all QCht samples for the transfection is not ruled out. Besides, the revealed ability of secondary amino groups of the polysaccharides to be protonated in slightly acidic media inherent in endosomes and lysosomes (Fig. 2) could promote the transfection according to proton sponge mechanism (Boussif et al., 1995). Finally, the size of positively charged polyplexes formed by "highly polymerized" DNA with QCht(250) or QCht(50) which was measured as described in Section 2 proved to be practically the same and equaled 100 ± 10 nm. This finding agrees well with the size of polyplexes and dendriplexes formed by "highly polymerized" calf thymus DNA and implies the availability of the cationic polysaccharide for delivery of gene material.

The results of the transfection reported below evidence the prospects for using of QCht as a vector.

Table 1
Values of IC_{50} (m kg/mL) measured in two cells lines after the cells treatment with PEI and QCht samples.

Cell culture	QCht(50)	QCht(100)	QCht(250)	PEI 25 kDa
CHO	46.8 ± 2.8	16.6 ± 1.8	6.2 ± 0.2	9.8 ± 3.1
COS-1	170 ± 22.0	91.3 ± 18.4	41.1 ± 8.8	19.4 ± 3.6

3.3. Cytotoxicity of the modified chitosans

Cytotoxicity of the polysaccharides was assessed using CHO and COS-1 cell lines as described in Section 2. The values of IC_{50}, i.e. the concentration of the polycation which corresponds to death of a half of the cells decreased in both cells cultures with the growth of QCht molecular weight (Table 1). This finding is in accordance with well-documented increase in toxicity of the polymer upon lengthening of the chains.

Judging from IC_{50} values determined for PEI, 25 kDa and the most long-chained polysaccharide QCht(250) (Table 1), in case of CHO cells the toxicity of both polyamines is comparable, whereas for COS-1 cell culture, the polysaccharide proved to be half as many toxic as PEI despite much higher molecular weight (nearly one order of magnitude) of the polysaccharide. The toxicity decreased with the shortening of QCht chains, and for the shortest QCht(50) molecules, IC_{50} value determined for CHO and COS-1 cell lines appears to be 5-fold and 9-fold lower than the toxicity of PEI measured under the same conditions (Table 1).

3.4. Efficiency of transfection by plasmid DNA polyplexes

COS-1 cells were treated with polyplexes formed by pGL3-Promoter plasmid and QCht (or PEI) as described in Section 2. Since for all polyplexes the best results were obtained at [N]:[P] = 16 (data not shown), this ratio was used in further experiments. Transfection efficacy was evaluated by measuring of luciferase activity in lysates of the transfected cells after 24 h of the cells treatment. The efficacy increased with increase in QCht molecular weight (Fig. 5). Highly

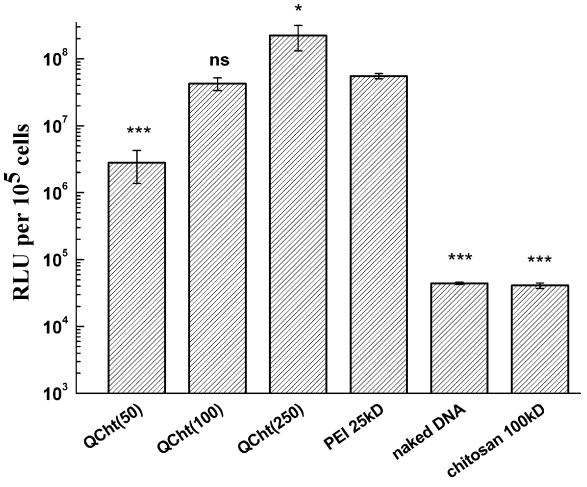

Fig. 5. Transfection efficacy of COS-1 cells treated with polyplexes formed by polycations and pGL3-Promoter plasmid with firefly luciferase reporter gene. Transfection efficiency is expressed as relative light units (RLU) per 10^5 cells. [N]:[P] = 16. Data obtained in three independent experiments. ***$p < 0.0001$ vs PEI 25 kDa; *$p < 0.05$ vs PEI 25 kDa; ns, no significant difference.

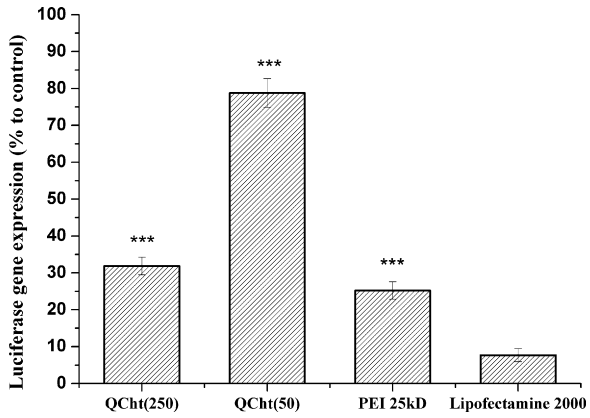

Fig. 6. The normalized residual activity of luciferase in CHO-luc cells transfected by polyplexes with siRNA. Data obtained in three independent experiments; [N]:[P] = 16. ***$p < 0.0001$ vs Lipofectamine 2000.

polymerized QCht(250) provided the highest level of the transfection being 5 and 80 times more active than QCht(100) and QCht(50), respectively, and even 4-fold more effective than PEI, 25 kDa that was used as a control.

However this advantage ceased to be true on shortening of the polysaccharides. Thus by the activity, PEI practically did not differ from QCht(100) and far exceeded (one order of magnitude) short chains of QCht(50) (Fig. 5). Noteworthy, the polyplex formed by the plasmid with non-modified chitosan that was prepared in slightly acidic media (pH 5.8) under the same conditions proved to be virtually ineffective vector which revealed the efficacy comparable with the transfection efficacy of naked DNA (Fig. 5).

3.5. Gene silencing with siRNA

CHO cells stably transfected by firefly luciferase gene (CHO-luc) were treated with siRNA polyplexes as described in Section 2. The polyplexes were prepared from the polycation and synthetic siRNA targeted to firefly luciferase gene. Data were presented as residual activity of luciferase that was normalized to the expression level of control cells treated with polyplexes based on nonspecific siRNA targeted to P-gene of respiratory-syncytial virus. Polyplex of siRNA with Lipofectamine 2000 which is extensively used as transfection agent was taken as a control. The polyplex inhibited the luciferase activity most efficiently (by 92%), whereas the polyplexes of PEI 25 kDa and highly polymerized QCht(250) revealed weaker capacity for the inhibition, i.e. 75% and 68%, respectively (Fig. 6). The shortening of QCht chains was accompanied by further lowering of the transfection efficacy that was indicated by decrease in the inhibition of luciferase expression from 68% for QCht(250) to 21% for QCht(50) (Fig. 6).

So, the results of both series of the experiments testify that highly polymerized QCht(250) exhibited the most pronounced transfection activity comparable with the activity of PEI 25 kDa in case of siRNA (Fig. 6) or even significantly higher as compared with this branched polyamine in case of plasmid (Fig. 5). Notice that the same regularity was established for PEI, i.e. the significant transfection efficacy with respect to DNA plasmid but far less effectiveness for siRNA transfection (Grayson, Doody, & Putnam, 2006). The relatively low transfection efficacy was markedly improved by introduction of hydrophobic groups into PEI chain (Creusat & Zuber, 2008) or chemical modifications that allowed decreasing of the toxicity (Zintchenko, Philipp, Dehshahri, & Wagner, 2008).

The suitability of highly polymerized QCht(250) for transfection is questionable due to toxicity that is the most pronounced among other QCht samples and even comparable with the toxicity of PEI (Table 1). The established oppositely directed influence of QCht molecular weight on toxicity and transfection efficacy allow us to assume that rational balance of these factors could be attained. QCht(100) sample appears to be the cationic polysaccharide which meets above requirement and hence, could form the basis for the development of effective transfection vector.

4. Conclusions

1. Primary amino groups of chitosan samples differed by a degree of polymerization were exhaustively alkylated with glycidyltrimethylammonium chloride. Each modified unit of the chains consisted of both quaternary amino group and secondary amino group that provided solubility of the polysaccharide in the whole pH range.
2. The intercalated dye displacement assay revealed relatively high stability of the modified chitosan polyplexes at physiological pH and ionic strength, whereas ζ-potential measurements indicated protonation of the secondary amino groups in slightly acidic media. These findings could be of particular importance for reinforced transfection of nucleic acids by the polyplexes, specifically in accordance with the proton sponge mechanism.
3. Although the cytotoxicity of the modified chitosans tended to increase with increase in the degree of polymerization, even highly polymerized samples proved to be less toxic on COS-1 cells than PEI 25 kDa (used as a control) in spite of one order of magnitude higher molecular weight of the polysaccharide.
4. The efficiency of transfection by either plasmid DNA or siRNA polyplexes also increased with lengthening of the modified chitosan. Compared to PEI, the highly polymerized sample revealed virtually the same and 4-fold more effective transfection of siRNA and plasmid DNA, respectively.
5. The established oppositely directed influence of the polysaccharide molecular weight on toxicity and transfection efficacy allows one to elaborate rational balance of these factors. The modified chitosan, 100 kDa met the requirement and hence, could form the basis for the development of effective transfection vector.

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