In Vitro Evaluation of Chitosan-EDTA Conjugate Polyplexes as a Nanoparticulate Gene Delivery System

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ABSTRACT

It was the purpose of this study to evaluate the potential of different molecular-weight chitosan-EDTA conjugates as a carrier matrix for nanoparticulate gene delivery systems. Covalent binding of EDTA to more than one chitosan chain provides a cross-linked polymer that is anticipated to produce stabilized particles. pDNA/chitosan-EDTA particles, generated via coazervation, were characterized in size and zeta potential by electrophoretic light scattering and electron microscopy. Stability was investigated at different pH values by enzymatic degradation and subsequent gel retardation assay. Lactate dehydrogenase assay was performed to determine toxicity. Furthermore, transfection efficiency into Caco-2 cells was assessed using a beta-galactosidase reporter gene. Chitosan-EDTA produced from low-viscous chitosan with 68% amino groups being modified by the covalent attachment of EDTA showed the highest complexing efficacy resulting in nanoparticles of 43 nm mean size and exhibiting a zeta potential of +6.3 mV. These particles were more stable at pH 8 than chitosan control particles. The cytotoxicity of chitosan-EDTA particles was below 1% over a time period of 4 hours. These new nanoplexes showed 35% improved in vitro transfection efficiency compared with unmodified chitosan nanoparticles. According to these results, the chitosan-EDTA conjugate may be a promising polymer for gene transfer.

KEYWORDS: nanoparticles, stability, cross-linking, gene transfer, cytotoxicity, Caco-2

INTRODUCTION

The past several years have witnessed the evolution of gene medicine from an experimental technology into a viable strategy for developing therapeutics for a wide range of human disorders. Numerous prototype DNA-based biopharmaceuticals can now control disease pro-

gression by induction and/or inhibition of genes¹; however, poor cellular uptake and rapid in vivo degradation of DNA-based therapeutics necessitate the use of delivery systems to facilitate cellular internalization and preserve their activity.¹ Since delivery systems based on viral elements provoked side reactions like immune response and mutagenesis, the trend is to improve efforts on nonviral delivery systems.²⁻⁴ Cationic polymers are commonly used as vehicles because of the ease of complex formation⁵ and higher stability compared with lipoplexes.⁶ Chitosan is one of the most used cationic polymers in gene delivery systems owing to its low toxicity and biocompatibility.^{7,8}

A further advantage is the easy manipulation of chitosan via modification with different reactive groups as side chains, allowing adaptation of physicochemical properties. Several derivatives of chitosan have been proposed for improving properties like transfection efficiency, stability, or solubility.⁹⁻¹²

The major drawback of chitosan-based particles, like all polymer-based particles, is the low transfection efficacy in comparison to viral transfection.^{13,14} One possible reason for low transfection efficacy could be the high stability of particles, which strongly limits the release of DNA after endosomal escape. Therefore, the best particles would be stable in the extracellular environment but would release DNA once they reach the cytoplasm.

Covalent coupling of EDTA to the chitosan backbone has the effect of reducing the cationic charge of the polymer, thus reducing the strength of binding to poly-anionic plasmid DNA (pDNA). In extracellular fluids, DNA needs to be protected against nuclease attack. At neutral pH, the chelator EDTA is able to bind divalent cations and in this way inhibit many enzymes that need these cations as cofactors. Moreover, as shown in previous studies with this polymer, a certain degree of cross-linking within and between polymer chains can be achieved¹⁵ providing a form of “branched” chitosan polymer and subsequently stabilized complexes with DNA.

The present study investigated the characteristics of chitosan-EDTA conjugates, the structures of which are shown in Figure 1, and examined if cross-linked chitosan-EDTA polymer could produce stabilized particles with efficient release of the payload in the cytoplasm to result in improved transfection. Therefore, chitosan-EDTA conjugates of

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Figure 1. Chemical structure of chitosan-EDTA conjugates and intermolecular cross-linking via covalent binding of EDTA to more than 1 amino group.

variable molecular weight and with different degrees of modification were synthesized and tested for their ability to build stable, nano-sized complexes with pDNA. Enzymatic digestion assay was performed to compare the protective properties of chitosan and chitosan-EDTA polymer. The release of pDNA was investigated in cell culture media. Subsequently, the most stable of these particles were examined with respect to their cytotoxicity and transfection efficiency. We also explored the possible effects of chitosan-EDTA on plasma membrane integrity as a potential reason for increased transfection efficiency.

MATERIALS AND METHODS

Chitosan-EDTA synthesis

Chitosan of different molecular weights (Fluka, Vienna, Austria) was used, including 10-kD chitosan (produced from medium viscous chitosan [Fluka] by a depolymerization method according to Huang et al¹⁶), low viscous chitosan (55 mPas⁻¹), and high viscous chitosan (≥400 mPas⁻¹), with a deacetylation-degree of >80%.

Chitosan-EDTA conjugates were synthesized in a slightly modified way from that described previously.¹⁵ In brief, 0.5 g of the different chitosans was suspended in 50 mL of 0.1% (vol/vol) acetic acid. To this solution different amounts of disodium EDTA were added to reach weight ratios from 1:40 to 1:1 of chitosan:EDTA. The pH was adjusted by the addition of 5 M NaOH. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was added in final concentrations ranging from 7.5 mM to 100 mM. The reaction mixtures (Table 1) were incubated at room temperature under constant stirring for 4 hours. The resulting conjugates were isolated by dialyzing against demineralized water, 2 times against 0.5 M NaCl 0.025 M NaOH, 2 times against 0.05 M NaOH, and once more against demin-

eralized water. The purified products were lyophilized and stored at 4°C.

Polymer characterization

The resulting chitosan-EDTA conjugates were characterized with 2,4,6-trinitrobenzen sulfonic acid (TNBS) to analyze the amount of free, unmodified amino groups. In brief, 1 mg of each chitosan conjugate was swelled in 250 μL of demineralized water and incubated with 250 μL of 4% NaHCO₃ and 250 μL of 0.1% TNBS reagent at 37°C for 2 hours. Thereafter, 250 μL 2 M HCl were added and absorbance was measured at 344 nm. The amount of remaining free amino groups was calculated using a standard curve obtained by the amino-group determination of a series of solutions with increasing amounts of unmodified polymer.

The complexing properties of the conjugate were determined via EDTA-titration. Xylenol orange indicator was added to 0.12 mM zinc chloride solution to achieve a violet color. Sodium acetate buffer, 1 M pH 5.5, was added. This solution was titrated with 0.4% (wt/vol) polymer solution and 0.01 M EDTA solution as reference. The complexing properties of the polymer solution were expressed as the molarity of the corresponding complexing properties of an EDTA solution.

Plasmid preparation

The plasmids (pSV-β-Galactosidase Control Vector [Promega, Madison, WI] and pEGFP-C2 Vector [Clontech, Mountain View, CA]) were amplified in *Escherichia coli* XL-1 competent cells, isolated, and purified according to a scaled up preparation method of alkaline lysis.¹⁷ Purified pDNA was resuspended in distilled water. The DNA content was quantified by photometric measurement at 260 nm. Plasmid-DNA solution was stored at -20°C.

Table 1. Polymer characterization

Polymer Name	Chitosan	Weight Ratio Chitosan:EDTA	pH	EDAC, M	NH ₂ -Groups Remaining, %	Complexing Properties of 0.4% Polymer Solution, mM EDTA	Properties
CE 1	High viscosity	1:40	6	0.100	—	5.0	High viscosity, precipitates at acidic pH
CE 2	Low viscosity	1:20	6	0.100	25 ± 2.5	5.7	Just in acidic pH soluble
CE 3	Low viscosity	1:10	6	0.100	10.7 ± 1.9	6.0	Low solubility, high viscosity
CE 4	Low viscosity	1:4	5	0.0125	32.5 ± 2.9	0.6	Soluble in water
CE 5	Low viscosity	1:1	4	0.0075	32.0 ± 1.2	0.2	Soluble in water
CE 6	10 kD	1:10	6	0.100	1.2 ± 0.2	6.7	Easily soluble in water
CE 7	10 kD	1:10	6	0.050	7.1 ± 0.6	8.6	Easily soluble in water

EDAC, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

Nanoparticle preparation*Chitosan nanoparticles*

A 0.02% (wt/vol) chitosan solution in 0.01% acetic acid was prepared. The pH was adjusted to 4 with acetic acid. This solution was sterile filtered. DNA solution was prepared in distilled water with a content of 100 µg pDNA/mL. The chitosan solution and DNA solutions were separately heated to 50°C for 10 minutes. To a 250-µL aliquot of pDNA solution, 400 µL of chitosan solution was added followed by immediately vigorous vortexing for 60 seconds. Before further use or characterization, the particle suspension was preincubated for at least 1 hour.

Chitosan-EDTA nanoparticles

Chitosan-EDTA nanoparticles were prepared similarly except that larger amounts of polymer were used. Chitosan-EDTA conjugate (CE 4, Table 1) was dissolved in demineralized water in order to obtain a final concentration of 0.4% (wt/vol); the pH was adjusted to 4 followed by sterile filtration. A volume of 80 µL of 0.4% (wt/vol) CE 4-solution was used to complex 25 µg pDNA.

Particle characterization

Nanoparticle suspension was characterized by measurement of size and zeta potential in a Nicomp particle sizer (PSS Nicomp 380 ZLS, Nicomp, Santa Barbara, CA). For size analyzing, the Nicomp number weighted distribution was

used since the particle distribution did not follow Gaussian distribution. A fixed angle of 90 degrees was used. The refractive index was 1.333, since particles were measured in aqueous solution. The results are presented as means of at least 3 samples measured each for 15 minutes. Determination of zeta potential was done with a dilution of 1:5 in distilled water. The measurement parameters were set as the following: 23°C constant temperature, scattering angle of 14.7° and electric field strength of 5 V/cm. Each sample was analyzed in 3 cycles, each 3 × 60 seconds.

Fluorescence binding titration

DNA condensation was measured by quenching of ethidium bromide fluorescence as described previously.¹⁸ Briefly, 30 µg pDNA was diluted in 10 mL of 1 mM HEPES, pH 7, containing 1 µM ethidium bromide. Aliquots of 200 µL of this solution were filled into 96-well plates and titrated in triplicate with increasing amounts of polymer in aqueous solution. The fluorescence was measured using Fluostar Galaxy plate reader (BMG Lab-tech GmbH, Offenburg, Germany) at 544/590 nm wavelength.

Transmission electron microscopy pictures

The prepared nanoparticle suspension was analyzed after 14 hours of incubation at room temperature. Drops of 10-µL particle solution were placed on pioloform-coated grids and air dried for 15 minutes. The dried nanoparticles were

examined by a ZEISS LIBRA 120 transmission electron microscope (Carl Zeiss, Göttingen, Germany) with a Carl Zeiss in-column energy filter (EFTEM). Digital micrographs were obtained from a ProScan Slow Scan CCD camera system (Proscan Electronic Systems GmbH, Scheuring, Germany) using iTEM 5.0 Software (Soft Imaging System GmbH, Münster, Germany).

Stability tests

Investigation of particle disintegration caused by pH changes were performed by adjusting the pH with 1 M HCl, 1 M NaOH, or addition of sodium acetate buffer (1 M, pH 5.5) and phosphate buffer (0.5 M, pH 8). After 30 minutes of incubation in an Eppendorf shaker at 37°C and at 400 rpm, the samples were run in an agarose gel electrophoresis with 0.7% agarose in 1×Tris-acetate-EDTA-buffer. The released DNA was quantified by measurement of fluorescence of the cut gel slice containing the band of free DNA and calculated as percent of initially loaded DNA.

RQ1 DNase I (Promega) nuclease was used for assessing the stability against enzymatic degradation. The nanoparticle suspension was mixed with trehalose to a final content of 5% (wt/vol) to protect against aggregation during the centrifugation step. Samples were centrifuged for 10 minutes at 15 000g. Supernatants were discharged and particles were resuspended in 1:10 dilution of enzyme reaction buffer provided with the Promega enzyme. The pH value of the samples was adjusted to pH 6 with 1 M HCl. An aliquot of 3 µL (3 units) DNase solution was added to a 100-µL sample. Digestion assay was performed at 37°C shaken at 400 rpm. Samples were taken at predetermined time points. The reaction was stopped with the manufacturer's stop-solution and samples were kept at 4°C until analysis via gel electrophoresis. Unprotected pDNA was digested as positive control for enzyme activity at pH 6.

In vitro DNA release

Chitosan/pDNA and chitosan-EDTA/pDNA complexes were incubated in minimal essential medium (MEM) at pH 7.4 without serum at 37°C, 400 rpm, up to 8 hours. Withdrawn samples were analyzed again via gel electrophoresis as described above. Bands of free pDNA were cut out, and the gel was melted and transferred into a microtiter plate. Measurement of ethidium bromide fluorescence was used to ascertain the amount of DNA. Release was calculated in percent of initial pDNA amount.

Maintenance of cell culture

Caco-2 cells were cultivated in MEM media supplemented with 2.2 g/L sodium bicarbonate, 2 mM L-glutamine,

penicillin/streptomycin (Sigma-Aldrich, Vienna, Austria), and 20% fetal bovine serum (Gibco, No. 26140–079, Invitrogen, Lofer, Austria). Propagation was done at 37°C, 95% humidity, and 5% CO₂ atmosphere. Cells were fed daily and split after trypsinization in a ratio 1:6 twice a week.

LDH test

A lactate dehydrogenase (LDH) test was performed on Caco-2 cells with a Cytotoxicity Detection Kit (LDH) from Roche (Vienna, Austria). Cells were seeded at a density of 1×10^5 cells/mL in 12-well plates. After 24-hour incubation, transfection was performed. Samples of 100 µL were taken after 0, 60, 120, and 240 minutes and analyzed according to the manufacturer's protocol, as low control cells were incubated with just medium. High control was prepared with medium plus 2% Triton-X100. Cytotoxicity was calculated by the following equation: cytotoxicity [%] = $100 \times (\text{experimental value} - \text{low control}) / (\text{high control} - \text{low control})$.

Transfection

Caco-2 cells (1×10^5) in 1 mL media were seeded into 12-well-plates and incubated for 24 hours. The medium was aspirated and cells were washed once with phosphate-buffered saline (PBS). The first 260 µL of nanoparticle suspension, containing 10 µg pDNA, was added to the cells and covered with 740 µL media containing 20% serum. Cells were incubated with the nanoparticles for 14 hours. After the transfection, cells were washed once with PBS, fed with 1 mL new medium, and incubated for another 24 hours. Cells were washed with PBS once. Thereafter, 100 µL lysis buffer (0.25 M Tris, pH 7.4, 0.25% [vol/vol] Triton X-100, and 2.5 mM EDTA) were added and the plate shaken for 5 minutes. To enhance cell lysis, a freezing-thawing cycle was performed. Cell lysate was transferred into tubes and cell debris was separated by centrifugation.

Beta-galactosidase assay

For the colorimetric beta-galactosidase assay, an aliquot of 50 µL of the cellular extract was mixed with 58 µL of o-nitrophenyl-beta-d-galactopyranoside (ONPG) buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl and 50 mM beta-mercaptoethanol) and 15 µL of ONPG solution (60 mM Na₂PO₄, 40 mM NaH₂PO₄, 14 mg/mL ONPG). The reaction mixture was incubated at 37°C for 2 hours. The reaction was stopped by adding 60 µL of 1 M Na₂CO₃ solution. The beta-galactosidase activity was determined by measuring the absorbance at 420 nm and 600 nm and the difference in optical density between these 2 wavelengths was calculated. Transfection efficiency was calculated by subtraction of background activity measured in untransfected

cells from the measured beta-galactosidase activity. The results were presented as means of 4 independent experiments each with 2 to 3 replicates of each sample.

Additionally, assay values of beta-galactosidase test were corrected for protein concentration. The total amount of protein in cell lysates of transfected cells was measured using a ProtQuant assay kit (Serva, Heidelberg, Germany) following the instruction manual. This protein quantification method was chosen because it is based on the precipitation of proteins as insoluble dye complexes with acidic, methaolic amido black 10B solution and is therefore not disturbed by detergents present in the lysis buffer.

Analysis of fluorescence from green fluorescence protein (GFP)

For fluorescence measurements of 50 μ L of cellular extract were transferred into microtiter plate and the fluorescence was measured at 485 nm excitation and 520 nm emission wavelength. Fluorescence of untransfected cells was subtracted as background.

Statistical data analyses

Statistical data analyses were performed using the F-test for analysis of variance consistence and Student *t* test, with $P < .05$ as the level of significance.

RESULTS AND DISCUSSION

Polymer characterization

Synthesized polymers were analyzed for content of unmodified amino groups via the TNBS test. The chelating properties of bound EDTA were assessed via titration with zinc ions. The correlation between these 2 properties, namely the amount of unmodified amino groups and the complexing properties resulting from immobilized free carboxylic groups, was statistically significant, with $R^2 = 0.754$. This observation is likely due to the cross-linking between the polymer chains via intra- and intermolecular binding of carboxyl groups of EDTA to more than 1 amino group as shown in Figure 1. A high degree of modification, in combination with low complexing properties, was therefore an indicator for high degree of cross-linking.

The challenge of chitosan-EDTA synthesis was to generate a polymer with the right balance between EDTA cross-linking for stabilization and sufficient unmodified amino groups to preserve the properties of chitosan. Moreover, a certain amount of free carboxylic groups was desired to enhance enzymatic stability. Protonated amino groups are responsible for many favorable properties of chitosan, such as solubility and cationic charge at acidic pH.¹⁹ The formation of chitosan particles

as carriers for nucleic acids is based on the interaction of the positively charged amino groups of the polymer with the negatively charged phosphate groups of nucleic acids.

The results of various polymer synthesis and their characterization is summarized in Table 1. Chitosan-EDTA conjugates synthesized in the presence of excess EDTA, eg, CE 1–3, reached a high degree of modification, but low cross-linking. These results are in accordance with previous studies.¹⁵ The low amount of free amino groups and the limited solubility of these polymers made them less valuable as nucleic acid vectors.

Short chitosan chains as backbones and less EDTA excess during coupling reaction (CE 6 and CE 7) also prevented cross-linking and created polymers of good solubility with the desired chelating properties. Nevertheless, these polymers were not able to condense pDNA, lacking the cationic charge of free amino groups. All attempts to use these polymers did not succeed. For example, a mixture of chitosan-EDTA conjugate with unmodified chitosan was analyzed as a complexing agent with the only result of destabilizing particles. In a separate experiment, coating of chitosan/pDNA nanoparticles with CE 7 polymer was performed. The change of zeta-potential from positive to negative charge, together with the increased size, was taken as evidence for an achieved coating; however, the resulting particles were not stable in aqueous solution and disintegrated completely with any changes in pH.

The key feature appeared to be the amount of amino groups left on the modified polymer. The only chitosan-EDTA conjugates with high enough amino-group content to complex pDNA on their own were CE 4 and CE 5. Their low chelating properties suggest a significant degree of cross-linking. CE 4, was tested to have the highest potential for complexation of pDNA and owning a higher amount of functional EDTA carboxyl groups, and it was chosen for further study.

Particle characterization

Chitosan-EDTA complexes exhibited just about one third of amino groups compared with unmodified chitosan. Consequently the amount of polymer needed for complex formation was comparatively higher for chitosan-EDTA. A weight ratio 1:12 instead of 1:2.5 was used for nanoparticle formation. Furthermore, the pDNA binding capacity of the chitosan-EDTA conjugate was lower and did not cover as wide a concentration range as for unmodified chitosan. The degree of DNA condensation was analyzed by ethidium bromide quenching assay. Ethidium bromide intercalates between the base pairs of the DNA double helix, forming a highly fluorescent complex. Polycations are able to displace the dye, resulting in a strong decrease of fluorescence. Hence the quenching of fluorescence provided information about the relative strength of the interaction between the

Figure 2. Titration of DNA in the presence of ethidium bromide. Data points represent the mean $\pm$ SD ($n = 3$).

DNA and the polymer. Results, shown in Figure 2, indicated that chitosan-EDTA was able to condense pDNA as tightly as unmodified chitosan but not in the same small amounts of polymer. To reach the same degree of condensation, a 4-fold higher amount of chitosan-EDTA conjugate is needed when compared with unmodified chitosan. In comparison, chitosan with a higher degree of acetylated units is reported to have also a less-pronounced decay of fluorescence.²⁰

The physicochemical properties of chitosan-pDNA and chitosan-EDTA-pDNA complexes were analyzed at pH 4 and 5 respectively and are listed in Table 2. All complexes were of nano-scaled size in the range of 27 to 88 nm. Particle size distribution did not follow Gaussian distribution, but showed a little tailing, indicating that more particles are bigger than the mean. Changing the pH of the polymer solution from 4 to 5 before complex formation caused the same tendency to form bigger complexes and a decrease in zeta-potential for both polymers. The extent of zeta-potential decrease was more prominent in unmodified chitosan particles compared with EDTA-coupled complexes. Particles generated at polymer:DNA ratios as shown in Table 2 resulted in cationic polyplexes. The resulting positive charge is favorable for the interaction with the negatively charged proteoglycans on the cell surface to improve polyplex binding and uptake.²¹

Aggregation in aqueous solution was monitored by measurement of the complex size after 24 hours' storage at

room temperature. All samples prepared in aqueous solutions have a tendency to form aggregates. The speed of aggregation was different depending on the pH of complex formation. Despite the smaller initial size and more positive zeta-potential, complexes produced at pH 4 revealed a more pronounced increase of mean size of plus 117.9% and 160.1% for chitosan complexes and chitosan-EDTA complexes, while at pH 5, the corresponding increases were 65.6% and 40.2%, respectively.

Electron microscopic images confirmed the nano-scaled size of chitosan/pDNA and chitosan-EDTA/pDNA complexes. Furthermore, they revealed a tendency to aggregation. After 14 hours of incubation, the recorded images of chitosan-EDTA/DNA complexes produced from CE 4 displayed an aggregated morphology and irregular shape. Aggregates sized approximately 100 to 250 nm were composed of smaller fragments with a size of approximately 50 nm.

Stability and release studies

Complex integrity and binding capacity in different solutions was investigated by electrophoretic mobility assay in agarose gel. In Figure 3 the disintegration of complexes at different pH values is demonstrated. Particles consisting of unmodified chitosan started to disintegrate at pH 8, showing a prominent band of released pDNA. While chitosan nanoparticles released $20.8\% \pm 6.8\%$ of DNA in 30-minute incubation at pH 8, the chitosan-EDTA nanoparticles only released $6.8\% \pm 3.4\%$. Apparently, the critical pH value for accelerated release of DNA was increased by the cross-linking modification of chitosan.

The higher stability of complexes using chitosan-EDTA was also evidenced by release profile in MEM medium, pH 7.4 (Figure 4). The amount of pDNA just loosely attached to the surface was released comparatively quickly. Consequently, the release profile showed a burst release within 30 minutes followed by a phase of slow sustained release. The release of control chitosan particles and chitosan-EDTA particles displayed comparable shapes. The difference was a little slower release in EDTA-modified chitosan complexes,

Table 2. Nanoparticle characterization. Particle size and zeta potential of nanocomplexes were measured by dynamic light scattering. Indicated values are means of at least 3 experiments ($\pm$ SD)

Polymer	Weight Ratio DNA: Polymer	pH	Mean Size, nm	ζ -potential, mV	Mean Size After 24 h, nm
Low-viscosity chitosan	1:2.5	4	26.8 ± 10.3	7.31 ± 1.7	$58.4 \pm 13.5^*$
Low-viscosity chitosan	1:2.5	5	88.0 ± 21.9	6.54 ± 1.5	$145.7 \pm 50.0^*$
CE 4	1:12	4	43.1 ± 21.4	6.33 ± 0.6	$112.1 \pm 61.2^*$
CE 4	1:12	5	77.6 ± 17.8	5.93 ± 0.1	108.8 ± 25.6

* $P < .05$.

Figure 3. Gel retardation assay in $1 \times$ TAE -agarose gel electrophoresis after 30-minute incubation at different pH values as indicated at the top of the lane. u indicates unchanged pH after particle preparation resulting in a pH of about 4.5. Bands in the gel were representing released pDNA, while complexed DNA was unable to leave the pockets.

apparently caused by the higher stability at pH 8 to 9. Since free pDNA possesses poor transfection efficiency, a low release in media would be favorable. Complexation decreases the size of nucleic acid molecules and neutralizes the negative net charge. Therefore, complexation facilitates

Figure 4. Release kinetics of pDNA in MEM cell culture medium, pH 7.4. Particle suspensions were mixed with medium at a ratio 1:2.85 and incubated at 37°C 400 rpm for the indicated time periods. Withdrawn samples were immediately analyzed via electrophoresis. Quantification of released DNA was done with ethidium bromide fluorescence measurement of the cut gel slice. Indicated values are means ($\pm$ SD) of 4 samples. *Significance level of $P < .05$.

the uptake into cells. Release of DNA before its internalization reduces the amount of DNA reaching the target.

The stability against enzyme was assessed via DNaseI digestion. Degradation of pDNA at pH 6 revealed a slightly better protection by unmodified chitosan (data not shown). DNaseI enzyme reaches the maximum of activity at pH 8. Neither chitosan nor chitosan-EDTA nanoparticles were capable of protecting against 1 unit DNaseI at pH 8 (data not shown). The compactness of particles formed by unmodified chitosan was thought to be higher compared with chitosan-EDTA polymer. In chitosan-EDTA/pDNA complexes, the partly negative charge and the sterical hindrance of side chains prevent tight binding. Pores in the complexes could allow enzyme to get access to inner parts of the complex, destroying them more quickly than by just access to the surface.

Cytotoxicity test

Cytotoxicity on Caco-2 cells was investigated by testing membrane integrity via the release of intracellular LDH. The effects of chitosan and modified chitosan polymer as well as chitosan particles and chitosan-EDTA particles were investigated. The measured cytotoxicity was low for all samples, polymers, and nanoparticles, respectively, as shown in Figure 5. Transfection with chitosan-EDTA complexes resulted in slightly higher amount of active LDH in supernatant after 2 hours than after 4 hours of exposure time. Chitosan-EDTA not incorporated in nanoparticles had no effect on LDH activity. Unmodified chitosan complexes caused comparatively higher but still moderate membrane damage, which did not change significantly between 2 and 4 hours of exposure time. Microscopic examinations during and after transfection studies revealed no changes in cell density and morphology indicating no severe damage over a time period of 14 hours of exposure.

Figure 5. LDH assay on Caco-2 cell culture with 260- μ L nanoparticle suspension (10 μ g pDNA) plus 740 μ L MEM medium and 0.005% polymer solution in MEM medium, respectively. Indicated values are means ($\pm$ SD) of 3 experiments. *Significance level of $P < .05$.

Table 3. Beta-galactosidase activity after transfection with different treatments, as determined by the difference in optical density (OD) between 420 and 600 nm, and after correction for protein content in lysate

Sample	Relative β -Galactosidase Activity, OD420-OD600	β -Galactosidase Activity, U/mg Lysate
pDNA	0.001 $\pm$ 0.004	6.38 $\pm$ 0.77
Chitosan NP	0.011 $\pm$ 0.003	31.34 $\pm$ 15.08
Chitosan-EDTA NP	0.025 $\pm$ 0.009*	48.04 $\pm$ 10.39*
Chitosan NP + EDTA	0.011 $\pm$ 0.001	not analyzed

*Significance level of $P < .05$ in comparison to chitosan/pDNA complexes. pDNA indicates plasmid DNA; NP, nanoparticles.

Transfection studies

The amount of beta-galactosidase expression after 14 hours of transfection with pure DNA, nanoparticles with unmodified chitosan, and nanoparticles with chitosan-EDTA were compared (Table 3). Chitosan-EDTA nanoparticles showed a significantly higher level of reporter gene expression than control chitosan nanoparticles. Pure pDNA resulted in very low transfection efficiency, since the utilized Caco-2 cell line is rather hard to transfect. Further, the presence of 20% serum in the transfection medium reduced the amount of active pDNA.

To test if the chelating agent EDTA was able to enhance transfection at the concentration present on the polymer, a control with unbound EDTA and control chitosan particles was performed. The addition of 1 mM EDTA to chitosan complexes did not change the transfection efficiency. Taken together with the data of the toxicity test we concluded that the higher transfection was not due to some damage of the cell membrane. The chelating properties of the EDTA, as such, were unlikely to be the reason for transfection enhancement since the chelating properties of the free EDTA were stronger when compared with immobilized EDTA.

The transfection results expressed as relative beta-galactosidase activity/mg lysate are listed in Table 3. Lysates of chitosan particles transfected cells contained a 3.98% lower amount of protein compared with untransfected cells. Reduced toxicity of chitosan-EDTA nanoparticles could be seen by less protein content reduction (-1.08%). Accordingly, the correction for protein content decreased the difference in transfection efficacy between unmodified and EDTA-modified nanoparticles. Nevertheless the beta-galactosidase activity/mg of chitosan-EDTA particles was 35% higher, a significant improvement in comparison to particles of unmodified chitosan.

The transfection with GFP plasmid complexed with chitosan-EDTA gave a dose-dependent result with higher amount of pDNA yielding in more transgene expression. The relative fluorescence observed after transfection of 2.5, 5.0, and 10.0 μg chitosan-EDTA/pDNA complexes were 604 ± 293 , $1.59 \pm 1.48 (\times 10^3)$ and $3.40 \pm 0.37 (\times 10^3)$ relative fluorescence units, respectively.

The physicochemical properties like DNA condensation, size, and zeta potential were in a comparable range and therefore not likely to provoke the difference. We propose that the apparently increased transfection efficiency was caused by the higher stability in medium pH 7.4 as demonstrated in the release experiment.

CONCLUSION

The described method is suitable to prepare cationic nanoparticles capable of effectively complexing pDNA. The main advantage seems to be the enhanced transfection efficiency. Moreover, the cytotoxicity of the chitosan-EDTA nanoparticle suspension is lower than that of unmodified chitosan nanoparticles. Covalent cross-linking of the polymer led to stabilization of complexes at moderate alkaline pH and thus resulted in a slower release in culture media.

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