Physicochemical and Transfection Properties of Cationic Hydroxyethylcellulose/DNA Nanoparticles

Farzaneh Fayazpour,[†] Bart Lucas,[†] Carmen Alvarez-Lorenzo,[‡] Niek N. Sanders,[†] Jo Demeester,[†] and Stefaan C. De Smedt*[,†]

Laboratory of General Biochemistry and Physical Pharmacy, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium, and Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Santiago de Compostela, Santiago de Compostela 15782, Spain

Received May 15, 2006; Revised Manuscript Received July 27, 2006

In this study the physicochemical and transfection properties of cationic hydroxyethylcellulose/plasmid DNA (pDNA) nanoparticles were investigated and compared with the properties of DNA nanoparticles based on polyethylene imine (PEI), which is widely investigated as a gene carrier. The two types of cationic hydroxyethylcelluloses studied, polyquaternium-4 (PQ-4) and polyquaternium-10 (PQ-10), are already commonly used in cosmetic and topical drug delivery devices. Both PQ-4 and PQ-10 spontaneously interact with pDNA with the formation of nanoparticles approximately 200 nm in size. Gel electrophoresis and fluorescence dequenching experiments indicated that the interactions between pDNA and the cationic celluloses were stronger than those between pDNA and PEI. The cationic cellulose/pDNA nanoparticles transfected cells to a much lesser extent than the PEI-based pDNA nanoparticles. The low transfection property of the PQ-4/pDNA nanoparticles was attributed to their neutrally charged surface, which does not allow an optimal binding of PQ-4/pDNA nanoparticles to cellular membranes. Although the PQ-10/pDNA nanoparticles were positively charged and thus expected to be taken up by cells, they were also much less efficient in transfecting cells than were PEI/pDNA nanoparticles. Agents known to enhance the endosomal escape were not able to improve the transfection properties of PQ-10/pDNA nanoparticles, indicating that a poor endosomal escape is, most likely, not the major reason for the low transfection activity of PQ-10/pDNA nanoparticles. We hypothesized that the strong binding of pDNA to PQ-10 prohibits the release of pDNA from PQ-10 once the PQ-10/pDNA nanoparticles arrive in the cytosol of the cells. Tailoring the nature and extent of the cationic side chains on this type of cationic hydroxyethylcellulose may be promising to further enhance their DNA delivery properties.

Introduction

The major progress in the field of genomics has encouraged numerous scientists to work on suitable carriers for delivering genes to target cells. Indeed, the biological efficacy (i.e., the production of a certain protein) of administered "naked" DNA is restrained as it cannot efficiently overcome cellular barriers on its way to the nucleus. For example, the cellular uptake of naked DNA is poor due to its large size, hydrophilicity, and negatively charged backbone. In addition, naked DNA is very susceptible to degradation by nucleases in the extracellular matrix and in the cells. Consequently, the efficient intracellular delivery of DNA is highly dependent on suitable carriers, which protect the DNA against degradation, allow the DNA to cross cellular membranes and escape from endosomes, and guide the DNA into the nuclei of the cells.

Worldwide, many laboratories are searching for appropriate pharmaceutical carriers for DNA. Different types of cationic lipids and cationic polymers are under investigation as nonviral DNA carriers.[1,2] Cationic lipids, as well as cationic polymers, spontaneously form interpolyelectrolyte complexes with negatively charged nucleic acids, called lipoplexes and polyplexes, respectively. The physicochemical features that govern the biological activity of lipo- and polyplexes are, however, not well understood, partly due to the complexity of the association and dissociation behavior of such complexes.

Cationic polysaccharides (like, e.g., cationic dextrans, cationic celluloses, cationic guar, and chitosan) are, for various reasons, widely used in both cosmetics and topical drug delivery systems.[3] Recently, cationic polysaccharides were also investigated for DNA delivery purposes. In particular, Azzam et al.[4] tested over 300 different types of cationic polysaccharides for gene transfection, starting from various polysaccharides (of various molecular weight) and oligoamines. Although most of the cationic polysaccharides formed stable complexes with various plasmids, only certain types of the dextran−spermine-based polyplexes transfected cells in vitro.[4,5]

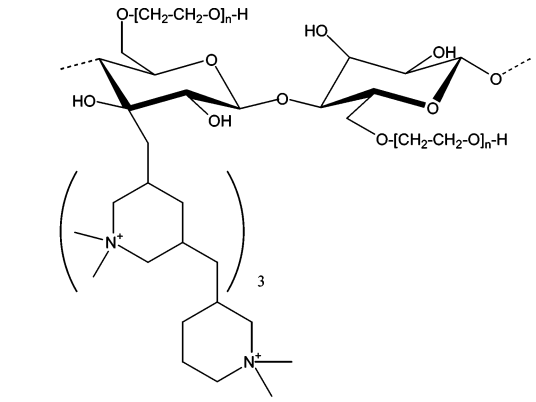
A major obstacle for in vivo gene delivery is the interaction of the DNA complexes with (extra)cellular fluids.[6−8] Indeed, binding of (extra)cellular proteins can induce aggregation, (partial) dissociation, and opsonization of the charged DNA complexes. It is well-known that pharmaceutical carriers bearing neutral, hydrophilic segments (such as poly(ethylene glycol) (PEG)) at their surface are attractive to avoid opsonization. Pegylated derivatives of dextran−spermine were reported to give efficient transfection in serum-rich media and to induce gene expression in the liver of mice after IV administration.[9] Transfection efficiency could be also increased by grafting fatty

* Corresponding author. Address: Laboratory of General Biochemistry and Physical Pharmacy, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium. Tel.: +32-9-2648076. Fax: +32-9-2648189. E-mail: stefaan.desmedt@UGent.be.

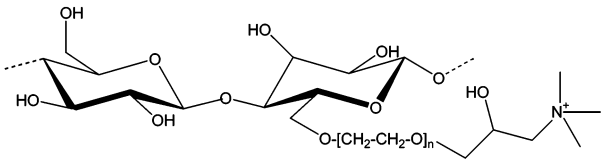
[†] Ghent University.

[‡] Universidad de Santiago de Compostela.

10.1021/bm060474b CCC: $33.50
Published on Web 09/07/2006



PQ-4



PQ-10

Figure 1. Schematic molecular structures of PQ-4 and PQ-10.

Table 1. Molecular Weight (MW), Intrinsic Viscosity in Water at 25 °C ([η]), the Number of PEG Chains Per Glucose Residue (X_{PEG}), the Average Number of Repeating Units of the Hydroxyethyl Chains (n), and the Number of Nitrogen Atoms Per Glucose Residue (X_N) of the Cationic Celluloses

polymer	MW (kDa)	[η] dL/g	X_{PEG}	n	X_N
PQ-4	1400	14.59	1.33	1.04	0.24
PQ-10	1700	70.09	0.51	1.52	0.51

acids on the dextran−spermine polymers. It was believed that hereby the surface of the particles becomes more hydrophobic, which would favor hydrophobic interactions with the cell membranes.[10] Cationic polysaccharides containing quaternary ammonium oligoamines were developed to make DNA particles with a highly positive surface, which would promote binding of the particles to the negatively charged cell membranes and would thus improve cellular uptake. However, quaternary ammonium polysaccharides as synthesized by Yudovin-Farber et al. were less efficient in gene transfer than were dextran−spermine-based carriers.[11]

The first aim of this study is to evaluate the DNA complexation properties of two types of cationic hydroxyethylcellulose. Polyquaternium-4 cellulose (PQ-4; Figure 1) and polyquaternium-10 cellulose (PQ-10; Figure 1) were previously characterized,[3,12] and already have applications in cosmetics and topical drug delivery devices.[13] In both PQ-4 and PQ-10, the sugar monomers are substituted with PEG. However, in PQ-4, the quaternary ammonium groups are directly attached to the cellulose backbone, while, in PQ-10, the quaternary ammonium groups are present at the end of the PEG chains.[14] Also, as Table 1 shows, compared to PQ-10, PQ-4 is less substituted with ammonium groups. The second aim of this study is to investigate whether quaternized cationic celluloses are suitable for gene delivery. The transfection properties of quaternized cationic cellulose/DNA nanoparticles were compared with the transfection properties of DNA nanoparticles based on branched and linear polyethylene imines (PEIs), which are widely investigated cationic polymers for DNA delivery purposes.

Materials and Methods

Materials. PQ-4 (Celquat H-100) and PQ-10 (Celquat SC-230M) were obtained from National Starch and Chemical Ltd. The physicochemical properties of PQ-4 and PQ-10 as provided by the supplier are shown in Table 1. Branched PEI (BPEI, MW = 25 kDa) was purchased from Sigma. Linear PEI (LPEI, MW = 22 kDa) was a kind gift from Professor Ernst Wagner (Ludwig Maximilian University, Munich, Germany). Secretory alkaline phosphatase (SEAP) plasmid DNA (pMet7-hβc-SEAP) was a kind gift from Professor Tavernier (Ghent University, Belgium). pMet7-hβc-SEAP was amplified in *Escherichia coli* and purified by the Qiagen Plasmid Mega Kit. Picogreen was purchased from Molecular Probes. Poly-L-aspartic acid (p(Asp)), dextran sulfate (DS), chloroquine, and ethidium bromide were purchased from Sigma. The cellulase used in this study was the Féderation Internationale Pharmaceutique (FIP) standard (0.147 U/mg). The fusogenic peptide INF-7, a 24 amino acid-containing peptide with fusogenic activity derived from the influenza virus, was a kind gift from Professor Hennink (Utrecht University, The Netherlands).

Preparation of Polyplexes. Stock solutions of the cationic polymers (PQ-4 (3.0 mg/mL), PQ-10 (3.0 mg/mL), BPEI (12.98 mg/mL), and LPEI (7.6 mg/mL)) were prepared in 20 mM HEPES buffer at pH 7.4. In general, polyplexes (varying in N/P ratio, see below) were prepared by adding (in one step) different volumes of the polymer stock solution (depending on the ratio) to a fixed amount of the DNA stock solution (1 μg, 1 mg/mL in HEPES buffer) and adding HEPES buffer to reach a final volume of 40 μL. The mixture was vortexed for 10 s, and the polyplexes were allowed to equilibrate for 30 min at room temperature prior to use.

In this study, the N/P ratio is the ratio of the number of nitrogen atoms on the cationic cellulose to the number of phosphorus atoms on the DNA. It was assumed that 1 μg of DNA contains 3.43 nmol of phosphorus and that 1 μg of PQ-4, PQ-10, and PEI contains 2.0, 3.2, and 23.3 nmol of nitrogen, respectively.

Polyplexes were also prepared from "low molecular weight PQ-10". The low molecular weight PQ-10 was obtained by incubating PQ-10 solutions (3 mg/mL in 20 mM HEPES buffer at pH 7.4) with cellulase at 37 °C. The reaction was terminated by heating the sample for 10 min at 95 °C.

Particle Size and ζ Potential Measurements on the Polyplexes. Dynamic light scattering measurements on the polyplexes were carried out on a Malvern 4700 instrument at 25 °C at an angle of 90°. The incident beam was a HeNe laser beam (633 nm). To avoid dust particles, the HEPES buffer used in the preparation of the polyplexes was filtered through a 0.45 μm membrane (Schleicher & Schuell). Polystyrene nanospheres (220 ± 6 nm; Duke Scientific) were used to verify the performance of the instrument. The particle size of each polyplex sample was measured three times.

The ζ potential measurements on the polyplexes were performed by electrophoretic light scattering at 25 °C on a Malvern Zetasizer 2000. Polystyrene nanospheres (−50 mV; Duke Scientific) were used to verify the performance of the instrument. The ζ potential of each polyplex sample was measured three times.

Gel Electrophoresis on the Polyplexes. Agarose gel electrophoresis experiments were performed to monitor the complexation of plasmid DNA (pDNA) to the cationic polymers. The slots of the agarose gel (1% agarose in Tris-borate EDTA buffer) were loaded with 40 μL of the sample. Electrophoresis was carried out at 100 V for 60 min. The bands containing pDNA were visualized by staining the gels with an ethidium bromide solution for 30 min at room temperature, followed by UV illumination.

Fluorescence Measurements on the Polyplexes in the Presence of Picogreen. The complexation of pDNA to the cationic polymers was further investigated by measuring the extent of the quenching of picogreen. Picogreen is only fluorescent when it can intercalate with pDNA, thus it is fluorescent when free (uncomplexed) pDNA is present. Briefly, 1 μg of pDNA was mixed with increasing amounts of cationic polymer in HEPES buffer (at pH 7.4) and incubated for 30 min at

room temperature to equilibrate. The fluorescence of the polyplex dispersions was measured 10 min after adding the picogreen to the dispersions. An SLM-Aminco Bowman spectrofluorimeter was used (λ_{ex} 480 nm, λ_{em} 520 nm).

Cell Viability and Cell Transfection Measurements. For evaluating the cytotoxicity and transfection properties of the polyplexes, COS-7 cells were cultured in Dulbecco's modified eagle medium (DMEM) containing glutamine (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin; Sigma). The cells were seeded in 24-well culture plates at a density of about 25 000 cells/cm^2. After 24−48 h of incubation at 37 °C in humidified air containing 5% CO_2, the cells were rinsed with phosphate-buffered saline. Subsequently, 500 μL of culture medium (without serum), containing 40 μL of polyplexes, was added to each well and incubated at 37 °C in a humidified air (5% CO_2) atmosphere for 2 h. Subsequently, the transfection medium was removed, and 500 μL of DMEM (containing 10% FBS) was added to each well. Two days later, SEAP activity was determined in the medium using 4-methylumbellifery phosphate (4-MUP).[15] Then the fluorescence due to conversion of 4-MUP to 4-methylumbelliferone was measured with a Wallac Victor 2 plate reader (Perkin-Elmer; λ_{ex} 360 nm, λ_{em} 449 nm). To take into account the number of cells per well, the measured SEAP activity was normalized; therefore, we divided the SEAP activity by the total protein content in the corresponding well, which was measured using the DC protein assay (BioRad). The cytotoxicity of the polyplexes was measured by the EZ4U assay (Lucron Bioproducts; Biomedica). COS-7 cells were seeded in 96-well culture plates at a density of about 7000 cells/well. The cells were incubated with polyplexes at different ratios. Consequently, the polyplex dispersions were removed from the cells in the wells. To each well 20 μL of EZ4U substrate in 200 μL of culture medium was added and incubated with the cells for 2−5 h. The absorbance of each well was measured with a Wallac Victor 2 plate reader (Perkin-Elmer; λ_{ex} 450 nm, λ_{em} 495).

Dissociation of the Polyplexes. To study the dissociation of the polyplexes by polyanions, an excess of DS or p(Asp) was added to the polyplex dispersions (at a ratio of 40:1 for DS/pDNA and 10:1 for p(Asp)/pDNA). The resulting mixtures were incubated for 30 min at room temperature. Gel electrophoresis and the picogreen fluorescence assay (see above) were used to determine the extent of dissociation of the polyplexes.

Confocal Laser Scanning Microscopy (CLSM). CLSM (MRC1024 Bio-Rad) was performed on COS-7 cells that were transfected by polyplexes containing fluorescently labeled pDNA (see below). The transfection of the cells with polyplexes occurred as described above. The cells were seeded in a glass culture dish to allow optical imaging.

pDNA Labeling. The pDNA labeling reagents were obtained from the Mirus Corporation. DNA (pMet7-hβc-SEAP) was modified using *Label* IT reagents according to the recommendations of the manufacturer. Briefly, *Label* IT reagent was added to a DNA solution (100 μg DNA/mL (20 mM) HEPES buffer at pH 7.4) at a 1:1 ratio (weight label/weight DNA) and incubated at 37 °C for 1 h. To remove unbound fluorescein, the pDNA was precipitated by adding 0.1 vol of 5 M NaCl and 2 vol of ice cold 100% ethanol to the pDNA. After centrifugation, the pallet was washed with 70% ethanol until the supernatant contained no fluorescein. Consequently, the pallet was dissolved in HEPES buffer and the concentration was determined by absorption at 260 nm.

Results and Discussion

pDNA Binds to Cationic Cellulose. As outlined above, the binding of pDNA to the cationic polymers was studied by gel electrophoresis. The gel electrophoresis measurements in Figure 2 clearly show that, at N/P ratios of 1 and higher, both the cationic celluloses and PEIs bind all the pDNA. The polymer/pDNA complexes remain in the slots as they are unable to migrate through the gel. Clearly, a difference between PQ-4 and PQ-10 is that, at an N/P ratio of 0.1, PQ-4 binds all the pDNA, whereas PQ-10 binds only a part of the pDNA.

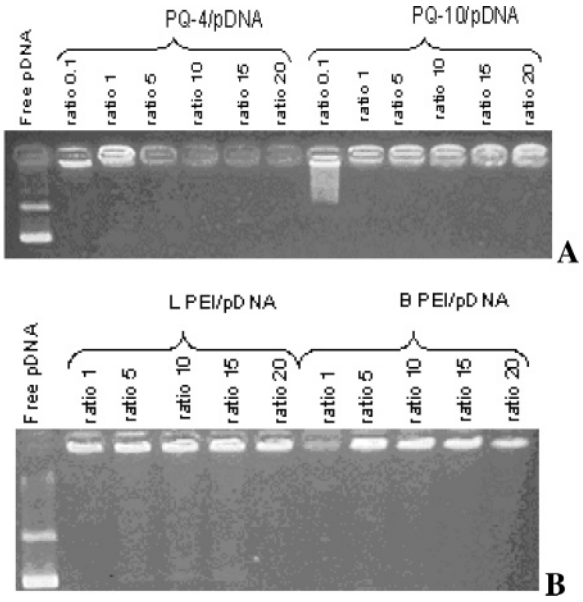


Figure 2. Gel electrophoresis on dispersions of (A) PQ-4/pDNA and PQ-10/pDNA complexes and (B) BPEI/pDNA and LPEI/pDNA complexes. The N/P ratio was varied.

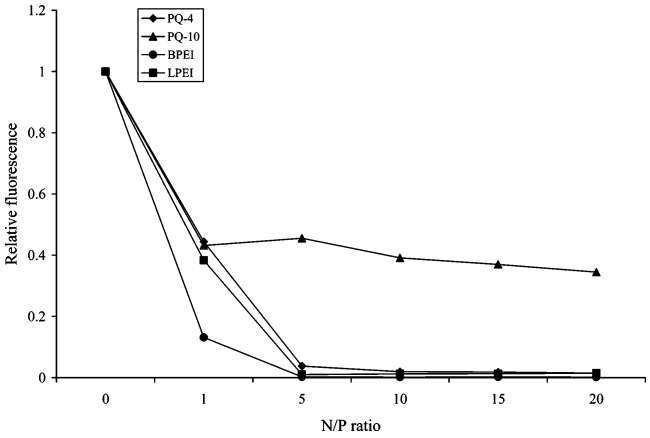


Figure 3. Relative fluorescence of cationic cellulose/pDNA and PEI/pDNA dispersions (as a function of the N/P ratio of the complexes) containing picogreen. The amount of pDNA was kept constant (1 μg), and the concentration of cationic polymers was increased. The fluorescence of the (free) pDNA solution was set to 1. The fluorescence of three samples was measured. The error bars were smaller than the size of the symbol.

Figure 3 shows the complexation between the cationic celluloses and pDNA as revealed from the fluorescence quenching assay. As explained in the Materials and Methods section, picogreen was added to the dispersions to detect free pDNA. As expected, the fluorescence decreases at higher concentrations of the cationic polymers: this indicates that the pDNA molecules not only bind to the cationic polymer chains but that pDNA molecules also condense to such an extent that picogreen has less access to/less intercalates in the double-stranded DNA, yielding less fluorescence. In the case of PQ-4, LPEI, and BPEI, the fluorescence is almost completely disappeared at an N/P ratio higher than 1. This confirms the gel electrophoresis data in Figure 2, as free pDNA was not detected in the dispersions. In the case of PQ-10, however, even at higher N/P ratio's, the fluorescence seemed to be only partially quenched when compared to the fluorescence of the corresponding free pDNA solution. However, the gel electrophoresis data showed that, at

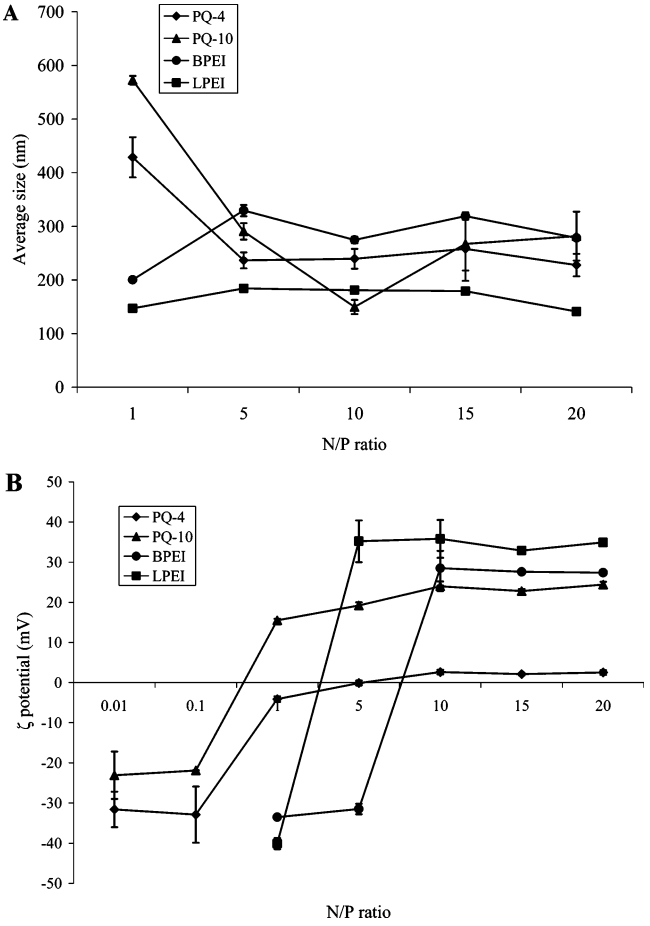


Figure 4. Hydrodynamic diameter (A) and ζ potential (B) of cationic cellulose- and PEI-based pDNA nanoparticles. Data represent the mean values obtained on five (independently prepared) samples.

these N/P ratios, PQ-10 binds all the pDNA. The partial quenching in Figure 3 probably indicates that PQ-10 does not condense pDNA as tightly as the other cationic polymers, still allowing picogreen to intercalate to a certain extent in the pDNA.

Size and ζ Potential of pDNA/Cationic Cellulose Complexes. Figure 4A shows that, at an N/P ratio of 1, the cationic cellulose/pDNA complexes are substantially larger than the PEI/pDNA complexes; at higher N/P ratios, all types of pDNA complexes are of similar size (between 150 and 300 nm). Figure 4B reveals that, at high N/P ratios, the PQ-10-based complexes are clearly positively charged, while the ζ potential of the PQ-4-based complexes is significantly lower. This can be explained by the structural differences between PQ-4 and PQ-10 (Figure 1). The sugar backbone of PQ-4 is substituted with both polycation chains (which bind the DNA) as well as neutrally charged PEG chains (which are not involved in the binding of pDNA). We assume that the PEG chains form a neutral shell around the inner core of the polyelectrolyte complexes. The resulting complexes might resemble the core−shell polyplexes formed between graft-polyelectrolytes and oppositely charged polymers or biomacromolecules.[16] In contrast, in PQ-10, the cationic groups are attached to the free end of the PEG side chains. Therefore, we assume that, upon binding the pDNA, the PEG strands are (sterically) not allowed to create a neutralizing PEG shell around the complexes.

Cytotoxicity of pDNA/Cationic Cellulose Complexes. As outlined in the Introduction, we are interested in how cationic cellulose-based DNA nanoparticles behave in a cellular environment. Clearly, an absolute requirement is that the nanoparticles should be not toxic for cells. Therefore, the cytotoxicity of the

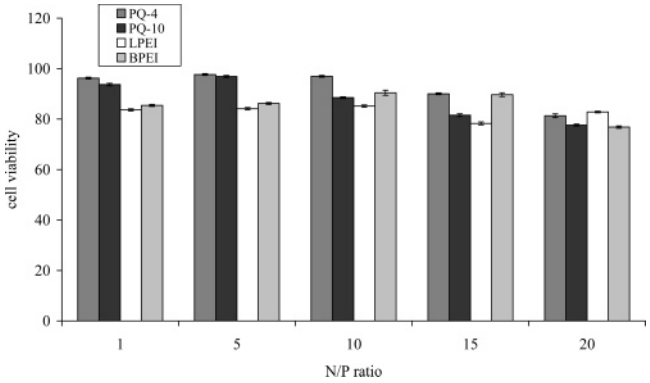


Figure 5. Viability of COS-7 cells after incubation with cationic cellulose- and PEI-based pDNA nanoparticles. The data are the mean values obtained on three (independently prepared) samples.

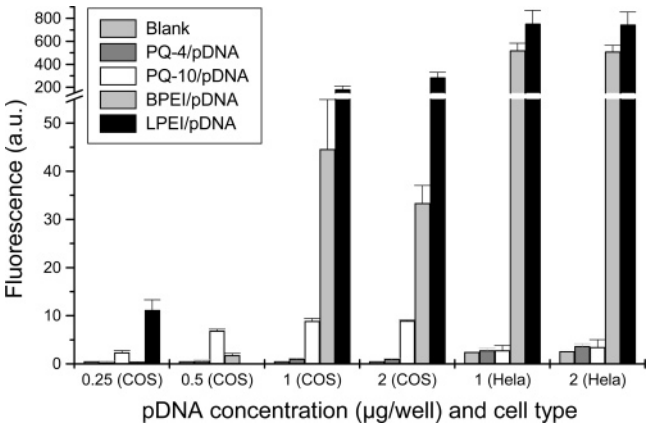


Figure 6. Transfection efficiency of cationic cellulose- and PEI-based pDNA nanoparticles in COS-7 cells and Hela cells as a function of the concentration of pDNA per well ($n = 6$). The N/P ratio of all the complexes equalled 10. (note: COS cells 0.5 μg/well for LPEI/pDNA, not determined).

cellulose-based polyplexes was studied using COS-7 cells. Figure 5 shows the results of the cell viability assay. PQ-4- and PQ-10-based pDNA complexes show low toxicity, as more than 90% of the cells remains alive. At higher N/P ratios the cationic cellulose/pDNA dispersions seemed to be slightly more toxic, which may be due to the presence of a higher amount of (free) cationic cellulose damaging the cellular membranes.[17] The PEI-based complexes demonstrated low cytotoxicity as well. Indeed, COS-7 cells seemed to tolerate well the PEI-based complexes used in this study, as the cell viability was around 80% compared to untreated cells. Although it has been reported that low molecular weight PEI shows low membrane damaging[18] and that cell damaging is more severe for free PEI compared to PEI/DNA complexes,[19] this low cytotoxicity of the PEI polyplexes was rather unexpected, as some studies reported significant cytotoxicity.[20,21] Also, the group of Kissel[22,23] showed that the molecular weight and type of PEI influences the cytotoxicity. From MTT assays [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], they concluded that the cytotoxicity of PEI is affected by polymer size and structure: high cationic charge densities, compact and highly branched structures, and high molecular weights render PEI less biocompatible.[18] Anyhow, the fact that the cationic cellulose-based polyplexes seemed to be well tolerated by cells was encouraging for further transfection experiments.

In Vitro Transfection of Cells by pDNA/Cationic Cellulose Complexes. Figure 6A shows the transfection efficiency of cationic cellulose- and PEI-based polyplexes in COS-7 cells as

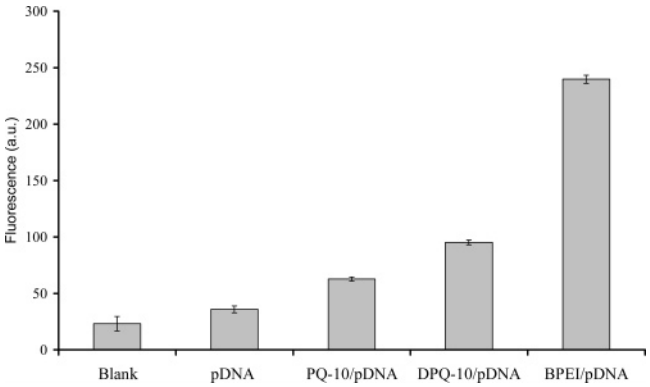


Figure 7. Transfection efficiency of cationic cellulose- and PEI-based pDNA nanoparticles in COS-7 cells. DPQ-10 denotes the complexes made from degraded PQ-10. The N/P ratio of the complexes is 10. The data are the mean values obtained on three (independently) prepared samples.

a function of the amount of pDNA per well. Note that the N/P ratio of all the complexes studied in Figure 6 equalled 10. Clearly, PEI-based polyplexes transfect significantly better than cationic cellulose/pDNA complexes. LPEI proves to be a better transfectant than BPEI, in agreement with earlier findings.[24,25] PQ-10/pDNA complexes show minor transfection efficiency, while PQ-4 does not transfect the COS-7 cells at all. As there is evidence that a positive surface charge promotes cellular uptake as a result of the binding of the complexes to the negatively charged cell membranes, we hypothesize that PQ-4/pDNA complexes do not transfect because of their neutral surface charge (Figure 4B). Indeed, confocal microscopy measurements did not evidence any binding or uptake of the PQ-4/pDNA nanoparticles (data not shown). We observed the same trend in transfection behavior when we used Hela cells instead of COS-7 cells (Figure 6B).

These results lead to the intriguing question of why the positively charged PQ-10/pDNA complexes fail in transfecting COS-7 and Hela cells efficiently, while PEI/pDNA complexes of similar surface charge successfully deliver the same pDNA in the cells. The molecular weight of PQ-10 is 1700 kDa, while the molecular weight of LPEI and BPEI is 22 and 25 kDa, respectively. To find out whether a lower molecular weight of PQ-10 would improve the transfection efficiency, we degraded the sugar backbone of PQ-10 by treating it with endocellulase prior to complexation with pDNA. Capillar viscosity measurements on PQ-10 solutions incubated with cellulase showed a significant decrease in viscosity, indicating that PQ-10 indeed degraded (data not shown). Figure 7 shows the transfection efficiency of the pretreated PQ-10: compared to high molecular weight PQ-10, lower molecular weight PQ-10 transfects better; however, it is still less efficient than BPEI.

It has been proposed in many studies that the good transfection properties of PEI are partly due to the fact that it facilitates the escape of pDNA from the endosomes. This is usually explained by the fact that, in the endosomes, PEI works as a buffer ("proton-sponge") based on the uniqueness of its chemical structure,[26] which eventually causes swelling and disruption of the endosomes. Consequently, PEI polyplexes in the endosomes can be efficiently delivered into the cytoplasm.[27] Because of quaternary ammonium, PQ-10 cannot buffer the endosomal pH. To investigate whether the low transfection efficiency of PQ-10 was in part attributed to a lack of endosomal escape of the polyplexes, we tested compounds known to improve the cytosolic delivery of macromolecules after their cellular uptake by the endosomes. Chloroquine is a lysosomotropic agent thought

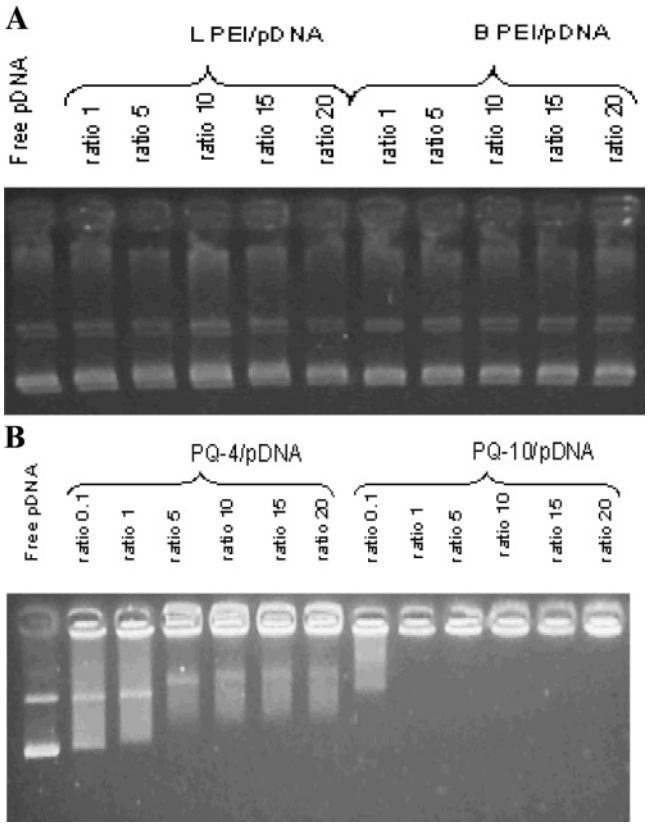


Figure 8. Gel electrophoresis on dispersions of (A) BPEI/pDNA and LPEI/pDNA complexes and (B) PQ-4/pDNA and PQ-10/pDNA complexes. p(Asp) was added to the dispersions. The N/P ratio of the complexes was varied.

to have a buffering capacity preventing endosomal acidification.[27] In addition, it can lead to swelling and bursting of the endosomes and was shown to enhance DNA transfection.[28−30] However, coincubation of the COS-7 cells with 100 μM chloroquine did not improve the transfection activity by PQ-10/pDNA complexes (data not shown). In a second approach, a membrane-disrupting peptide derived from the influenza virus (INF-7) was used, which is known to destabilize endosomal membranes (facilitating the escape of the viral particles from the endosomes).[31] While other studies have shown that INF-7 significantly enhances the transfection activity in COS-7 cells,[31,32] the addition of this peptide to PQ-10/pDNA polyplexes did not improve their transfection behavior (data not shown). These data indicate that the lack of endosomal escape is probably not the major reason for the low transfection activity of PQ-10/DNA nanoparticles.

pDNA Release from Cationic Cellulose. Another critical step for obtaining successful transfection is the intracellular release of the pDNA from its carrier. While the pDNA has to remain associated with its carrier as long as it is outside the cells, intracellularly it has to release its carrier. In other words, a critical balance between being associated extracellularly and becoming dissociated intracellularly has to be maintained. To obtain information on "the ease of disassembling" of the complexes, we studied in buffer the extent to which pDNA could be displaced from the complexes by the polyanions p(Asp) and DS. Their disassembling behavior was studied using agarose gel electrophoresis and a fluorescence dequenching assay.

After incubation of the polyplexes with an excess of p(Asp), gel electrophoresis revealed that both LPEI and BPEI almost completely released the pDNA (Figure 8A). In contrast, PQ-4 only partially released the pDNA, whereas PQ-10 did not release pDNA at all (Figure 8B), suggesting a stronger binding of

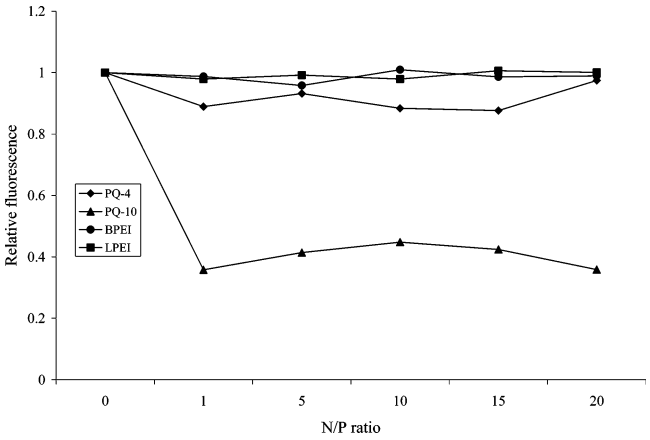


Figure 9. Fluorescence of (picogreen-containing) cationic cellulose/pDNA and PEI/pDNA dispersions after adding p(Asp). The N/P ratio of the complexes was varied.

pDNA to cationic celluloses than to PEI. Similar results were obtained when DS was added to the cationic cellulose/pDNA nanoparticles (data no shown).

Fluorescence dequenching experiments (Figure 9) show that, upon adding p(Asp), all the pDNA is released from the PEI-based complexes, in agreement with the gel electrophoresis results in Figure 8A. Also, p(Asp) (almost) completely releases pDNA from the PQ-4/pDNA complexes, which fully agrees with the outcome of the gel electrophoresis experiments in Figure 8B, which suggests that the pDNA was only partially released at higher N/P ratios. Possibly, p(Asp) only "partially" disassembles PQ-4/pDNA complexes in the sense that the pDNA molecules become fully accessible for picogreen without really becoming released from the cationic cellulose polymers. Importantly, the fluorescence dequenching experiments in Figure 9 indicate that PQ-10/pDNA complexes cannot be destabilized by p(Asp), in full agreement with the observations in the gel electrophoresis experiments in Figure 8B.

Conclusions

We have studied the physicochemical and transfection properties of PQ-4/pDNA and PQ-10/pDNA nanoparticles and compared them with DNA nanoparticles based on BPEI and LPEI. Gel electrophoresis and fluorescence experiments indicated that PQ-4 can bind and condense pDNA through stronger interactions than PEI. The resulting PQ-4/pDNA nanoparticles are approximately 200 nm in size and have a neutral ζ potential, which is explained by the fact that the sugar backbone of PQ-4 bears, besides polycation side chains, PEG side chains. We observed that PQ-4/pDNA nanoparticles do not transfect Cos-7 and Hela cells, which we attribute to their neutrally charged surface, which does not allow an optimal binding of PQ-4/pDNA nanoparticles to cellular membranes, thus preventing an efficient cellular uptake. Developing a PQ-4-like cationic cellulose with a lower degree of pegylation might improve the transfection properties of PQ-4/pDNA nanoparticles.

PQ-10/pDNA nanoparticles have a positively charged surface. Gel electrophoresis experiments indicated that PQ-10 binds pDNA stronger than PEI and PQ-4. However, upon binding to PQ-10, pDNA does not completely condense, as picogreen can still, to a certain extent, intercalate in the pDNA. Although the PQ10/pDNA nanoparticles are positively charged and thus expected to be taken up by cells, they are much less efficient in transfecting cells than PEI/pDNA nanoparticles. Agents known to enhance the endosomal escape (like chloroquine and the INF-7 peptide) were not able to improve the transfection properties of PQ-10/pDNA nanoparticles, indicating that a poor endosomal escape is, most likely, not the major reason for the low transfection activity of PQ-10/pDNA nanoparticles. On the basis of our findings, we hypothesize that the strong binding of pDNA to PQ10 prohibits the release of pDNA from PQ-10 once the PQ-10/pDNA nanoparticles arrive in the cytosol of the cells. Since the PQ-10/pDNA nanoparticles did transfect the cells to a certain extent, although less efficiently than PEI/pDNA nanoparticles, tailoring the nature and extent of cationic side chains on this type of cationic hydroxyethylcellulose may be promising to further enhance their DNA delivery properties.

Acknowledgment. The authors thank Ghent University (BOF) for a scholarship to F.F. The members of the Laboratory of General Biochemistry and Physical Pharmacy are acknowledged for many helpful comments and discussions. We would like to acknowledge Professor Ernst Wagner (Ludwig Maximilian University, Munich, Germany) for the kind gift of LPEI.

References and Notes

(1) Brown, M. D.; Schatzlein, A. G.; Uchegbu, I. F. *Int. J. Pharm.* **2001**, *23*, 1−21.
(2) Wagner, E.; Ogris, M.; Zauner, W. *Adv. Drug Delivery Rev.* **1998**, *30*, 97−113.
(3) Rodriguez, R.; Alvarez-Lorenzo, C.; Concheiro, A. *Biomacromolecules* **2001**, *3*, 886−93.
(4) Azzam, T.; Eliyahu, H.; Shapira, L.; Linial, M.; Barenholz, Y.; Domb, A. J. *J. Med. Chem.* **2002**, *45*, 1817−24.
(5) Azzam, T.; Raskin, A.; Makovitzki, A.; Brem, H.; Vierling, P.; Lineal, M.; Domb, A. J. *Macromolecules* **2002**, *35*, 9947−53.
(6) Ruponen, M.; Honkakoski, P.; Ronkko, S.; Pelkonen, J.; Tammi, M.; Urtti, A. *J. Controlled Release* **2003**, *93*, 213−7.
(7) Wiethoff, C. M.; Middaugh, C. R. *J. Pharm. Sci.* **2003**, *92*, 203−17.
(8) Sanders, N. N.; De Smedt, S. C.; Demeester, J. *J. Controlled Release* **2003**, *87*, 117−29.
(9) Hosseinkhani, H.; Azzam, T.; Tabata, Y.; Domb, A. J. *Gene Ther.* **2004**, *11*, 194−203.
(10) Azzam, T.; Eliyahu, H.; Makovitzki, A.; Linial, M.; Domb, A. J. *J. Controlled Release* **2004**, *96*, 309−23.
(11) Yudovin-Farber, I.; Yanay, C.; Azzam, T.; Linial, M.; Domb, A. J. *Bioconjugate Chem.* **2005**, *16*, 1196−203.
(12) Rodriguez, R.; Alvarez-Lorenzo, C.; Concheiro, A. *Eur. J. Pharm. Biopharm.* **2003**, *56*, 133−42.
(13) Brode, G. L.; Goddard, E. D.; Harris, W. C.; Salensky, G. A. In *Cosmetic and Pharmaceutical Applications of Polymers*; Gebelein, C. G., Cheng, T. C., Yang, V. C., Eds.; Plenum Press: New York, 1991; pp 117−28.
(14) Rodriguez, R.; Alvarez-Lorenzo, C.; Concheiro, A. *J. Controlled Release* **2003**, *86*, 253−65.
(15) Berger, J.; Hauber, J.; Hauber, R.; Geiger, R.; Cullen, B. R. *Gene* **1988**, *66*, 1−10.
(16) Petersen, H.; Fechner, P. M.; Martin, A. L.; Kunath, K.; Stolnik, S.; Roberts, C. J.; Fischer, D.; Davies, M. C.; Kissel, T. *Bioconjugate Chem.* **2002**, *13*, 845−54.
(17) Choksakulnimitr, S.; Masuda, S.; Tokuda, H.; Takakura, Y.; Hashida, M. *J. Controlled Release* **1995**, *34*, 233−41.
(18) Kunath, K.; von Harpe, A.; Fischer, D.; Petersen, H.; Bickel, U.; Voigt, K.; Kissel, T. *J. Controlled Release* **2003**, *89*, 113−25.
(19) Godbey, W. T.; Wu, K. K.; Mikos, A. G. *J. Controlled Release* **1999**, *60*, 149−60.
(20) Godbey, W. T.; Wu, K. K.; Mikos, A. G. *Biomaterials* **2001**, *22*, 471−80.
(21) Putnam, D.; Gentry, C. A.; Pack, D. W.; Langer, R. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 1200−5.
(22) Fischer, D.; Bieber, T.; Li, Y.; Elsasser, H. P.; Kissel, T. *Pharm. Res.* **1999**, *16*, 1273−9.
(23) Fischer, D.; Bieber, T.; Li, Y.; Elsasser, H. P.; Kissel, T. *Eur. J. Cell Biol.* **1998**, *75*, 108−16.
(24) Wightman, L.; Kircheis, R.; Rossler, V.; Carotta, S.; Ruzicka, R.; Kursa, M.; Wagner, E. *J. Gene Med.* **2001**, *3*, 362−72.
(25) Itaka, K.; Harada, A.; Yamasaki, Y.; Nakamura, K.; Kawaguchi, H.; Kataoka, K. *J. Gene Med.* **2004**, *6*, 76−84.

(26) Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297−301.
(27) Funhoff, A. M.; van Nostrum, C. F.; Koning, G. A.; Schuurmans-Nieuwenbroek, N. M.; Crommelin, D. J.; Hennink, W. E. *Biomacromolecules* **2004**, *5*, 32−9.
(28) Ciftci, K.; Levy, R. J. *Int. J. Pharm.* **2001**, *218*, 81−92.
(29) Erbacher, P.; Roche, A. C.; Monsigny, M.; Midoux, P. *Exp. Cell Res.* **1996**, *225*, 186−94.
(30) Mellman, I.; Fuchs, R.; Helenius, A. *Annu. Rev. Biochem.* **1986**, *55*, 663−700.
(31) Plank, C.; Oberhauser, B.; Mechtler, K.; Koch, C.; Wagner, E. J. *J. Biol. Chem.* **1994**, *269*, 12918−24.
(32) Zuidam, N. J.; Posthuma, G.; de Vries, E. T.; Crommelin, D. J.; Hennink, W. E.; Storm, G. *J. Drug Targeting* **2000**, *8*, 51−66.

BM060474B