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International Journal of Pharmaceutics 225 (2001) 97–111

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Cationized human serum albumin as a non-viral vector system for gene delivery? Characterization of complex formation with plasmid DNA and transfection efficiency

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Received 5 February 2001; received in revised form 23 February 2001; accepted 4 June 2001

Abstract

Cationized human serum albumin (cHSA) could serve as a potential non-viral vector system for gene delivery. Native human serum albumin was cationized by covalent coupling of hexamethylenediamine to the carboxyl groups resulting in a shift of the isoelectric point from pH 4–5 to 7–9. The cationized albumin underwent spontaneous self-assembly with DNA as demonstrated by retardation of CMV-nlacZ plasmid in agarose gel electrophoresis. Photon correlation spectroscopy showed a decrease of complex size with increasing cHSA/plasmid ratios. Under optimized conditions complexes were formed with 230–260 nm mean diameter and a homogenous, narrow size distribution. At room temperature complexes were stable in 0.9% sodium chloride solution pH 7.4 for 1 h without aggregation. Process parameters such as albumin concentration, incubation time, temperature, pH, order of reagent addition, the presence of bivalent ions and the ionic strength of the complexation medium all influenced the complex size. Confocal laser scanning microscopy showed interactions of a Texas Red labeled cationized albumin with cell membranes of ECV 304 cells and an enhanced endocytic uptake compared to native albumin. The potential for introducing exogeneous DNA into cells was shown using NIH 3T3 fibroblasts. Successful, albeit low reporter gene expression could be achieved in the presence of chloroquine. Under in vitro conditions no toxic effect could be observed. In conclusion, cationized albumin may have promise as a non-toxic vector for gene delivery, especially for DNA vaccination.

Keywords: Cationized human serum albumin; Gene transfer; Confocal laser scanning microscopy; Absorptive endocytosis

1. Introduction

Many reports describe the local and systemic administration of naked DNA resulting in transgene expression levels that are insignificant or too

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0378-5173/01/$ - see front matter
PII: S0378-5173(01)00765-7

low for clinical applications (Mulligan, 1993; Ledley, 1995). The high net negative charge density and a hydrodynamic diameter of about 100–300 nm leads to opsonization of plasmids and clearance from circulation by the reticuloendothelial system. Also uptake by their target cells is an unlikely event due to electrostatic repulsion with the cell membranes (Ledley, 1996). Naked plasmid DNA could successfully be transferred by direct injection into skeletal muscles of rodents and primates (Wolff et al., 1990; Shiver et al., 1996). Gene expression has been shown to last for several months, but generally at relatively low levels (Tomlinson and Rolland, 1996), since the p-DNA was degraded by nucleases and removed from muscle. Diffusion from the site of injection was low. Improved cellular uptake and tissue dispersion after i.m. injection were achieved by complexation of DNA with the synthetic polymers polyvinyl pyrrolidone and polyvinyl alcohol (Mumper et al., 1996).

The administration of DNA into muscle could be therapeutically useful both for local treatment of diseases as well as depot for systemic protein delivery, e.g. for vaccination purposes (Corr et al., 1996). Controlled release of DNA, continuous transgene expression and the protection of the genetic material from enzymatic degradation over longer periods are important prerequisites for the use of polymers as non-viral carriers for plasmids and oligonucleotides. Additionally, to allow prolonged residence times of the devices in the tissue, the materials need to be safe and biocompatible. Nanoparticles, microparticles and devices made of biodegradable and non-biodegradable polymers of synthetic or natural origin (gelatine, collagen) have been investigated as sustained-release delivery systems (Labhasetwar and Levy, 1996). Controlled delivery of plasmid and continuous transfection for at least 60 days could be achieved using an atelocollagen matrix as delivery system (Ochiya et al., 1999).

Human serum albumin (HSA) (Peters, 1985) has been widely used as material for nano- and micro-particulate drug carrier systems (Roser and Kissel, 1993; Müller et al., 1996). Under physiological conditions albumin has a net negative charge and consequently HSA cannot react electrostatically with plasmids or oligonucleotides to form complexes for gene transfer. However, the charge of the native albumin can be modified by converting anionic side chain carboxylic groups with hexamethylenediamine (Hoare and Koshland, 1967; Kumagai et al., 1987), thereby producing a highly cationic derivative. Cationized human serum albumin (cHSA) produced by this simple and highly reproducible technique (Bass et al., 1990) demonstrated low cytotoxicity and good biocompatibility both in vitro and in vivo, as well as enzymatic biodegradability (Pardridge et al., 1990a; Choksakulnimitr et al., 1995; Fischer et al., 1997). Furthermore, cationized albumin contains many primary amino groups which can be employed for conjugation with lipids and ligands such as antibodies (Ouellette et al., 1996), viral proteins (Wagner et al., 1992a; Sedlacek et al., 1997), sugars (Midoux et al., 1993) and lectins (Monsigny et al., 1988). Modification of cHSA can induce tissue- and cell-specific targeting (Miller and Vile, 1995) or a fusogenic activity of the cHSA/DNA complexes (Wagner et al., 1992b). Huckett et al. (1990) used insulin-bound cationized albumin for targeted gene transfer by receptor-mediated endocytosis. By this approach they produced stable transfected HepG2 clones. However, a protocol for stable transfection was used which is not comparable with procedures necessary for in vitro and systemic or local in vivo gene transfer.

In the present study, we investigated the feasibility of cHSA as a non-viral vector system for in vitro gene transfer and focused our interest on the physicochemical aspects of complex formation and process parameters influencing the characteristics of cHSA/DNA complexes.

2. Materials and methods

2.1. Synthesis and characterization of cationized human serum albumin

Native human serum albumin (nHSA) was cationized by covalent coupling of hexamethylenediamine to carboxyl groups according to the method of Kumagai et al. (1987). Briefly, we

added 5 ml of a 20% (w/v) solution of nHSA (Hoechst Marion Roussel, Frankfurt/Main, Germany) in water to 67 ml of 2 M hexamethylenediamine (Fluka, Neu-Ulm, Germany) and the pH of the solution was adjusted to 7.8 with 1 N HCl. After stirring for 30 min at room temperature, 1 g 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Merck, Darmstadt, Germany) was added, and the pH readjusted to 7.8. The cationization was allowed to proceed at room temperature for 4 h. Excess reagent was removed by repeated centrifugation (3000 × *g*, 45 min, 20 °C) using Centriplus 30 concentrators (cutoff: 30,000 g/mol, Millipore, Eschborn, Germany). The final solution of cHSA was lyophilized and stored in a desiccator over silica gel at 4 °C.

The molecular mass and the isoelectric point of nHSA and cHSA were determined by SDS-PAGE and isoelectric focussing using the PhastSystem flat bed electrophoresis unit (Pharmacia, Freiburg, Germany). Separations were carried out according to standard protocols (PhastSystem Separation Technique File No. 100 and No. 110, Pharmacia). The gels were fixed, Coomassie blue stained and preserved as detailed in PhastSystem Development Technique File No. 200. The molecular mass of the albumins was calculated in comparison with the Pharmacia Calibration Kits (Pharmacia).

The cationization procedure resulted in the substitution of carboxylic groups by hexamethylenediamide groups (Fig. 1). cHSA was readily soluble in water. The molecular weights (67,000 g/mol) of nHSA and cHSA were not different according to their mobility in SDS-PAGE. High molecular weight aggregates or low molecular weight fragments could not be detected. The isoelectric point was shifted from pH 4.58–4.89 (nHSA) to 7–9 (cHSA) by cationization (data not shown).

2.2. Preparation of plasmid

pCMV-nlacZ plasmid contains the bacterial lacZ gene preceded by a nuclear localization signal under control of the CMV promotor/enhancer. The plasmids were replicated in *E. coli*, isolated by alkaline lysis followed by anion exchange chromatography using Qiagen tip 500 according to the manufacturers protocol (Qiagen, Hilden, Germany). Purity of the plasmid and the integrity of the cDNA insert were determined by agarose gel electrophoresis and UV spectroscopy (E 260 nm/280 nm ratio).

2.3. Synthesis and characterization of Texas Red labeled albumin

The bovine albumin-sulforhodamine 101 acid conjugate (Texas Red-nBSA) was purchased from Sigma (Deisenhofen, Germany). According to the manufacturer's specification, the labeled albumin contained approximately 2–3 mole sulforhodamine 101 acid chloride per mol protein.

Five milligrams cHSA were dissolved in 1 ml 0.1 M sodium carbonate pH 9. One milligram Texas Red sulfonyl chloride (Pierce, KMF Laborchemie Handels GmbH, St. Augustin, Germany) in 100 μl acetonitrile was added to the polymer solution in the dark. To enhance protein coupling relative to hydrolysis, protein solution must be kept on ice. After 1 h stirring at room temperature, excess fluorophore and reaction by-products

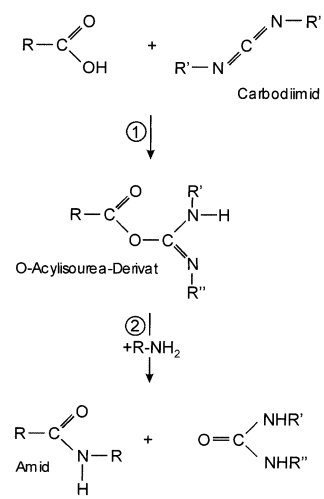

Fig. 1. Cationization of nHSA by modification of the carboxylate groups with a diamine. Initially, the *N*-substituted carbodiimide reacts with the carboxylic acids to form a highly reactive, short-lived *O*-acylisourea derivative (step 1). The following nucleophilic attack of the primary group results in the formation of an amide bond and the release of the carbodiimide as isourea derivative (step 2).

were removed by centrifugation ($3000 \times g$, 30 min, 20 °C) using Centricon-30 concentrators (cutoff: 30,000 g/mol, Millipore) as described above. The coupling ratio with cHSA (F/P ratio) was determined by measuring the absorbance of the labeled protein at 520 and 280 nm. Molecular mass, isoelectric point and DNA-binding were determined as described above.

2.4. Preparation of plasmid/cHSA complexes

5.73 μg CMV-nlacZ plasmid were equilibrated in 350 μl 0.9% sodium chloride solution (NaCl) pH 7.4 at room temperature for 10 min. Eighty three milligram of cHSA were dissolved in 9 ml 0.9% NaCl, adjusted to pH 7.4 with 1 N HCl, brought to a final volume of 10 ml and sterile filtered (0.2 μm, Schleicher and Schuell, Dassel, Germany). Two hundred and fifty microliter of the cHSA solution were rapidly pipetted to the plasmid solution. The mixture was allowed to equilibrate for further 5 min at room temperature, before the complexes were used for analysis. For all physicochemical studies 5.73 μg plasmid and 0.9% sodium chloride pH 7.4 as complexation medium were used, except where otherwise stated.

2.5. Agarose gel electrophoresis

Fifty microliter aliquots of the complex solution were mixed with 5 μl loading buffer consisting of 50% (v/v) glycerol, 1 mM EDTA and 40 mM Tris-base pH 7.4 (Gibco, Eggenstein, Germany) and loaded onto a 1% agarose gel (Roth GmbH, Karlsruhe, Germany). Electrophoresis (Blue Marine 200, Serva, Heidelberg, Germany) was carried out with a current of 80 V (LKB 2197 Power Supply, Pharmacia) for 2 h in TAE running buffer solution (40 mM Tris-base, 1% (v/v) acetic acid, 1 mM EDTA). Eight microliter 1% (w/v) ethidium bromide solution (Roth GmbH) were included into the gel to visualize the localization of the DNA by UV transillumination (TC-254A Transilluminator, 254 nm). Gels were documented directly after electrophoresis using a Video Copy Processor P67E (Mitsubishi). In a subsequent staining step the protein bands were detected by staining with Coomassie blue. The gel was incubated with a solution of 1 g Serva Blue (Serva) in a mixture of 225 ml 96% ethanol, 225 ml distilled water and 50 ml glacial acetic acid at room temperature for 3–5 min. Excess Coomassie dye was removed from the gel matrix using a 7.5% (v/v) acetic acid solution in distilled water leaving the dark blue bands against a clear background. For the destaining procedure the agarose gel was shaken overnight in a horizontal shaker at room temperature and detected photographically.

2.6. Particle size determination by photon correlation spectroscopy

Measurements of size and polydispersity indices with cHSA/DNA complexes in 0.9% NaCl, pH 7.4 were performed by photon correlation spectroscopy (PCS) at 25 °C using the Malvern Zetasizer 4 (Malvern Instruments, Herrsching, Germany) with a nominal 5 mW helium neon laser and the Malvern software version 1.26, 1992. Samples were measured in the AZ 110 cell at 633 nm and a scattering angle of 90°. For data analysis, the viscosity (0.88 mPa s) and the refractive index (1.33) of distilled water at 25 °C were used. All measurements were carried out at least in triplicate. The instrument was routinely calibrated using Standard Reference latex particles (AZ 55 Electrophoresis Standard Kit, Malvern Instruments) at 25 °C. Measured data were in good agreement with the nominal values. The mean diameter of the free plasmids was measured with a Autosizer LoC (15 mW, 633 nm, Malvern Instruments) in 0.9% NaCl, pH 7.4, since the laser power of the Zetasizer 4 was insufficient to detect the optical transparent plasmid DNA.

2.7. Confocal laser scanning microscopy

ECV 304 cells from human urinary bladder carcinoma (ATCC, Rockville, MD) were cultured in Lab-Tek chamber slides (Nunc, Wiesbaden, Germany) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 5% horse serum and 1% *N*-acetyl-L-alanyl-L-glutamine (all from Gibco) at 37 °C, 95% relative humidity and 5% CO_2. After 24 h cells were washed with phosphate buffered saline (PBS) pH

7.4 and incubated with 150 μg/ml native or cationized albumin complexed with 3.3 μg pSV-β-Gal plasmid in DMEM for 0–60 min at 37 °C. Cells were rinsed eight times with ice-cold PBS, fixed with a freshly prepared solution of 4% paraformaldehyde in PBS for 30 min at room temperature and washed again. Excess paraformaldehyde was removed by 50 mM ammonium chloride solution for 30 min. Cells were mounted on slides with Slow Fade Antifade (Molecular Probes, Leiden, Netherlands) for microscopic analysis. Confocal microscopic images were acquired on a Zeiss confocal laser scanning microscope (LSM 410 invert, Zeiss, Jena, Germany) using 543 nm laser light for excitation and a 570 nm long pass emission filter.

2.8. Transfection experiments

NIH 3T3 mouse fibroblasts (ATCC, Rockville, MD) were used growing in DMEM (Gibco) supplemented with 10% FCS (Gibco) without antibiotics at 37 °C, 5% CO_2 and 89% relative humidity. Twenty four hours before transfection, 200000 cells were seeded in gelatine-coated 8.8 cm^2 tissue culture plates (Nunc). The medium was removed and the monolayer was washed with PBS pH 7.4 shortly before transfection. Cells were incubated with the CMV-nlacZ/albumin complexes (10 μg plasmid/8.8 cm^2 dish) for 1 h without serum in 150 mM NaCl/10 mM HEPES pH 7.4. Afterwards cells were treated with 100 μM chloroquine in DMEM with 10% FCS for further 3 h. Cells were washed and reincubated in the presence of 10% serum in DMEM for additional 48 h. Expression of the CMV-nlacZ gene was assessed in intact cells by an enzymatic cleavage reaction of a chromogenic substrate. Cells were washed with PBS pH 7.4 and fixed with 0.1% (v/v) glutaraldehyde in PBS for 10 min. The cells were washed again with PBS and subsequently incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as substrate for at least 12 h. The substrate solution consisted of 0.08% mg/ml X-Gal stock solution (2% in dimethylformamide) (Sigma), 3 mM $K_3Fe(CN)_6$ and 3 mM $K_4Fe(CN)_6$ in PBS. After a final wash with PBS, cells were fixed again with 3% (v/v) formaldehyde and examined by phase contrast microscopy (Nikon TMS). Cells actively expressing β-galactosidase were identified by their intensive blue staining. Cultures treated only with plasmids served as control. The degree of expression was given as the percentage of blue cells from all cells per 8.8 cm^2 petri dish.

Table 1
Influence of different process parameters on the size of plasmid/cHSA complexes measured by PCS

Process parameter	Complex size (nm) ± S.D.
Velocity of adding albumin	
50 μl/10 s	Aggregation
50 μl/1 s	370.73 ± 34.50
At once	213.98 ± 7.46
pH of complexation medium	
pH 4	520.51 ± 77.25
pH 7.4	456.98 ± 37.48
pH 9	No complex formation

Complexes were prepared according to the standard protocol described in Section 2. Results are expressed as mean values (± S.D.) of three independent experiments.

3. Results

3.1. Complex formation

CMV-nlacZ plasmid was complexed with cHSA under different conditions as indicated in Table 1. To determine whether the DNA remained intact during the complexation, the complexation was carried out using DNA without albumin. With agarose gel electrophoresis no degradation, damage or fractionation of the plasmid could be detected (data not shown).

The order of adding the components strongly influenced the characteristics of the resulting complexes. Adding the plasmid to the polycation solution led to irreversible aggregation, whereas addition of cHSA to plasmid resulted in opalescent, homogeneous preparations. Continuous, very slow stirring facilitated the formation of monodispersly distributed particles (data not shown). To estimate the relationship between complex size and the velocity of reagent addition,

250 μl cHSA solution were pipetted to the slowly stirred plasmid solution (5.73 μg) for five consecutive times in 50 s (50 μl/10 s), 5 s (50 μl/1 s) and completely in one portion. At faster addition of cHSA smaller complex sizes were obtained (Table 1).

The volume of the complexation medium for 1 μg plasmid was varied from 5 μl up to 1 ml. Whereas complex formation in small volumes resulted in strongly aggregated particles, preparations in larger volumes led to an average size of 230–260 nm. The preparation of DNA/cHSA particles was carried out at pH 4.0, 7.4 and 9.0. At pH 4 complex formation occurred, and the mean diameter of complexes did not differ significantly from complexes formed at pH 7.4. At pH 9.0 no interactions between the cationic protein and the negatively charged polynucleotides could be detected (Table 1). Due to the isoelectric region of 7–9 most amino residues of the cHSA are not protonated at pH 9 and cannot interact electrostatically with the polynucleotides. Finally, we tested the temperature dependency on DNA/cHSA complexation. Whereas at 25 °C the complex formation occurred within 2–3 min indicated by opalescence, no opalescence or aggregation could be observed at 4 °C after 30 min. When these mixtures were warmed to 25 °C, interaction between the components started slowly (data not shown) after 10–15 min as indicated by the formation of opalescence. Complex sizes measured after 30 min by PCS were polydispersly distributed in the range from 320 to 950 μm.

3.2. Agarose gel electrophoresis

The interaction between cHSA and DNA was analyzed by electrophoresis on an agarose gel containing ethidium bromide. Additionally, gels were stained with Coomassie blue to visualize the localization of both complex components (Fig. 2). Complexes of DNA and various amounts of cHSA were prepared in 0.9% NaCl pH 7.4 as described in Section 2. The analysis of the free expression vector (lane 8a) revealed two fluorescent bands corresponding to the supercoiled and nicked circular forms of the plasmid. At 0.25 μg cHSA/μg plasmid (lane 7a) and 2.5 μg cHSA/μg plasmid (lane 6a) a fraction of plasmid was still migrating in the gel in the same way as the non-complexed DNA. Simultaneously, retention of the complex at the origin could be observed. The ability of cHSA to immobilize the plasmid on gel suggests that the cHSA/DNA complexes were too large to migrate into the gel and/or less negatively charged than free DNA. Total retention of the plasmid was observed at $\geqslant 12.5$ μg cHSA/μg plasmid (lane 1–5a). However, a complete inaccessibility of complexed DNA to ethidium bromide due to the exclusion of the intercalant dye could not be achieved even at higher cHSA concentrations (Fig. 2(b)). The detection of the protein by Coomassie blue staining demonstrated that preparations up to 50 μg cHSA/μg DNA are oriented to the anode and therefore, carried a net negative charge (lane 1b, 2c). Using higher cHSA concentrations (lanes 2–4b, 3–5c) orientation of the complexes to the cathode could be observed suggesting that the cationic protein masked the anionic nature of the DNA completely.

3.3. Variation of cHSA concentration

The size of DNA/polycation complexes is an important parameter that should control not only the deposition and body distribution of complexes after their in vivo administration, but also their uptake by target cells. The effect of cHSA/DNA ratio on the complex size was studied by dynamic light scattering (Fig. 3). The cHSA concentration was increased from 36.17 μg to 720 μg/μg DNA, while the plasmid concentration was kept constant. Naked CMV-nlacZ plasmid in 0.9% NaCl showed a mean diameter of 103 nm representing the supercoiled form. At 36.17 μg cHSA/μg DNA large particles with an average diameter of 1829.51 nm ($\pm$ 940.69 nm) and a polydisperse size distribution were obtained. An increase in the amount of cHSA caused a continuous decrease of the complex size reaching a constant value of about 260 nm at 220–360 μg cHSA/μg DNA (Fig. 3(a)). The size distribution was rather homogeneous as demonstrated by the polydispersity index (Fig. 3(b)). From electrophoresis results, these complexes had a net positive charge prevent-

ing particles from aggregation by charge–charge-repulsion. The polydispersity decreased with increasing cHSA concentration, also increasing the homogenicity of the preparations (Fig. 3(b)). At >720 μg cHSA/μg plasmid the complex size showed a tendency to increase again. Macroscopically, rapidly sedimenting aggregates were formed, too large for adequate size determination by PCS.

3.4. Time dependent stability of the complexes

Plasmid/cHSA complexes were incubated in 0.9% NaCl pH 7.4 for 5, 30, 60, 120 and 240 min at room temperature to test the stability over time (Fig. 4). As measured by PCS the plasmid/albumin complexes were found to be stable up to 1 h without aggregation or sedimentation. The mean diameter of the complexes was maintained at

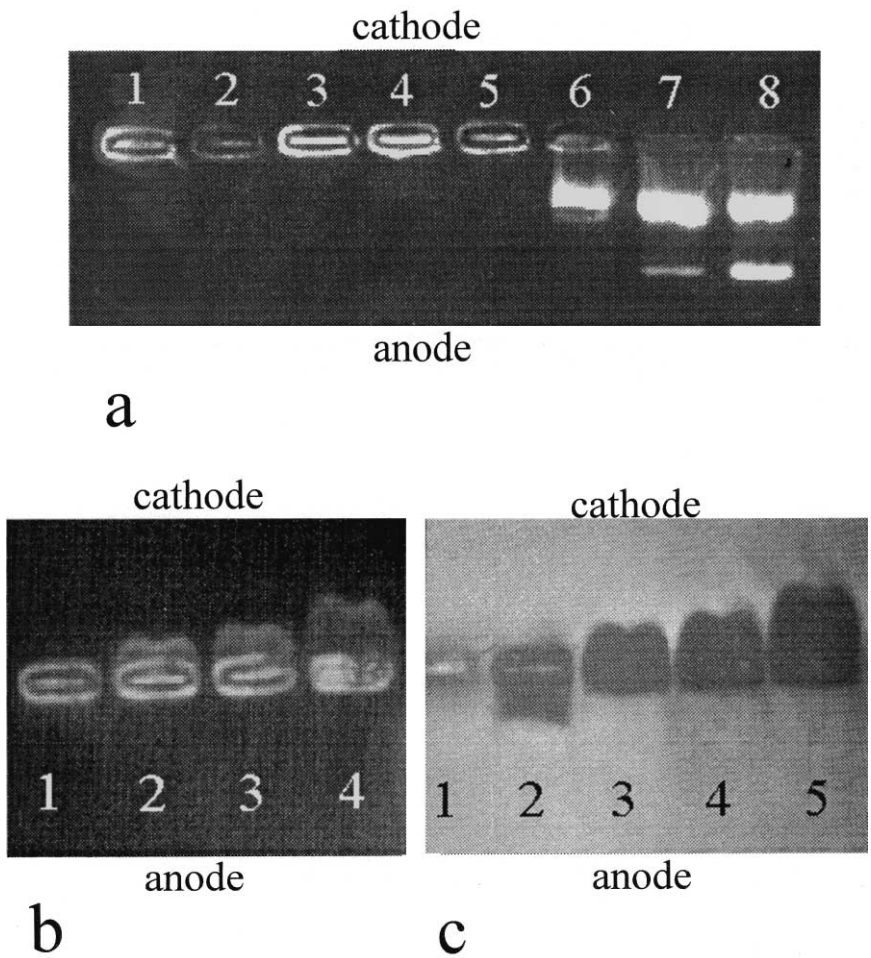

Fig. 2. Effect of increasing cHSA concentrations on the electrophoretic migration of CMV-nlacZ plasmid through an 1% agarose gel. Increasing amounts of cHSA were added as indicated to a constant amount of plasmid as described under Section 2. (a) As detected by ethidium bromide staining, total retention of DNA could be observed at $\geqslant 12.5$ μg cHSA/μg DNA, but no complete condensation up to 70 μg cHSA/μg plasmid: Lanes: (1–7) 1 μg CMV-nlacZ/70, 60, 50, 37.5, 12.5, 2.5, 0.25 μg cHSA, (8) CMV-nlacZ (no protein). (b) Even at higher amounts cHSA no ethidium bromide exclusion indicating condensation of the plasmid could be detected: Lanes (1–4) 1 μg DNA/36.17, 110, 220, 360 μg cHSA. (c) Afterwards the same gel was stained with Coomassie Blue, indicating a partial orientation of the complexes to the cathode: Lanes (1) non-complexed DNA, (2–5) 1 μg DNA/36.17, 110, 220, 360 μg cHSA.

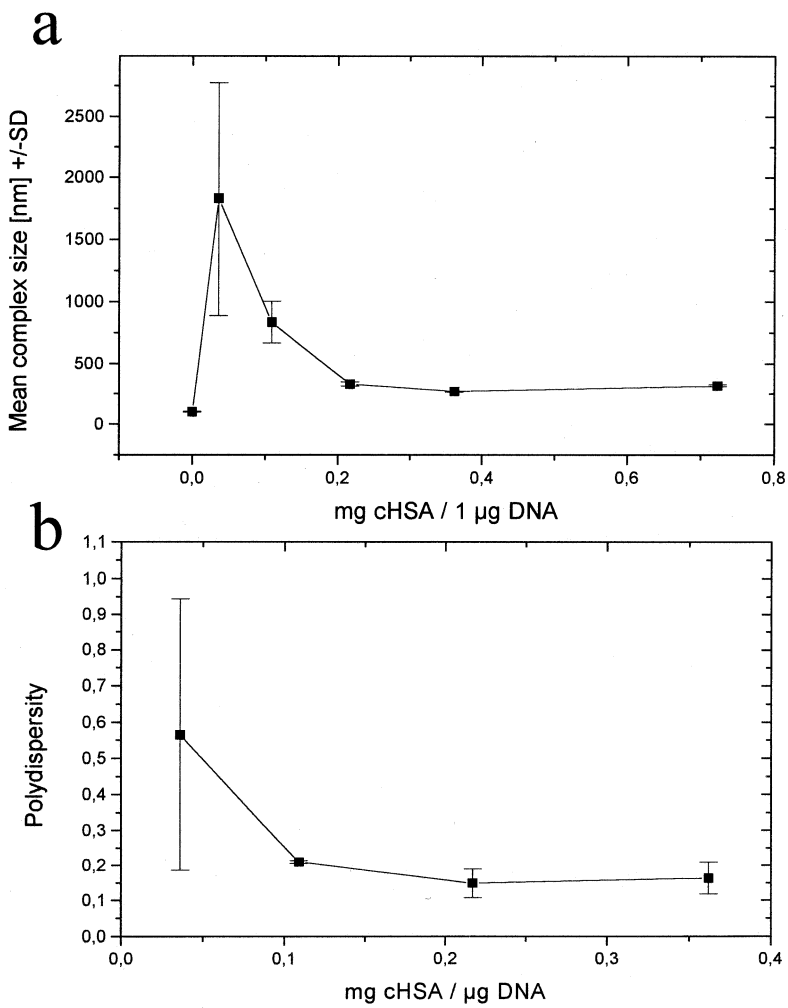

Fig. 3. (a) Mean size and (b) polydispersity of cHSA/CMV-nlacZ complexes as determined by PCS at a fixed plasmid concentration and varying cHSA concentration. Complexes were prepared in 0.9% sodium chloride solution pH 7.4 as described under Section 2. Results are expressed as mean values (± S.D.) of three experiments.

250–270 nm with a narrow size distribution. A substantial increase in size was found with longer incubation times (120–240 min), transforming the initially homogenous, opalescent mixture to a cloudy, non-homogeneous suspension with macroscopically visible aggregates. This observation could be confirmed by the PCS measurements, detecting highly variable mean diameters from 600 to 2400 nm after 4 h incubation time.

3.5. Influence of complexation medium on complex size and stability

Complexes of plasmids with cationized albumin were prepared in bidistilled water, PBS with calcium and magnesium pH 7.4, 0.9% sodium chloride pH 7.4 and 150 mM NaCl/10 mM HEPES pH 7.4. Immediately after addition of the polycations to the DNA solution, in all cases no clouding could be observed. After 3–5 min all solutions

became slightly turbid. After 30 min PCS size measurements displayed complex dimensions between 200 and 350 nm (Fig. 5). In contrast, complexes formulated in PBS with calcium and magnesium showed a slightly increased size after 30 min and an intensive aggregation and sedimentation after 1 h probably caused by electrostatic interactions of the components with bivalent ions (data not shown). Complexes prepared in bidistilled water, which were characterized by low sizes after 30 min, displayed also aggregation after 1 h. In contrast, complexes in 0.9% NaCl or 150 mM NaCl/10 mM HEPES remained stable without aggregation. After 4 h all solutions formed large macroscopically visible complexes.

Therefore, the following experiments were carried out in 0.9% sodium chloride pH 7.4 or 150 mM NaCl/10 mM HEPES. Complexes were used for physicochemical and in vitro studies 5 min after the addition of the cHSA solution to the plasmid, which had been equilibrated in the solvent for 10 min at room temperature.

3.6. Efficiency of cell transfection

NIH 3T3 mouse fibroblasts were exposed to DNA/cHSA complexes in 150 mM NaCl/10 mM HEPES and incubated for 1 h. The selection of the DNA/albumin ratio of the complexes were based on the results of the physicochemical experiments. Complexes characterized by a small complex size and orientation to the cathode in electrophoresis experiments were used. Since in preliminary studies no significant expression of the reporter gene could be observed, cells were treated with chloroquine to facilitate the release of the complexes from lysosomes. Since, the plasmid contained the reporter gene for β-galactosidase under the control of the cytomegalovirus promotor, transfected cells were visualized by staining with the chromogenic substrate X-Gal. The analysis by light microscopy revealed transfection rates $\leqslant 1\%$ (Fig. 6), which were comparable with data using DEAE-dextran (data not shown). No change in cell morphology and viability could be observed by microscopic observations. Cells treated only with DNA, which were used, as control showed no blue staining.

3.7. Fluorescent labeling of albumin with Texas Red

Texas Red sulfonyl chloride rapidly reacted with the albumin's amino groups to form stable sulfonamide bonds. Excess fluorophore hydrolyzed in the presence of moisture and was removed by centrifugation. The extent of conjugation was described as F/P ratio. Titus et al. (1982)

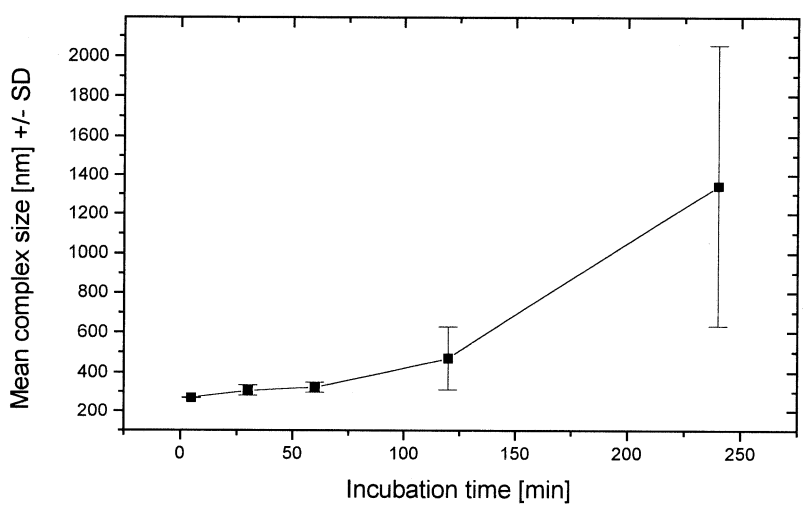

Fig. 4. Mean particle size of cHSA/CMV-nlacZ complexes as function of the incubation time. Mean values ($\pm$S.D.) of three independent experiments are given. Complexes were prepared in 0.9% sodium chloride solution pH 7.4 according to the protocol described in Section 2 and stored at room temperature for 5, 30, 60, 120 and 240 min.

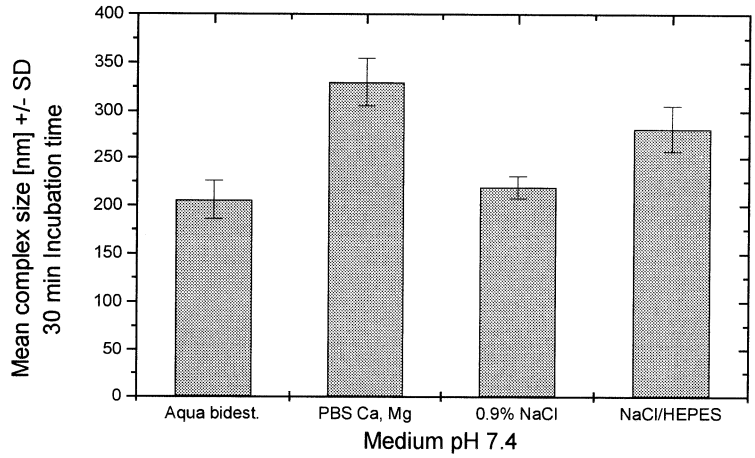

Fig. 5. Mean complex diameter (± S.D.) of cHSA/CMV-nlacZ complexes measured by PCS. Complexes were prepared in different complexation media as indicated. The incubation time was 30 min. Results are mean values of three independent measurements.

reported that labeled proteins having a 520/280 nm ratio of absorbance of 0.3–0.8 should perform well in most applications. Commercially available TR-nBSA showed F/P ratios of about 0.32 in agreement with product specifications, corresponding to 3.1 moles fluorophore per mol albumin. The F/P ratio of our Texas Red conjugated cHSA was determined to be 0.35–0.41. As shown by SDS-PAGE and isoelectric focussing the molecular weight and the isoelectric point of cHSA were not changed by the covalent binding of the fluorescent marker (data not shown). The maxima of emission and excitation of Texas Red were not influenced by conjugation to albumin (data not shown). As determined by electrophoretic mobility shift assay, the marker did not change the DNA-binding characteristics of the cationized protein (data not shown).

3.8. Cell uptake experiments

ECV 304 cells were incubated with Texas Red labeled nBSA and cHSA for 5, 15, 30 and 60 min. The studies were carried out at 37 and 4 °C to distinguish between active and passive uptake mechanism. When cells were incubated with the DNA/albumin complexes at 4 °C, no intracellular fluorescence could be observed. The fluorescence at the surface of the cells after the 4 °C incubation was slightly increased using cHSA compared to nBSA, but in general compared to the following experiments negligible low (data not shown). In contrast, ECV 304 cells treated either with the fluorescent nBSA or cHSA complexed with plasmid at 37 °C showed an uptake of both proteins. After 5 min cells exhibited small fluorescent granules localized throughout the whole cytoplasm (Fig. 7(a)). With longer incubation times fluorescence intensity increased and appeared to be concentrated in discrete and larger perinuclear vesicles (Fig. 7(b) and (c)). The time dependent uptake profile and the cytosolic distribution of the fluorescent vesicles were similar for nBSA and cHSA. However, the granular fluorescence intensity was stronger with cHSA compared to nBSA, presumably due to electrostatic interactions of cHSA with the negatively charged cell membranes. This observation might be explained by an absorptive endocytic uptake mechanism for the internalization of the different albumins, as suggested by different authors (Kumagai et al., 1987; Vorbrodt and Trowbridge, 1991). Similarly, results were obtained with primary capillary endothelial cells from porcine brains (data not shown). Free, non-complexed cHSA was also strongly internalized by the cells and presented intracellular distribution pattern and fluorescence intensities comparable to the cHSA/plasmid complexes. However, as indicated by aggregates and highly fluorescent layers on the cell surface cHSA

showed a higher affinity to the plasma membranes compared to the complexes presumably due to the higher amount of free charges (data not shown).

4. Discussion

An ideal gene delivery vector system should (i) be easy to produce, (ii) compact the DNA into nanoscale structures and protect it from degradation, (iii) overcome biological barriers and (iv) be non-toxic and non-immunogenic. We were interested in the feasibility of cHSA as a non-viral vector system for plasmid DNA with regard to DNA-binding, physicochemical stability of the complexes, parameters influencing the complex characteristics and the transfection efficiency.

As demonstrated by gel electrophoresis, complexes between the negatively charged DNA and the cationized HSA were formed by spontaneous electrostatic self-assembly as known from other polycationic macromolecules used for gene transfer (Tang and Szoka, 1997). The complexation resulted in masking the anionic nature of the plasmids and should therefore facilitate interactions of the carriers with cell membranes as observed for free Texas Red labeled polyethylenimine by CLSM (Fischer et al., 1998). However, complete exclusion of ethidium bromide even at high cHSA/plasmid ratios could not be attained, indicating incomplete condensation of the plasmid. In contrast, other cationic transfection reagents like DOTMA (Behr, 1994), poly-L-lysine (Vitiello et al., 1996), methacrylate derivatives (Wolfert et al., 1996) and polyethylenimine (Fischer et al., 1999) demonstrated complete exclusion of ethidium bromide which was assumed to be an important indicator for the stability of the complexes against enzymatic and non-enzymatic attacks. The stability of our plasmid/cHSA complexes against nucleases, serum proteins and mechanic shear stress in correlation with the complex composition is currently under investigation. Preliminary results suggests a insufficient protection of DNA by the cationized albumin against DNase. However, we were able to demonstrate the stability of our complexes over 1 h without aggregation and sedimentation. In comparison, the size of poly((2-dimethylamino)ethyl methacrylate/plasmid complexes was found to increase fast with increasing incubation time (30–135 min) (Cherng et al., 1996).

The charge and dimension of our complexes can be varied by altering different process parameters. The smallest mean complex size of about 230–260 nm measured by PCS is comparable with the diameter of a wide variety of polycation/DNA complexes such as DOSPA/DOPE/DNA (165–231 nm) for lipofection (Felgner et al., 1994) and the complexes with polymethacrylate (150–200 nm) for polyfection (Cherng et al., 1996). However, 800 kDa

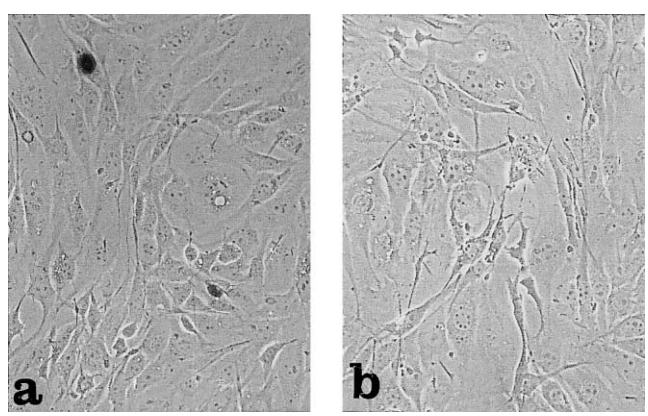

Fig. 6. (a) Phase contrast microscopy of β-galactosidase expression in NIH 3T3 mouse fibroblasts incubated with 10 μg DNA/3600 μg cHSA complexes per 8.8 cm^2 dish. (b) Control cells incubated with 10 μg plasmid/8.8 cm^2 dish.

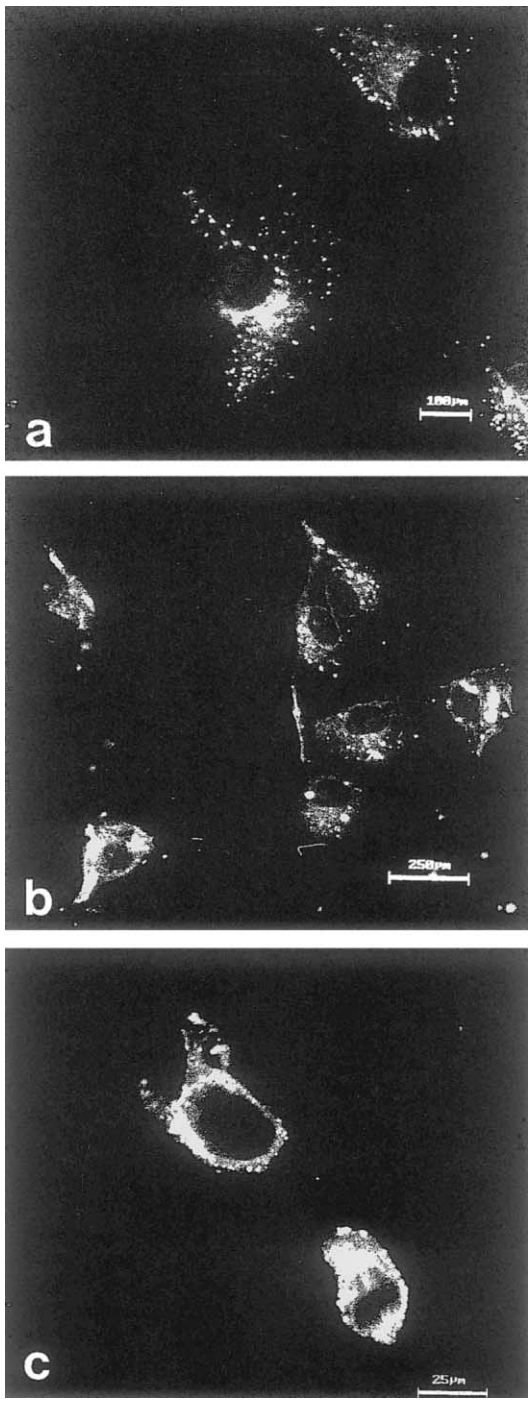

Fig. 7.

polyethylenimine, poly-L-lysine and dendrimers were found to form smaller toroidal or doughnut-like structures in the range of 30–100 nm (Tang and Szoka, 1997). Both electrophoretic shift assay and PCS measurements demonstrated that a high excess of cHSA was necessary to achieve small sized complexes. The results from agarose gel electrophoresis were in good agreement with the observations of Huckett et al. (1990) for insulin-cBSA. The low cationic charge density of the cationized albumin compared to polymers like polyethylenimine and poly-L-lysine, the non-flexible globular structure presumably hindering efficient interactions with DNA and only partial protonation of the amino groups at pH 7.4 could be responsible for these effects (Choksakulnimitr et al., 1995).

The potential of cationic albumin as a vehicle for peptide delivery has been shown by different authors (Pardridge et al., 1990a,b). As demonstrated by electron microscopy and quantitative uptake experiments the cationization of albumin resulted in enhanced binding and transport of the protein across cellular barriers (Vorbrodt and Trowbridge, 1991). Chimeric peptides formed by the covalent coupling of β-endorphin to cationic albumin underwent absorptive-mediated endocytosis into the brain (Kumagai et al., 1987). The transcytosis through the blood–brain barrier was mediated by electrostatic interactions with the negative charges lining the glycocalyx of the endothelium (Pardridge et al., 1985). We demonstrated the enhanced cellular uptake of plasmids complexed with the cationized albumin compared to the native form by CLSM confirming an endocytic uptake mechanism for cHSA according to the previously published observations. The intra-

Fig. 7. Confocal laser scanning microscopy of ECV 304 cells which had been incubated either with plasmid complexes with Texas Red labeled native bovine or cHSA at 37 °C. Incubations were performed with 150 μg/ml albumin for time periods indicated. cHSA was complexed with 3.3 μg pSV-β-Gal plasmid DNA. As shown for nBSA in (a) cells exhibited a pattern of small granular fluorescent structures distributed throughout the cytoplasm after 15 min of incubation. After 60 min larger granules appeared which accumulated in the perinuclear space (b, c). This was observed for (b) nBSA as well as for (c) cHSA. Usually, the fluorescence intensity of the granular structure was higher with cHSA, compared to nBSA.

cellular distribution and processing visualized by CLSM is comparable to the distribution pattern of 800 kDa polyethylenimine (Fischer et al., 1998). However, cytotoxic effects and membrane damage as noted for the high molecular weight polyethylenimine after 1 h incubation time could not be observed using cHSA. In vitro cytotoxicity studies using endothelial cells, primary macrophages (Choksakulnimitr et al., 1995) and mouse fibroblasts (Fischer et al., 1997) as well as in vivo experiments with rats (Pardridge et al., 1990a) also demonstrated the low cytotoxicity and good biocompatibility of cationized albumin compared to other polycationic transfection reagents such as poly-L-lysine, polyethylenimine and cationic methacrylate derivatives (Choksakulnimitr et al., 1995; Cherng et al., 1996; Fischer et al., 1997).

The transfection experiment was used to investigate the possibility of cHSA as gene vector system. Under our experimental conditions expression of the marker gene was possible, albeit to a low extent. One reason is presumably the relatively low complex release from endosomal compartments in the cell cytoplasm, since the lysosomotropic agent chloroquine increased the transfection efficiency, an effect which was also found for poly-L-lysine (Cotten et al., 1990) and methacrylate derivatives (Wolfert et al., 1996). Dendrimers and polyethylenimine exhibiting a considerably buffer capacity due to their order of amines, seem to inhibit the endosomal acidification after endocytosis of the complex (Tang and Szoka, 1997) and transfect cells without the addition of lysosomotropic agents. However, the minor increase of the transfer rate by chloroquine treatment suggests that the low efficiency can additionally be explained by other factors. The limited stability of the genetic material under physiological conditions could also be an important factor causing the low expression of the marker gene. Due to the incomplete condensation of the DNA by cHSA it is conceivable that the poor transfection rate may also be related to inadequate protection of plasmid from nucleases. Using cHSA/plasmid complexes cytotoxic effects could not be observed, in contrast to polyethylenimine (Fischer et al., 1999) and methacrylate derivatives (Cherng et al., 1996).

Although cHSA achieved only low transfection efficiencies, it has the potential to serve as a vehicle for the DNA and RNA delivery, especially with regard to its good biocompatibility. To improve the capability to express transgenes, further modifications are necessary and possible, whereby especially the limited stability and incomplete condensation seem to be a good starting point. As known from various liposomes the protection of the genetic material presumably could be increased by precondensation of the plasmids using polycationic polymers.

5. Conclusion

cHSA formed complexes with plasmid DNA leading to a particle size in the range of 250 nm. The low transfection efficiency, requiring chloroquine shock treatment, suggests that lysosomal escape and enzymatic protection are inferior to for example polyplexes based on polyethylenimine. The cytotoxicity profile of cHSA is, however, an attractive feature, which could be possibly exploited for plasmid DNA/cHSA complexes used for DNA vaccination.

Acknowledgements

Financial support of the project by Hoechst Marrion Roussell (Frankfurt a. M., Germany) is gratefully acknowledged. We thank Prof Dr H.H. Sedlacek for helpful discussions and the gift of human serum albumin.

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