Preparation and Characterization of Chitosan and Trimethyl-chitosan-modified Poly-(ε-caprolactone) Nanoparticles as DNA Carriers

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ABSTRACT

The purpose of this research was to prepare poly-(ε-caprolactone) (PCL) particles by an emulsion-diffusion-evaporation method using a blend of poly-(vinyl alcohol) and chitosan derivatives as stabilizers. The chitosan derivatives used were chitosan hydrochloride and trimethyl chitosans (TMC) with varying degrees of quaternization. Particle characteristics–size, zeta potential, surface morphology, cytotoxicity, and transfection efficiency–were investigated. The developed method yields PCL nanoparticles in the size range of 250 to 300 nm with a positive surface charge (2.5 to 6.8 mV). The cytotoxicity was found to be moderate and virtually independent of the stabilizers' concentration with the exception of the highly quaternized TMC (degree of substitution 66%) being significantly more toxic. In immobilization experiments with gel electrophoresis, it could be shown that these cationic nanoparticles (NP) form stable complexes with DNA at a NP:DNA ratio of 3:1. These nanoplexes showed a significantly higher transfection efficiency on COS-1 cells than naked DNA.

KEYWORDS: biocompatible, cytotoxicity, gene transfer, nanoparticles, trimethyl chitosan, AFM

INTRODUCTION

Nonviral gene transfer systems have become increasingly popular as an alternative to viral vectors.[1] While the latter ones offer a high transfection efficacy owing to highly specialized intrusion mechanisms, they also suffer from a certain risk of backwards mutation toward the infectious wild type. In contrast to this, artificial transfection agents are considered a much safer alternative; they can be administered repeatedly with a low risk for immune response, can be "tailored to measure" by chemical and immunological modifications and can be easily produced in large quantities. Because of these advantages they are enjoying an increasing interest in gene transfer research. Among the strategies that have been employed to condense DNA can be found dendrimers,[2,3] cationic peptides,[4,5] cationic polymers,[6-9] cationic lipids,[10-12] as well as liposomes.[13-18] Even if nonviral gene transfer systems have been much improved during the past decade, they still cannot compete with viral vectors in terms of transfection efficiency, so research activities continue in order to unite the advantages of both approaches. DNA could be transferred to the cells either by encapsulating in or by surrounding the particles[11,19–21] or by having the DNA complexed on the outer surface of particles.[22,23] However, one cannot ignore the possibility of damaging the active substance during the encapsulation process; above all there is no immediate availability of the DNA for the cells.[24] Investigations were performed to encapsulate DNA-cation-complexes into microspheres, but the preparation is complicated and yields microscaled particles.[25] Successful transfection in vitro and in vivo has been achieved with positively charged silica nanoparticles;[21,26] however, the fate of the inorganic carrier substance silica may remain a problem. In order to address this issue it was decided to develop nanoparticulate DNA carriers based on biodegradable polymers.[22,23] Poly-(ε-caprolactone) (PCL) was chosen because of its biocompatibility, lipophilicity (to support passive uptake processes), and cost-effectiveness compared with other polyesters such as poly-(lactic-co-glycolic acid) (PLGA).

Besides the common parameters for particle characterization (ie, size, zeta potential, and microscopy), the biocompatibility of the carriers was investigated by cytotoxicity studies, since some nonviral transfection agents (notably short linear poly-[ethylene imine], PEI) are characterized by a cytotoxicity, which limits their use.[27,28] Since nanoparticles were prepared using amphiphilic molecules or surfactants as excipients and stabilizers, possible effects on the cell membrane are an important parameter to investigate.

Finally, the ability of the nanoparticles to complex DNA and transfect cells was tested in COS-1 cells, since this

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parameter can be regarded as the pivotal point for their suitability as transfection agents.

MATERIALS AND METHODS

Materials

PCL with an average molecular weight of 10 000 Da was purchased from Fluka (Deisenhofen, Germany). The poly-(vinyl alcohol) (PVA) used was Mowiol 4-88 (88% hydrolysis) from Hoechst (Frankfurt/Main, Germany). Seacure CL 210 chitosan hydrochloride (CS, 83% deacetylated) was obtained from Pronova Biopolymer (Drammen, Norway). Trimethyl chitosan (TMC) with varied degrees of substitution (4%, 10%, 18%, 66%) were synthesized at Leiden University, The Netherlands. The COS-1 cells and the beta-galactosidase expression plasmid pCMVβ were obtained from ATCC (Manassas, VA). We further prepared the plasmid; it was transformed into *E. coli* DH5α and a Gigaprep from 2500 mL of an overnight culture was performed according to instructions provided by the supplier (Qiagen, Hilden, Germany). The lactate dehydrogenase (LDH), detection Kit, was obtained from Roche Applied Sciences (Penzberg, Germany, catalog no. 1644793) as was the WST-1 cell proliferation reagent (catalog no. 1644807). Cell culture media were obtained from Promocell (Heidelberg, Germany) and Sigma-Aldrich (Taufkirchen, Germany). All other chemicals were purchased from Sigma-Aldrich. All solvents were of high-performance liquid chromatography (HPLC) grade, and reverse osmosis treated water (Millipore, Schwalbach, Germany) was used.

Particle Preparation

Particles were prepared by an emulsion-diffusion-evaporation method.[22] A 100-mg amount of PCL was dissolved in 10 mL organic phase consisting of 9 mL ethyl acetate (EA) and 1 mL acetone (AC) for 1 hour under mild heating at 30°C (water bath) in a closed container. As for the aqueous phase, 100 mg PVA and 15 mg CS or TMC were stirred in 5 mL water for 2 hours at room temperature until a clear solution was obtained. The organic phase was passed through a 0.22-μm syringe filter to remove any undissolved solids and subsequently added drop wise to the aqueous phase under constant stirring. The resulting microemulsion was kept under constant agitation on magnetic stirrer at 1000 rpm for 1 hour and was subsequently homogenized with an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany) at 13 500 rpm for 10 minutes. This colloidal preparation was diluted to a volume of 50 mL by adding water drop wise under stirring conditions (1000 rpm, magnetic stirrer), which resulted in nanoprecipitation. In order to remove the organic solvent and to harden the nanospheres, the suspension was treated with a rotary evaporator at 50 mbar at 40°C (water bath) for 20 minutes. The finished nanoparticle suspension was stored at 4°C for further use. To determine the concentration of nanoparticles (weight per volume), 2 mL of this suspension was spun down in Eppendorf tubes at 20 000*g* for 30 minutes; the supernatant was removed and the pellet was allowed to dry under a nitrogen stream before weighing.

Determination of Particle Size and Zeta Potential

Nanoparticles and NP-DNA complexes were analyzed for their size and zeta potential with a Malvern Zetasizer 3000 photon correlation spectroscopy (PCS) system (Malvern Instruments, Southborough, UK). The nanoparticle and NP-DNA suspensions were diluted 100-fold with a 20-mM N-(2-hydroxyethyl)piperazine-N′-(2-ethane sulfonic acid) (HEPES) buffer to minimize particle interactions and multiple scattering and were adjusted to pH 7.4 to maintain a constant dispersion medium. For particle size determination, 3 mL diluted suspension was placed in a disposable polystyrene cuvette and measured at room temperature using a scattering angle of 90°. For zeta potential determination, samples were measured in a fixed-glass cell; and the instrument was calibrated with −50 mV latex standard. Samples were analyzed using Malvern PCS software. The signal intensity mean was used to calculate the mean particle diameter, and all measurements were performed in duplicate. DNA-modified particles were prepared by mixing equal parts of a particle suspension of 60 μg/mL with a 20 μg/mL DNA solution and incubating for 15 minutes minimum, thus keeping the 3:1 NP:DNA ratio described below. The product was diluted 1:100 with 20 mM HEPES buffer as described above prior to PCS measurements. Arithmetic mean and SD were calculated from 3 consecutive runs, and samples were analyzed using Malvern PCS software.

Gel Electrophoresis and Determination of Unbound DNA

NP-DNA complexes were prepared by mixing 25 μL of a serial dilution of NP in water with 25 μL DNA in 50 mM HEPES buffer. Plasmid DNA was kept at a constant concentration of 10 μg/mL throughout the experiment; the particle suspension varied from 100 μg/mL to 0 μg/mL. The particles were allowed to incubate and complex DNA for 15 minutes at room temperature. Ten microliters of this suspension was added to 2 μL of a loading buffer containing coomassie blue dye for monitoring. Ten microliters of this blend were electrophoresed in a 1% agarose gel, stained with 1% ethidium bromide for better visualization; the electrophoresis chamber (Bio-Rad, Munich, Germany) was set to 90 minutes and 90 V (ie, 10 V/cm). A control was done by electrophoresing a DNA "ladder" mix with DNA fragments of different molecular masses. Data

analysis was done by acquiring the images on a Geldoc 2000 gel documentation system with a UV transilluminator (Bio-Rad, Munich, Germany) and analyzing the images with Molecular Analyst 1.1 software (Bio-Rad).

Nanoparticle Characterization by Atomic Force Microscopy

The nanoparticle formulations were prepared as described above and diluted in demineralized water. After 1 hour, the particles or particle-DNA complexes were directly transferred onto a silicon chip by dipping into the nanoplex solution. Atomic force microscopy was performed on a Digital Nanoscope IV Bioscope (Veeco Instruments, Santa Barbara, CA) as described elsewhere.[21] The microscope was vibration damped. Commercial pyramidal Si_3N_4 tips (NCH-W, Veeco Instruments) on a cantilever with a length of 125 μm, a resonance frequency of ~220 kHz, and a nominal force constant of 36N/m were used. All measurements were performed in tapping mode to avoid damage of the sample surface. The scan speed was proportional to the scan size, and the scan frequency was between 0.5 and 1.5 Hz. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals being simultaneously recorded. The results were visualized either in height or in amplitude mode.

Cytotoxicity Studies

COS-1 cells were cultured in Dulbecco's modified eagle's medium (DMEM, ready-made liquid media from Promocell, Heidelberg, Germany) with 4.5 g/L glucose, supplemented with 1% nonessential amino acids (MEM-NEAA); pH was adjusted to 7.4. Subculture was performed by detaching the cells with trypsine-EDTA solution and splitting them at a 1:10 ratio. Double concentrated DMEM was prepared by using powdered media (Sigma-Aldrich) dissolved in half the required amount of water.

For cytotoxicity studies, COS-1 cells were seeded onto 96-well plates with a density of 10 000 cells per well and allowed to adhere and grow for 24 hours. Serial dilutions of the nanoparticle suspensions were prepared in DMEM in a concentration range from 2000 μg/mL to 31.25 μg/mL; the first dilution was prepared by mixing the original suspension (4 mg/mL) with double-concentrated DMEM. Cells were washed with phosphate-buffered saline (PBS) prior to addition of 100 μL of the nanoparticle suspensions. Negative control was performed by incubating cells with medium only, positive control by 0.1% Triton X-100 in medium. The samples were allowed to incubate for 4 hours at 37°C at 5% CO_2. After incubation, 50 μL of the supernatant was removed for determination of LDH release, 50 μL of LDH reagent mixture (prepared according to manufacturer's instructions) was added, and absorbance was read at 492 nm immediately after addition of reagent and after 60 minutes of incubation at room temperature under light protection. Cells were washed again with PBS and 100 μL fresh medium was added; then cells were kept in an incubator for another 48 hours to simulate the conditions in a transfection study. After this period, 10 μL of WST-1 proliferation agent was added and samples were analyzed using a UV/Visible photometric plate reader at 450 nm. Data were corrected for blank samples (no cells) and expressed in percentage of survival compared with a positive control.

To dissect the influence of the used excipients from a whole particle suspension, cytotoxicity studies were also performed with PVA and the various chitosan derivatives. A stock solution of PVA was prepared starting with the concentration present in the native particle suspension by dissolving PVA in water (1.43 mg/mL) and mixing with double-concentrated DMEM. A serial dilution of this was prepared in DMEM and added to the cell monolayers. Further treatment was performed according to the procedures mentioned above. Chitosan and trimethyl chitosan derivatives were equally investigated, starting with stock solutions of 428 μg/mL. Both excipients were applied in the same concentrations as in corresponding particle preparations.

Transfection Studies

To examine the actual suitability of the prepared nanoparticles for gene transfer, complexes of particles and the plasmid DNA were prepared and added to cell cultures. Cos-1 cells were cultured as described above and seeded at a density of 10 000 cells per well on 96-well plates, and cells were used for transfection after 24 hours of settling and adhesion. The DNA concentration was kept constant at 10 μg/mL; particles were used in 2 concentrations deemed optimal from binding and electrophoresis experiments (10 and 30 μg/mL, respectively). Complexes of nanoparticles and plasmid DNA were prepared by adding freshly prepared particle suspension to 2× concentrated DMEM to obtain the desired concentration. This suspension was mixed with an equal volume of a solution of DNA in 50 mM HEPES buffer and complexes were allowed to incubate and form for 15 minutes. Cells were washed with warm PBS prior to addition of the transfection agent. A solution of DNA in medium was used as a control. The transfection agent was allowed to incubate for 4 hours at 37°C. After incubation, the cells were washed once with PBS and fresh medium was added. Subsequently cells were kept for another 48 hours in an incubator to allow gene expression. Controls were performed by incubating cells with either naked DNA or DNA with a commercial transfection agent, PolyFect.

To assay the cells content of β-galactosidase, cells were lysed with a solution of 0.1% Triton X-100 in PBS

Table 1. Photon Correlation Spectroscopy Analysis of Trimethyl Chitosan-modified Nanoparticles*

Batch	Size [nm] Without DNA	Polydisp Index	Size [nm] With DNA	Polydisp Index	ζ pot [mV] Without DNA	ζ pot [mV] With DNA
Chitosan	280.9	0.10	298.8	0.07	+ 2.6 ± 0.5	−6.8 ± 0.4
TMC, 4%	302.5	0.13	514.6	0.52	+ 2.5 ± 1.2	n/d
TMC, 10%	279.0	0.09	293.2	0.08	+ 4.0 ± 0.2	−6.8 ± 0.5
TMC, 18%	261.5	0.03	352.1	0.15	+ 6.0 ± 0.9	−4.5 ± 0.3
TMC, 66%	245.0	0.04	268.6	0.10	+ 6.8 ± 1.2	−5.5 ± 0.6

*Polydisp index indicates polydispersity index; ζ pot, zeta potential; TMC, trimethyl chitosan; n/d, not determined. Size is given as diameter in nm; zeta potential is noted as mean ± SD.

(100 μL/well) for 30 minutes at 4°C. Fifty microliters of this cell lysate was mixed with an equal amount of reaction mixture, prepared from 5 mM 4-methyl-umbelliferyl-β-D-galactoside, 100 mM D-galactose and 2 mM $MgCl_2$ in PBS. After an incubation period of 4 hours at 37°C, the fluorescence was determined with a Cytofluor II microplate fluorescence reader at λ_{ex} = 360 nm and λ_{em} = 460 nm (PE Biosystems, Weiterstadt, Germany).

Statistical Analysis

Statistical analysis and determination of significance was performed with SigmaStat software (SPSS Inc., Chicago, IL). Data sets were tested using Student *t* test on raw data, and differences were considered statistically significant if $P < .05$.

RESULTS

Particle Preparation

The preparation resulted in a colloidal dispersion of nanoparticles in water measuring 35 mL in total after the final solvent evaporation step. In order to accurately prepare particle-DNA-complexes the concentration of solid matter in the dispersions was determined. After drying the pellets under nitrogen to a constant weight, concentration was found to be 4 mg/mL for all preparations (or 140 mg solids in 35 mL). Since the polymer, PCL, is considered insoluble in water, it can be assumed that only 40 mg or ∼30% of the particles' dry matter is made up from the water-soluble excipients PVA and TMC, while the remaining 75 mg of the excipient blend (ie, 115 mg in total) remain dissolved in the aqueous phase.

Determination of Particle Size and Zeta Potential

The PCS analysis of particle sizes showed a unimodal size distribution in most cases. All batches ranged in size between 250 and 300 nm with low polydispersity indices (0.03 to 0.13) indicating a narrow size distribution. The TMC formulations with higher degrees of substitution yielded slightly smaller particles with a lower polydispersity. The addition of DNA increases the measured diameter of the particle/DNA nanoplexes by 20 to 200 nm.

The zeta potential of freshly prepared particles was always found to be in a slightly positive range, between +2.5 and +6.8 mV. After incubation with DNA, the zeta potential dropped to negative values in the range of −4.5 to −6.8 mV, also indicating that anionic DNA molecules had attached to the cationic surface. Results from PCS measurements are compiled in Table 1 .

Atomic Force Microscopy Analysis of Nanoparticles and DNA-nanoplexes

Atomic Force Microscopy (AFM) imaging showed all particles to be of an isodiametric shape. The preparation with plain chitosan yielded particles with highly spherical shape (Figure 1A). Comparison with the results from PCS measurements showed the particles to be larger in diameter, between 300 and 500 nm. The preparations with the lower substituted TMC derivatives (ie, 4% and 10%) were characterized by particles embedded in a matrix of excipients more than the homogeneously distributed chitosan preparation. In the case of TMC 4%, a spherical shape was observed as well, with some particles surrounded by a halo-like structure (Figure 1B). Size is similar to those prepared with chitosan and hence ∼1.5 to 2 times as much as PCS measurements. With an increasing degree of substitution, the discrete particles become less clearly visible and are to an increasing extent embedded in a matrix, as can be seen in Figure 1 (comparison of images B to D). Preparations with TMC 10% showed a trend toward less agglomeration of particles, while a preparation with TMC 18% showed hardly any particles at all. Particles prepared using TMC 66% showed a different result: all particles appear clustered together in rows, and their size was found to be in a similar range as PCS measurements (Figure 1E). However, there is no spherical shape to be seen; furthermore many particles seem to have collapsed.

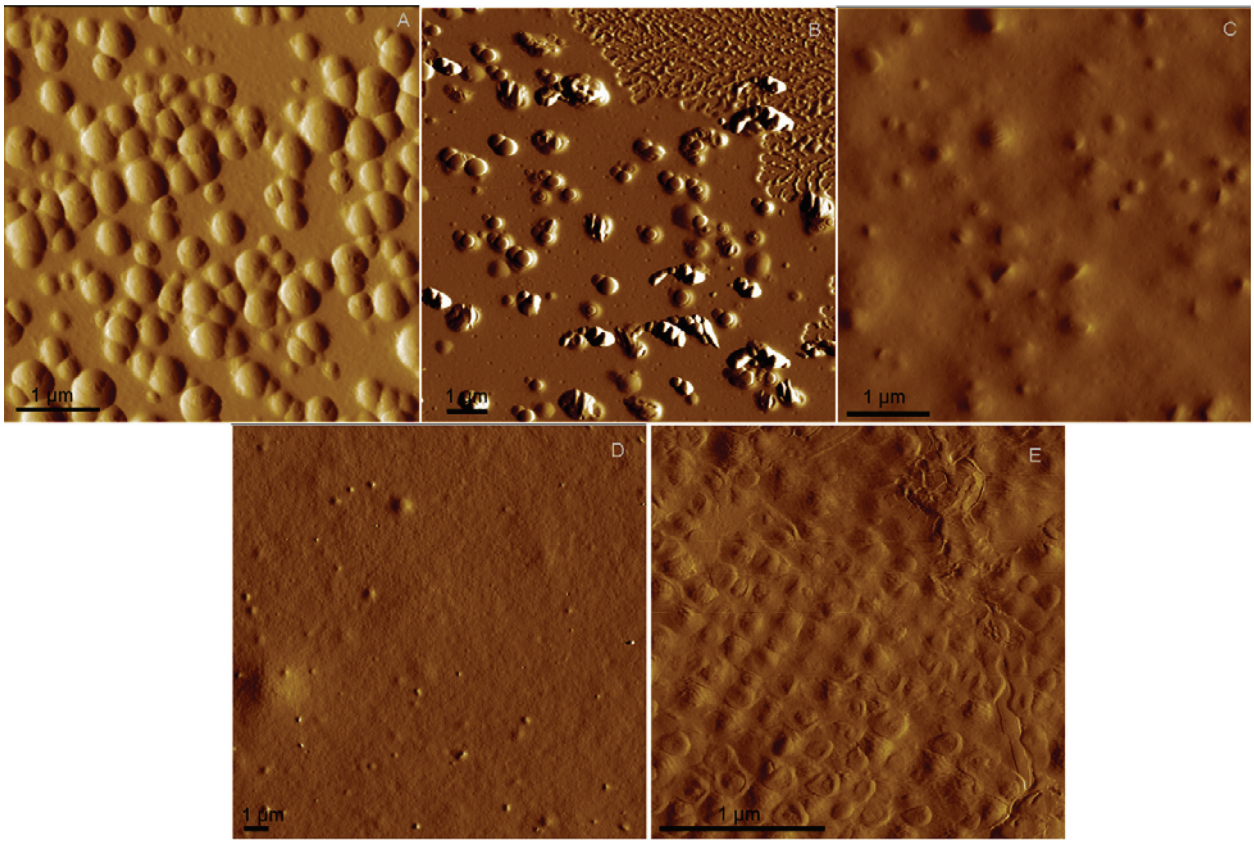

Figure 1. AFM micrograph, particles prepared with chitosan derivatives: (A) chitosan hydrochloride; (B) trimethyl chitosan, 4% degree of substitution; (C) trimethyl chitosan, 10% degree of substitution; (D) trimethyl chitosan, 18% degree of substitution; and (E) trimethyl chitosan, 66% degree of substitution. Length of scale bar = 1 µm.

Gel Electrophoresis and Determination of Unbound DNA

The binding capacity of cationic nanoparticles for plasmid DNA was investigated by assessing the complexes' electrophoretic mobility in an agarose gel. If a complex is formed efficiently, that is, all DNA is bound to the nanoparticles, no bands for free DNA can be observed, and the immobile complex remains in the starting zone in the same way as the much larger particles do. Judging from the binding curves, an efficient binding started at a particle concentration of 20 µg/mL (NP:DNA ratio of 2:1), and at a concentration of 30 µg/mL (3:1) the DNA was nearly fully complexed. Therefore, concentrations of 10 µg/mL and 30 µg/mL were chosen as appropriate for transfection studies. Results from DNA binding measured by gel electrophoresis are compiled in Table 2.

Cytotoxicity Studies

The effects of chitosan-modified particles on COS-1 cells were investigated by testing membrane integrity via the LDH release and metabolic activity via mitochondrial enzymes.

The effect on LDH release is only little dependent on the particle concentration (Figure 2). For all nanoparticle formulations, survival rates range between 40% and 80% whether at higher concentrations of particles or at the lower end. Only in case of particles modified with TMC (66% modification) a different effect was observed: it showed a direct correlation between the particles concentration and the survival rate.

The incubation of cells with nanoparticles over a period of 4 hours has an influence on the cells as shown by the metabolic activity (Figure 3). However, there is no indication as to a correlation between particle concentration and survival rate. As mentioned above in the LDH assay, the amount of viable cells ranged between 40% and 80% survival rate of a negative control. While the particles modified with chitosan or TMC of a low-substitution degree (4%) showed a minimal survival at medium concentrations (see dip at 250 µg/mL), the TMC derivatives of an intermediate substitution degree (10% and 18%) seemed to influence the cell growth even positively at higher concentrations. As in case of the LDH release, only the highly substituted TMC 66% shows a direct relation between dose and effect, with a metabolic activity higher than an untreated control at the

Table 2. Immobilization of Plasmid DNA by Cationic Nanoparticles*

Concentration NP [µg/mL]	Chitosan	TMC 4%	TMC 10%	TMC 18%	TMC 66%	Ratio NP:DNA
0	100.00	100.00	100.00	100.00	100.00	0:1
1	68.27	95.45	133.95	118.20	64.64	0.1:1
10	≤0	57.88	103.80	105.20	23.94	1:1
20	≤0	23.04	29.77	45.40	≤0	2:1
30	≤0	≤0	2.58	13.67	≤0	3:1
50	≤0	≤0	18.95	7.35	≤0	5:1
100	≤0	5.16	15.33	13.19	≤0	10:1

*NP indicates nanoparticles; TMC, trimethyl chitosan. Numbers indicate percentage of free, uncomplexed DNA.

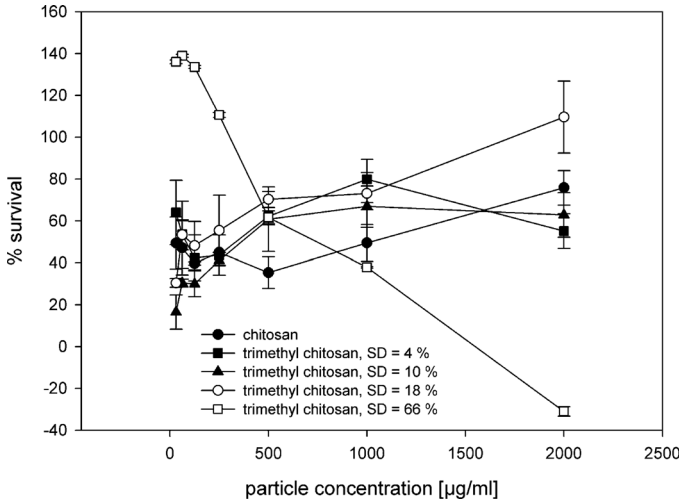

Figure 2. Cytotoxicity of trimethyl chitosan-modified nanoparticles–membrane integrity measured in release of LDH. Error bars represent standard deviation of the mean.

lower end of the concentration range and a total inhibition at the upper end.

Statistical analysis revealed no significant differences between different preparations, either for LDH test or for WST-1 assay.

The membrane degrading dose-effect relation of pure chitosan derivatives was more pronounced than of whole particle preparations. With the notable exception of TMC 66%, all chitosans affected the cells' membranes in a similar fashion and intensity, with a survival rate of around 90% at the lowest concentrations and 20% to 40% at the highest. The curves ran closely parallel with marginally higher variations in the upper concentration range (see Figure 4).

In contrast to this, PVA showed no linear relations between dose and cytotoxicity. The effect of PVA on the cells' metabolism was not discernible, since at all concentrations the survival rate ranged around a 100% value, thus indicating no effect on mitochondrial activity at all (Figure 5). The LDH release followed no discernible pattern and could therefore only be explained by an influence of handling stress or the general surface-active properties of the substance, which might disturb the bilayer structures (Figure 5).

Transfection Studies

After 4 hours of transfection and 48 hours of gene expression, cells were still viable as could be expected from the cytotoxicity studies with particle suspensions. First results indicated the particle-DNA-complexes to be suitable for transfection but much less effective than the commercial transfection agent, PolyFect. Batches prepared with CS, TMC 4%, and TMC 10% showed expression levels of β-galactosidase (in fluorescence units) significantly higher than naked DNA (see Figure 6); while nanoplexes prepared with TMC 18% and TMC 66% remained below the chosen level of significance. Further experiments are in progress to demonstrate the potentiality of the new PCL nanoparticles coated with chitosan and modified chitosans.

DISCUSSION

The preparation process resulted in a homogenous colloidal suspension of particles, which was stable for up to 7 days. Visualization via AFM showed that the particles' morphologies in the prepared batches are different. A direct look at the particles revealed differences between the preparations. While some appeared as discretely distributed and spherically shaped particles, as in the case of the preparations with chitosan hydrochloride, others (eg, TMC 10% and 18% degree of substitution) showed a thick matrix covering sparsely visible and irregularly shaped particles. This finding could perhaps be explained by the increasing solubility in water or the polarity of the chitosan derivatives. While pure chitosan is the least polar

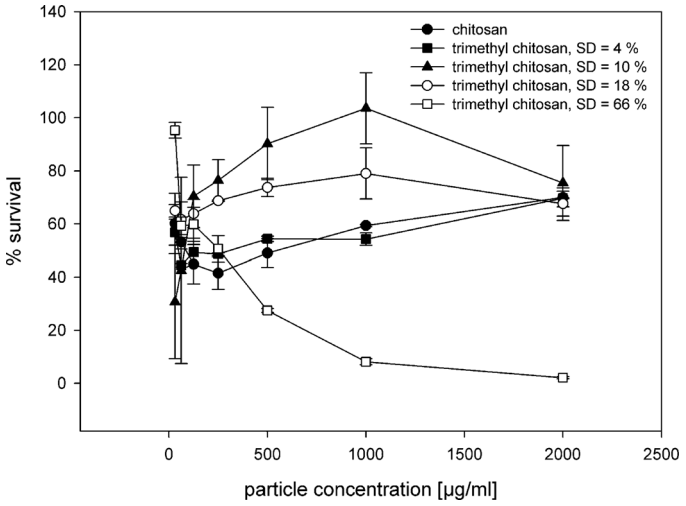

Figure 3. Cytotoxicity of trimethyl chitosan-modified nanoparticles–metabolic activity of mitochondria measured by WST-1 assay. Error bars represent standard deviation of the mean.

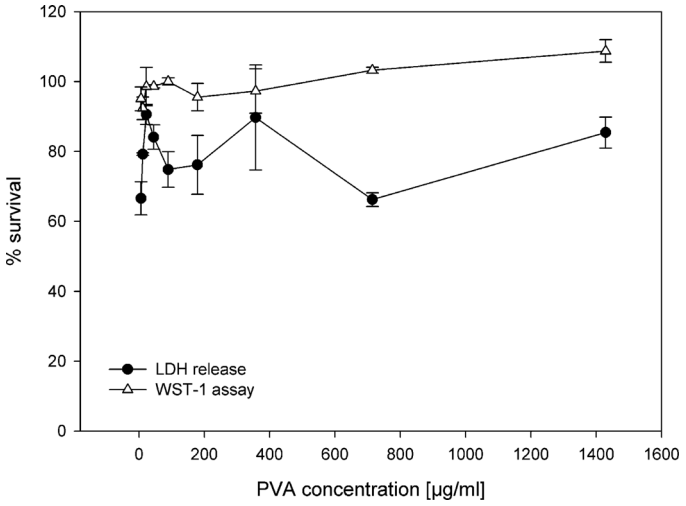

Figure 5. Cytotoxicity of PVA. Black circles: membrane integrity measured in release of LDH. Open triangles: metabolic activity of mitochondria measured by WST-1 assay. Error bars represent standard deviation of the mean.

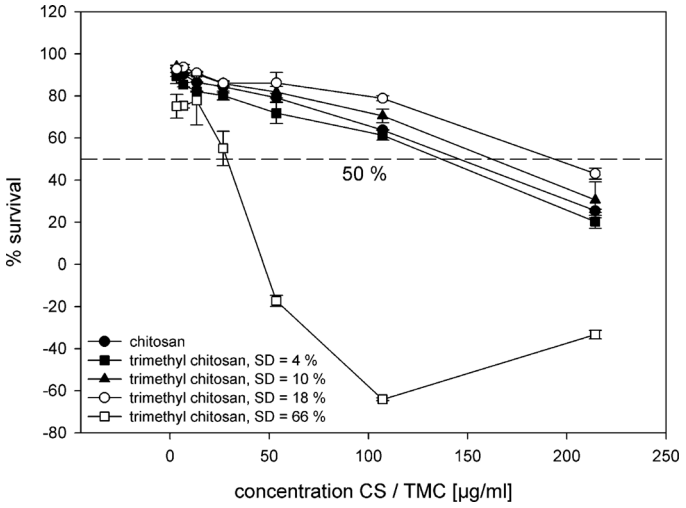

Figure 4. Cytotoxicity of chitosan and trimethyl chitosan derivatives–membrane integrity measured in release of LDH. Error bars represent standard deviation of the mean.

of the excipients, the polarity increases with the percentage of cationic moieties in the molecule. Therefore, chitosan should adsorb better than other derivatives to the lipophilic polymer, a property that is reduced with the degree of hydrophilicity (see Figure 1). These effects are reduced when the suspensions are highly diluted for PCS measurements, hence the similar sizes as determined with this method. The lower particle sizes and polydispersity indices of particles prepared with high-substitution TMC (see Table 1) can be explained by a higher surface activity; the TMC derivatives can be regarded as surfactants similar to other cationic amphiphiles (eg, cetrimide).

The plasmid chains associate themselves with the oppositely charged particles by wrapping around them, forming a new outer layer. This phenomenon should lead to an increase in particle size, which was observed (see Figure 7). Supercoiled plasmid DNA chains show a diameter of ∼8 nm, so the particle diameter should increase by ∼16 nm as was observed in case of batches prepared with chitosan, TMC 10%, and TMC 66%. The other batches (TMC 4% and 18%) were found to have increased much more in terms of particle size after incubation; this result was attributed to particle agglomeration.

The positive zeta potentials were considered sufficient for binding DNA long enough to safely transport it to the desired site of action and subsequently release it. Particulate DNA carriers are reported[20] with a zeta potential of up to +50 mV, binding DNA to a higher extent than the ones described here, but the question arises whether these highly charged carriers are actually capable of completely releasing their drug load at the target cells at all. It is interesting to note that while the unmodified particles showed an interdependence of substitution degree and zeta potential, no correlation of this kind could be detected with the DNA-modified particles. It was concluded that the surface was saturated with DNA chains, while additional free phosphate groups determined the measured surface charge.

Insights into the cytotoxic effects of transfection agents are essential when dealing with new developments in this area. Since one of the best-known and widely used transfection agents–PEI–is known for both its efficiency and toxicity, the development of less harmful alternatives is still interesting. The impact on membrane integrity and metabolic activity were investigated, but no correlation or interdependence could be detected for particle concentration and cytotoxicity. The different behavior of particles pre-

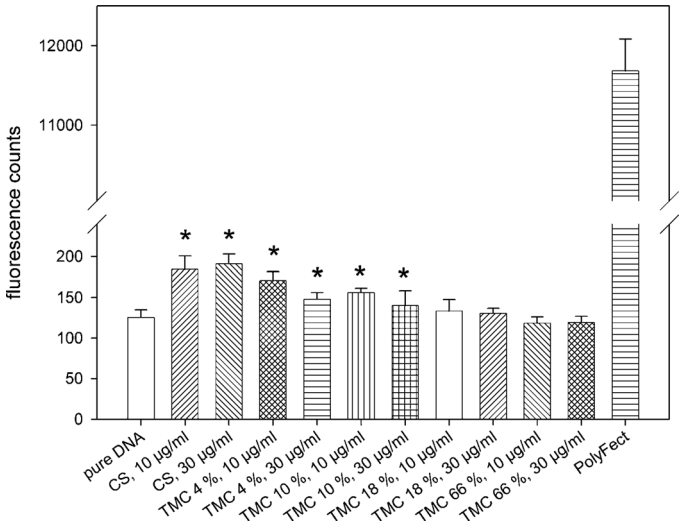

Figure 6. Transfection efficiency of nanoparticle-DNA-complexes compared with pure DNA and a commercial transfection agent, PolyFect. Data are presented as fluorescence units (mean ± standard deviation). Asterisks (*) denote a statistically

pared with TMC 66% with its direct correlation can be explained by the high amount of cationic moieties (see below). The pure excipients seem to be less cytotoxic than the particle preparations when applied in the same concentrations but in absence of any nanoparticles. PVA is little or not at all toxic to the cell cultures (Figures 4), while chitosan and its trimethyl derivatives with a low and intermediate degree of substitution show a linear correlation between dose and effect (Figure 3). The substances are however less toxic than the particles at corresponding concentrations. The different behavior of highly substituted TMC 66% both pure and in a particle preparation can be explained by the high degree of quaternization: cationic amphiphilic molecules like benzalkonium chloride or cetyl trimethyl ammonium bromide (cetrimide) have been widely used as preservatives owing to their membrane-disrupting properties. Concluding from this, TMC 66% seems to show much more amphiphilic properties than the other trimethyl chitosans and hence shows a higher cytotoxicity; therefore its use as an excipient in a biological matrix should be considered carefully, and it should be replaced by lower substituted derivatives when possible.

First experiments aimed at transfection showed the particle-DNA-complexes as being superior to naked DNA; however, experiments are underway aiming at the improved transfection rates, whereas similar studies using chitosan-modified PLGA nanoparticles showed transfection in vitro and in vivo. [23]

CONCLUSION

The described method seems suitable to prepare cationic nanoscale carriers for nucleic acids. The main advantage of the method can be seen in the fact that all degrading influences such as contact of the sensitive DNA with extreme pH values or organic solvents during the preparation process is avoided; the DNA is added after all preparation steps are finished. The particles' diameter depends only to a

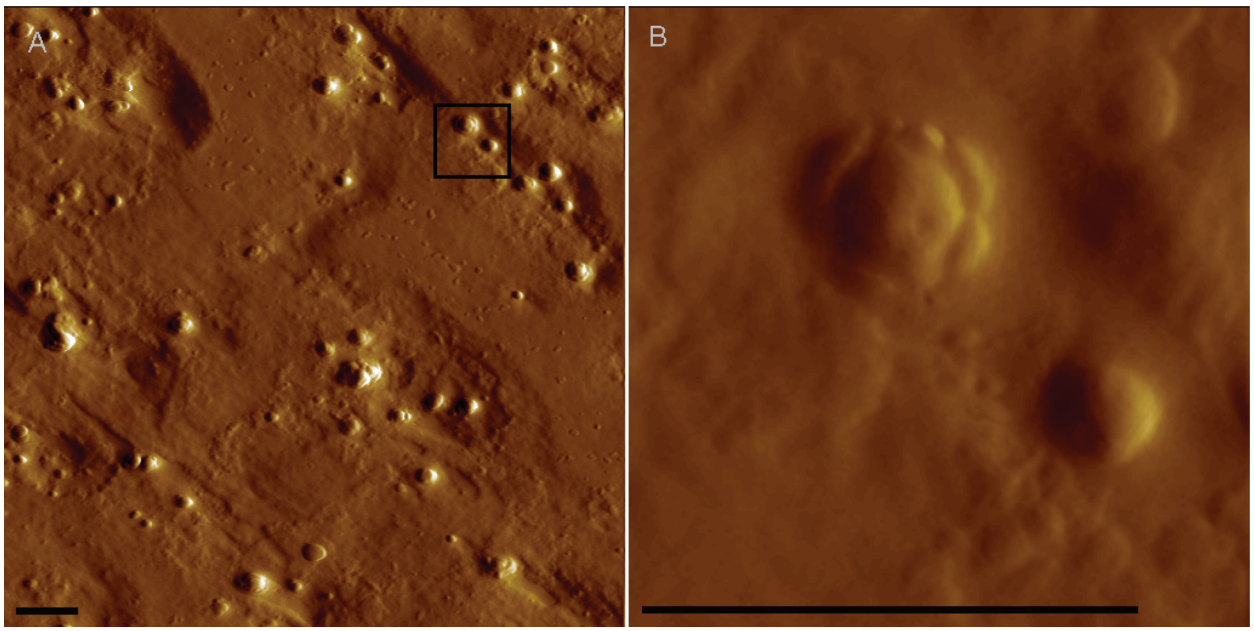

Figure 7. Nanoparticle-DNA complex; particles prepared with trimethyl chitosan 4% degree of substitution. (A) amplitude signal, lateral resolution 10 µm; (B) amplitude signal, lateral resolution 1.25 µm. Black square in panel A is zoomed in as panel B. Length of scale bar = 1 µm.

minor extent from the used chitosan derivative, which allows this parameter to be varied in a relatively broad range. The cytotoxicity of the nanoparticle suspensions appears to be moderate and independent from the concentration (with the notable exception of 66% substituted TMC, which appears to be significantly more toxic). Transfection ability seems to be present but needs further investigation.

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REFERENCES

1. Crystal RG. The gene as drug. *Nat Med.* 1995;1:15-17.

2. Florence AT. Sakthivel T, Toth I. Oral uptake and translocation of a polylysine dendrimer with a lipid surface. *J Control Release.* 2000;65:253-259.

3. Ramaswamy C, Sakthivel T, Wilderspin AF, Florence AT. Dendriplexes and their characterization. *Int J Pharm.* 2003;254:17-21.

4. Pouton CW. Lucas P, Thomas BJ, Uduehi AN, Milroy DA, Moss SH. Polycation-DNA complexes for gene delivery: a comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids. *J Control Release.* 1998;53:289-299.

5. Ramsay E, Gumbleton M. Polylysine and polyornithine gene transfer complexes: a study of complex stability and cellular uptake as a basis for their differential in-vitro transfection efficiency. *J Drug Target.* 2002;10:1-9.

6. Oupicky D, Konak C, Ulbrich K, Wolfert MA, Seymour LW. DNA delivery systems based on complexes of DNA with synthetic polycations and their copolymers. *J Control Release.* 2000;65:149-171.

7. Wagner E, Plank C, Zatloukal K, Cotten M, Birnstiel ML. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc Natl Acad Sci U S A.* 1992;89:7934-7938.

8. Fischer D, Bieber T, LiY, Elsasser HP, Kissel T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm Res.* 1999;16:1273-1279.

9. Kunath K, von Harpe A, Fischer D, et al. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. *J Control Release.* 2003;89:113-125.

10. Smith JG, Wedeking T, Vernachio JH, Way H, Niven RW. Characterization and in vivo testing of a heterogeneous cationic lipid-DNA formulation. *Pharm Res.* 1998;15:1356-1363.

11. Olbrich C, Bakowsky U, Lehr CM, Muller RH, Kneuer C. Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA. *J Control Release.* 2001;77:345-355.

12. Oberle V, Bakowsky U, Zuhorn IS, Hoekstra D. Lipoplex formation under equilibrium conditions reveals a three step mechanism. *Biophys J.* 2000;79:1447-1454.

13. Mahato RI, Kawabata K, Nomura T, Takakura Y, Hashida M. Physicochemical and pharmacokinetic characteristics of plasmid DNA/cationic liposome complexes. *J Pharm Sci.* 1995;84:1267-1271.

14. Farhood H, Serbina N, Huang L. The role of dioleyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim Biophys Acta.* 1995;1235:289-295.

15. Sternberg B, Hong K, Zheng W, Papahadjopoulos D. Ultrastructural characterization of cationic liposome-DNA complexes showing enhanced stability in serum and high transfection activity in vivo. *Biochim Biophys Acta.* 1998;1375:23-35.

16. Meyer O, Kirpotin D, Hong K, Sternberg B, Park JW, Woodle MC, Papahadjopoulos D. Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides. *J Biol Chem.* 1998;273:15621-15627.

17. Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc Natl Acad Sci U S A.* 1989;86:6982-6986.

18. Torchilin VP, Levchenko TS. Rammohan R, Volodina N, Papahadjopoulos-Sternberg B, D'Souza GG. Cell transfection in vitro and in vivo with nontoxic TAT peptide-liposome-DNA complexes. *Proc Natl Acad Sci U S A.* 2003;100:1972-1977.

19. Cui Z, Mumper RJ. Plasmid DNA-entrapped nanoparticles engineered from microemulsion precursors: in vitro and in vivo evaluation. *Bioconjug Chem.* 2002;13:1319-1327.

20. Kneuer C, Sameti M, Haltner EG, Schiestel T, Schirra H, Schmidt H, Lehr CM. Silica nanoparticles modified with aminosilanes as carriers for plasmid DNA. *Int J Pharm.* 2000;196:257-261.

21. Kneuer C, Sameti M, Bakowsky U, et al. A nonviral DNA delivery system based on surface modified silica-nanoparticles can efficiently transfect cells in vitro. *Bioconjug Chem.* 2000;11:926-932.

22. Ravi Kumar MNV, Bakowsky U, Lehr C-M. Preparation and characterization of cationic PLGA nanospheres as DNA carriers. *Biomaterials.* 2004;25:1771-1777.

23. Ravi Kumar MNV, Mohapatra SS, Kong X, Jena PK, Bakowsky ULehr C-M. Cationic poly(lactide-co-glycolide) nanoparticles as efficient in vivo gene transfection agents. *J Nanosci Nanotechnol.* 2004;4:990-994.

24. Leong KW, Mao HQ, Truong Le VL, Roy K, Walsh SM, August JT. DNA-polycation nanospheres as non-viral gene delivery vehicles. *J Control Release.* 1998;53:183-193.

25. Capan Y, Woo BH, Gebrekidan S, Ahmed S, DeLuca PP. Preparation and characterization of poly (D,L-lactide-co-glycolide) microspheres for controlled release of poly(L-lysine) complexed plasmid DNA. *Pharm Res.* 1999;16:509-513.

26. Ravi Kumar MNV, Sameti M, Mohapatra SS, Kong X, Lockey RF, Bakowsky U, Lindenblatt G, Schmidt H, Lehr CM. Cationic silica nanoparticles as gene carriers: synthesis, characterization and transfection efficiency in vitro in vivo. *J Nanosci Nanotechnol.* 2004;4:876-881.

27. Florea BI, Meaney C, Junginger HE, Borchard G. Transfection efficiency and toxicity of polyethylenimine in differentiated Calu-3 and nondifferentiated COS-1 cell cultures. *AAPS PharmSci.* 2003;4:E12.

28. Merdan T, Kopecek J, Kissel T. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. *Adv Drug Deliv Rev.* 2002;54:715-758.