Influence of Chitosan Structure on the Formation and Stability of DNA–Chitosan Polyelectrolyte Complexes

ARTICLE *in* BIOMACROMOLECULES · NOVEMBER 2005

Impact Factor: 5.75 · DOI: 10.1021/bm0503726 · Source: PubMed

4 AUTHORS:

Sabina Strand
Norwegian University of Science and Techno…
25 PUBLICATIONS **943** CITATIONS
SEE PROFILE

Signe Danielsen
St. Olavs Hospital
18 PUBLICATIONS **751** CITATIONS
SEE PROFILE

Bjørn E. Christensen
Norwegian University of Science and Techno…
93 PUBLICATIONS **2,332** CITATIONS
SEE PROFILE

Kjell M Varum
Norwegian University of Science and Techno…
110 PUBLICATIONS **6,477** CITATIONS
SEE PROFILE

Influence of Chitosan Structure on the Formation and Stability of DNA−Chitosan Polyelectrolyte Complexes

Sabina P. Strand,*,† Signe Danielsen,‡,§ Bjørn E. Christensen,† and Kjell M. Vårum†

NOBIPOL, Department of Biotechnology, and Biophysics and Medical Technology, Department of Physics, The Norwegian University of Science and Technology, NTNU, NO-7491 Trondheim, Norway

Received June 3, 2005; Revised Manuscript Received August 10, 2005

The interactions between DNA and chitosans varying in fractional content of acetylated units (F_A), degree of polymerization (DP), and degree of ionization were investigated by several techniques, including an ethidium bromide (EtBr) fluorescence assay, gel retardation, atomic force microscopy, and dynamic and electrophoretic light scattering. The charge density of the chitosan and the number of charges per chain were found to be the dominating factors for the structure and stability of DNA−chitosan complexes. All high molecular weight chitosans condensed DNA into physically stable polyplexes; however, the properties of the complexes were strongly dependent on F_A, and thereby the charge density of chitosan. By employing fully charged oligomers of constant charge density, it was shown that the complexation of DNA and stability of the polyplexes is governed by the number of cationic residues per chain. A minimum of 6−9 positive charges appeared necessary to provide interaction strength comparable to that of polycations. In contrast, further increase in the number of charges above 9 did not increase the apparent binding affinity as judged from the EtBr displacement assay. The chitosan oligomers exhibited a pH-dependent interaction with DNA, reflecting the number of ionized amino groups. The complexation of DNA and the stability of oligomer-based polyplexes became reduced above pH 7.4. Such pH-dependent dissociation of polyplexes around the physiological pH is highly relevant in gene delivery applications and might be one of the reasons for the high transfection activity of oligomer-based polyplexes observed.

Introduction

Self-assembly of polycations (PC) and polyanions (PA) yielding polyelectrolyte complexes (PEC) through a cooperative system of electrostatic interactions is widely exploited both in nature and in technological applications. Among many systems studied, the condensation of DNA by PC has received considerable attention due to its application in nonviral gene delivery.[1−3] Although the collapse of extended DNA chains into compact particles seems striking, it is just one example of PA−PC interactions leading to a transition from the extended to the compacted state, driven by the overall increase in the entropy of the system due to the released counterions. Regardless of the nature of the condensing agent, the resulting compact forms adopt a limited range of morphologies, generally a mixture of toroids, rods, and globules, although in different relative amounts.[4−7] The prominent toroidal morphology of condensed DNA particles is not restricted to DNA but has also been observed for other polyanions with sufficiently large persistence length, such as xanthan.[8]

The development of safe and effective vectors for delivery of therapeutic genes remains a central challenge in the field of gene therapy. The safety concerns associated with viral vectors have triggered increasing interest toward vectors based on cationic polymers.[9−12] Despite the similar principles of formation and the similar morphology of complexes formed by different PC, the size, stability, and colloidal properties of the complexes strongly depend on the PC used and, likewise, their performance as nonviral gene delivery systems.[3,11,13,14] In addition to the chemical structure of the repeating unit of the PC, other factors such as molecular weight, polymer/DNA mixing ratio, and details of preparation have been shown to impact transfection efficiency.[14−17]

Chitosans, a family of linear binary polysaccharides consisting of (1 → 4)-β-linked 2-acetamido-2-deoxy-D-glucose (GlcNAc) and its de-N-acetylated analogue (GlcN), have emerged as a biocompatible alternative to synthetic polycations, suitable for in vivo gene delivery to mucosal tissues.[17−23] Besides biodegradability[24] and low toxicity,[17,25,26] chitosan also offers an advantage inherent to synthetic PC; its properties may be tuned through the fraction of acetylated units (F_A), degree of polymerization (DP), and its polydispersity, as well as the pH-dependent degree of ionization. Tailoring of chitosans with respect to F_A, DP, and polydispersity provides a tool for controlling the functional properties of chitosans.[27]

In the field of gene delivery, the F_A and DP of chitosan are reported to affect the transfection efficiency of DNA−chitosan polyplexes.[17−19,28] Recently, the low molecular weight chitosan-based polyplexes appeared especially promising, showing a 120−260-fold higher gene expression in

* Corresponding author. E-mail: sabina.strand@biotech.ntnu.no.
† NOBIPOL, Department of Biotechnology.
‡ Biophysics and Medical Technology, Department of Physics.
§ Present address: Department of Radiation Oncology, University Hospital of St. Olav, 7006 Trondheim, Norway.

10.1021/bm0503726 CCC: $30.25
Published on Web 09/10/2005

Table 1. Chitosan and Chitosan Oligomers Used in This Study

fraction of acetylated units (F_A)	intrinsic viscosity η (mL/g)	degree of polymerization (DP)	comment
<0.002	600	900	
0.01	620	900	
0.15	740	1000	
0.35	760	900	
0.49	900	1000	
<0.002		4, 6	commercial samples, monodisperse
<0.002		9, 15	enzymatic hydrolysis, monodisperse
<0.002		16, 18, 25	HONO depolymerization, polydisperse

the mouse lung in vivo compared to that of chitosan with high molecular weight.[19] Such increased performance of the oligomers is believed to be related to the better ability to release the DNA from the polyplexes.[15,19] In agreement with this notion, the stability of chitosan−DNA polyplexes after exposure to competing polyanions increased with increasing DP of chitosan and the mixing ratio of polyions.[29] However, considerable differences between the transfection efficiency of oligomer fractions with slightly different DP and polydispersity suggest that a subtle balance between stability and dissociation of complexes might be crucial.[19]

The intriguing dependence of the transfection efficiency of chitosans on their structure illustrates the need for further studies addressing the link between chitosan structure and properties of DNA−chitosan nanoparticles. This has motivated us to perform a systematic study where chitosans varying widely in chemical composition and degree of polymerization were applied to reveal differences in their interaction with DNA that may be of relevance for the observed differences in their biological activity. We demonstrate that both the charge density and the number of charges of the chitosan are key factors for the properties of polyplexes. Our study also emphasizes the importance of thoroughly characterized chitosan samples in terms of F_A, DP, and polydispersity prior to their application as nonviral gene delivery agents.

Materials and Methods

Buffers and Reagents. Sodium acetate buffer (HAc/NaAc, 20 mM, pH 5.0), 3-(*N*-morpholino)propanesulfonic acid buffer (MOPS, 20 mM, pH 6.5 and 7.4, Sigma-Aldrich), or 2-(*N*-morpholino)ethanesulfonic acid (MES, 20 mM, pH 5.5 and 6.5, Sigma-Aldrich) were used in this study. The ionic strength (*I*) of the buffers was adjusted to 50−500 mM by adding the corresponding amount of NaCl. Agarose (A 9539) and ethidium bromide (EtBr, E 1510) were purchased from Sigma-Aldrich.

DNA. Linear calf thymus DNA (ctDNA, ∼15 kbp, D-1501 Sigma-Aldrich) and the plasmid pBR322 (∼4.4 kbp, Promega) were used in this study. The concentration of DNA in the solution was determined by UV absorbance at 260 nm from the relationship $1A = 50$ mg/mL $= 0.146$ mM DNA phosphate.

Chitosans PLL and PEI. All chitosan samples were prepared by heterogeneous de-N-acetylation of shrimp chitin as previously described,[30] converted to HCl salts, and lyophilized. An overview of all chitosans used in this study is given in Table 1. All samples were characterized by ^{1}H NMR to determine the fraction of acetylated units (F_A) and, in case of chitosan oligomers prepared by depolymerization, also the number-average degree of polymerization (DP_n). The DP_n of highly polymerized samples was estimated from intrinsic viscosity measurements.[31] The water content in chitosan samples was determined by thermal gravimetric analysis (TGA) on a Netzsch STA 449C and was found to be 11−13%. The following highly polymerized chitosans with increasing fractional content of acetylated units were used in this study: $F_A < 0.002$, F_A 0.01, F_A 0.15, F_A 0.35, and F_A 0.49. The completely de-N-acetylated chitosan $F_A < 0.002$ was further depolymerized with nitrous acid to obtain three polydisperse oligomer samples with number-average degrees of polymerization of 16 (DP_n 16), 18 (DP_n 18), and 25 (DP_n 25). Chitosan tetramer (DP 4) and hexamer (DP 6) were obtained from Seikagaku Inc., Japan. The longer oligomers, nonamer (DP 9) and 15-mer (DP 15), were prepared by enzymatic hydrolysis as previously described.[18]

PLL of average molecular weight of 22 kDa (P-2658) and PEI, a branched sample of average molecular weight of 25 kDa, were purchased from Sigma-Aldrich.

Formulation of Polyplexes for Gel Electrophoresis, Size, and Zeta Potential Measurements. Chitosan stock solutions (0.2−1 mg/mL) were prepared by dissolving chitosan in sterile filtered MQ grade water or 20 mM HAc/NaAc buffer of pH 5.0. ctDNA stock solutions (0.2−1 mg/mL) were prepared by dissolving the DNA in appropriate buffer (MES, MOPS, or HAc/NaAc). The stock solution of pDNA (1 mg/mL) was diluted to the working concentration of 0.05 mg/mL in 20 mM HAc/NaAc buffer of pH 5.0. DNA−chitosan complexes were prepared by adding the DNA to the chitosan followed by intense stirring on vortex mixer for 10 s (Heidolph, KEBO Lab, Sweden). The final concentration of DNA in the formulations ranged from 5 to 10 μg/mL, depending on the assay of interest. The mixing ratio between chitosan and DNA is expressed as the molar ratio between all protonable amino groups of chitosan and the phosphate groups of DNA and is referred to as the A/P ratio.

Ethidium Bromide Displacement Assay. A solution of ctDNA (10 μg/mL) in the appropriate buffer was mixed with ethidium bromide (EtBr) in molar ratio EtBr/DNA phosphate of 1:20, corresponding to one molecule of intercalated EtBr per every 10th base pair. This low EtBr/DNA ratio, instead of the common 1:4 ratio, was applied in order to minimize the possible influence of the intercalated dye on the electrostatic interaction of DNA with polycations.[32] DNA−

chitosan complexes were formed directly in the 3 mL cuvette by stepwise addition of polycation to the DNA solution. The total volume of the polymer solution added was no more than 7% of the final total volume of solution. The fluorescence of samples was measured 4 min after addition of polycation at excitation and emission wavelengths of 511 and 603 nm, respectively (Perkin-Elmer LS50B). The recorded fluorescent intensities (FI) were expressed relative to the fluorescence intensity of the DNA−EtBr solution in the absence of polycation (FI_0), after subtracting the fluorescence of EtBr in the absence of DNA under the same buffer conditions (FI_{buff}):

$$FI\ (\%) = [(FI - FI_{buff})/(FI_0 - FI_{buff})] \times 100 \qquad (1)$$

pH Stability of Polyplexes. The pH stability of DNA−chitosan polyplexes was studied by titrating the polyplex solution with 0.1 M NaOH in the presence of EtBr and recording the fluorescence. The polyplexes were formed in 25 mM MOPS (pH 6.3, $I = 100$ mM) by adding ctDNA to the chitosan solution, yielding a final DNA concentration of 10 μg/mL. The amount of chitosan added was adjusted to achieve an A/P ratio of 3. The complexation was left to proceed for 30 min. EtBr was then added in the molar ratio of EtBr/DNA phosphate 1:10, and samples were further incubated for 30 min. A volume of 3 mL of the polyplex solution were then titrated by stepwise addition of aliquots of 50 μL of 0.1 M NaOH, and the pH and fluorescence of the sample were recorded 4 min after each addition. The fluorescence intensity was normalized by the intensity of fluorescence of the DNA−EtBr solution without chitosan, titrated with NaOH.

Ionic Strength Stability of Polyplexes. Dissociation of DNA−chitosan polyplexes was monitored by recording the fluorescence of DNA−EtBr as a function of increasing ionic strength. The polyplexes were formed in 20 mM MES buffer of pH 5.5 or 6.5 by adding ctDNA to the chitosan solution, yielding a final DNA concentration of 10 μg/mL. Samples with an A/P ratio in the range 1−10 were prepared. After 30 min of incubation time, EtBr was added in the molar ratio of EtBr/DNA phosphate 1:10 and the samples were further incubated for 30 min. Aliquots of 150 μL of polyplexes were pipetted on 96-well plates, and 50 μL of NaCl solution of appropriate concentration was added to each well. After 2 h of incubation, the fluorescence of the samples was measured using a Spectra MAX Gemini XS plate reader (Molecular Devices). Results are expressed relative to the fluorescence of the DNA−EtBr complex at the same ionic strength in the absence of chitosan.

Atomic Force Microscopy. Dried samples for AFM were prepared as previously described and imaged by tapping mode AFM (TM−AFM) in air using a Digital Instrument Multimode IIIa.[6,8] DNA−chitosan complexes were prepared by adding the chitosans F_A 0.01/$DP_n \sim 900$ and $F_A < 0.002$/DP_n 16, respectively, to the pDNA solution to achieve an A/P ratio of 60. The complexation was performed at pH 7.4 and 150 mM ionic strength.

Gel Retardation Assay. Gel electrophoresis was performed in 0.8% agarose gel in 40 mM Tris−acetate−EDTA (TAE) buffer at pH 8 and 5. The complexes of pBR322 with different chitosans at A/P ratios of 1−60 were prepared as described above at a constant DNA concentration of 10 μg/mL and incubated for 30 min. Next, 10 μL of the complex solution was mixed with 5 uL of loading buffer and loaded into the agarose gel wells. After electrophoresis (80 V, 1.5 h), the DNA was stained by incubating the gels in TAE buffer of pH 8 containing EtBr (1 μg/mL) for 15 min.

Zeta Potential and Size of Complexes. The zeta potential and mean hydrodynamic diameter (*z*-average) of polyplexes were determined by photon correlation spectroscopy on a Zetasizer Nano ZS (Malvern Instruments, Malvern, U.K.). The polyplexes were prepared at an A/P of 5 and final ctDNA concentration of 5 μg/mL in 20 mM HAc/NAc buffer of pH 5.0 or 24 mM MOPS of pH 7.4, both with an ionic strength of 13 mM. All measurements were carried out at 25 °C.

Results

Characterization of Interaction between DNA and Chitosan by EtBr Displacement Assay. Ethidium bromide (EtBr) intercalates between the base pairs of the DNA double helix, yielding a highly fluorescent DNA−EtBr complex.[33] Upon polycation binding to DNA, EtBr is expelled from the DNA−EtBr complex, resulting in a decrease in fluorescence.[34,35] The degree of displacement of the dye thus provides an indirect measure of the binding affinity, indicating the relative strength of the interaction between the DNA and the polycation. Figure 1 A−C summarizes binding of high molecular weight chitosans with similar DPs (DP range 900−1000), but with different fractions of acetylated units (F_A 0−0.49), to calf thymus DNA (ctDNA) at different pH values and an ionic strength of 100 mM. To allow direct comparison between PCs, all titration curves are presented as a function of the molar ratio of the amino groups of the PC to the phosphate groups of DNA (A/P ratio). To illustrate the differences between different PCs, poly-L-lysine (PLL) and polyethylenimine (PEI) were also included in Figure 1A. Figure 1A shows the titration curves obtained at pH 5.0, where most of the amino groups of chitosan, PLL, and PEI are protonized.[13,17] The A/P ratio at pH 5.0 may therefore be considered close to the effective charge ratio. Despite the similar total amount of charges available for interaction with DNA, the ability to displace EtBr varied strongly among the different polycations. Addition of PLL to the DNA−EtBr complex resulted in a rapid displacement of EtBr, reaching a sharp saturation point around A/P 1. On the contrary, titration with PEI resulted in a more gradual, but still the most efficient, displacement of the intercalator as shown from the lowest level of residual fluorescence (Figure 1A). The affinity of the chitosans was strongly dependent on F_A: the higher the F_A, the less-pronounced decay of fluorescence was observed and the higher fraction of EtBr remained intercalated in the polyplexes, as shown from the values of fluorescence intensity reached at A/P 3.

The degree of ionization of amino groups of chitosans depends strongly on the pH of the solution. Parts B and C of Figure 1 show binding of high molecular weight chitosans

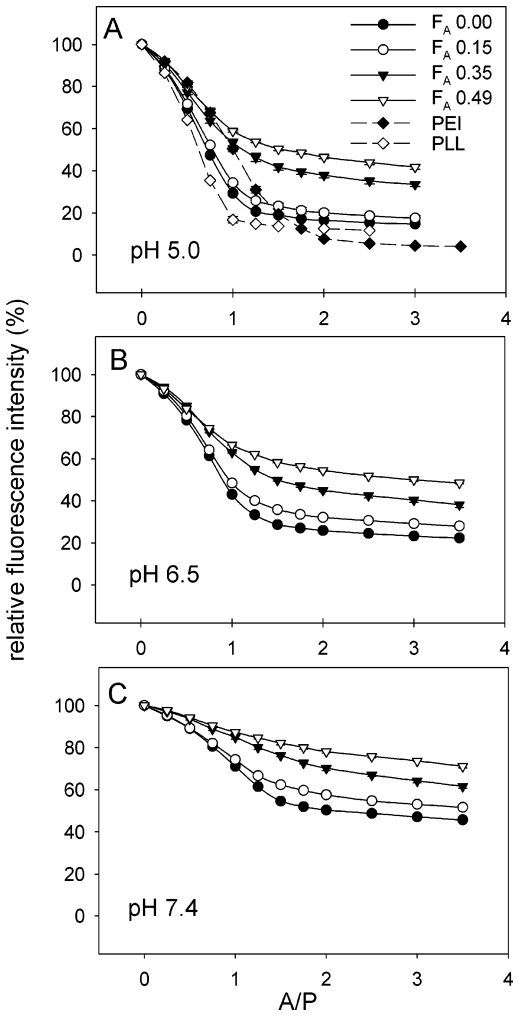

Figure 1. Titration of ctDNA−EtBr solution by high molecular weight chitosans with different fractions of acetylated units (F_A), poly-L-lysine, and polyethylenimine. The fluorescence intensity relative to the fluorescence of ctDNA−EtBr in the absence of polycation is plotted as a function of amino/phosphate (A/P) ratio. Titration curves grouped in each panel A−C were performed from a single preparation of DNA−EtBr solution (10 μg/mL DNA and EtBr/DNA phosphate 1:20). Data points represent the mean ± SD ($n = 3$). The following buffers were used: (A) HAc/NaAc pH 5.0, (B) MOPS pH 6.5, and (C) MOPS pH 7.4; ionic strength 100 mM.

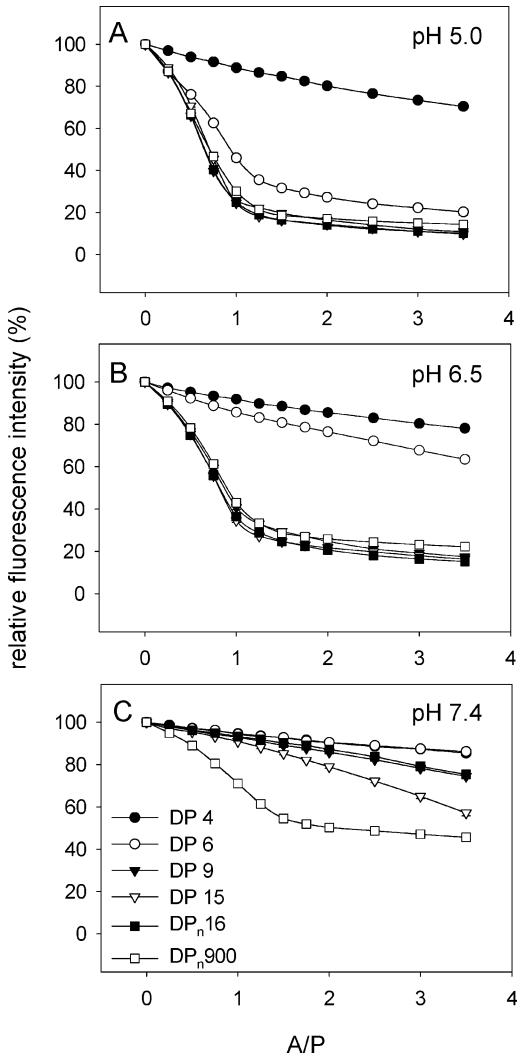

Figure 2. Titration of ctDNA−EtBr solution by completely de-N-acetylated chitosan (F_A 0) with different degrees of polymerization (DP). The fluorescence intensity relative to the fluorescence of ctDNA−EtBr in the absence of polycation is plotted as a function of amino/phosphate (A/P) ratio. Titration curves grouped in each panel A−C were performed from a single preparation of DNA−EtBr solution (10 μg/mL DNA and EtBr/DNA phosphate 1:20). Data points represent the mean ± SD ($n = 3$). The following buffers were used: (A) HAc/NaAc pH 5.0, (B) MOPS pH 6.5, and (C) MOPS pH 7.4; ionic strength 100 mM.

with F_A 0−0.49 at pH 6.5 and 7.4, respectively, where the ionization of the amino groups is suppressed and the charge density of chitosans is thus substantially reduced. The fluorometric curves at pH 6.5 were similar to those at pH 5.0, showing the same F_A dependence, but the residual amount of EtBr estimated from the fluorescence levels at A/P 3 was 10% higher for each chitosan (Figure 1B). The titration curves obtained at pH 7.4 demonstrate that all chitosans lost most of their ability to displace intercalated EtBr from DNA (Figure 1C). The low degree of ionization of the amino groups at pH 7.4 resulted in the degeneration of the sigmoidal shape of the titration curves and about 30% increase in the amount of intercalated EtBr at A/P 3 compared to that at pH 5.0.

As the next step, the same fluorescence assay was employed to evaluate the effect of the DP of chitosan on the binding to ctDNA. Figure 2 shows titration of DNA−EtBr by completely de-N-acetylated chitosan samples ($F_A < 0.002$) ranging from DP 4 to $DP_n \sim 900$ at different pH values and a constant ionic strength of 100 mM. Comparing fully charged oligomers at pH 5.0 (Figure 2A), the extent of EtBr expulsion increased strongly from tetramer to hexamer and further to nonamer, but no significant differences were observed above DP 9. Reduction of the degree of ionization of the oligomers by increasing the pH to 6.5 (Figure 2B) influenced mainly the binding of the hexamer as shown by the loss of the sigmoidal form of the titration curve. Finally, at pH 7.4, only the chitosan sample with $DP_n \sim 900$ exhibited the characteristic sigmoidal form of the titration curve. The short oligomers up to DP 9 showed very low displacement with approximately linear titration curves, whereas the binding of DP 15 increased somewhat with increasing A/P (Figure 2C). As noted in the figure texts, the curves plotted in each panel A−C of Figures 1 and 2 were performed on a single preparation of DNA−EtBr solution, giving standard deviations between the repeats of less than 1% (error bars not visible). Reproducibility between independent experi-

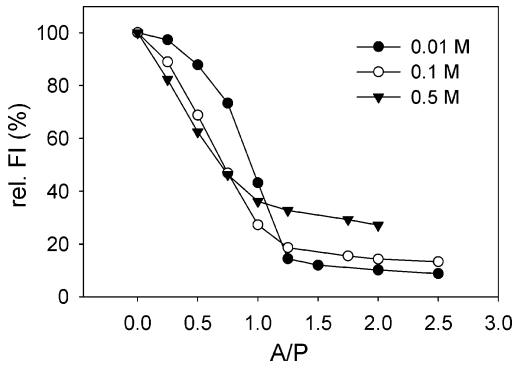

Figure 3. Titration of ctDNA−EtBr by chitosan $F_A < 0.002$ ($DP_n \sim 900$) in 20 mM HAc/NaAc pH 5.0 with different ionic strengths adjusted by addition of NaCl. Titrations were performed from a single preparation of DNA−EtBr solution (10 μg/mL DNA and EtBr/DNA phosphate 1:20). Data points represent the means of 3 repeats.

ments was typically around ±5%; however, the relative differences between the chitosans were almost identical.

To examine the effect of ionic strength, the DNA−chitosan interactions were also studied at 10 mM and at 500 mM. Figure 3 shows binding of chitosan with $F_A < 0.002$ and $DP_n \sim 900$ at pH 5 and different ionic strengths. Two main differences in binding pattern were observed: lower plateau values and a shift of curves toward higher A/P ratios for lower ionic strengths. Similar ionic strength effects were also observed for other chitosan samples and other pH values (data not shown).

Characterization of Polyplexes by Zeta Potential Measurements and AFM. The formation of DNA−chitosan complexes was also monitored by measurements of the changes in zeta potential of polyplexes as a function of A/P ratio. Upon self-assembly of DNA and chitosan, the highly negative charge of DNA is rapidly neutralized, and the surface charge of the polyplexes is turned into positive values at higher A/P ratios. Figure 4A shows the zeta potential of complexes formed in 20 mM HAc/NaAc buffer of pH 5.0 ($I = 13$ mM) as a function of A/P ratio. At these conditions, chitosans are fully charged, and consequently, the molar A/P ratio corresponds to the ± charge ratio. All chitosans studied showed similar behavior: the negative charge of the DNA was rapidly neutralized around A/P 1, and further addition of chitosan resulted in rapid overcharging of the polyplexes. The zeta potential increased steeply up to A/P 2, and above that, only a moderate increase was observed. Polyplexes formed by completely de-N-acetylated chitosan ($F_A < 0.002$) with high molecular weight ($DP_n \sim 900$) acquired the highest zeta potential.

Figure 4B shows the zeta potential of polyplexes formed at pH 7.4, where the charge density of the chitosans is much lower and consequently, much higher amounts of chitosans were required to neutralize the DNA phosphates. The formation of polyplexes at pH 7.4 depended strongly on the type of chitosan used. In the case of F_A 0.49 with $DP_n \sim 1000$, the amount of chitosan corresponding to an A/P ratio of 2 was sufficient to neutralize the phosphates of the DNA, whereas for the oligomer DP_n 16 ($F_A < 0.002$), an A/P ratio of 10 was required. When the two deacetylated chitosans with different molecular weights are compared, the oligomer DP_n 16 showed a markedly lower affinity for DNA at pH

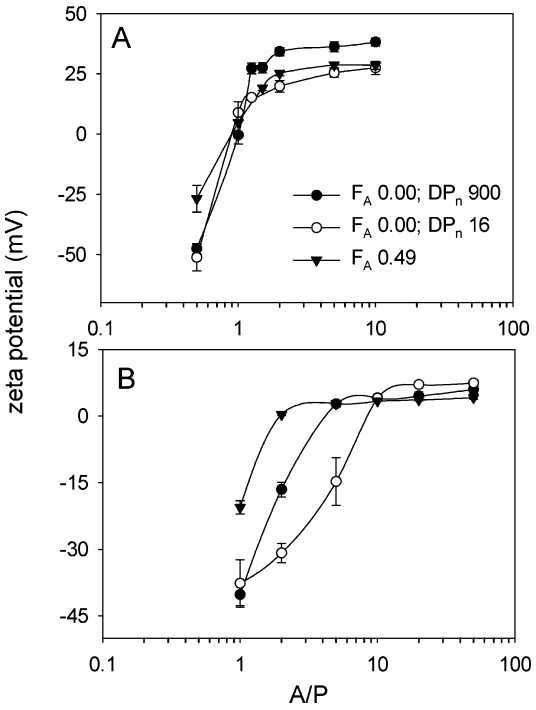

Figure 4. Zeta potentials of DNA−chitosan complexes as a function of amino/phosphate ratio (A/P). Complexes were prepared in 20 mM HAc/NaAc of pH 5.0 (A) or MOPS pH 7.4 (B), with a constant ionic strength of 13 mM. Results are expressed as the mean ± SD of 3 samples, with at least 3 readings of each.

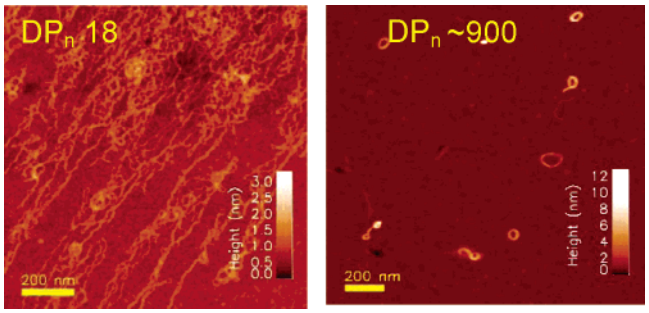

Figure 5. Tapping mode AFM height topographs of pBR322 complexed with chitosan F_A 0.01/$DP_n \sim 900$ (A) and $F_A < 0.002$/DP_n 18 (B) at pH 7.4 and an ionic strength of 150 mM.

7.4; the amount of amino groups necessary to neutralize DNA was about 2-fold higher than that of the chitosan with $DP_n \sim 900$. With the exception of chitosan F_A 0.49, polyplex formation at pH 7.4 was generally accompanied by aggregation and precipitation. The latter was especially pronounced in the case of oligomers; size measurements revealed large aggregates on the micrometer scale (data not shown).

Tapping mode AFM topograps of pDNA complexed by chitosans F_A 0.01/$DP_n \sim 900$ and $F_A < 0.002$/DP_n 18, respectively, at pH 7.4 and 150 mM are shown in Figure 5. Whereas the former chitosan formed characteristic polyplexes at pH 7.4, large amounts of clusters of uncondensed DNA were observed when mixing DP_n 18 oligomer with the DNA (Figure 5). This also illustrates the limited ability of the oligomers to compact DNA at pH 7.4, despite the high A/P ratio of 60.

Stability of DNA−Chitosan Complexes. DNA−chitosan polyplexes are typically prepared at low pH and ionic strength and are subsequently exposed to physiological pH

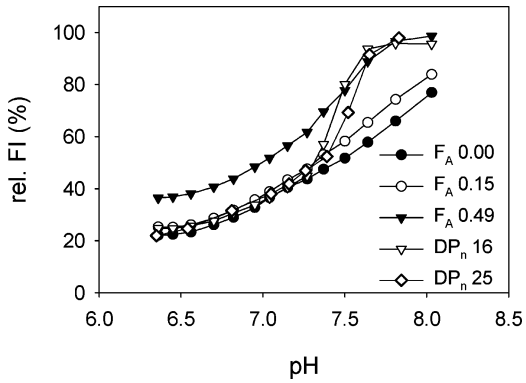

Figure 6. Stability of DNA−chitosan polyplexes as a function of pH. Polyplexes were prepared in 20 mM MOPS pH 6.3 and $I = 100$ mM at an A/P ratio of 3:1 and titrated with 0.1 M NaOH. Data points represent the means of 3 repeats with the SD within 1%.

and ionic strength during transfection experiments. Therefore, the stability of polyplexes following an increase in pH and ionic strength is of interest. DNA−chitosan complexes prepared in 20 mM HAc/NaAc buffer of pH 5.0 remained stable up to pH 6, which is the upper limit of the buffering range of this buffer, thereby making further measurements difficult (data not shown). Figure 6 shows recovery of fluorescence as a function of pH for complexes of ctDNA and five different chitosans formed in 25 mM MOPS pH 6.3 ($I = 100$ mM) at an A/P ratio of 3:1 and then subjected to increasing pH. All complexes started to dissociate around pH 6.5, and the trend of dissociation remained similar for all chitosans up to pH 7.4. Above pH 7.4, complexes based on the two $F_A < 0.002$ oligomers with DP_n 16 and DP_n 25 rapidly dissociated, as shown by the abrupt increase in DNA−EtBr fluorescence.

The stability of complexes formed by chitosans varying in F_A and DP was also assessed in a gel retardation assay. Figure 7 compares the stability of polyplexes subjected to gel electrophoresis at pH 5 and 8, respectively. At pH 5, all polyplexes remained stable, and only the complexes with A/P 1 showed a slight tendency to release a fraction of DNA as indicated by a smear. At A/P > 1, no DNA was released from the complexes, irrespective of the F_A and DP of the chitosan used. When the same polyplexes were subjected to electrophoresis at pH 8, clear differences between complexes based on high molecular weight chitosans and chitosan oligomers were observed. All oligomer-based complexes released a significant portion of DNA even at high A/P ratios of 10−50, whereas no DNA was released from complexes formed by the chitosans with high DP (Figure 7). When the different oligomers are compared, an increase in average chain length from DP_n 16 to DP_n 25 as well as an increase in A/P ratio yielded increased stability against dissociation. In addition, gel electrophoresis did not reveal significant differences in the stability of polyplexes formed by high DP chitosans with F_A in the range of 0−0.49.

The dissociation of polyplexes as a function of increasing ionic strength, monitored by changes in DNA−EtBr fluorescence, is shown in Figure 8. Addition of NaCl to the polyplexes preformed in 20 mM MES buffers either of pH 5.5 or 6.5 and an A/P of 3:1 in the presence of EtBr resulted in an approximately linear increase in the fluorescence intensity (Figure 8). Although the slopes of the curves, and thereby the degree of dissociation, varied among chitosans, the fluorescence increased monotonically without any abrupt dissociation in the ionic strength range studied. The polyplexes formed by the chitosan with $F_A < 0.002$ and $DP_n \sim 900$ were the most stable, whereas the oligomer-based polyplexes, represented here by $F_A < 0.002$ with DP_n 16, dissociated most easily (Figure 8). Generally, the complexes prepared at pH 5.5 showed higher salt stability compared to those prepared at 6.5. This effect was particularly pronounced for the oligomer-based complexes.

Size of DNA−Chitosan Polyplexes. The size of DNA−chitosan polyplexes, given in Figure 9 as the *z*-average of the hydrodynamic radius, was affected by both the F_A and DP of the chitosan. As shown in Figure 9, the higher the F_A, the larger were the structures that formed; the size of complexes formed in 20 mM acetate buffer of pH 5 ($I = 13$ mM) by high DP chitosans with $F_A < 0.002$ and F_A 0.49 at A/P 5 was ca. 70 and 250 nm, respectively. Despite the low ionic strength of 13 mM, the size of oligomer-based

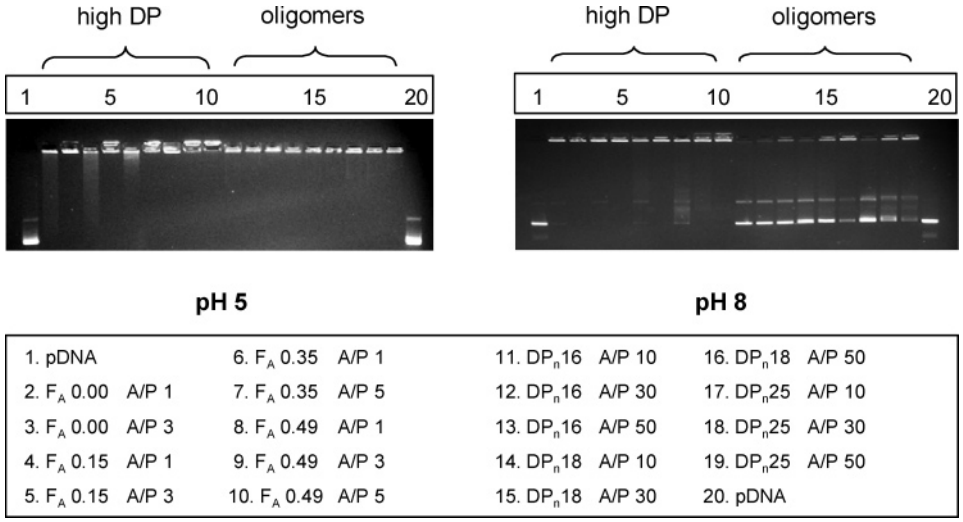

Figure 7. Physical stability of chitosan-based polyplexes in gel retardation assay at pH 5 and 8. Polyplexes were prepared in 20 mM HAc/NaAc pH 5.

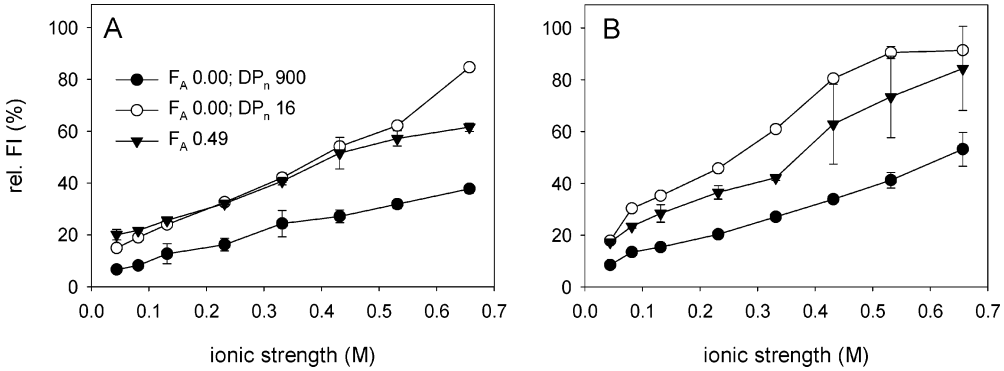

Figure 8. Stability of chitosan−DNA polyplexes as a function of increasing ionic strength. Polyplexes were prepared at an A/P ratio of 3:1 in MES buffer pH 5.5 (A) or MES buffer 6.5 (B), and ionic strength was increased by addition of NaCl. Results are expressed as the mean ± SD of 3−4 samples.

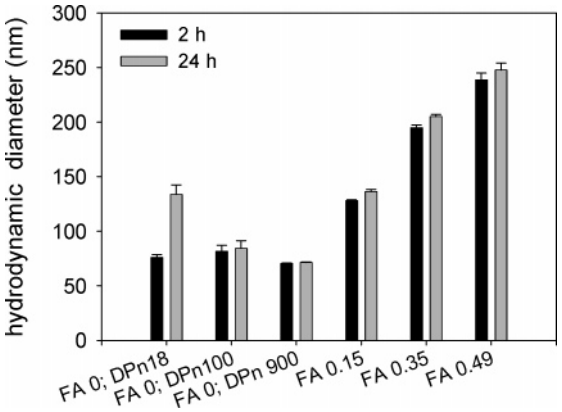

Figure 9. Hydrodynamic diameters of DNA−chitosan polyplexes prepared at A/P 5 in 20 mM HAc/NaAc buffer pH 5, $I = 13$ mM. Results are expressed as the mean ± SD of 3 samples, with at least 3 readings of each.

polyplexes was found to increase over time, as represented by the DP_n 18 sample in Figure 9.

Discussion

The ability of various species to displace intercalators such as EtBr from DNA, leading to a loss of fluorescence, is well established as an indirect method for monitoring the binding affinity of biologically active compounds to DNA.[36,37] In the past decade, the EtBr displacement assay has been extensively applied to study the binding of DNA condensing agents used in nonviral gene delivery system, such as polycations and cationic lipids,[13,34,35,38,39] as well as the stability of the DNA complexes.[29,32,40,41] Despite the extensive use of the assay, the exact mechanism of the exclusion of the intercalated dye from DNA is still not completely understood. There is increasing evidence that apart from a simple competitive binding of the polycation, the conformational changes of DNA during condensation are also responsible for EtBr release.[36,42,43] Regardless of the exact mechanism of EtBr release, the differences in the ability of chitosans to displace the dye from DNA provide detailed insight into the distinctive features of the self-assembly as well as the structure of the complexes themselves.

Generally, the binding affinity of a polycation to DNA is determined by the number of interacting charges per molecule, i.e., the valence, as well as the distribution of charges along the chain, i.e., the charge density.[39,41,44] The effect of charge density was investigated by using the high molecular weight chitosans varying in F_A and varying the degree of ionization of the amino group by pH (Figure 1). When trying to keep the molecular dimensions of the chitosans in the same range by keeping the DP_n between 900 and 1000, it was not possible to vary the charge density of chitosans without also affecting the valence. However, as expected and justified below, the binding of chitosans with such a high number of charged residues to DNA is essentially independent of DP. As shown in Figure 1, at the same total amount of charges available for interaction with DNA, the ability of chitosan to displace EtBr varied strongly among the different chitosans. Apparently, the degree of displacement of EtBr increased with increasing charge density of chitosan proportional to $(1 - F_A)$. A similar correlation between charge density and EtBr displacement has been established also for other cationic species.[34,39,43] Since the charge density of PLL is higher than that of chitosan, the higher affinity of PLL also supports this interpretation. The weaker affinity of PEI at A/P < 1.5 is believed to result from difficulties in polyion coupling due to steric hindrance and seems to be a general feature of branched polymers compared to linear polymers.[34,42,45] On the other hand, the lowest plateau values of the PEI titration curves correlated well with the highest charge density of this polymer.

Chitosan is a weak polyelectrolyte with a pK_a value of 6.5−6.6, and its charge density may be controlled by the pH of the solution.[46] Since the pK_a value was found to be independent of F_A,[46] the effective charge ratio at the same A/P is expected to be equal for all chitosans. The low degree of EtBr displacement by chitosans as the pH increased from 5 to 7.4 provides additional support that the extent of EtBr release is related to the charge density of PC. Chitosans of low charge density bind to DNA with reduced intersegment interaction strength, as indicated by the presence of sites still occupied by the intercalator within the polyplexes. The extent of EtBr displacement may also reflect differences in conformational changes of DNA during compaction by chitosans with varying charge densities. It has been previously shown in AFM studies that the F_A of chitosans influenced the morphology of DNA−chitosan complexes, with an increasing fraction of rods observed as F_A increases.[6]

To investigate the interaction between DNA and chitosan as a function of the number of cationic residues of chitosan, while keeping the charge density constant, a series of completely deacetylated oligomers with increasing DP was employed. The binding constants of charged oligomers have been found to increase with increasing number of charged residues per chain.[47,48] Correspondingly, the degree of EtBr displacement has generally been found to increase with the DP of PLL and other PC.[10,34] Here, fully protonized chitosan oligomers with 9 and 15 repeating units showed similar titration curves as the high molecular weight chitosan (Figure 2A). On the other hand, comparison of the titration curves of fully charged tetramer and hexamer (Figure 2A) showed that at least 6 charged residues per molecule were required to achieve efficient exclusion of EtBr. This suggests that a transition to polycation-like character of binding occurs when the number of positive charges reaches 6−9. Interestingly, this number seems to be in agreement with another study showing similar change in the binding affinity of peptides when the number of cationic amino acids increased from 4 to 8.[42]

It has been postulated that the displacement of EtBr is quantitatively determined by the degree of cooperativity in the electrostatic interactions of DNA with the polycation.[34] Here, the term cooperativity refers to intramolecular cooperativity where numerous groups of the same molecule interact with the matrix.[48] A degeneration of the sigmoidal shape of the titration curve has been interpreted by low cooperativity of binding between the DNA and the polycation, generally attributable to low charge density of the polycation, low DP, or steric hindrance.[34] On the basis of this assumption, 6−9 charged residues seem necessary to yield cooperative electrostatic interaction in our system. The existence of a minimum number of charges per oligomer required for efficient EtBr removal is further supported by the shift in the profile of the titration curves of the hexamer at pH 5.0 and 6.5 (Figure 2, parts A and B). The absence of the region of pronounced EtBr displacement at pH 6.5 suggests that the number of charges per molecule is no longer sufficient to expel EtBr. The longer oligomers retained the characteristic binding profile, implying that their number of charges was still sufficient. Assuming a pK_a of the amino group of 6.5−6.6, the number of protonized GlcN residues of the nonamer at pH 6.5 should theoretically lie below the critical value of 6 identified above. This discrepancy may reflect an increased protonation of the GlcN residues in the presence of the negatively charged phosphates. Such shifts in pK_a values due to polyion coupling have been reported for many different PA−PC systems.[34,50,51] Further suppression of the ionization of the amino groups by increasing the pH to 7.4 showed that only the chitosan with $DP_n \sim 900$ seemed to possess sufficiently charged segments to expel EtBr in a cooperative manner (Figure 2C). Interestingly, the difference in the affinity of monodisperse DP 15 and polydisperse DP_n 16 further supports the notion of a critical number of charges, as the DP_n 16 sample contains chain lengths down to DP 8, i.e., with the number of charges well below 6−9 at pH 7.4.

As expected for electrostatic interactions, EtBr displacement by chitosan was clearly affected by ionic strength (Figure 3). The profiles of the titration curves in Figure 3 illustrate that the extent of EtBr release at A/P < 1 increased with increasing ionic strength, whereas the total amount of EtBr removed decreased with increasing ionic strength. From the thermodynamic point of view, the PEC formation is favored at low ionic strength due to the lower screening of Coulombic interactions as well as larger entropic contribution from released counterions. Consequently, the binding constants of charged ligands have been reported to decrease with increasing ionic strength.[47,48] Similarly, the binding constant of EtBr for DNA was also reported to decrease with increasing ionic strength.[33] Moreover, the mode of binding of EtBr to DNA is also dependent on ionic strength; at low ionic strength (I < 10 mM), EtBr can bind outside the helix in addition to intercalation.[33,52]

Summarizing the results of EtBr assay presented in Figures 1−3, it has been shown that the amount of the residual intercalated EtBr within the polyplexes increased with increasing F_A and pH (Figure 1A−C), increasing ionic strength (Figure 3), and partly also DP (Figure 2B). The variation in the levels of residual intercalated dye within the polyplexes may point toward differences in the structure of polyplexes.[49] Generally, the alignment of the complementary polyions is never optimal but leaves out some of the ionic groups uncoupled, precluding successful coupling of other ligands.[49] As these uncoupled regions may still be accessible for EtBr intercalation, the amount of residual EtBr will increase with increasing difficulties in alignment between the polyions. Such hindrances in the coupling of DNA and chitosan may originate from the presence of acetylated residues, low degree of ionization of the amino groups, high degree of screening of Coulombic interactions, or irreversible nonoptimal binding of long highly charged chains. As shown from the plateau levels of fluorescence in Figures 1−3, all these parameters were virtually shown to increase the levels of intercalated EtBr in the complexes with DNA.

Monitoring the interaction between chitosan and DNA by recording the changes in the zeta potential of polyplexes revealed noticeable pH-dependent differences in the behavior of chitosans. A good agreement between the stoichiometric A/P ratio and zero mobility of polyplexes at pH 5.0 (Figure 4A) confirmed that all amino groups participated equally in charge neutralization. Apparently, irrespective of the F_A and DP of the chitosan, the same amounts of GlcN residues were bound to DNA. The higher positive surface charge of polyplexes formed by high DP chitosan with F_A < 0.002 may be qualitatively explained by the nonoptimal alignment of long and highly charged chains leading to the accumulation of the excess charge. Similarly as in the EtBr assay, polyplex formation at pH 7.4 strongly reflected the dramatic decrease of the protonation of the amino groups and thereby the low ability of the chitosans to neutralize the DNA phosphate. The weak binding of the oligomer DP_n 16 may again be interpreted in terms of an insufficient number of charges per chain. The relatively high affinity of the chitosan F_A 0.49 may be related to the higher solubility of this sample at pH 7.4.[27]

When the information given in Figures 1 and 4 is combined, the results emphasize that the EtBr displacement assay is not a direct measure of the binding affinity but rather an indication of interaction providing also information about the structure of the DNA−chitosan complexes. Apparently, the zeta potential measurement given in Figure 4A revealed that F_A 0.49 bound to DNA in a similar amount as $F_A <$ 0.002, but it expelled much less EtBr (Figure 1A). Similar limited correlation between the EtBr assay and other assays measuring binding affinity has also been reported elsewhere.[36]

There is increasing evidence that a subtle balance between the stability and dissociation of polyplexes is one of the key determinants of their transfection efficiency.[15,19,40,41] It has been shown here and elsewhere that the stability of polyplexes increases with increasing DP,[15,19,29,41,53] and this relationship is particularly pronounced for short oligomers.[41] As shown in Figures 6 and 7, in contrast to the high molecular weight chitosan, the oligomer-based complexes showed pH-triggered dissociation at pH > 7.3. Due to the low degree of ionization of the amino groups above this pH, the number of charges per oligomer chain drops below a minimum number of charges required for association, and the polyplexes rapidly dissociate. If not fractionated, the chitosan oligomer samples are highly polydisperse, containing chains with DP values both below and above the limiting number of charges. The stability of the polyplexes may thus be expected to reflect the detailed distribution of the chain length as confirmed by the observed increase in the stability of polyplexes with increased A/P and DP (Figure 7) and previously reported results.[19,29] Besides the chitosan characteristics such as DP and polydispersity, the formulation conditions such as A/P ratio, pH, ionic strength, and the transfection protocol will also affect the amount of chains possessing sufficient binding strength for interaction with DNA, and thereby the dissociation behavior of polyplexes. The relationship between the chain length distribution and the stability of polyplexes may be of relevance for interpretation of the reported data on the variable transfection efficiency of chitosan samples. Compared to the high molecular weight chitosan, the weaker association of oligomers with DNA enabled efficient release of DNA in transfection experiments and faster onset of gene expression.[18,19] Whereas no effect of molecular weight was observed for chitosans 30−190 kDa,[17] the significant differences in the transfection efficiency of polydisperse oligomers with DP_n 18, DP_n 25, and the fractionated oligomers[19] indicates the critical importance of the chain length and the chain length distribution in this range. While the shorter polycations may dissociate too easily to protect the DNA, the presence of long chains may result in excessively stable polyplexes with limited DNA release. The polyplexes with a proper balance between DNA protection and DNA release, i.e., with the optimal chain length distribution, will be the most efficient in gene delivery. As the stability of polyplexes will also vary with the actual physicochemical conditions during transfection experiment, the performance of a given chitosan oligomer may vary in vitro and in vivo as often observed.[19]

Finally, the increase of the hydrodynamic radii of the polyplexes with increasing F_A of chitosan (Figure 9), also reported elsewhere,[28] can possibly be related to the amount of chitosan bound within the polyplexes. As the number of charged residues of chitosan is inversely proportional to F_A, the actual amount of chitosan necessary to achieve a given A/P ratio increases with F_A. The increase in the size of the oligomer-based complexes, indicating proceeding aggregation at the present buffer condition, is a subject of ongoing studies concerned with the colloidal properties of DNA−chitosan complexes.

Conclusions

Our data demonstrate that, although all chitosans complex DNA, the properties of DNA−chitosan complexes depend strongly on the structure of chitosans. In case of high molecular weight chitosans with a high number of cationic residues, increasing the F_A was found to increase the size and reduce the compactness of the polyplexes formed. The strength of interaction of the chitosan oligomers with DNA was critically dependent on the number of cationic residues per chain. Accordingly, the stability of oligomer-based polyplexes depended strongly on DP and pH, affecting the number of protonized amino group. Varying the F_A, DP, and polydispersity of the chitosans thus provides a tool for controlling the properties of DNA−chitosan polyplexes. As different gene delivery applications and routes of administration may require vectors of different properties, different chitosans may be used to construct tailor-made gene delivery systems.

References and Notes

(1) Gebhart, C. L.; Kabanov, A. V. *J Bioact. Compat. Polym.* **2003**, *18*, 147−166.
(2) Anwer, K.; Rhee, B. G.; Mendiratta, S. K. *Crit. Rev. Ther. Drug* **2003**, *20*, 249−293.
(3) Vijayanathan, V.; Thomas, T.; Thomas, T. J. *Biochemistry* **2002**, *41*, 14085−14094.
(4) Arscott, P. G.; Li, A. Z.; Bloomfield, V. A. *Biopolymers* **1990**, *30*, 619−630.
(5) Bloomfield, V. A. *Biopolymers* **1997**, *44*, 269−282.
(6) Danielsen, S.; Varum, K. M.; Stokke, B. T. *Biomacromolecules* **2004**, *5*, 928−936.
(7) Tang, M. X.; Szoka, F. In *Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial*; Kabanov, A. V., Felgner, P. L., Seymour, L. W., Eds.; Wiley: Chichester, U.K., 1998.
(8) Maurstad, G.; Danielsen, S.; Stokke, B. T. *J. Phys. Chem. B* **2003**, *107*, 8172−8180.
(9) Akinc, A.; Anderson, D. G.; Lynn, D. M.; Langer, R. *Bioconjugate Chem.* **2003**, *14*, 979−988.
(10) Read, M. L.; Bremner, K. H.; Oupicky, D.; Green, N. K.; Searle, P. F.; Seymour, L. W. *J. Gene Med.* **2003**, *5*, 232−245.
(11) Anderson, D. G.; Akinc, A.; Hossain, N.; Langer, R. *Mol. Ther.* **2004**, *11*, 426−434.
(12) Putnam, D.; Gentry, C. A.; Pack, D. W.; Langer, R. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 1200−1205.
(13) Tang, M. X.; Szoka, F. C. *Gene Ther.* **1997**, *4*, 823−832.
(14) Jones, N. A.; Hill, I. R. C.; Stolnik, S.; Bignotti, F.; Davis, S. S.; Garnett, M. C. *Biochim. Biophys. Acta* **2000**, *1517*, 1−18.
(15) Schaffer, D. V.; Fidelman, N. A.; Dan, N.; Lauffenburger, D. A. *Biotechnol. Bioeng.* **2000**, *67*, 598−606.
(16) Godbey, W. T.; Wu, K. K.; Mikos, A. G. *J. Biomed. Mater. Res.* **1999**, *45*, 268−275.
(17) Koping-Hoggard, M.; Tubulekas, I.; Guan, H.; Edwards, K.; Nilsson, M.; Varum, K. M.; Artursson, P. *Gene Ther.* **2001**, *8*, 1108−1121.
(18) Köping-Höggård, M.; Mel'nikova, Y. S.; Vårum, K. M.; Lindman, B.; Artursson, P. *J. Gene Med.* **2003**, *5*, 130−141.

(19) Koping-Hoggard, M.; Varum, K. M.; Issa, M.; Danielsen, S.; Christensen, B. E.; Stokke, B. T.; Artursson, P. *Gene Ther.* **2004**, *11*, 1441−1452.
(20) Roy, K.; Mao, H.-Q.; Huang, S. K.; Leong, K. W. *Nat. Med.* **1999**, *5*, 387−391.
(21) MacLaughlin, F. C.; Mumper, R. J.; Wang, J. J.; Tagliaferri, J. M.; Gill, I.; Hinchcliffe, M.; Rolland, A. P. *J. Controlled Release* **1998**, *56*, 259−272.
(22) Kai, E.; Ochiya, T. *Pharm. Res.* **2004**, *21*, 838−843.
(23) Iqbal, M.; Lin, W.; Jabbal-Gill, I.; Davis, S. S.; Steward, M. W.; Illum, L. *Vaccine* **2003**, *21*, 1478−1485.
(24) Nordtveit, R. J.; Vårum, K. M.; Smidsrød, O. *Carbohydr. Polym.* **1996**, *29*, 163−167.
(25) Lee, M.; Nah, J. W.; Kwon, Y.; Koh, J. J.; Ko, K. S.; Kim, S. W. *Pharm. Res.* **2001**, *18*, 427−431.
(26) Corsi, K.; Chellat, F.; Yahia, L.; Fernandes, J. C. *Biomaterials* **2003**, *24*, 1255−1264.
(27) Vårum, K. M.; Smidsrød, O. In *Polysaccharides: Structural Diversity and Functional Versatility*; Dumitriu, S., Ed.; Marcel Dekker: New York, 2004; pp 625−642.
(28) Romoren, K.; Pedersen, S.; Smistad, G.; Evensen, O.; Thu, B. J. *Int. J. Pharm.* **2003**, *261*, 115−127.
(29) Danielsen, S.; Strand, S. P.; Davies, C. d. L.; Stokke, B. T. *Biochim. Biophys. Acta* **2005**, *1721*, 44−54.
(30) Schipper, N. G. M.; Vårum, K. M.; Artursson, P. *Pharm. Res.* **1996**, *13*, 1686−1692.
(31) Anthonsen, M. W.; Vårum, K. M.; Smidsrød, O. *Carbohydr. Polym.* **1993**, *22*, 193−199.
(32) Izumrudov, V. A.; Wahlund, P. O.; Gustavsson, P. E.; Larsson, P. O.; Galaev, I. Y. *Langmuir* **2003**, *19*, 4733−4739.
(33) Lepecq, J. B.; Paoletti, C. *J. Mol. Biol.* **1967**, *27*, 87.
(34) Izumrudov, V. A.; Zhiryakova, M. V.; Kudaibergenov, S. E. *Biopolymers* **1999**, *52*, 94−108.
(35) Parker, A. L.; Oupicky, D.; Dash, P. R.; Seymour, L. W. *Anal. Biochem.* **2002**, *302*, 75−80.
(36) Basu, H. S.; Schwietert, H. C. A.; Feuerstein, B. G.; Marton, L. J. *Biochem. J.* **1990**, *269*, 329−334.
(37) Hsieh, H. P.; Muller, J. G.; Burrows, C. J. *Bioorg. Med. Chem.* **1995**, *3*, 823−838.
(38) Izumrudov, V. A.; Zhiryakova, M. V.; Goulko, A. A. *Langmuir* **2002**, *18*, 10348−10356.
(39) Geall, A. J.; Blagbrough, I. S. *J. Pharm. Biomed. Anal.* **2000**, *22*, 849−859.
(40) Ruponen, M.; Yla-Herttuala, S.; Urtti, A. *Biochim. Biophys. Acta* **1999**, *1415*, 331−341.
(41) Zelikin, A. N.; Putnam, D.; Shastri, P.; Langer, R.; Izumrudov, V. A. *Bioconjugate Chem.* **2002**, *13*, 548−553.
(42) Plank, C.; Tang, M. X.; Wolfe, A. R.; Szoka, F. C. *Hum. Gene Ther.* **1999**, *10*, 319−332.
(43) Wiethoff, C. M.; Gill, M. L.; Koe, G. S.; Koe, J. G.; Middaugh, C. R. *J. Pharm. Sci.* **2003**, *92*, 1272−1285.
(44) Vijayanathan, V.; Lyall, J.; Thomas, T.; Shirahata, A.; Thomas, T. J. *Biomacromolecules* **2005**, *6*, 1097−1103.
(45) Mannisto, M.; Vanderkerken, S.; Toncheva, V.; Elomaa, M.; Ruponen, M.; Schacht, E.; Urtti, A. *J. Controlled Release* **2002**, *83*, 169−182.
(46) Strand, S. P.; Tømmeraas, K.; Vårum, K. M.; Østgaard, K. *Biomacromolecules* **2001**, *2*, 1310−1314.
(47) Mascotti, D. P.; Lohman, T. M. *Biochemistry* **1992**, *31*, 8932−8946.
(48) Stigter, D.; Dill, K. A. *Biophys. J.* **1996**, *71*, 2064−2074.
(49) Kriz, J.; Dautzenberg, H. *J. Phys. Chem. A* **2001**, *105*, 3846−3854.
(50) Rmaile, H. H.; Schlenoff, J. B. *Langmuir* **2002**, *18*, 8263−8265.
(51) Kabanov, V. A.; Zhiryakova, M. V.; Kargov, S. I.; Zezin, A. B.; Izumrudov, V. A. *Dokl. Akad. Nauk.* **1993**, *332*, 722−726.
(52) Nordmeier, E. *J. Phys. Chem.* **1992**, *96*, 6045−6055.
(53) Papisov, I. M.; Litmanovich, A. A. *Adv. Polym. Sci.* **1982**, *90*, 140−179.

BM0503726