Long-Term Stability of Chitosan-Based Polyplexes

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Purpose. There is a lack of information about the long-term stability of chitosan-based polyplexes although a large amount is known as regards transfection efficiency and physicochemical characteristics. The aim of this work is to study the transfection efficiency and physicochemical properties of chitosan-based polyplexes over time when stored at different temperatures in an acetate-buffer at pH 5.5.
Methods. Aqueous samples of chitosan-based polyplexes were aged at 4°C, 25°C, and 45°C for up to 1 year. Samples were taken at predetermined time-points and evaluated for *in vitro* transfection efficiency and physiochemical properties (particle size, zeta potential).
Results. One year of storage at 4°C did not result in any major changes in the properties of the polyplexes. At 25°C there were minor changes in the physicochemical characteristics of the polyplexes, and the *in vitro* transfection efficiency was reduced at 1 year of storage. Storage at 45°C altered both the *in vitro* transfection efficiency and the physicochemical properties of the polyplexes after a short time.
Conclusions. The biological and physicochemical stability of the chitosan-based polyplexes are maintained for 1 year of storage in acetate-buffer at 4°C. The changes in the polyplex characteristics at elevated temperatures may be explained by degradation of both plasmid and chitosan.

KEY WORDS: chitosan; degradation; DNA; long-term stability; polyplex.

INTRODUCTION

Chitosan is a biodegradable polysaccharide composed of the subunits D-glucosamine and *N*-acetyl-D-glucosamine, linked together by β(1,4)-glycosidic bonds. The polymer is attractive for a variety of biomedical and pharmaceutical applications due to its advantageous biocompatibility and biodegradability profile (1–3). Chitosan was first described as a delivery system for plasmids by Mumper and co-workers (4). Since then, effort has been made to explore the biological and physicochemical characteristics of chitosan-based polyplexes (5–9). Only a few studies addressing the stability of chitosan polyplexes during long-term storage has previously been published. This is surprising, as pharmaceutical products need to remain stable for extended periods under commercial and practical conditions. Leong and co-workers demonstrated that chitosan-based polyplexes stored in phosphate-buffered saline or serum dissociated after a few hours, while cross-binding with transferrin increased the stability (10). Chitosan-based polyplexes conjugated with polyethylene glycol (PEG), maintained the transfection potency for at least one month of storage in 50% sucrose or in a freeze-dried condition at 4°C (8,11).

Both the drug (DNA) and the carrier (chitosan) may be degraded when the polyplexes are stored. Naked plasmids exist in three major tertiary structures: i) supercoiled representing the most compact stable isomer, ii) open circle occurring when there are breaks in the phosphate backbone of the double-stranded molecule, and iii) linear, indicating breaks at a single site in both strands of the circular plasmid (12,13). Degradation of DNA in aqueous environment are mainly caused by hydrolysis (depurination and β-elimination reactions) and oxidation (via free radicals) (13,14) and a degradation of the plasmid may be seen as a change in the tertiary structure. Degradation pathways for chitosan involve acid hydrolysis or, to a lesser extent, alkaline degradation (15).

From an industrial/production point of view, as few steps as possible in the manufacturing process is desirable and a possibility to avoid complicated and expensive purification and lyophilization steps would be advantageous. Degradation of chitosan and/or plasmid may alter the properties of the polyplexes. The aim of this study was to follow the characteristics (*in vitro* transfection efficiency and physicochemical properties such as size, zeta potential, and degree of association) of the chitosan-based polyplexes over time at different temperatures when the complexes are stored in the solution in which they were made (i.e., 25 mM sodium acetate buffer, pH 5.5). The samples were stored at 4°C, 25°C, and 45°C for a period of up to 1 year. The change in characteristics of chitosan- and plasmid-solutions at these three temperatures over time and the effects of these changes on the ability to form complexes and the properties of these were assessed by complexation of aged chitosan and DNA with fresh DNA and chitosan, respectively.

MATERIALS AND METHODS

Materials

The plasmid construct pcDNA3-luc was obtained from GeneCare (Lyngby, Denmark). In this construct, the reporter gene encoding firefly luciferase was inserted into the polylinker of pcDNA3 (Invitrogen, Groningen, The Netherlands) and the expression of the luciferase gene is under the control of a cytamegalovirus (CMV) promoter. The endotoxin content (72 EU/mg) and DNA homogeneity (>95% covalently closed circular DNA) were specified from the manufacturer. The purity and integrity of plasmid DNA was assessed by UV-spectroscopy (A_{260}/A_{280} ratio) and by agarose gel electrophoresis. The concentration of the plasmid was determined by UV-spectroscopy at 260 nm (1 OD = 50 μg/ml) (16). Chitosans with different number–average molecular weights (M_n) and degree of actetylation (F_A), SeaCure 113 CL (M_n = 6.6 kDa, F_A = 0.17, SC113), SeaCure 211 CL (M_n 90 kDa, F_A = 0.32, SC211), SeaCure 214 CL (M_n 160 kDa, F_A = 0.15, SC214), and SeaCure 312 CL (M_n 160 kDa, F_A = 0.25, SC312) were kindly provided by Pronova Biopolymers (Drammen, Norway). Fetal bovine serum (FBS), gentamicin, L-glutamine, and Earle's Minimum Essential Medium (EMEM) containing 25 mM Hepes were purchased from BioWhittaker (Verviers, Belgium). Poly-L-aspartic acid (MW 15,000–50,000) was obtained from Sigma-Aldrich Chemicals

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(St. Louis, MO, USA). The luciferase assay was obtained from Promega (Madison, WI, USA), the PicoGreen assay from Molecular Probes (Leiden, The Netherlands), and the protein assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other agents used were of analytical grade.

Preparation of Chitosan Solutions and Polyplex

Chitosan samples were dialyzed against distilled, deionized water to remove excess chloride ions. The dialyzed chitosan solutions were filtered (0.8 μm, Millipore AA filter), freeze-dried and characterized as described earlier (17). The dialyzed and freeze-dried chitosans were dissolved overnight in sterile 25 mM sodium acetate buffer (pH 5.5). The chitosan solutions were diluted and added to an equal volume of plasmid solution (50 μg/ml), to give polyplexes with a charge ratio of 2. The concentrations of the chitosan solutions were 72 μg/ml, 89 μg/ml, 67 μg/ml, and 82 μg/ml for SC113, SC211, SC214, and SC312, respectively. The polyplexes were further vortexed for 20 s and left to settle for 30 min before use. The charge ratio was defined according to recent recommendations (16) as the ratio between the maximum number of protonable primary amines in the chitosans and the number of negative phosphates in the plasmid DNA.

Ageing of Polyplexes, Plasmid DNA, and Chitosan

Aqueous samples of polyplexes, plasmid DNA, and chitosan, 3 parallels of each, were aged at 4°C, 25°C, and 45°C for up to 1 year. Samples were taken at predetermined timepoints and evaluated for *in vitro* transfection efficiency and physiochemical properties as described below. The aged chitosan and plasmid-solutions were complexed with fresh solutions of plasmid and SC312, respectively, before analysis.

In Vitro Transfection Efficiency

Epithelioma papulosum cyprini (EPC) cells (ECACC no. 93120820), a cell line from carp (18), were grown in EMEM/Hepes supplemented with 10% FBS, 3.5 mM L-glutamine, and 50 μg/ml gentamicin at 25°C. The cells were seeded at a density of 50,000 cells per well in a 96-well plate. After 24 h, the polyplex formulations were added to the wells together with serum-free medium and kept on the cells for 24 h. The cells were analyzed for luciferase activity after an additional 2 days of incubation. The complete medium was then removed and a lysating buffer was added (25 mM Tris-phosphate pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% glycerol, 1% Triton X-100). The supernatants were analyzed for luciferase in a Berthold EG&G luminometer according to the manufacturers instructions. The relative light units (RLU) were normalized to the protein concentration as determined by the BioRad assay.

Physicochemical Characterization

Particle Size Measurements

The size of the particles was determined by photon correlation spectroscopy at 25°C using a Coulter N4 MD (Hialeah, FL, USA) at a 90° angle. The refractive index and viscosity of pure water were used as calculation parameters and each sample was measured in triplicate using the unimodal model for size distribution.

Zeta Potential

The zeta potential was measured by micro-electrophoresis at 25°C (Zetasizer 2000 HS, Malvern Instruments, Great Britain). The viscosity and dielectric constant of pure water were used as calculation parameters. All samples were diluted in a 25 mM sodium acetate buffer at pH 5.5, to a chitosan concentration of 0.5 μg/ml.

Association Efficiencies and Remaining dsDNA in the Polyplexes

After storage, the remaining dsDNA in the polyplexes was determined with the PicoGreen assay. Briefly, 5 μl poly-L-aspartic acid (5 mg/ml) was added to 100 μl aliquots of polyplexes (diluted to a DNA concentration of 1 μg/ml in 20 mM sodium acetate, pH 6) to facilitate the release of DNA from the polyplexes. After 5 min of incubation, 100 μl PicoGreen reagent (diluted 1:200 in 20 mM sodium acetate, pH 6) was added. After 2–5 min of incubation, the fluorescence of the mixture was measured (Ex. 480 nm, Em. 520 nm, Luminiscence Spectrophotometer LS50B, PerkinElmer, Bodenseewerk, Germany). To determine the association efficiency between the aged chitosan solutions and the fresh DNA solutions, the poly-L-aspartic acid step was omitted.

Kinematic Viscosity Measurements

The change in kinematic viscosity of the chitosan solutions over time at 5 weeks, 32 weeks, and 52 weeks, respectively, and viscosity was determined with a Micro-Ostwald viscosimeter (Schott-Geräte, Hofheim, Germany) at 20°C. The concentration of the chitosan solutions was 10 mg/ml, 1.5 mg/ml, 1.0 mg/ml, and 1.0 mg/ml for SC113, SC214, SC214, and SC312 respectively. The chitosans were dissolved in 25 mM sodium acetate buffer at pH5.5 and the kinematic viscosities for the chitosan solutions at the start of the experiment were 1.66 mm^2/s, 1.68 mm^2/s, 1.92 mm^2/s, and 2.00 mm^2/s, respectively.

Gel Electrophoresis

Fresh and aged plasmid solutions were applied in the wells of an agarose gel (0.8% in Tris-Acetate-EDTA (TAE)-buffer) and with ethidium bromide in the gel. After electrophoresis (80 V, 2.5 h), the bands in the gel were visualized by ultraviolet illumination.

RESULTS

In Vitro Transfection Efficiency

The relative transfection efficiency of aged chitosan-based polyplexes is shown in Fig. 1 (for absolute numbers of the transfection efficiency of freshly prepared polyplexes, see Table I). The polyplexes almost fully preserved their transfection potential after ageing at 4°C, which also was the case if the plasmid was aged unformulated (i.e., naked DNA). At 25°C, the transfection efficiency decreased rapidly for the SC113 and SC211-based polyplexes. Polyplexes made of SC214 and SC312, showed high transfection efficiency even

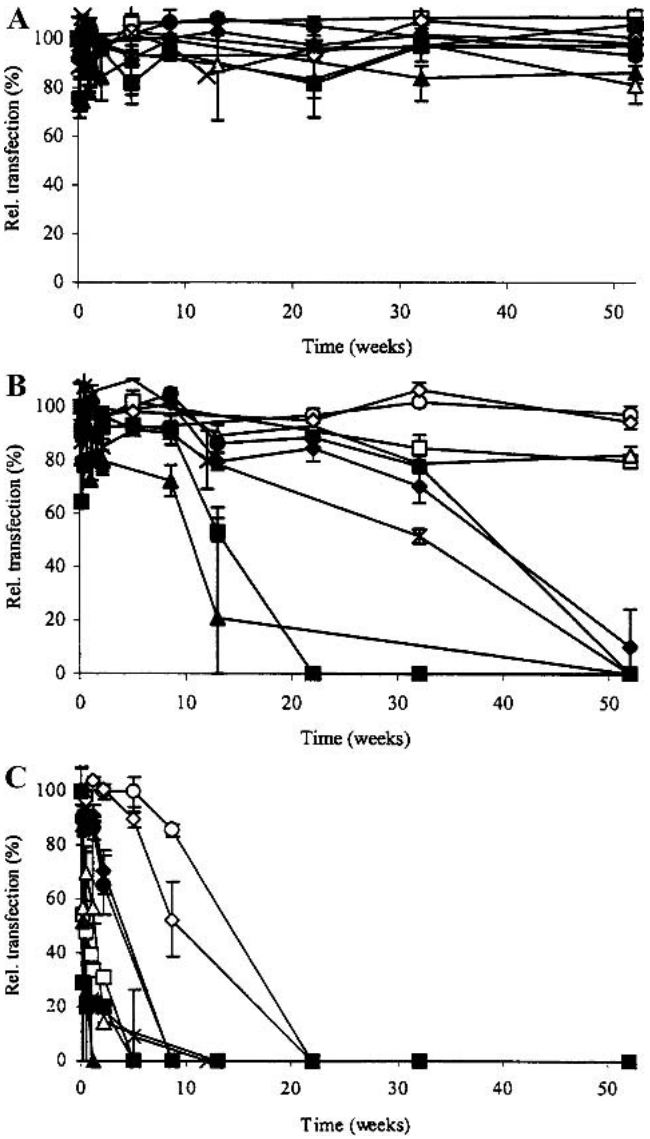

Fig. 1. Relative transfection efficiency of aged chitosan/plasmid complexes at 4°C (A), 25°C (B), and 45°C (C). Four different types of chitosan were used throughout the study, SC113 (■ chitosan aged in polyplexes/□ chitosan aged in solution), SC211 (▲ chitosan aged in polyplexes/Δ chitosan aged in solution), SC214 (● chitosan aged in polyplexes/○ chitosan aged in solution), and SC312 (♦ chitosan aged in polyplexes/◇ chitosan aged in solution). The relative transfection efficiency of plasmid DNA aged in water and complexed with fresh SC312 is also included in the figures (x). The results are expressed as mean values ± max/min values of 3 parallels.

after 32 weeks of storage (>70%), however, low levels (SC312) or no (SC214) luciferase was detected following transfection with polyplexes stored for 52 weeks. The transfection efficiency obtained with aged plasmids complexed with fresh chitosan SC312 decreased with 50% when stored for 32 weeks at 25°C. For all formulations, the transfection efficiencies of formulations stored at 45°C decreased rapidly, and no luciferase activity could be detected following 10 weeks of storage. Chitosan solutions aged separately and complexed with fresh DNA prior to transfection showed a similar pattern of reduced transfection efficiency when stored at 45°C, however, for the other temperatures, the transfection efficiency remained high for all types of chitosan.

TABLE I. The *in vitro* Transfection Efficiency, Size, Zeta Potential, and Association Efficiency of the Freshly Prepared Polyplexes Used for the Aging Study

Chitosan	Transfection efficiency (log RLU/mg protein)	Size (nm)	Zeta potential (mV)	Association efficiency (%)
SC113	7.5 ± 0.61	148 ± 16	15.9 ± 2.6	97.4 ± 0.10
SC211	6.3 ± 0.57	265 ± 24	21.3 ± 1.8	94.3 ± 0.65
SC214	7.5 ± 0.13	223 ± 18	23.5 ± 4.5	97.2 ± 0.46
SC312	7.9 ± 0.20	231 ± 19	22.4 ± 1.9	96.8 ± 0.39

The results are expressed as means ± SD (n = 3 to 9).

Physicochemical Characterization

Particle Size Measurements

Dynamic light scattering measurements revealed that the size of the polyplexes, regardless of type of chitosan, remained stable for 1 year during storage at 4°C (Fig. 2; Table I presents the size of freshly prepared polyplexes). After storage at 25°C, changes in the size were observed, and the changes were dependent on the type of chitosan used in the preparation of the polyplexes. The size of the SC113-based polyplexes increased with 40% over 1 year storage, while the size decreased with 14%, 5%, and 3% for SC211, SC214, and SC312, respectively. A massive increase in size of the polyplexes was observed after storage for a few weeks at 45°C, independent of chitosan used. Aging of the DNA did not affect the size of fresh complexes made of the aged DNA and fresh chitosan SC312. Fresh complexes made with aged chitosan solutions complexed with fresh DNA showed a similar shift in size as observed for the aged polyplexes.

Zeta Potential Measurements

The zeta potential of all the different polyplexes stored at 4° and 25°C decreased with around 5 mV over the course of the experiment (Table II). The zeta potential of the polyplexes stored at 45°C changed dramatically, and the net charge of the polyplexes shifted from positive to neutral or even a negative charge within 22 weeks of storage. The zeta potential of polyplexes made of aged DNA and fresh chitosan SC312 remained constant. In contrast, the zeta potential of aged chitosans complexed with fresh DNA changed similarly to the aged polyplexes

Remaining dsDNA in the Polyplexes and Degree of Association

The remaining dsDNA in aged SC214-based polyplexes as assessed by the PicoGreen assay is shown in Fig. 3. The temperature was of importance and dsDNA was better preserved at 4°C than the other temperatures. The curve profiles for the other types of chitosan were similar to the SC214 curve. Yet, the remaining dsDNA in the chitosans with a low F_A, that is, SC113 and SC214 (49% and 55%, after 1 year storage at 4°C) was lower than using chitosans with a higher F_A (80% and 73% for SC211 and SC314, respectively). As much as 97% dsDNA was maintained after storage of the naked DNA solution at 4°C for 1 year. At 25°C, the SC113-based polyplexes retained less dsDNA (17%) than the other

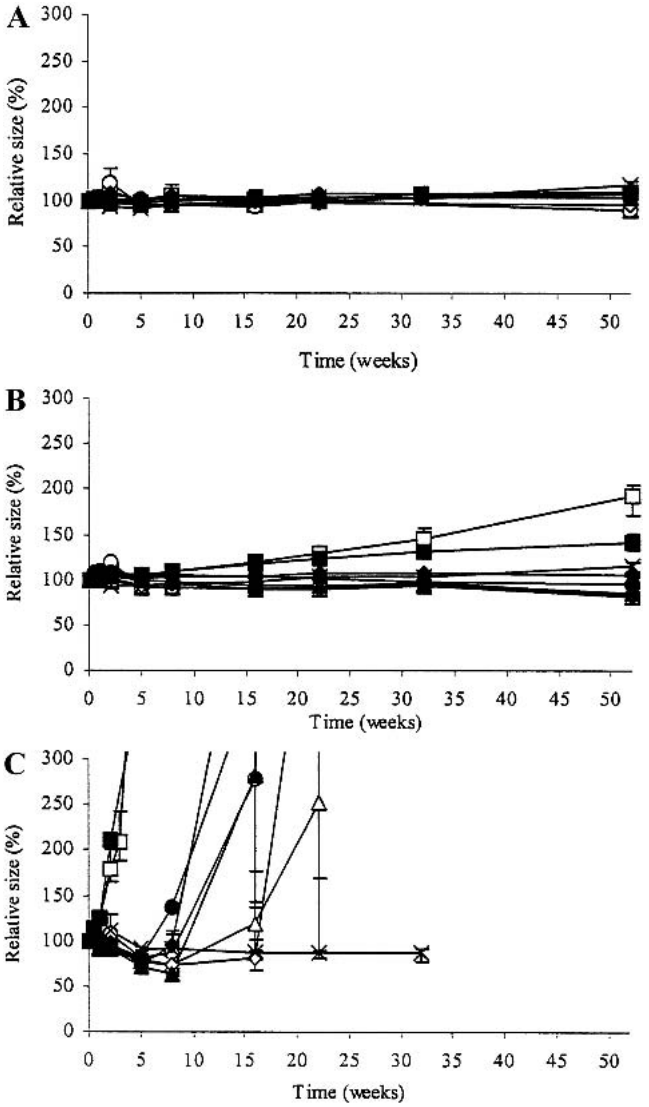

Fig. 2. Relative size of aged chitosan/plasmid complexes at 4°C (A), 25°C (B), and 45°C (C). Four different types of chitosan were used throughout the study, SC113 (■ chitosan aged in polyplexes/□ chitosan aged in solution), SC211 (▲ chitosan aged in polyplexes/Δ chitosan aged in solution), SC214 (● chitosan aged in polyplexes/○ chitosan aged in solution), and SC312 (♦ chitosan aged in polyplexes/◇ chitosan aged in solution). The relative size of plasmid DNA aged in water and complexed with fresh SC312 is also included in the figures (52 weeks not determined) (x). The results are expressed as mean values ± max/min values of 3 parallels.

formulations (37–50%) when stored for 1 year. After 19 weeks of storage, no dsDNA was detected in the polyplexes kept at 45°C.

The PicoGreen assay was also used to determine the degree of association when fresh DNA was complexed with aged chitosans. Of the added DNA, 88–94% associated to chitosans aged at 4°C and 25°C for 1 year. At 45°C, the association efficiencies were high for all chitosans but SC113 (data not shown).

Kinematic Viscosity of Aged Chitosan Solutions

The changes in relative kinematic viscosity of chitosan solutions aged at 4°C, 25°C and 45°C is shown in Table III, a typical curve is shown in Fig. 4. Similar to earlier observations, also the change in kinematic viscosity was dependent on the temperature. The reduction in aged SC211, SC214, and SC312 viscosities were similar after storage for 1 year (30–38%, 61–66% and 97–99% at 4°C, 25°C, and 45°C, respectively). The reduction in the kinematic viscosity of the aged SC113 solutions was (9%, 25%, and 76%).

Gel Electrophoresis

Freshly prepared DNA solutions gave rise to the appearance of one band on the agarose gel (Fig. 4, lane 2). Ageing resulted in the appearance of more bands (lanes 3, 4, 6, 7, and 9), indicating the presence of other tertiary structures in the plasmid solutions. No bands were detected in lanes 5, 8, 10, and 11, plasmid samples stored at 45°C (2, 9, and 32 weeks) and at 25°C for 32 weeks, only a smear of low molecular plasmid fragments was observed.

DISCUSSION

This study demonstrates that the *in vitro* transfection efficiency and physicochemical properties of aqueous dispersions of chitosan-based polyplexes are preserved over a 1-year storage period when kept at 4°C. The changes in the physicochemical characteristics of the polyplexes during storage at 25°C for 1 year are minor, however, the *in vitro* transfection efficiency was dramatically reduced. Storage at 45°C altered both the *in vitro* transfection efficiency and physicochemical properties of the polyplexes after a short time. A possible reason for the changes in the polyplex characteristics is degradation of both plasmid DNA and chitosan.

With regard to stability, DNA has distinct advantages over protein-based pharmaceuticals, which are susceptible to a loss in biologic activity caused by small changes in the tertiary or quaternary structure (13). Polyplexes kept at 4°C retained their transfection efficiency during 1 year of storage. This is in accordance with other long-term storage studies of DNA complexes (12,19) and it is even suggested that plasmid DNA will remain stable for 2 years at 5°C assuming that depurination and β-elimination are the only degrading mechanisms occurring (13). Chitosan-based polyplexes stored in a freeze-dried condition at 4°C also retain their properties when subjected for long-term storage (8,11). Also for other gene delivery systems, for example, lipid-based gene delivery systems, the *in vitro* transfection efficiency showed that such formulations were stable for at least 90 days when stored at 4°C (20) or for 1 year when stored at –20°C (21).

In concordance with ageing of poly((2-dimethylamino) ethyl methacrylate)-based polyplexes (19), the transfection efficiency was not retained for the chitosan-based polyplexes when stored at temperatures ≥25°C. The decrease in transfection efficiency may be a sign of degradation of the plasmid DNA. The degradation of chitosan, however, may not be detected as a decrease in transfection efficiency as long as the chitosan chains remain sufficiently long to form stable complexes with plasmid DNA. Already mentioned, plasmid DNA exists in the three major isoforms: supercoiled, nicked/open, and linear (13). In our studies, the gel electrophoresis of aged naked DNA showed a gradual transition from supercoiled to open and linear isoforms of the DNA. For DNA in polyplexes, the PicoGreen assay showed a gradual transition from

TABLE II. The Zeta Potential (mV) of the Aged Chitosan-Based Polyplexes

	Zeta potential (mV)								
	4°C			25°C			45°C		
Chitosan/DNA	0 w	22 w	52 w	0 w	22 w	52 w	0 w	22 w	52 w
SC113									
PP	15.9 ± 2.6	17.8 ± 1.1	11.7 ± 1.9	15.9 ± 2.6	17.5 ± 0.9	14.8 ± 2.4	15.9 ± 2.6	–6.1 ± 0.7	ND
Sol	15.9 ± 2.6	19.7 ± 3.0	12.1 ± 1.3	15.9 ± 2.6	16.1 ± 2.2	15.2 ± 1.4	15.9 ± 2.6	–27.1 ± 0.3	ND
SC211									
PP	21.3 ± 1.8	19.6 ± 0.5	14.3 ± 3.4	21.3 ± 1.8	19.3 ± 0.7	13.7 ± 2.9	21.3 ± 1.8	1.6 ± 1.8	ND
Sol	21.3 ± 1.8	19.4 ± 0.9	10.9 ± 3.5	21.3 ± 1.8	18.3 ± 1.8	10.3 ± 1.8	21.3 ± 1.8	0.8 ± 4.8	ND
SC214									
PP	23.5 ± 4.5	22.9 ± 1.3	16.7 ± 3.4	23.5 ± 4.5	21.7 ± 0.2	17.9 ± 0.6	23.5 ± 4.5	–0.5 ± 0.3	ND
Sol	23.5 ± 4.5	22.1 ± 0.7	13.6 ± 3.0	23.5 ± 4.5	22.2 ± 0.3	14.3 ± 3.4	23.5 ± 4.5	1.6 ± 0.8	ND
SC312									
PP	22.4 ± 1.9	23.3 ± 1.3	17.5 ± 2.8	22.4 ± 1.9	21.3 ± 1.3	17.9 ± 3.2	22.4 ± 1.9	–4.1 ± 5.7	ND
Sol	22.4 ± 1.9	22.7 ± 1.1	18.5 ± 1.4	22.4 ± 1.9	23.1 ± 0.8	14.3 ± 3.1	22.4 ± 1.9	8.9 ± 3.43	ND
DNA									
Sol	22.4 ± 1.9	23.5 ± 1.3	23.5 ± 1.7	22.4 ± 1.9	22.9 ± 1.0	22.9 ± 0.3	22.4 ± 1.9	22.1 ± 1	ND

w, weeks; ND, not determined; PP, chitosan aged in polyplexes; sol, chitosan/DNA aged in solution.
The results are expressed as means ± SD (n = 3).

dsDNA to ssDNA during storage, indicating that plasmid DNA is not completely protected from degradation under the present storage conditions. The major degradation pathway of DNA, the two-step process of de-purination and β-elimination, resulting in cleaving of the phosphodiester backbone in the molecule (22) may therefore be a likely cause of reduced transfection efficiency at elevated temperatures. Other degradation reactions of DNA may involve the free radicals superoxide, hydrogen peroxide and hydroxyl radicals (13). Furthermore, care must be taken when handling naked DNA solutions as nucleases from the skin easily degrade the plasmids. Although such enzymes normally are not present at *in vitro* storage conditions, traces may influence the stability.

Despite the fact that a fraction of the aged DNA was converted from supercoiled to open and linear isoforms (gel retardation assays) and converted from dsDNA to ssDNA isoform (as measured by the PicoGreen assay) following ≥32 weeks of storage at 4°C, the samples did not show a reduction in the level of biologic activity (i.e., the transfection efficiency). It is previously shown that a change in the chemical structure of plasmid DNA not necessarily is detected as a change in the biologic activity (12). In order to better compare biologic and analytical assays it is necessary to develop quantitative measures of structural stability that do not rely on dye intercalation, as different isoforms of DNA will also produce different levels of staining with for instance the indicator dye ethidium bromide (12).

The physicochemical properties of the polyplexes were maintained when stored for a year at 4°C, but changed rather rapidly at 45°C. Polyplexes prepared of aged chitosan solutions and fresh plasmid solutions exhibited similar characteristics like the aged polyplexes. In contrast, ageing of naked DNA before complexation with fresh SC312 did not result in detectable changes in the physicochemical characteristics of the polyplexes. It is therefore likely that the changes in the physicochemical properties reflect a degradation of chitosan in the complexes while a degradation of plasmid DNA not can be detected by such methods. Separate storage of chitosan solutions showed that the kinematic viscosity of all the chitosan types decreased when stored under the present conditions, most markedly at 45°C. A direct comparison between degradation of chitosans in solution and of chitosans complexed with plasmid DNA cannot be drawn. Koping-Hoggard and co-workers (7) have shown that incubation with the chi-

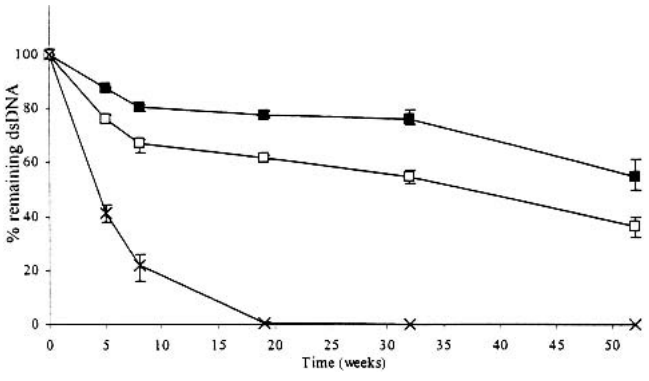

Fig. 3. Percentage remaining dsSNA in aged SC214 polyplexes at 4°C (■), 25°C (□), and 45°C (x). The results are expressed as mean values ± max/min values of 3 parallels.

TABLE III. The Percentage Reduction in Kinematic Viscosity of Aged Chitosan Solutions

	Reduction in kinematic viscosity (%)								
	4°C			25°C			45°C		
Chitosan	5 w	32 w	52 w	5 w	32 w	52 w	5 w	32 w	52 w
SC113	2	7	9	11	21	25	34	68	76
SC211	10	24	30	28	54	61	76	95	97
SC214	17	33	38	38	59	64	81	99	99
SC312	14	27	33	37	60	66	84	98	98

w, weeks.
The results are expressed as means of 2–3 parallels.

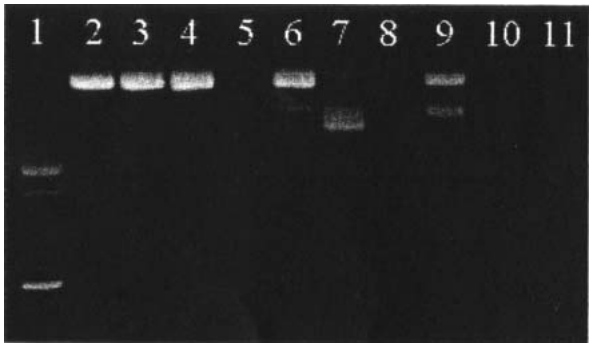

Fig. 4. Gel electrophoresis of naked DNA solutions stored at different temperatures: Molecular weight standard (lane 1), fresh DNA (lane 2), DNA stored for 2 weeks at 4°C (lane 3), 25°C (lane 4), and 45°C (lane 5), DNA stored for 9 weeks at 4°C (lane 6), 25°C (lane 7), and 45°C (lane 8), and DNA stored for 32 weeks at 4°C (lane 9), 25°C (lane 10), and 45°C (lane 11).

tosan-degrading enzyme chitosanase released all plasmid DNA from polyplexes. As chitosan is degraded under the storage condition used in our studies, the possibility that degradation of chitosan may take place in the polyplex as well should not be disregarded. Data from the zeta potential measurements supports this possibility (Table II): The zeta potential of aged chitosan solutions complexed with fresh DNA change similarly to the zeta potential of aged polyplexes (where chitosan is aged in the polyplex).

In our studies, the transfection efficiencies of the polyplexes made from the low molecular weight chitosans (SC113 and SC211) decreased most markedly upon storage. The degradation of DNA was also most pronounced in the SC113 polyplexes, and the association efficiency using aged SC113 for complex formation was poor. In addition the increase in size during storage was dependent on the initial molecular weight of the chitosans and the size of the SC113 polyplexes increased most during the 1-year storage. Köping-Höggård and co-workers demonstrated recently that chitosan oligomers (6-14-mers) with a molecular weight $\leq$2.8 kDa were not efficient as gene delivery systems (23). The gene transfection efficiencies of chitosan-DNA complexes are related to the physical shape and stability of the complexes. The complexes have various physical shapes depending on the experimental conditions, and the fraction of complexes with the favorable nonaggregated, globular structure increase with increasing chain length of chitosan oligomer, increasing charge ratio and reduction in pH. This is explained by the effects these parameters have on the entropic gain of globule formation (the cooperative effect). Oligos with 14 monomer units (14-mer, ~2.8 kD) and less did not form stable complexes and were inefficient with respect to gene expression. The 24-mer (~4.7 kD), however, formed stable complexes that gave high level of gene expression comparable to high molecular weight chitosan *in vitro* and *in vivo*. Although changes in physical shape and stability of polyplexes upon storage have not yet been explored, the theory of minimum chain length and cooperativity may be used to explain the observations in our study. Using low molecular weight chitosan, unfavorable chain lengths leading to destabilization of the complex are more rapidly obtained. Thus, polyplexes made from SC113 with a molecular weight of 6.6 kDa transfection may be more sensitive to chitosan degradation than polyplexes made from chitosans with a higher molecular weight (SC214, $\bar{M}$n 160 kDa).

No microbial growth was detected in the aged polyplex samples, which may be attributed to the aseptic handling of the samples as well as the antimicrobial activity of chitosans (27,28). A yellow coloration was observed in the chitosan solutions stored at 45°C. Such coloration has previously been shown in chitosan solutions being exposed for a short time to dry heat $\geq$120°C. It is believed that the heat produced chromophores, which may be related to interchain cross-link formation involving the NH_2 groups (25). A yellow coloration is also observed in aged chitosan films. In the latter case, polymorphism of chitosan acetate crystals is the suggested explanation (29).

In our studies, the theoretical charge ratio of the complexes were kept constant at 2, as previous studies showed that the transfection efficiency of polyplex 2 was high for all 4 chitosans (9). It is not unlikely that the stability at higher temperatures could have been improved at other charge ratios as various types of chitosans form optimal complexes at different charge ratios (23,24). Furthermore, it has been shown that the thermal stability of DNA, as determined by differential scanning calorimetry, complexed with the cationic polymers polyethylenimine and poly-L-lysine resulted in a decreased stability of supercoiled DNA in the polyplexes when DNA was in charge excess. When the polymers were in charge excess, all polymers stabilized supercoiled DNA (30). Thus, charge ratios higher than 2, may improve the stability, but the exact optimal charge ratio must be determined for each type of chitosan.

The process of DNA and polymer mixing is a simple and straightforward two-step procedure. Accordingly, the characterization and manufacturing are easily controlled, and there is a potential for scaling up. The biological and physicochemical stability of the chitosan-based polyplexes are maintained for 1 year of storage at 4°C. It is however desirable to increase the stability also at higher temperatures. Possibly, small pH adjustments of the polyplex solutions and inclusion of radical scavengers or chelating agents in the formulation may increase stability at elevated temperatures.

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