



Quaternized starch-based carrier for siRNA delivery: From cellular uptake to gene silencing

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ABSTRACT

RNAi therapeutics is a powerful tool for treating diseases by sequence-specific targeting of genes using siRNA. Since its discovery, the need for a safe and efficient delivery system for siRNA has increased. Here, we have developed and characterized a delivery platform for siRNA based on the natural polysaccharide starch in an attempt to address unresolved delivery challenges of RNAi. Modified potato starch (Q-starch) was successfully obtained by substitution with quaternary reagent, providing Q-starch with cationic properties. The results indicate that Q-starch was able to bind siRNA by self-assembly formation of complexes. For efficient and potent gene silencing we monitored the physical characteristics of the formed nanoparticles at increasing N/P molar ratios. The minimum ratio for complete entrapment of siRNA was 2. The resulting complexes, which were characterized by a small diameter (~30 nm) and positive surface charge, were able to protect siRNA from enzymatic degradation. Q-starch/siRNA complexes efficiently induced P-glycoprotein (P-gp) gene silencing in the human ovarian adenocarcinoma cell line, NCI-ADR/Res (NAR), over expressing the targeted gene and presenting low toxicity. Additionally, Q-starch-based complexes showed high cellular uptake during a 24-hour study, which also suggested that intracellular siRNA delivery barriers governed the kinetics of siRNA transfection. In this study, we have devised a promising siRNA delivery vector based on a starch derivative for efficient and safe RNAi application.

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1. Introduction

RNA interference (RNAi) is a natural process of sequence-specific post-transcriptional gene silencing by which gene expression is inhibited. RNAi therapeutics holds a promising future for therapeutic application due to its capability to target almost any gene and its therapeutic potential for treating previously untreatable diseases such as HIV infection [1], cancer [2–4], and neurogenetic disorders [5]. The RNAi mechanism pathway utilizes small interfering RNA (siRNA) molecules (21–23 bp long duplex RNA fragments) to degrade mRNA transcripts before translation into peptide sequences [6]. These siRNA molecules can be custom designed for a variety of gene targets with relatively strong reduction in gene expression [7]. Although siRNA is being tested in a variety of clinical trials [8,9], the remaining challenge before widespread clinical use is developing an efficient and safe non-viral delivery mechanism for siRNA [9,10]. Naked siRNA delivery is unstable in vivo due to enzymatic degradation and immunological responses. The efficacy of siRNA that does reach the target cells is further limited by poor

cellular uptake as both the oligo fragments and cellular membrane are negatively charged [7]. To compensate, studies have used a combination of a carrier and siRNA to generate nano-scale delivery particles [7]. The carrier is designed and can be easily manipulated so that the nanoparticles can assist in overcoming delivery barriers and deliver its siRNA cargo safely into the desired target cells. Such a platform should be able to sufficiently bind siRNA in order to avoid enzymatic degradation of siRNA moieties, cross the anionic cellular membrane, and release siRNA into the cytoplasm in order to integrate into the RNAi pathway.

Since the necessity for an efficient delivery system was recognized, many carriers have been developed and reported in the literature. Among them, some of the most studied non-viral carriers are cationic polymers [11,12] and lipids [13,14]. Common synthetic polymeric carriers include polyethyleneimine (PEI) [15], poly(L-lysine) (PLL) [16], and PLGA [17], which offer a wide diversity in delivery functionality. While some of these were found highly efficient in vitro, a significant concern for synthetic polymers' (e.g. PEI and PLL) applicability as delivery carriers was mainly associated with their extreme toxicity [18]. Therefore, there is a constant need not only to develop new delivery systems that are as efficient as the existing ones but also take into consideration safety issues upon drug administration.

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The use of polysaccharides as delivery systems addresses these issues and is considered advantageous due to their natural characteristics such as biodegradability, biocompatibility, low immunogenicity, and minimal cytotoxicity [19,20]. Pectin was recently studied in our lab [21] as a gene delivery vector with cell-specific ligands and was shown to be a non-toxic efficient carrier for targeted gene delivery. Chitosan, another polysaccharide, is intensively studied for its potential as siRNA carrier. It was reported to be highly efficient and non-toxic in inducing gene silencing [22–24], but has solubility limitations since it is soluble only in acidic aqueous solutions below pH 6.5 [24]. Therefore, its application in siRNA delivery is limited and requires manipulations such as modifications that also present significant cytotoxicity [20]. Here we propose an alternative approach of using starch for the first time, as far as we know, as siRNA delivery carrier. Starch has been studied in our lab as a drug [25] and gene delivery carrier [26] and was found to be a promising delivery vector. It can be carefully designed and characterized in terms of molecular weight and modifications in order to address safety and efficiency issues in siRNA delivery.

In this study, we would like to present the first step in the development of a new strategy for siRNA delivery based on low molecular weight modified starch (90 kDa). Starch polysaccharide is a biodegradable polymer composed of α -D-glucose building blocks. The two polymer components of starch are amylose, the linear α (1–4) linked glucose; and amylopectin, α (1–4) linked glucose with α (1–6) branch points [27]. Amylose constitutes about 80% of most common starches from different plant origins [28]. In order to validate starch as an siRNA carrier, modification with a cationic group is essential. We suggest that modified starch with a quaternary amine [29] would be able to interact with negatively charged siRNA nucleic acid to generate self-assembled complexes, which allows siRNA entrapment regardless of the pH of the surrounding media. We hypothesize that with suitable characteristics (such as surface charge and size), modified starch-based complexes will induce efficient gene silencing *in vitro* while maintaining minimum toxicity. We also believe that further investigation of the transfection process will enlighten us with the rate limiting steps of siRNA delivery for future development of the delivery system.

2. Materials and methods

2.1. Materials

Soluble starch (101252) was purchased from Merck. Sodium hydroxide (S-0399), 3-chloro-2-hydroxypropyltrimethylammonium chloride (348287), dialysis cellulose membrane (D9652), phosphate buffered saline (PBS) (P4417), Tris base (T6791), ethylenediaminetetraacetic acid (EDTA) pH = 8 (E1644), Thiazolyl Blue Tetrazolium Bromide (MTT) (M2128), methyl sulfoxide- d_6 (DMSO- d_6) (547239), deuterium oxide – D_2O (151882), trypan blue (T6146), and KBr (221864) were purchased from Sigma-Aldrich Inc. Hydrochloric acid 32% (08460201), acetone (01030521), and ethanol (05250502) were purchased from Bio-Lab. Loading buffer (G190A) was purchased from Promega. DNA ladder mixed 25/100 bp (IM-D-1020) was purchased from Hy-Labs. Agarose powder (50004) was purchased from Ornat. Microscope slides 76 × 26 mm, Non-targeting siRNA (control non-silencing, sense-5'-UGGUUACAUGUCGACUAA-3') (Non-targeting siRNA #5, D-001210-05-50), siRNA targeting against the human gene ABCB1 that encodes for P-glycoprotein (sense-5'-ACCAUAAUGUAAGGUUU-3') (D-003868-05-005), and the same siRNA fluorescently labeled by DY677 (siP-gp^{lab}-cy5 alternative) (especially constructed for our lab) were purchased from Thermo Scientific. RPMI 1640 medium (01-104-1A), fetal bovine serum (FBS) (04-121-1A), trypsin ethylenediaminetetraacetic acid (EDTA) (03-052-1B), L-glutamine (03-020-1B), penicillin-streptomycin (03-031-1B), and DEPC-treated water (01-825-1A) were purchased from Biological Industries. Multidish 6 (NUNC) 6-well plates (140675) were purchased from Daniel Biotech. Lipofectamine 2000® (11668-027), wheat germ agglutinin (WGA) Alexa Fluor 555 conjugate

(W32464), Hank's balanced salt solution (HBSS) (14175–079), and Pro-Long® gold antifade reagent with DAPI (P36935) were purchased from Invitrogen. Antibodies: primary antibody – mouse monoclonal to P-glycoprotein (ab10333), secondary antibody – goat polyclonal secondary antibody to mouse IgG-H&L (FITC) (ab6785), and isotype control – mouse IgG2a [ICIGaA] (ab91361), were purchased from Abcam.

2.2. Starch modification

Starch modification with quaternary amine groups to obtain quaternized starch (Q-starch) was carried out as previously described in Geresh et al. [29]. Briefly, 500 mg of commercial available low molecular weight soluble native potato starch (0.0030 mol anhydroglucose units) were dissolved in 10 mL of sodium hydroxide solution (0.19 g/mL, 0.0475 mol) to obtain 50 mg/mL starch concentration. The solution was then stirred continuously with a magnetic stirrer for 30 min at room temperature (RT). 9 g (7.8 mL, 0.0478 mol) of the quaternization reagent, 3-chloro-2-hydroxypropyltrimethyl-ammonium chloride (CHMAC), was mixed with 20 mL of double distilled water (DDW) (0.32 g/mL) and then added to the starch solution. The reaction volume was continuously stirred with a magnetic stirrer for 20 h at RT. For product precipitation, one volume of product was precipitated by adding 4 volumes of an acidified (1% HCl) mixture of ethanol and acetone (1:3 vol.%). The precipitate was washed 4 times with 25 mL of 80% ethanol (100 mL total washing volume), dissolved in a small volume (1–2 mL) of DDW, and poured into a 14 kDa cutoff dialysis bag that was placed in a vessel containing 5 L of DDW. The water was replaced 4 times with fresh DDW during 48 h of dialysis. The dialyzed product was then dried by lyophilization for 72 h.

2.3. Chemical analysis of quaternized starch

The structural elucidation of cationic starch was confirmed by FT-IR, ¹H NMR and elemental analysis. The nitrogen content (%N weight) of Q-starch was also measured by Kjeldahl method [30]. For more details see supplementary material.

2.4. Preparation of Q-starch/siRNA complexes

Complexes of Q-starch and siRNA were prepared at various N/P molar ratios (molar ratio between positive nitrogen groups on Q-starch and negative phosphate groups on siRNA backbone). To determine N/P ratio, Q-starch dissolved in DEPC-treated water (sterile, RNase- and DNase-free water) (0.4 mg/mL) was added in aliquots to solutions containing 0.5 μ g siRNA (20 μ M). The quantity of Q-starch added to the siRNA was determined by the desired N/P ratio. Following gentle vortexing, the samples were incubated at RT for 40 min before use to allow self-assembly formation of the complexes.

For complex characterization and *in vitro* experiments, Q-starch (15 μ L) dissolved in DEPC-treated water (sterile, RNase- and DNase-free water) was added to solutions containing 185 μ L siRNA (0.27 μ M). The concentration of the Q-starch solutions was determined by the desired N/P ratio and was in the range of 0.05–0.15 mg/mL.

2.5. Agarose gel electrophoresis

The desired N/P ratio for full complexation of siRNA was evaluated by gel electrophoresis assay. Samples containing 0.5 μ g of non-targeting siRNA (nt-siRNA), either naked or complexed with Q-starch at a desired N/P ratio, were mixed with $\times 6$ loading buffer and loaded (15 μ L) onto 3% agarose gel containing (0.2 μ g/mL) ethidium bromide for siRNA staining. The gel was placed in a horizontal electrophoresis apparatus (Wide Mini-Sub cell GT, BioRad) containing $\times 1$ Tris acetate EDTA (TAE) buffer solution (prepared in our lab as $\times 50$ stock solution), exposed to an electric field (160 V) for 40 min, and then visualized by

UV illumination (Visible and Ultraviolet Transilluminator, DNR Bio-Imaging Systems).

2.6. Q-starch/siRNA complex characterization

Q-starch/siRNA complexes were characterized using the methods below. Complexes of Q-starch and non-targeting siRNA (nt-siRNA) were prepared as described above.

2.6.1. Atomic force microscopy (AFM)

Complex size and geometry were visualized by AFM. Before imaging, 5 μL from each sample (Q-starch, siRNA, or Q-starch/siRNA complexes) was dispensed onto freshly cleaved mica surfaces, incubated at RT for 20 min, and dried with nitrogen gas. AFM measurements were performed at ambient conditions at RT using a Digital Instrument Dimension 3100 mounted on an active anti-vibration table. A 100 μm scanner was used (Microfabricated Si oxide NSC11/50 type Ultrasharp with integrated pyramidal tip). The 512 \times 512 pixel images were taken in tapping mode with a scan size of up to 5 μm at a scan rate of 1 Hz.

2.6.2. Dynamic light scattering (DLS) & zeta potential

The hydrodynamic size distribution of the complexes was measured by DLS. Spectra were collected using CGS-3 (ALV, Langen, Germany). The laser power was 20 mW at the He-Ne laser line (632.8 nm). Correlograms were calculated by ALV/LSE 5003 correlator, which were collected at 90°, during 10 s, 20 times, at 25 °C. The correlograms were fitted with a version of the program CONTIN [31]. Each complex sample was measured twice and solutions were further diluted until results were independent of dilution rate. Complex size is presented as the average of triplicates \pm standard deviation.

The surface charge of the complexes was evaluated by zeta potential. Samples from DLS were transferred to U-tube cuvette (DTS1060C, Malvern) for subsequent zeta potential measurements using Zetasizer (ZS-NanoSizer, Malvern, England). Each free siRNA, Q-starch, and complex sample was measured in automatic mode, at 25 °C, and the Smoluchowski model was used to calculate the zeta potential. For each sample the zeta potential value was presented as the average value of three runs, and the average value of each N/P ratio is presented as the average of triplicates \pm standard deviation.

2.7. Complex stability in human serum

Complex stability in human serum was evaluated using gel electrophoresis. Samples of nt-siRNA naked or complexed with Q-starch at N/P 2 were mixed with fresh human serum to give 50% (vol%) serum concentration and incubated at 37 °C. The serum was donated by two unrelated volunteers from our lab, and two sources were used due to innate differences in serum activity between sources. At each time interval (10 min, 30 min and 1 h for naked siRNA and 0, 0.5, 1, 3, and 24 h for complexes) the samples were removed, incubated at RT with 0.5% sodium dodecyl sulfate (SDS) for 15 min in order to disassemble the protected siRNA from Q-starch. The samples were then loaded into agarose (3%) gel electrophoresis in which intact siRNA that was protected from degradation migrates as a clearly visible band after staining with ethidium bromide.

2.8. Cell culture

In this research, the human ovarian adenocarcinoma cell line NCI-ADR/Res (NAR), which highly expresses the P-glycoprotein (P-gp) extrusion pump, were used as the cancer cell model. NAR cell line is highly resistant to chemotherapeutic drugs and expresses a high level of the trans-membrane protein P-gp extrusion pump as part of its drug-resistance mechanism. NAR cell line overexpresses the gene ABCB1, which encodes for the expression of P-gp [32].

NAR cells were used for all experiments and were cultured in RPMI growth media containing 1% L-glutamine, 1% penicillin-streptomycin, and 10% fetal bovine serum (FBS) in a sterile incubator at 5% CO₂ and 37 °C.

2.9. Gene knockdown by Q-starch/siRNA complexes

NAR cells were seeded in a 6-well plate 24 h before transfection in RPMI growth media at a density of 1.5×10^5 cells/well. On the day of transfection, the culture medium was removed, the cells were washed once with PBS, and 800 μL of serum- and antibiotic-free media were added to each well. Q-starch/siRNA complexes, either with nt-siRNA or with siRNA targeting P-gp (siP-gp), were prepared at N/P ratios 1, 2, and 3 with siP-gp and N/P ratio 2 with nt-siRNA as described above to reach 50 nM siRNA concentration in each well. Lipofectamine 2000® was used as a positive control carrier and prepared according to the manufacturer's procedure. Untreated cells (negative control) were supplemented with 200 μL of serum- and antibiotic-free RPMI media. Control cells treated by uncomplexed Q-starch were supplemented with 200 μL of Q-starch aqueous solution (7.6 $\mu\text{g}/\text{mL}$). The transfection complexes (200 μL) were added to the cells and incubated at 37 °C and 5% CO₂. After 4 h, 500 μL of growth medium containing 30% serum without antibiotics was added and further incubation was continued (24 or 72 h) until fluorescence activated cell sorter (FACS) analysis was conducted.

2.10. Flow cytometry & analysis

Gene silencing efficiency was quantified by labeling the protein P-gp with a fluorescent antibody as previously described [33]; FACS analysis was then performed. Following transfection (after 24 or 72 h of incubation) the medium was aspirated from the wells, and cells were washed once with PBS, trypsinized, and pelleted by centrifugation for 5 min at 250 g and 25 °C in 5 mL tubes. Each tube was re-suspended in FACS buffer (1% FBS in PBS) containing an anti-Pgp polyclonal antibody diluted 1:20 or an isotype-matched negative control diluted 1:100 for 30 min on ice. The cells were washed with FACS buffer and treated for 30 min on ice in the dark by an FITC-labeled anti-goat antibody diluted 1:400. The cells were pelleted and re-suspended in 450 μL of FACS buffer. P-gp expression was measured by flow cytometry using FACS caliber instrument (BD) equipped with a 488 nm Argon laser and a 530/30 band pass filter; the data analysis was done with BD Cellquest pro™ software version 5.1.1. The relative fluorescent unit (RFU) of the labeled and treated cells was normalized relative to the RFU of the labeled and untreated cells. From both RFU values the RFU of non-labeled cells (auto-fluorescence) was reduced (Eq. (1)). Untreated cells were considered as 100% of P-gp expression.

$$\% \text{ gene expression} = \frac{\text{RFU}_{\text{treated}} - \text{RFU}_{\text{unstained}}}{\text{RFU}_{\text{untreated}} - \text{RFU}_{\text{unstained}}} \quad (1)$$

Unstained: non-labeled cells (auto-fluorescence).

Untreated: untreated and labeled cells.

Treated: treated and labeled cells.

2.11. Cytotoxicity of Q-starch/siP-gp complexes

The cytotoxicity of Q-starch/siRNA complexes was evaluated by MTT assay in NAR cell line. NAR cells were seeded in a 6-well plate in RPMI growth media at a density of 4×10^5 cells/well and cultured overnight. The following day, complexes (200 μL) were prepared as for transfection (as described above) to reach 50 nM siRNA concentration in each well. Following 72 h incubation with each treatment, a standard MTT assay was performed according to manufacturer's procedure. Briefly,

cell medium was replaced with 1 mL of fresh starvation medium (1% L-glutamine, 1% penicillin–streptomycin, and 5% FBS) and 100 μ L of MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole)) solution (5 mg/mL in PBS) was added to each well. Cells were incubated for 2 h at 37 °C and 5% CO₂ after which 1 mL of MTT solvent (acidified isopropanol 0.04 M) was added to dissolve the formazan crystals produced. The cell plates were incubated for an additional 24 h at RT in a sterile hood. The absorbance of each well was read at test wavelength of 570 nm and reference wavelength of 650 nm (the blank – MTT + RPMI medium + acidified isopropanol – was read at the reference wavelength).

2.12. Cellular uptake of Q-starch/siRNA complexes

Cellular uptake of Q-starch/siRNA complexes was visualized using a confocal microscope. NAR cells were seeded in a 12-well plate on an 18 mm glass coverslip 24 h before transfection in RPMI growth media at a density of 1.2×10^5 cells/well. On the day of transfection the culture medium was removed, the cells were washed gently once with PBS, and 400 μ L of serum- and antibiotic-free RPMI medium was added. Complexes were prepared as described above at N/P ratios of 1 and 2 using fluorescently labeled siP-gp^{lab} to give Q-starch/siP-gp^{lab} complexes at 250 nM (to reach 50 nM final concentration inside the well). The cells were supplemented by 100 μ L of Q-starch/siP-gp^{lab} complexes at N/P 1 or 2, and as a control by 100 μ L of naked siP-gp^{lab}, after which the cells were incubated at 37 °C and 5% CO₂ for 1, 4, 8, or 24 h. At each time interval the cells were removed from the incubator washed twice with HBSS and fixed with 4% paraformaldehyde. The cell membrane was labeled by WGA Alexa Fluor 555 conjugate (excitation 555 nm, emission 565 nm) according to the manufacturer's procedure. The cell coverslip was mounted by fluorescent mounting medium containing DAPI for nucleus staining (ProLong® Gold antifade reagent with DAPI). Confocal microscopy was performed on a FluoView FV-1000 (Olympus) spectral confocal laser-scanning microscope using excitation of 405 nm, 559 nm, and 635 nm for DAPI, Alexa 555, and DY677 fluorophores, respectively. Images were processed using FV10-ASW 4.0 Viewer browser software and for each treatment the presented images represent the entire slide.

3. Results and discussion

Gene silencing therapy emerged as a clinical tool more than a decade ago and a few major challenges still remain to be resolved before clinical use. Developing siRNA delivery strategy is one of them. Drug delivery in a wide aspect is a major hurdle not only in gene silencing but also in other drug development fields as well. The delivery carrier should be able to protect siRNA from nuclease degradation and allow efficient gene knockdown, while avoiding toxicity effects. Based on the above needs, we focused our research on developing and characterizing the

modified starch as an siRNA delivery carrier due to its natural properties, biocompatibility, and biodegradability.

3.1. Starch modification

Starch modification is essential in order to add a positive charge to the polymer backbone. The modification in our study was done by substituting a quaternary amine group on the polymer (quaternization). This modification changes starch's functionality so that the resulting polycation would be able to interact with siRNA while maintaining its natural properties. The introduction of the cationic quaternary group onto the polysaccharide was done as described by Geresh et al. [29]. In this study the author stated that when the amount of cationic reagent is increased with respect to given weight of polysaccharide, the insertion of cationic group onto the polymer backbone also increased. Since NaOH hydrolyzes the cationic reagent (CHMAC) into its corresponding glycol as side product [34]. Keeping the above facts in our mind, we followed their procedure for the quaternization of starch by using excess amount of cationic reagent (CHMAC); the reaction scheme is presented in Fig. 1.

As seen in Fig. 1, the starch is treated with 3-chloro-2-hydroxypropyl trimethyl ammoniumchloride (CHMAC) in the presence of aqueous sodium hydroxide solution at room temperature for 24 h. Under the present reaction condition, the incorporation of quaternary ammonium group onto starch backbone proceeded smoothly and provided required cationic starch. After completion of the reaction, unreacted cationic reagent was removed by dialysis process through a molecular weight cut-off dialysis tube (14 kDa) for 2 days. It is very important to note that the parameters that influence the quaternization reaction are sodium hydroxide, CHMAC and starch concentrations, temperature, and reaction time. The obtained cationic starch was characterized by FT-IR, ¹H NMR and elemental analysis. From the FT-IR spectrum of the cationic starch (Fig. S8, see supplementary materials), the strong and new absorption bands appeared at 3027 and 1479 cm^{−1}. These bands are related to the C–H and C–N stretching vibrations of quaternary ammonium group, respectively. The rest of the bands are similar when compared with FT-IR spectrum of the unmodified starch (Fig. S4, see Supplementary materials). The appearance of new bands at 3027 and 1479 cm^{−1} in modified starch is due to the incorporation of cationic group onto the polymer backbone. Further, it was characterized with ¹H NMR analysis. A typical ¹H NMR spectrum of the cationic reagent (Fig. 2A and a more detailed spectra in Fig. S1, see supplementary materials) showed most significant peak at $\delta = 3.2$, which belongs to the 9 hydrogen of methyl quaternary ammonium group (⁺N(CH₃)₃). From the ¹H NMR spectrum of the obtained cationic starch (Fig. 2C and a more detailed spectra in Fig. S9, see Supplementary materials), the peak represents to the 9 hydrogen of methyl quaternary ammonium group (⁺N(CH₃)₃) appeared at same chemical shift ($\delta = 3.2$), which was not appeared in the spectrum of native starch (Fig. 2B and a more detailed spectra in Fig. S5, see Supplementary materials), which clearly indicates the incorporation of quaternary ammonium group onto the polymer

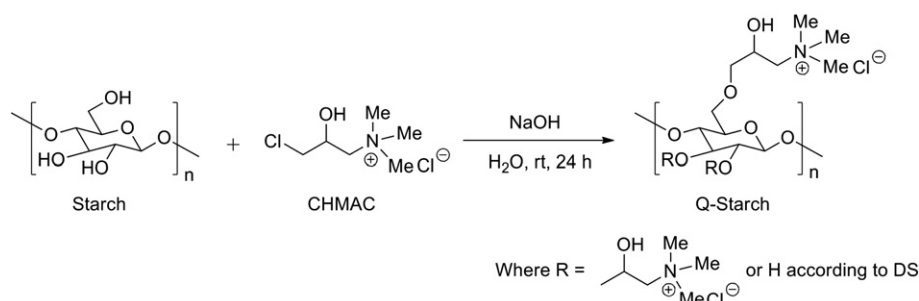


Fig. 1. Q-starch synthesis by the quaternary reagent CHMAC.

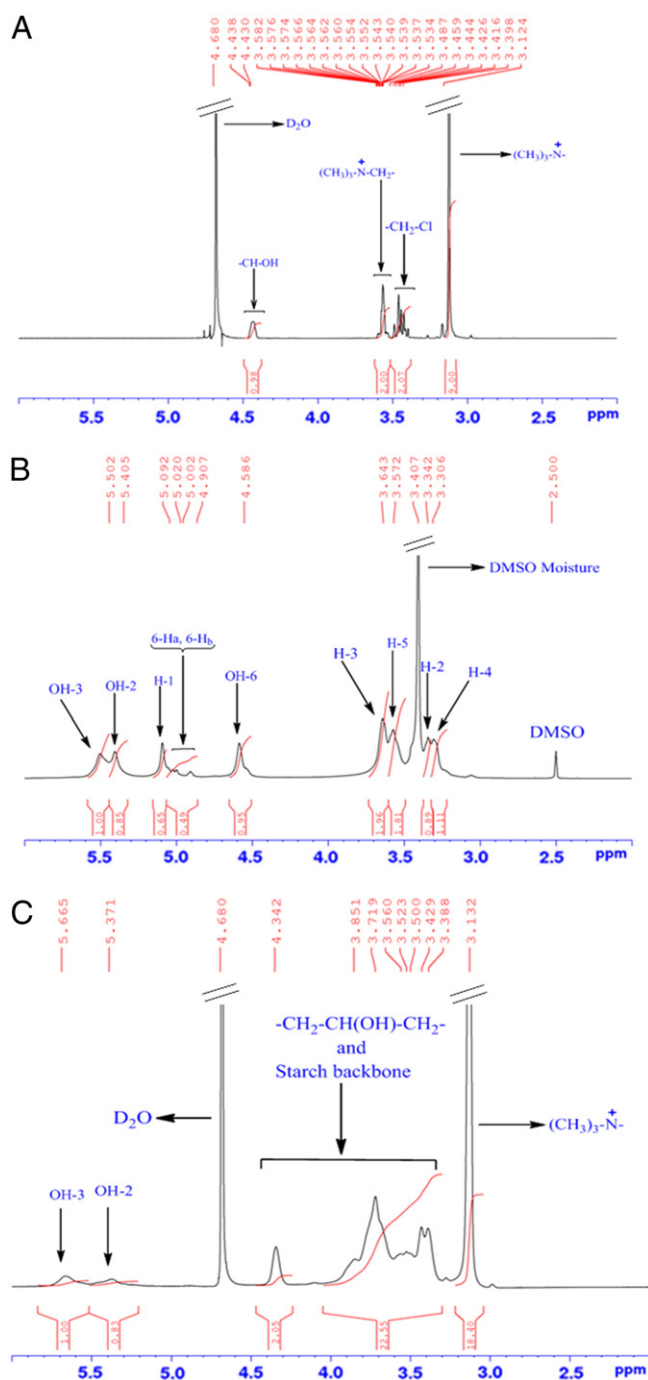


Fig. 2. Q-starch synthesis, ^1H NMR spectra of the quaternization reagent 3-chloro-2-hydroxypropyltrimethylammonium chloride (CHMAC) (A), potato starch (B), and Q-starch (C).

backbone. Finally, it was confirmed by the elemental analysis, presented in Table 1, that starch does not contain any significant amount of nitrogen or chloride. Whereas, considerable amount of nitrogen (3.18%) and chloride (8.28%) is present in the cationic starch (Q-Starch). The presence of nitrogen and chloride is the proof of incorporation of quaternary ammonium group onto the starch backbone. We proved the

Table 1
Elemental analysis results of starch and cationic starch.

Polymer	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Chloride (%)
Starch	39.18	6.06	0.00	0.00
Q-starch	40.92	7.71	3.18	8.28

incorporation of quaternary ammonium group onto the starch backbone based on the FT-IR, ^1H NMR and elemental analysis results.

The nitrogen content of Q-starch was also analyzed by the Kjeldahl method. Following the substitution, nitrogen weight percent was $3.5 \pm 0.5\%$. According to calculations, the degree of substitution of the starch, which was calculated based on the Kjeldahl results (see supplementary materials), is 0.65; therefore, it could be deduced that the substitution reaction yields modification of almost every starch glucose unit.

This synthesis yields a polycation that will be able to interact with the negatively charged polyanionic siRNA based on electrostatic interactions. The most significant advantage to using a quaternary amine as the substituted molecule is the independence of the polymer charge on the solution's pH. The quaternary starch retains its positive charge throughout the pH range (which varies along siRNA transfection in vitro and in vivo) and its interactions with siRNA are pH independent, unlike chitosan complexes, which are highly dependent on pH and remain stable mainly under acidic conditions [24].

3.2. Q-starch/siRNA complex formation and characterization

Q-starch/siRNA complex formation is based on electrostatic interactions between positively charged Q-starch and negatively charged siRNA. Q-starch was added in aliquots to siRNA to form complexes at various N/P molar ratios (as described in Materials and methods). Primarily, we determined the minimal ratio for full complexation with siRNA by gel retardation assay (results presented in Fig. 3).

The desired N/P ratio is the one at which Q-starch will be able to form a condensed complex with siRNA and none of the siRNA remains free. Free siRNA (lane B) migrates under the influence of the electric field during gel electrophoresis. Upon addition of Q-starch to siRNA, free fragments of siRNA interact with Q-starch (by electrostatic interactions), thus neutralizing the negative charges of siRNA. As the N/P ratio increases from 0.5 to 3 (lanes C–H), the positive charges of Q-starch available to interact with siRNA increase; therefore, as seen in Fig. 3, the quantity of free siRNA entrapped within the Q-starch/siRNA complexes increases inside the well, while the remaining free siRNA fragments that are attracted towards the positive electrode decrease. Complete entrapment of siRNA was detected when N/P ratio reached 2. At an N/P ratio of 1.5, although the free siRNA band cannot be detected, the complex band at the well is less bright than at an N/P ratio of 2, suggesting the complexation is not full at this ratio.

There are two limitations when deciding on the optimal N/P ratio for transfection experiments. First, the optimal ratio will be the one that enables full complexation of siRNA (when a free siRNA band cannot be detected) and second, the minimal N/P ratio will be chosen from those that allow full complexation because highly positive-charged complexes might strongly interact with the anionic plasma membrane and could induce membrane damage that initiates apoptosis [35]. We therefore concluded that complexes of N/P 2 and 3 will be further investigated for efficient Q-starch/siRNA transfection.

3.3. Physical characterization of Q-starch/siRNA complexes

The physical properties of drug nanoparticles influence their cellular internalization and biodistribution. It was previously shown that the size, shape, and charge of the complexes influence their ability to transfer siRNA into the cells [11]. These properties are of importance in overcoming delivery barriers along the transfection route. Before testing for gene silencing efficiency, we determined the physical properties of Q-starch/siRNA complexes by several methods, and accordingly determined the optimal N/P ratio for efficient transfection.

Mean size and size distribution of the complexes in suspension were measured by dynamic light scattering (DLS) using the Stokes–Einstein equation. Size measurements in suspension can demonstrate the complex's hydrodynamic diameter during transfection, which was

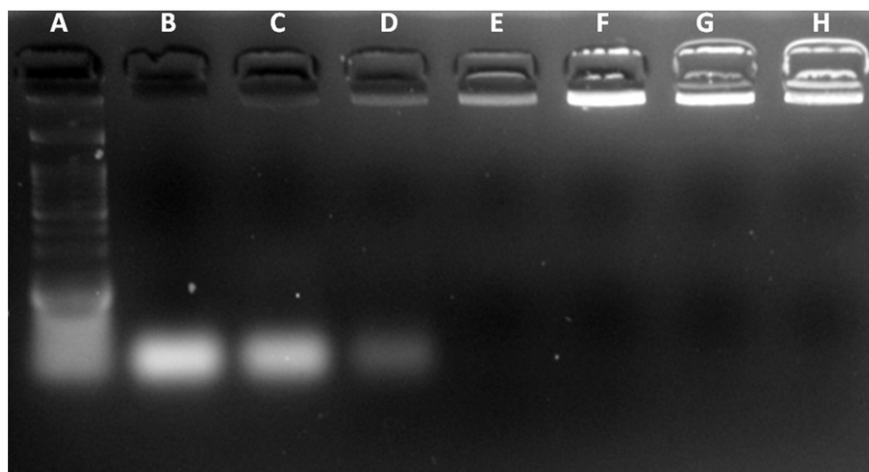


Fig. 3. Q-starch/siRNA complex formation demonstrated by agarose (3%) gel electrophoresis at different N/P ratios. Lanes from left to right represent: DNA marker (A), naked siRNA (B), complexes at N/P ratios of 0.5 (C), 1 (D), 1.5 (E), 2 (F), 2.5 (G), and 3 (H).

also done in a liquid medium. The complex size strongly influences their therapeutic potential since it affects the complex's biodistribution upon systemic administration *in vivo* [7]. Moreover, previously reported studies [11,13] suggested that nano-sized complexes have a relatively high uptake compared to microparticles. According to Fig. 4A, the formulated complexes were accepted in the nano-size range. The mean diameter of self-assembled complexes was found to be between 28.3 and 34.0 nm and independent of N/P ratio. We also found that 99.41% of the complex population at N/P 2 ranged between 26 and 100 nm (Fig. 4B), suggesting that the self-assembly formation of the complexes gave a relatively narrow distribution of diameter.

Zeta potential results (Fig. 5) gave indication of the surface charge of the complexes and the complexation process. As expected, naked siRNA had a negative zeta potential due to the phosphate groups of the polynucleotide backbone. Mammalian cells do not bear the natural machinery for double stranded RNA uptake, and because of its negative charge and large size, siRNA typically cannot cross the plasma membrane by simple free diffusion [36]. Therefore it is most likely to enter cells via passive endocytosis (similarly to pDNA) when associated with a carrier. The siRNA-carrier system can associate with the anionic plasma membrane by bearing an excess positive surface charge. Taken together, we evaluated the complexes' surface charge by measuring the zeta potential of the modified polysaccharide and its complexes with siRNA. Q-starch had a positive zeta potential of +27.7 mV, confirming the substitution of the quaternary amine on starch. Moreover, complexes at N/P 1

had a zeta potential of −24 mV. This solution was probably a non-homogenous solution composed of free fragments of siRNA and complexes as was shown in the gel electrophoresis results (Fig. 3). The negative zeta potential indicates the presence of both naked siRNA and particles with a certain density of negative charges on their surface. The zeta potential, which turns positive to +25 mV and +15 mV at N/P 2 and 3, respectively, demonstrated that the complexes are characterized by a positive surface charge. Positive surface charge of the Q-starch/siRNA complexes could encourage electrostatic interaction with the anionic plasma membrane for sufficient cellular uptake.

Further characterization was done in order to reach a better understanding of the morphology of the complexes. For that purpose, Q-starch/siRNA complexes were analyzed by atomic force microscopy (AFM). As opposed to DLS, the AFM scanning was done on a dried mica sheet. Although the dried form of the complexes in AFM does not give as accurate information about the complexes' size as in a suspension (as was done in DLS), it can give visual insight of the complexes' morphology and size. First, a sample of Q-starch (7.5 ng/μL, the same concentration used for N/P 2) was scanned (Fig. 6a) in order to visualize the Q-starch before complexation. It seems that the Q-starch spreads well on the mica and there are no particles in the sample. Upon addition of siRNA to the Q-starch solution (at quantities that result in N/P ratio 2), it can be clearly seen (Fig. 6b) that spherical complexes are formed. The diameter of the complexes at N/P 2 is in the range of 30–70 nm and the mean diameter is ~44.4 nm. In order to verify that the particles seen

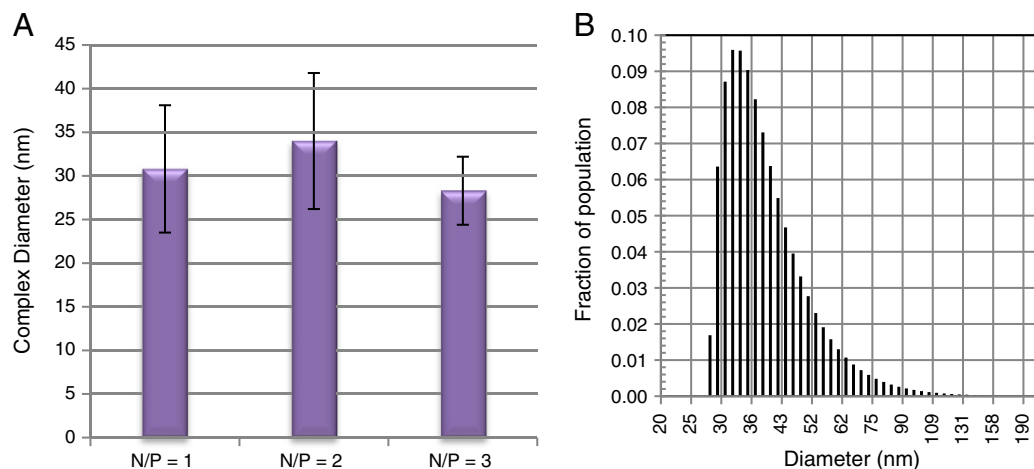


Fig. 4. Dynamic light scattering measurements: Q-starch/siRNA complexes' diameters at N/P ratios 1, 2, and 3 (A). Vertical bars represent mean \pm s.d. of three replicates. (B) Representative size distribution of complexes at N/P 2.

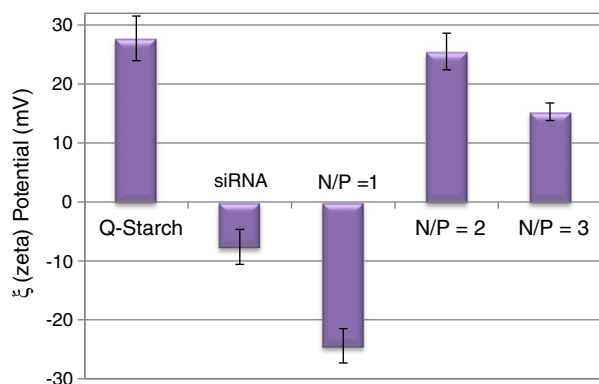


Fig. 5. Zeta potential of Q-starch, free siRNA, and Q-starch/siRNA complexes at different N/P ratios.

in Fig. 6b aren't free siRNA molecules, control images of free siRNA (Fig. 6c) were scanned. Because the siRNA molecule has a negative charge it can be excluded from the mica. Thus, when dispensed onto the mica, the sample was not washed with DDW (as was done with the rest of the samples) and was left to dry for 20 min in RT. Fig. 6c shows small particles of ~9 nm, which corresponds to the length of 21 base-paired siRNA as reported in the literature [13]. This size is smaller than that of the particles observed in Fig. 6b; therefore, the complexes observed in Fig. 6b are necessarily complexes formed by interaction between siRNA and Q-starch.

Comparison to results with gene delivery by DNA-polycation complexes indicate that the general concept of the complexation process is different from siRNA-polycation complexes, as was reported in the literature [37,38]. In general, increasing the N/P ratio for DNA complexes increases the amount of positive groups that are able to interact with the negative phosphate groups on the DNA backbone and condense the large plasmid DNA (~3000 kDa) to a smaller particle. The size of the formed particles decreases with increasing N/P molar ratio until any additional polycation will not allow further condensation. Based on data previously reported in our lab, pDNA was fully condensed by Q-starch into small particles (150–200 nm) that were able to be taken up by cells at N/P ratios of 3 and above, and the size decreased with increasing N/P ratio. However, based on the present results, complex size shows that the carrier does not have the same effect in the case of siRNA (~13 kDa). As can be seen from Fig. 4, the size of Q-starch/siRNA complexes was independent of N/P ratio (in the range tested). The reason is that siRNA is already small (~9 nm) and constitutes a rigid molecule that is unable to be condensed to a smaller size, with persistent length of 70 nm [39]. It appears that in the case of siRNA, the carrier in fact is the molecule to be condensed, as opposed to complexes with DNA in which Q-starch condenses the DNA to a smaller size. However, in both

cases the carrier helps screen the negative charge of the polynucleotide in order for it to be effectively taken up by cells.

Complexes' characterization gave information on their size, surface charge, and geometry. Since the interactions between siRNA and Q-starch are most likely not siRNA sequence specific, and because siRNA length is considered relatively constant (between 19 and 23 base pairs), the features of these complexes are independent of the siRNA in use, and can be used for multiple RNAi therapeutic purposes, i.e., an siRNA delivery platform for siRNA-based therapeutics.

3.4. Complex stability test in human serum

Development of a delivery vehicle for siRNA should take into consideration future obstacles in in vivo application of RNAi therapy. For optimal gene silencing during in vivo administration, Q-starch must be able to form a stable complex with siRNA that will not decomplex in the presence of blood serum, and will protect siRNA from nuclease degradation (by RNases found in blood serum). We established a stability study in order to test the lability of free siRNA and Q-starch/siRNA complexes in human serum. Free siRNA and Q-starch/siRNA complexes at N/P 2 were incubated with human serum (50 vol.%) for several time periods after which the samples were incubated with SDS in order to disassemble siRNA from Q-starch or any attachment to serum proteins and visualize it in gel electrophoresis apparatus. The results are presented in Fig. 7.

The control band at Fig. 7A represents naked siRNA that was not incubated with the serum and indicates the typical migration of intact siRNA. According to the results in Fig. 7A, incubation of free siRNA in the presence of serum results in gradual degradation over time until complete degradation is visualized after 1 h. This is indicated by the intensity of the band, which reduces over time until it completely disappears. Apparently, complete degradation of siRNA occurred during the incubation and even fragments of siRNA smaller than 21 bp weren't visualized. It appears that siRNA is highly sensitive, and if not protected by other means such as siRNA modifications or suitable carrier it immediately degrades [40,41]. Association of siRNA with a polymeric delivery system was shown to enhance the stability of siRNA in such a violent environment [42]. Thus in order to examine its suitability as a legitimate carrier, the ability of Q-starch to protect siRNA was examined over a 24-hour study. Complexes were incubated with serum after which SDS was added in order to release the complexed siRNA and run as a free band. The migration of released siRNA from complexes that were incubated for 0 h in the serum can be detected in Fig. 7B (0 h). Further incubation in the serum (Fig. 7B 0.5–24 h) shows that complexation of siRNA with Q-starch at N/P 2 protects siRNA from nuclease activity for the entire length of study. The brightness intensity of the bands gets slightly lighter after 24 h of incubation with the serum, indicating that a partial amount of the loaded siRNA was degraded, but even after 24 h of incubation, significant non-degraded siRNA could be detected. It

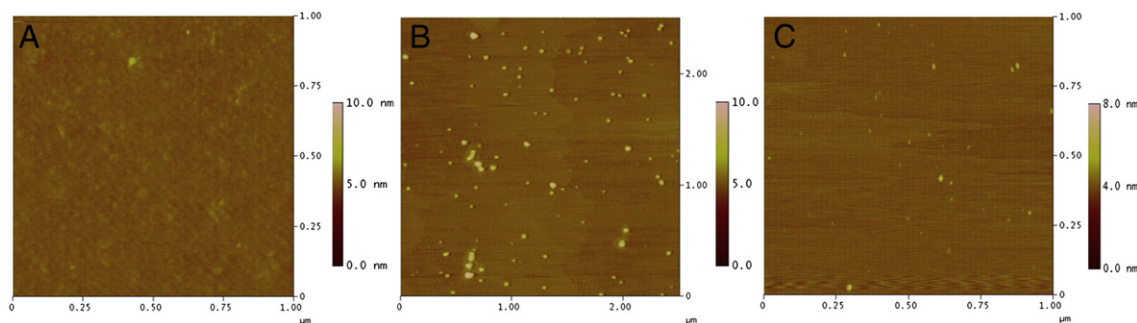


Fig. 6. Atomic Force Microscopy (AFM) images of pure Q-starch (7.5 ng/μL) (a), Q-starch/siRNA complexes at N/P 2 (prepared as for transfection, 250 nM siRNA and 7.5 ng/μL Q-starch) (b), and free siRNA (250 nM) (c).

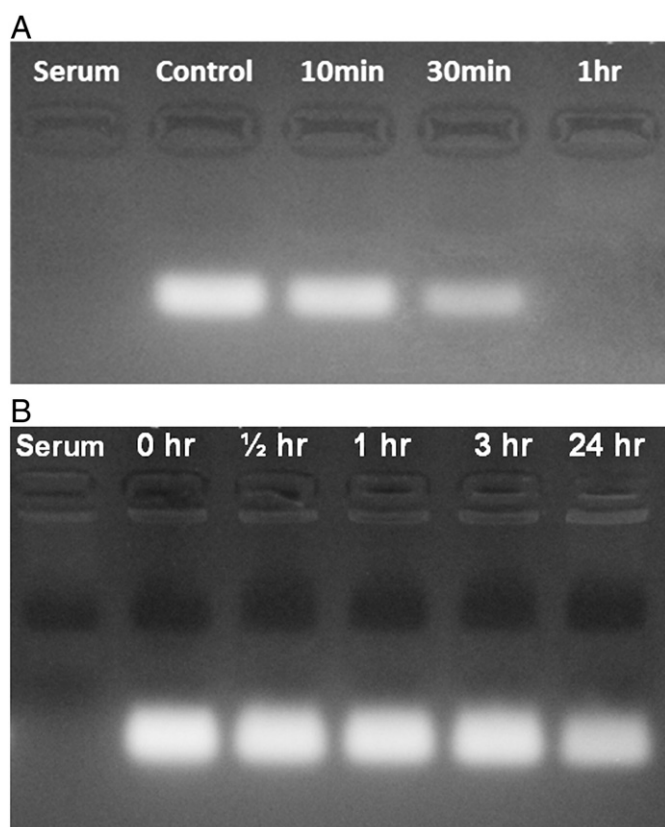


Fig. 7. Gel electrophoresis results of stability test samples of naked siRNA and Q-starch/siRNA complexes at N/P 2 in human serum. (A) Incubation of naked siRNA in human serum for 10 min, 30 min, and 1 h. Serum and naked siRNA which was not incubated in the serum (referred as control) served as controls. (B) Incubation of Q-starch/siRNA complexes (at N/P ratio 2) in human serum for 0, 1/2, 1, 3, and 24 h after which SDS (0.5%) was added to run free protected siRNA.

should be noted that the slight thickness of the control and experimental bands in Fig. 7B could result from attachment of positively charged serum components to siRNA and result in band spreading. In addition, one might be concerned that during complex's incubation in the serum, siRNA molecules could have decomplex from Q-starch complexes and be protected during the course of study by complexing serum proteins with $pI > 7$. However, this possible protection did not last more than 1 h since control experiment of naked siRNA incubation (Fig. 7A) showed that complete degradation of siRNA was observed after 1 h of incubation in the serum and only protection by Q-starch was able to maintain a substantial amount of intact siRNA during the whole study. Thus it can be deduced that the significant protection of siRNA during the 24 hour study was due to the formation of Q-starch/siRNA complexes.

The stability of the complexes is determined by the strength of ionic interaction between the siRNA and Q-starch. A balance in ionic strength should be obtained for successful transfection, since the complexes should be stable during transport through the blood and during cellular internalization, but afterwards, decomplexation should occur in order to release siRNA to the RNAi machinery.

3.5. P-glycoprotein gene silencing using Q-starch/siP-gp complexes

The effectiveness of the developed Q-starch-based delivery system was investigated by measuring the gene silencing efficiency in transfection experiments. The *in vitro* study was conducted utilizing the model cell line NCI-ADR/Res (human ovarian cancer cell line NAR), which highly expresses the P-glycoprotein (P-gp) extrusion pump, the silenced protein in the transfection experiments. The targeted gene,

ABCB1, which expresses P-gp, was silenced by using a sequence-specific siRNA (siP-gp).

In the transfection experiments, the cells were treated with different formulations containing 50 nM of free siP-gp or siRNA (siP-gp or nt-siRNA) complexed with Q-starch. The transfection efficiency was measured by FACS 24 and 72 h post-treatment. For FACS measurements, the silenced protein, P-gp, in each experimental group was fluorescently labeled by two antibodies, a primary P-gp-specific antibody and a secondary antibody labeled by FITC. In order to reduce non-specific attachments of the antibodies to cellular components, the cells were also treated by a control mixture of secondary and isotype control antibodies. The fluorescence intensity detected from these cells was equal to the intensity of cells that were not treated with antibodies, indicating that the non-specific attachment of the antibodies was negligible. The efficiency of gene silencing is presented by percentage of gene expression and was calculated according to Eq. (1) (Materials and methods). The transfection results are presented in Fig. 8.

Gene expression percentage is normalized to the untreated cell group, which was considered as 100% of gene expression. As shown in Fig. 8, for each group exposed to complexes of Q-starch/siP-gp, as time of incubation increases, gene silencing increases (gene expression decreases). Compared to untreated cells, P-gp expression levels were effectively reduced to $49 \pm 16\%$ and $52 \pm 4\%$ after 72 hours incubation with complexes at N/P ratios of 2 and 3, respectively. Cells exposed to free Q-starch or naked siP-gp did not induce significant gene silencing and the reduction in P-gp expression seen in these groups ($\sim 15\%$) is due to non-specific silencing events that are also seen in the Q-starch/nt-siRNA experimental group. According to Fig. 8, complexes of Q-starch with nt-siRNA at N/P 2 (nt-siRNA at Fig. 8) resulted in 20–25% off-target gene silencing in both 24 and 72 h of incubation. The nt-siRNA does not target the P-gp gene, while according to Fig. 8 it inhibits its expression. These complexes indicated the off-targeting effects that are known to be expressed by unintentional up or down regulation of genes [43]. In order to evaluate Q-starch transfection efficiency, the off-target gene silencing was compared to the transfection efficiency of complexes at N/P 2. Using a paired *t*-test, a statistically significant difference was observed ($p < 0.01$) and it seems that the complexes at N/P 2 caused a significant sequence-specific gene silencing.

Complexes at N/P ratio 1 were significantly less effective in gene silencing in comparison to compounds at N/P ratios 2 and 3. In Fig. 8B the representative histograms of the various experimental groups are presented for 72-hour studies. As can be seen, the expression of the fluorescently labeled P-gp is reduced in the NAR cell population treated by the complexes compared to control untreated cells. In addition, P-gp silencing by complexes at N/P 2 and 3 showed a shift down in fluorescence of the cells compared to complexes at N/P 1. P-gp expression by complexes at N/P 1 was slightly reduced and only $68 \pm 12\%$ of gene expression was accomplished at maximum incubation time with the complexes (Fig. 8). We have already shown that at this ratio full siRNA complexation with Q-starch has not been established; therefore, the uptake of the uncomplexed fraction of siP-gp and the negatively charged complexes was insufficient. Lipofectamine 2000, a commercially available transfection reagent, served as a positive control and resulted in $66 \pm 19\%$ and $24 \pm 8\%$ of gene silencing after 24 and 72 h of incubation with NAR cells, respectively. Although highly efficient, Lipofectamine 2000 is not a therapeutic agent because of its previously reported toxicity that interferes with its therapeutic application [44].

3.6. Cytotoxicity of Q-starch/siP-gp complexes

The toxic effect of a delivery system is one of the main parameters that should be evaluated when developing a delivery carrier. The safety issues regarding non-viral carriers were previously discussed in the literature, namely polymers and cationic lipids [45]. The leading motivation for using polysaccharides as delivery carriers is to address these issues. Polysaccharides are known as biocompatible

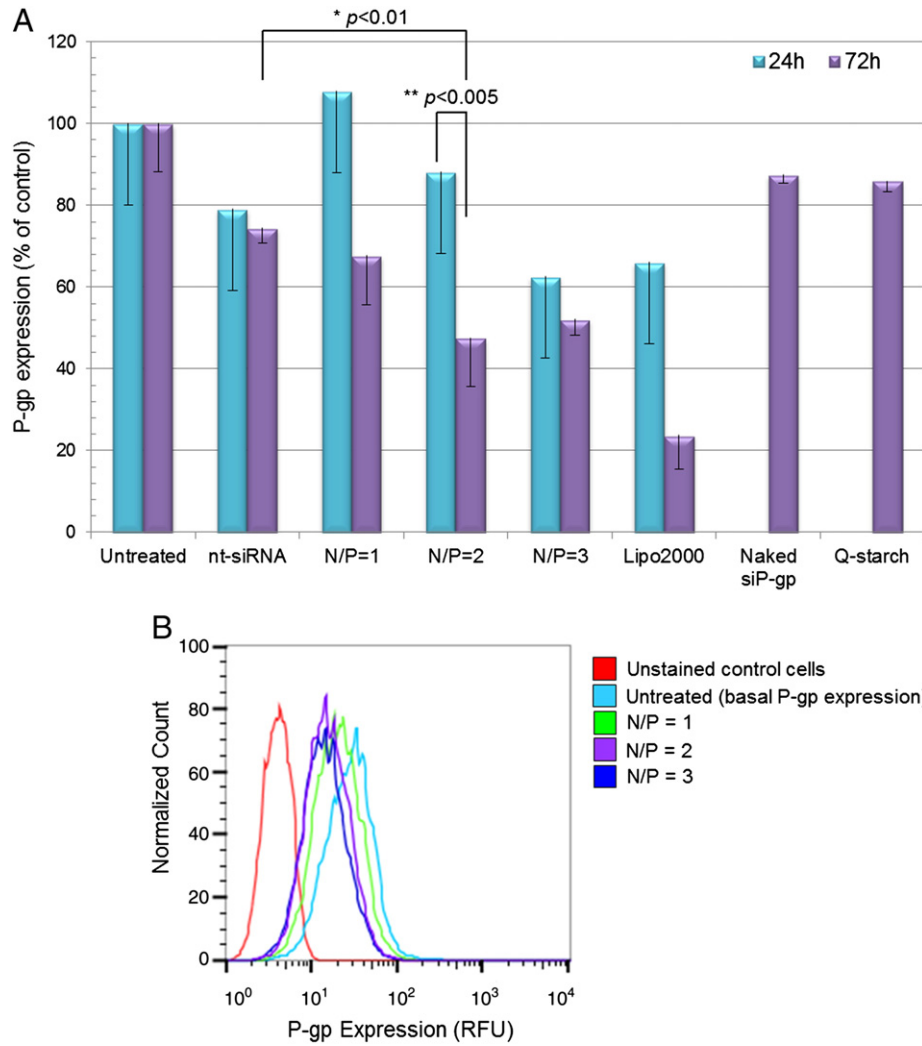


Fig. 8. P-gp knockdown by siP-gp (50 nM) delivery into NAR cells using Q-starch/siP-gp complexes. (A) Gene silencing by Q-starch/siP-gp at N/P ratios of 1, 2, and 3 for 24 and 72 h courses of treatment. Percentage of P-gp expression was normalized to the expression in the untreated group (expressed as 100%) and vertical bars represent mean \pm s.d. of minimum three experiments; * $p < 0.01$ comparing N/P 2 to nt-siRNA for same time point (72 h), ** $p < 0.005$ comparing 24 h and 72 h of the same application (N/P 2). Lipofectamine 2000 (Lipo2000) was used as positive control. (B) Representative histogram of P-gp silencing in NAR cells incubated for 72 h with Q-starch/siP-gp complexes at N/P ratios of 1, 2, and 3.

materials that have low toxicity effects. Chitosan and its derivatives were well studied for that reason [46]. Since we established in this study a delivery platform for siRNA based on modified starch, it is necessary to evaluate its cytotoxicity. For toxicity evaluation of Q-

starch/siP-gp complexes, NAR cells were incubated with complexes or complex components (siRNA and Q-starch separately) for 72 h, after which an MTT cell viability assay for cellular proliferation was conducted. The percentage of cell viability was normalized to the

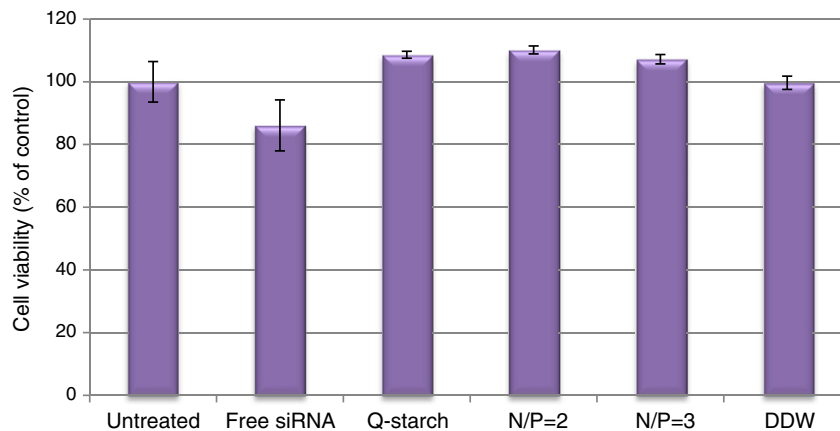


Fig. 9. NAR cell viability post-72 h of treatment by Q-starch/siRNA complexes (N/P 2 and 3) and their components (siRNA and Q-starch separately) measured by MTT cell viability assay. siRNA concentration 50 nM ($n = 3$). Vertical bars represent mean \pm s.d. of 3 experiments.

control group of untreated cells (100% viability); the results are summarized in Fig. 9.

We found that exposure to Q-starch (1.5 ng/ μ L) and Q-starch complexes (50 nM) did not result in cell death and even cell proliferation was observed (>100% viability). Cells that were treated with naked siRNA at the same concentration used in the complexes established 86% viability. It appears that siRNA's toxic effect was reduced when it was associated with Q-starch. We therefore devised a delivery vehicle based on a starch derivative that did not influence its biocompatibility for this application.

The major limitation of the compared commercial carrier Lipofectamine is its reported toxicity and poor stability in serum environment [47]. Thus, while presenting higher efficiency compared to Q-starch, the advantage of using Q-starch/siRNA complexes is their ability to resolve these disadvantages while demonstrating a sequence specific gene silencing. Additional study would be required to further improve gene silencing efficiency in future study. Overall, we present a proof of concept of modified starch as a legitimate potential carrier for siRNA.

3.7. Cellular uptake of Q-starch/siRNA complexes

The site of action of RNAi is the cytosol, because of which, along the transfection process siRNA complexes face multiple delivery barriers

including the non-permeable plasma membrane (extra-cellular barrier), endosomal escape, and decomplexation of siRNA complexes (intra-cellular barriers). According to Fig. 8, NAR cells exposed to complexes at N/P 1 didn't induce significant P-gp silencing through the whole transfection timeline (until 72 h of incubation with the complexes). Also, cells exposed to complexes at N/P 2 didn't induce significant P-gp silencing after 24-hour incubation with the complexes, while further incubation time with the complexes efficiently induced P-gp silencing. Our initial hypothesis was that the plasma membrane was a key barrier for siRNA delivery by Q-starch, and poor cellular uptake was presented through the whole assay for complexes at N/P 1 and through the first 24 h for complexes at N/P 2. We therefore examined the cell uptake of fluorescently labeled naked siP-gp^{lab} and complexes of Q-starch/siP-gp^{lab} at N/P 1 and 2 by a confocal microscope. During the experiment, NAR cells were treated with different formulations and monitored over a 24-hour course of treatment (siRNA concentration was the same as in transfection – 50 nM). Representative confocal images are shown in Fig. 10.

DAPI (blue) staining defines the nucleus area and cell borders are labeled by WGA Alexa 555® (yellow) staining in order to demonstrate intracellular trafficking of the complexes. Fig. 10A indicates that complexes at N/P 1 have relatively low uptake capabilities and only a few complexes (siRNA labeled in red and pointed to by a white arrow

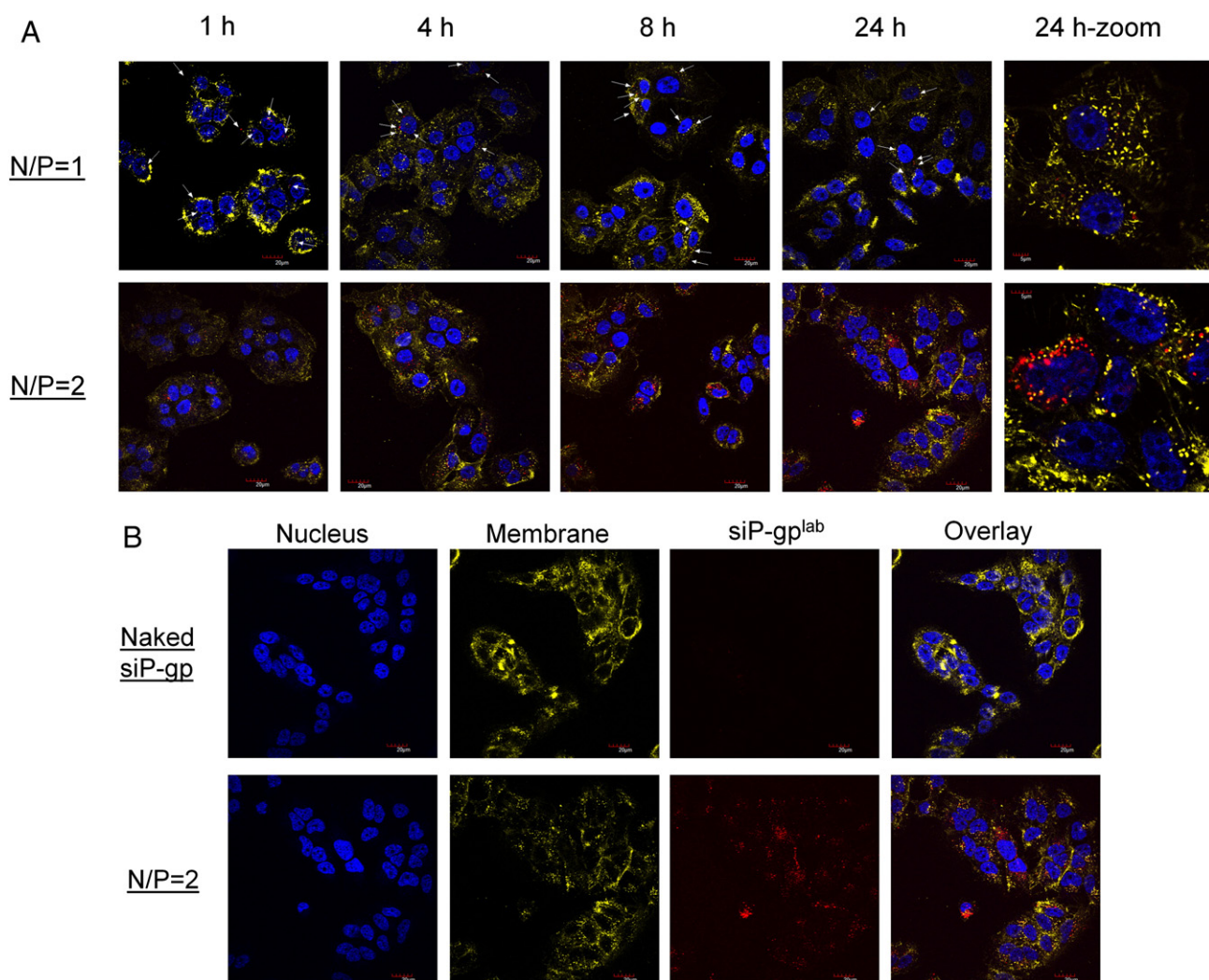


Fig. 10. Cellular uptake of Q-starch/siP-gp^{lab} complexes. Confocal microscopic images of NAR cells treated by Q-starch/siP-gp^{lab} complexes (50 nM). (A) Representative images at 1, 4, 8, and 24 h of incubation with the complexes. The nucleus was stained with DAPI in blue, the membrane was stained with WGA Alexa Fluor® 555 in yellow, and siP-gp was labeled by DY677 dye (cy5 alternative) in red. (B) Representative images of 24 h incubation with naked siP-gp and complexes at N/P 2 and overlay example of channels in use. Scale bar = 20 μ m except for zoom in images in which scale bar = 5 μ m.

in the images at Fig. 10A, N/P = 1, 1–24 h) were visualized inside the cell cytoplasm after 24 h of study. As mentioned above, the uptake of complexes at N/P 1 is insufficient and the plasma membrane constitutes a key barrier for negatively charged particles that don't bear targeting ligands. The association of these particles with the anionic membrane is a limiting step in the transfection and our hypothesis was confirmed. On the other hand, complexes at N/P 2 showed a substantial uptake by NAR cells. After 1 h of complex incubation (Fig. 10A, N/P = 2) we observed that not all the cells showed uptake and the scanned fields were non-homogenous regarding complex uptake. Comparing it to images of treatment by N/P 1 along all 24 h treatments, complexes at N/P 2 showed a significant entrance after only 1 h of incubation. However, after 4 h incubation with the complexes, most of the cells presented uptake, and after 24 h, significant complex penetration was shown. In order to emphasize the necessity of Q-starch as an siRNA delivery vector, Fig. 10B shows a side-by-side comparison of cells treated by naked siP-gp^{lab} and by Q-starch/siP-gp^{lab} at N/P 2 after 24 h of incubation. Primarily, one can see that by separating the fluorescence channels, the red signal (complexes) is screened by the membrane dye (yellow) highlighting the massive uptake shown and that the complexes are mainly detected inside the cytoplasm. Second, it seems that negligible naked siP-gp^{lab} entrance was accepted, and entrapment by Q-starch significantly improved its potential to enter NAR cells and finally integrate into the RNAi mechanism. These results suggest, in contrast to our hypothesis, that the membrane barrier didn't inhibit the gene silencing process that was observed during the first 24 h of treatment by complexes at N/P 2 (Fig. 8). This could imply that the rate limiting step for the transfection was intracellular. Either the complexes that entered the cells escaped the endosome compartment 24–72 h post-exposure, or dissociation of siRNA from its carrier limited the transfection kinetics.

4. Conclusions

In this study, we devised a modified cationic starch as an siRNA carrier. We have demonstrated that formulations in which full entrapment of siRNA was observed, showed significant gene silencing due to high cellular uptake. This platform was shown as safe and efficient in gene knockdown and presented promising potential as a wide platform for siRNA transport into cancer cells.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2014.04.031>.

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