Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmaceutics and Biopharmaceutics 59 (2005) 419–429
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Albumin–protamine–oligonucleotide nanoparticles as a new antisense delivery system. Part 1: Physicochemical characterization

Dirk Lochmann[a], Jörg Weyermann[a], Christiane Georgens[a], Ruth Prassl[b], Andreas Zimmer[c,*]

[a]*Institute for Pharmaceutical Technology, Johann Wolfgang Goethe-University, Frankfurt am Main, Germany*
[b]*Institute of Biophysics and X-ray Structure Research, Austrian Academy of Science, Graz, Austria*
[c]*Institute of Pharmaceutical Sciences, Karl-Franzens-University Graz, Austria*

Received 19 November 2003; accepted in revised form 2 April 2004

Available online 7 June 2004

Abstract

In this paper, a ternary system of albumin–protamine–oligonucleotide nanoparticles (AlPrO-NP) recently developed by Vogel et al. [V. Vogel, D. Lochmann, J. Weyermann, G. Mayer, C. Tziatzios, J.A. van den Broek, W. Haase, D. Wouters, U.S. Schubert, J. Kreuter, A. Zimmer, D. Schubert, Oliganucleotide–protamin–albumin nanoparticles: preparation, physical properties and intracellular processing, J. Controlled Rel. (in press)] [1] which could serve as a potential drug delivery system for antisense oligonucleotides. Former studies of binary protamine-oligonucleotide nanoparticles showed two main disadvantages: (i) aggregation of the particles within a few minutes in the presence of salt; (ii) low intracellular dissociation between protamine and oligonucleotide, especially phosphorothioates. To overcome these problems, human serum albumin (HSA) as a non-toxic, biodegradable macromolecule was introduced as protective colloid. The assembly process of AlPrO-NP was investigated by small angle X-ray scattering (SAXS), fluorescence correlation spectroscopy (FCS), photon correlation spectroscopy (PCS) measurements and scanning electron microscopy (SEM). 'Initial complexes' of HSA and protamine sulphate with a mean hydrodynamic diameter (d_h) of about 10–14 nm were found. After adding oligonucleotides (unmodified, phosphorothioate DNA and small interfering RNA), nanoparticles (NPs) were assembled in water and in isotonic media with a d_h in a range of 230–320 nm for most preparations. The chemical composition of the particles was investigated by high performance liquid chromatography and fluorescence spectrometry. The whole amount of oligonucleotides (30 μg) was entrapped into the particles at a 1:2 mass ratio (oligonucleotide/protamine). Approximately 7–10% (w/w) of the HSA was bound to the particles. The surface charge of the particles ranged from about +12 to −60 mV depending on the protamine concentration and the ionic conditions. The size and the molecular weight of the components, initial complexes and two model NP preparations were calculated from FCS data. These data verified the PCS, SEM and SAXS measurements.

Keywords: Nanoparticles; Albumin; Protamine; Oligonucleotides; Small interfering RNA; Antisense

1. Introduction

Studies on protamine started in the year 1868 by Friedrich Miescher [2]. A long period of research work was necessary to characterise the group of arginine rich, strongly basic, aliphatic peptides, present in the sperm cell nuclei of fish with a molecular mass of approximately 4000–6000 Da [3]. Now protamine and its salts are well established as a pharmaceutical excipient of sustained release formulations of insulin and further, it is used as stabilizer in vaccines. Additionally, protamine represents an antidote for heparin intoxication [4].

In the last three decades protamine salts are often used often in combination with liposomal preparations to deliver plasmid DNA into cells [4–7]. After the invention of antisense technology [8,9] oligonucleotides and their chemical modifications became a new group of agent in pharmaceutics [10]. The principle of antisense strategy is the sequence specific binding of an antisense oligonucleotide to target mRNA preventing gene translation. Native unmodified phosphodiester oligonucleotides are highly susceptible towards nuclease degradation. To overcome this problem, chemical modifications of the DNA were applied [11]. However, besides degradation by nucleases, an even more important problem in handling oligonucleotides both, in vitro and in vivo, is their poor bioavailability [12]. Nanoparticles (NPs) were introduced in

* Corresponding author. Institute of Pharmaceutical Sciences Dept. of Pharm. Technology, Karl-Franzens-University graz, Schubertstraße 6, 8010 Graz, Austria. Tel.: +43-316-380-8881; fax: +43-316-380-9100.
E-mail address: andreas.zimmer@uni-graz.at (A. Zimmer).

0939-6411/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2004.04.001

the past as drug delivery systems to solve these multiple problems of oligonucleotide application [1,11,13–19].

In a recent study from our group as well as from other researchers, NPs consisting of protamine and oligonucleotides [20,21] were developed to deliver oligonucleotides (ON) and phosphorothioate modified oligonucleotides into mammalian cells [16,17,22,23].

So far, the characterisation of these NP was focused on the physicochemical stability, which showed that NP are stable in high purified water for days [17,24] but after adding salt to the colloidal system the NP aggregated rapidly. To get more salt tolerant particles PEG 20,000 was introduced as a third component into the system [24]. It was shown that these macromolecules can stabilise the NP suspension, but due to the unphysiological conditions of 15% (m/v) PEG 20,000 more biocompatible systems have to be developed. A further disadvantage of these first generation protamine–oligonucleotide–nanoparticles, so called 'proticles' [17,22], was the low intracellular dissociation of the oligonucleotides which was observed by laser scan microscopy and a limited antisense effect [25].

To overcome both problems, in our recent work different groups of biodegradable, non-toxic macromolecules were investigated to prevent NP aggregation. Additionally, the intracellular dissociation between the oligonucleotides and protamine should be enhanced with amphiphilic biopolymers. Human serum albumin (HSA) was found by Vogel et al. to be such a promising candidate to solve these problems [1]. HSA with a molecular weight of approximately 65,000 Da [26,27] and a slightly negative surface charge under physiological conditions is a non-toxic macromolecule which was widely used in nano- and microparticle preparations as well as in other pharmaceuticals before [28].

In context to these earlier findings the objective of this study was to characterise a novel colloidal delivery system [1] for antisense oligonucleotides based on protamine sulphate (PS), HSA and oligonucleotides (ON), respectively, oligonucleotides with different amounts of phosphorothioate modifications. Information about such albumin–protamine–oligonucleotide nanoparticles (AlPrO-NP) regarding their composition, size, shape and surface charge, under salt-free and isotonic assembly conditions, were derived from the presented physicochemical characterisation.

2. Materials and methods

2.1. Materials

Unmodified oligonucleotides (ONs), partly thioated and fully thioated oligonucleotides, used in this study, were synthesised by MWG Biotech (Ebersberg, Germany), 'high purity salt-free' (HPSF®-quality) and were analysed by mass spectroscopy (MALDI-TOF MS documentation). Small interfering RNA (siRNA) was synthesised by Dharmacon (Lafayette, CO, USA). Detailed information on the sequences are given in Table 1. Sodium perchlorate was obtained from Merck (Darmstadt, Germany). The fluorescent dye tetramethylrhodamine isothiocyanate (TRITC) was purchased from Molecular Probes (MoBiTec, Göttingen). Protamine free base from salmon, PS from salmon, human serum albumin fraction V powder (HSA) [29] and all other chemicals were obtained from Sigma (Steinheim, Germany) in the highest available purity. Water (MQ-water) was purified with a Milli-Q Plus system from Millipore (Schwalbach, Germany).

2.2. Methods

2.2.1. Particle preparation

2.2.1.1. Particles assembled under salt-free conditions. Based on a preparation method of our group [1,17,22], PS, HSA and oligonucleotide stock solutions were made each with a concentration of 2500 μg/ml in MQ-water. Different amounts of HSA (100–2000 μg) were added to various quantities of PS (15–150 μg) followed by mixing this solution for 5 s. Afterwards, 30 μg ON were added by reaching a final volume of 1 ml in MQ-water and for a second time the mixture was vortexed for 5 s.

2.2.1.2. Particles assembled under isotonic conditions. PS, HSA and ON stock solutions were prepared each with a concentration of 2500 μg/ml in an isotonic NaCl solution (NaCl 140 mM), PBS-buffer (NaCl 136.9 mM, KCl 2.68 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.47 mM; pH 7.4) or cell medium RPMI 1640 without phenol red (CM). Again, different amounts of HSA (100–2000 μg) were added to various quantities of PS (15–150 μg) followed by mixing this solution. Afterwards 30 μg ON were added to a final

Table 1
Oligonucleotides used in this study

Oligonucleotide ($n \times s$)	Sequences	MW (Da)
ON	5′-CpTpApGpGpApTpCpTpApCpTpGpGpCpTpCpCpApT	6068
ON (4)	5′-CsTsApGpGpApTpCpTpApCpTpGpGpCpTpCpCsAsT	6132
ON (6)	5′-CsTsAsGpGpApTpCpTpApCpTpGpGpCpTpCsCsAsT	6164
ON (8)	5′-CsTsAsGsGpApTpCpTpApCpTpGpGpCpTsCsCsAsT	6196
ON (20)	5′-CsTsAsGsGsAsTsCsTsAsCsTsGsGsCsTsCsCsAsT	6373
siRNA	5′-UpApApApGpCpUpUpGpCpCpUpUpGpApGpUpGpCpTpT	6648
	TpTpApUpUpUpCpGpApApCpGpGpApApCpUpCpApCpG-5′	6654

Phosphate (p) and phosphorothioate modifications (s) of the oligonucleotides are displayed in the sequences.

volume of 1 ml either in NaCl solution, PBS buffer or cell medium (CM) and again the mixture was vortexed for 5 s.

2.2.2. Small angle X-ray scattering

Small angle X-ray scattering (SAXS) measurements were performed with an integrated SWAXS camera system (HECUS Graz X-ray Systems, Austria) based upon the Kratky line collimation, equipped with a position-sensitive detector, a semitransparent Ni-filtered beam-stop and an automatic temperature/time programmer and data collection unit. The camera was attached to a conventional Cu-anode generator (Seifert Analytical Systems, Austria) operated at 2 kW. The measurements were performed at 20 °C with exposure times of 1–4 h. The data were buffer background corrected, normalized to intensity and corrected for slit collimation geometry. Indirect Fourier transform of the data was performed with the program GIFT [30]. The desmeared scattering function and the electron pair distance distribution function $p(r)$ are calculated. The $p(r)$ function contains information about the spatial distance distribution and hence about the shape of the molecule. The maximum particle diameter (d_{max}) is represented as the point where the $p(r)$ function vanishes and corresponds to the maximum distance between scattering centres of the molecule. The geometric mean value of the size distribution function was given as the d_{gm} parameter.

2.2.3. Fluorescence correlation spectroscopy

All measurements were performed with a Zeiss ConfoCor II (Jena, Germany). We used a HeNe laser ($\lambda = 543$ nm) to detect molecules, complexes or NPs, which were labelled with the fluorescent dye TRITC. The NP hydrodynamic diameter (d_h) was characterised by the determination of the diffusion coefficient (D, m^2/s), which was used to calculate the hydrodynamic radius r_h (nm)

$$r_h = \frac{kT}{6\pi\eta D} \quad (1)$$

In this equation, k stands for the Boltzmann constant (1.38×10^{-23}, J/K), T (K) for the absolute temperature and η (g/cm s) indicates the viscosity of the solvent. Hereby $2r_h$ is according to d_h.

The hydrodynamic radius r_h (nm) is related to the molecular weight MW (g/mol) of the particles:

$$MW = \frac{4\pi r_h^3 N_A \rho}{3} \quad (2)$$

N_A denotes the Avogadro's number (6.023×10^{23} mol^{-1}) and ρ (g/cm^3), the mean density of the molecules, complexes and NPs.

2.2.4. Scanning electron microscopy

Fifty microlitres of the particle preparation were freeze dried on a polished aluminium surface. After drying, the sample was sputtered with gold for 30 s under argon atmosphere (Agar sputter coater). Afterwards, scanning electron microscopy (SEM) was performed with a S-4500 Hitachi field emission electron microscope (Krefeld, Germany) with the upper detector at 15 kV. The magnification was set to 65,000. The tilt of the sample plate was adjusted to 20°.

2.2.5. Oligonucleotide loading

To determine the oligonucleotide loading of the NP and to ensure the stability of the oligonucleotides in the particle preparation, strong anion exchange high performance liquid chromatography (HPLC) was performed after the assembly procedure as described before [24,31–33].

In detail, the NP were centrifuged for 1 h at 20,000 × g, 25 °C and the supernatants were separated from the pellet. Five hundred microlitres of a 20% (w/w) solution of polyphosphate in 2N sodium hydroxide were added to dissolve the pellet samples and afterwards the solutions were heated for 3 h in a thermomixer with 1000 rpm at 60 °C. Fifty microlitres of each sample were injected into the HPLC system. This system uses a Dionex DNApac™ PA-100 (4 × 250 mm) column (Idstein, Germany) and a Merck-Hitachi HPLC system (Darmstadt, Germany). The elute solutions used were (a) 25 mM sodium hydroxide in double distilled water and (b) 25 mM sodium hydroxide, 800 mM sodium perchlorate in double distilled water. The gradient used was 0% (b) for 1 min, 0–100% (b) linear in 10 min, 10 min 100% (b), 100–0% (b) linear in 5 min, with a flow rate of 1 ml/min. The DAD signal was integrated between 250 and 270 nm. The column temperature was set to 60 °C.

2.2.6. Protamine and albumin quantification

Based on the labelling procedure of clupeine with fluorescein isothiocyanate [34], protamine free base and HSA was tagged with TRITC in aqueous solution (0.1N borate buffer, pH 9.2) and afterwards purified by dialysis in a Pierce Slide-A-Lyser Dialysis cassette (MWCO 3500 Da for protamine, respectively, MWCO 10,000 Da for HSA; Perbio Science Bonn, Germany).

Particles were prepared with different amounts of PS/protamine-TRITC 10:1 (m/m) mixture and, additionally with a HSA/HSA-TRITC 10:1 (m/m) mixture. The NP were centrifuged at 20,000 × g for 2 h. Afterwards, the supernatants were separated from the pellets and transferred into a black 96-well microtiter plate (NUNC, Wiesbaden, Germany). The fluorescence measurements were performed by excitation at 540 nm and by emission at 590 nm with a FLUOstar Galaxy microplate reader (BMG-Labtechnologies, Offenburg, Germany). The amount of protamine and HSA entrapped in the NP was calculated as the difference between the amount found in the supernatant and the initial content of protamine and HSA. The pellet samples were dissolved in a 10% sodium laurylsulphate solution, heated at 60 °C for 1 h and afterwards were measured as described above.

2.2.7. Surface charge

The surface charge (zetapotential) was determined by measuring the electrophoretic mobility in a microelectrophoresis flow cell [35]. A Malvern Zetasizer 3000 HSA (Herrenberg, Germany) was used. All samples were measured in MQ-water and were carried out after 1 h of incubation of the HSA/PS mixture with ON.

2.2.8. Particle size determination

The particle size of the resulting NPs was determined by photon correlation spectroscopy (PCS) [36–40] with a Malvern Zetasizer 3000 HSA (Herrenberg, Germany). The particle size was expressed by the mean hydrodynamic diameter (d_h) [40] and the width of the size distribution was expressed by the polydispersity index (PDI) [41]. The software data analysis for calculation of the size distribution of the NP samples was based on the fitting method of non-negative constrained least-squares (NNLS) [37]. The measurements were carried out after 1 h of incubation of the HSA/PS mixture with the ON.

3. Results and discussion

3.1. Characterisation of macromolecular components

To get a better understanding of the assembly process of the NPs the three macromolecular components were characterised by SAXS and fluorescence correlation spectroscopy (FCS) measurements.

3.1.1. Small angle X-ray scattering

By small angle X-ray scattering (SAXS) technique it was possible to determine the maximal particle diameter d_{max} of protamine (0.17 mM) and HSA (0.08 mM) in aqueous buffered solutions (pH 7.4). Fig. 1 shows the desmeared

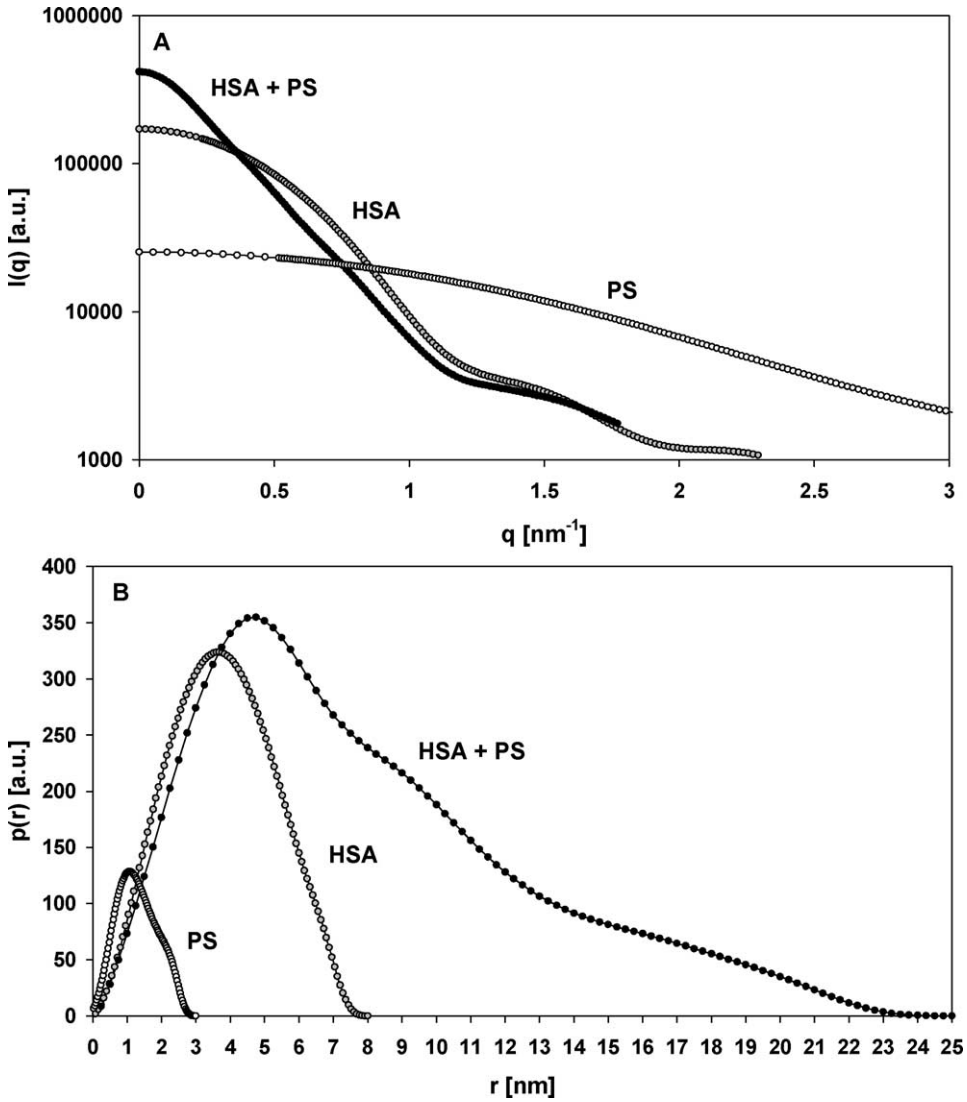

Fig. 1. Small angle X-ray scattering (SAXS) measurements: (A) Desmeared scattering curves for PS, HSA and the complex (2:1, mol/mol) and (B) the corresponding $p(r)$ functions are shown.

Table 2
Fluorescence correlation spectroscopy (FCS) measurements of the different macromolecular components, initial complexes and two model nanoparticle preparations

Sample	Medium	Hydrodynamic diameter (d_h)		Molecular weight (MW)	
		(nm)	SD	Dalton	SD
Protamine	MQ	2.7	± 0.2	5.9×10^3	$\pm 3.3 \times 10^2$
Human serum albumin (HSA)	MQ	5.9	± 0.4	6.6×10^4	$\pm 4.8 \times 10^3$
Oligonucleotides (ON)	MQ	2.8	± 0.4	6.8×10^3	$\pm 9.7 \times 10^2$
100 μg HSA +90 μg protamine	MQ	12.7	± 5.8	6.5×10^5	$\pm 2.9 \times 10^5$
1000 μg HSA +90 μg protamine	CM	9.8	± 2.6	3.7×10^5	$\pm 2.5 \times 10^5$
100 μg HSA +30 μg ON	MQ	n.d.	–	–	–
1000 μg HSA +30 μg ON	CM	n.d.	–	–	–
NP-MQ	MQ	232	± 32	2.7×10^9	$\pm 8.6 \times 10^8$
NP-MQ	CM	325	± 22	1.1×10^{10}	$\pm 3.8 \times 10^9$

To detect the samples, the components were labelled with the fluorescence dye tetramethylrhodamine (443.5 Da) ($n = 5$, except protamine $n = 10$).

scattering curves and the corresponding $p(r)$ functions. d_{max} values of $d_{max} = 2.5$ and 7 nm were found for protamine and HSA, respectively. Both values confirmed earlier data taken from literature [26,27].

3.1.2. Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) measurements were carried out to determine the mean hydrodynamic diameter (d_h) and to estimate the mean molecular weight (MW) of the different components of our study. For this approach oligonucleotides (ON), HSA and protamine free base were labelled with the fluorescent dye tetramethylrhodamine (TRITC). The d_h was calculated (Eq. (1)) and additionally the MW was calculated as described before (Eq. (2)). The results are shown in Table 2.

The d_h of the single compounds was found to be about 2.7 nm for protamine, 5.9 nm for HSA and 2.8 nm for the ON. The size of protamine and HSA confirmed the SAXS data. The MW of the components was also calculated from FCS measurements. The HSA samples showed a MW of approximately 66,000 Da. Considering the mass of the fluorescent dye of about 500 Da, this value is in a close correlation to the mass of 65,000 Da described before in the literature [26,27,29].

The theoretical mass (6783 Da) of the ON was confirmed by MALDI-TOF mass analysis. In addition, the MW was calculated to be 6820 Da based on the FCS measurements. These findings verify the MALDI-TOF data.

Our main interest was focused on the MW estimation of protamine. This strongly basic peptide could not be analysed by MALDI-TOF mass spectroscopy, due to its ratio between mass and charge of the arginine rich molecules (data not shown). To overcome this problem the diffusion time of the labelled protamine in the confocal volume was determined and afterwards the mean MW was calculated to be about 5900 Da. In consideration of the mass of the fluorescent dye, the mean MW for protamine can be estimated with approximately 5400 Da, which confirms the findings of earlier studies with other molecular weight determination methods [2].

3.2. Initial complex

In the particle preparation procedure first PS was added to a HSA solution. Thus, the interaction between the cationic protamine and the HSA was investigated. Additionally, we examined the interaction of HSA and the oligonucleotides, both to get information about the composition of the solution before adding a third component. The mixture of protamine and ON forms spontaneously NPs, which was described before [22]. PCS measurements revealed that both, the HSA/protamine and the HSA/ON mixture formed small complexes with a size of approximately 13 nm. However, it was not clear if these complexes consists of one component like a HSA dimer [26,27] or a complex of two components (HSA and PS). Thus, SAXS and FCS measurements were carried out.

3.2.1. Small angle X-ray scattering

By mixing HSA and PS, a significant shift of d_{max} to larger distances as compared to the pure components was observed (see Fig. 1). A d_{max}-value of about 50 nm was obtained. Obviously, the formed complexes between HSA and protamine resulted a brought multimodal size distribution. This polydispersity can be attributed at the smaller fraction to a molecular association between one HSA and two or more PS molecules, and at the larger fraction to an association between two HSA and one or more PS molecules.

3.2.2. Fluorescence correlation spectroscopy

To get more detailed information about the 'initial complexes' between protamine and HSA, we performed fluorescence correlation spectroscopy (FCS) measurements in situ, meaning that the measurements were carried out under the same conditions as the assembly procedure.

After the exchange of 10% (m/m) of the PS with protamine-TRITC (10 μg), the solution was added to a 100 μg/ml HSA (in salt-free MQ-water dissolved) and 1000 μg/ml HSA solution (in isotonic cell medium dissolved). Both concentrations were found to be useful for further in vitro experiments due to their stability in aqueous media (see also Section 3.3). The HSA/PS complex showed a d_h = 12.7 nm in MQ-water which is in a close correlation to the mean value of the SAXS size distribution data determined with d_{gm} = 14.0 nm. The isotonic solution (CM) obtained a d_h = 9.8 nm for the FCS measurements (Table 2).

Additionally, no complexation between HSA and ON without protamine was detected in MQ-water and in CM (Table 2). These findings also confirm the results of previous studies [28].

Summarising the findings of the SAXS and FCS techniques an 'initial complex' of HSA and PS was found. This complex could have an influence on the spontaneous self-assembly process after adding the oligonucleotides.

3.3. Nanoparticles

AlPrO-nanoparticles were prepared by adding ONs or phosphorothioate modified oligonucleotides to an aqueous mixture of HSA and PS. Complex formation spontaneously occurred within 2–3 min indicated by opalescence. After 10 min, the colloidal solution was dried and scanning electron microscope images were taken from the samples. The particles showed a round shape and a smooth surface. The diameter of the NPs were less than 300 nm (typical micrograph is shown in Fig. 2).

As demonstrated in this paper before, in a first step an initial complex of HSA and PS was formed. After the addition of the strong negatively charged oligonucleotides NP self-assembly occurred (Fig. 3). The following studies were performed to yield more information about these NPs.

3.3.1. Influence of HSA on the particle size

NPs consisting of protamine and ON can be assembled in MQ-water as described in different studies previously [16,17,22,23]. By isotonisation of the colloidal solution these NPs aggregate into large structures [24]. This aggregation was also seen when NP consisting of ON and protamine were assembled in isotonic NaCl solution, isotonic PBS buffer and a standard CM (Fig. 4). The d_h was found to be greater than 1 μm with a broad size distribution shown by a PDI larger than 0.4 determined by PCS measurements.

Adding HSA in various amounts (100–500 μg) to 90 μg PS and afterwards mixing with 30 μg ON in MQ-water, the d_h was found to be in a range of 250–280 nm with a PDI of ≤0.1. Increasing the HSA amount (1000–2000 μg) in the preparation leads to a larger d_h and a PDI higher than 0.2. These findings indicate that HSA in higher concentrations has a negative influences on the assembly procedure in MQ-water resulting in less stable NPs.

In contrast, increasing amounts of HSA added to preparations with 90 μg PS and 30 μg ON improved the stability under isotonic conditions. The d_h of these ternary particles decreases from 1–2 μm to 250–400 nm depending on the used isotonic solution (Fig. 4). The best results with a d_h = 245 nm and a PDI of ≤0.15 were found for isotonic preparations in cell medium RPMI 1640 without phenol red (CM). This isotonic solution, containing a complex mixture of salts, sugars, amino acids, vitamins and buffer substances, is well known as a physiological

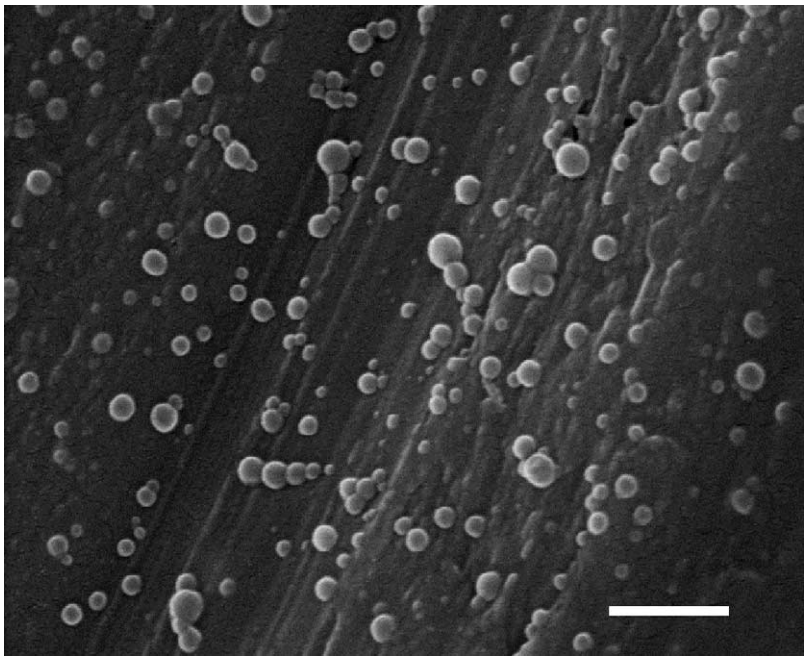

Fig. 2. Scanning electron micrograph of albumin–protamine–oligonucleotide nanoparticles (AlPrO-NP). The scale bar represents 1 μm.

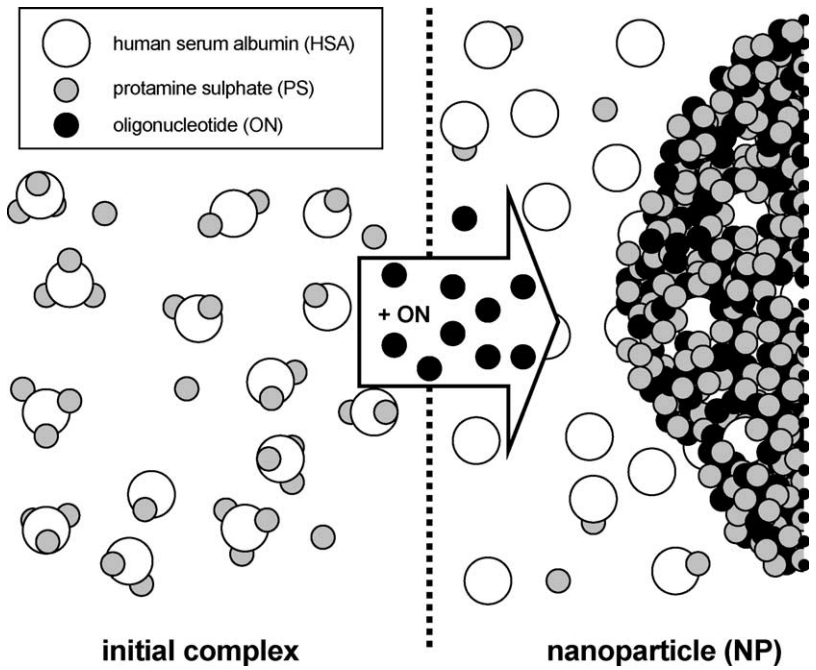

Fig. 3. Schematic illustration of the self-assembly process in the AlPrO-NP formation via an initial complex.

medium in cell culture for years [42]. It can be assumed that in addition to HSA these nutritive compounds have a synergistic effect on the NP size and stability.

3.3.2. Influence of protamine sulphate on the particle size

In the following part of our study we focused on the optimised HSA concentrations in water (100 μg/ml, NP-MQ) and under physiological conditions in CM (1000 μg/ml, NP-CM). The influence of protamine was studied with various amounts of protamine sulphate (PS) (15–150 μg). The d_h and the PDI of the NPs were determined by PCS measurements (Fig. 5).

Starting with the NP-MQ preparations, at low PS concentrations very small particles but large size distributions were observed (30:15/ON:PS). By increasing the PS concentration at an ON:PS ratio of about 30:45 particle aggregation was observed. Most favourable, stable preparations with small size distributions could be assembled at an ON:PS ratio higher than 30:60 (Fig. 5). In contrast to these data resulted in MQ-water, at low PS concentrations, NPs assembled in CM showed a large size distribution

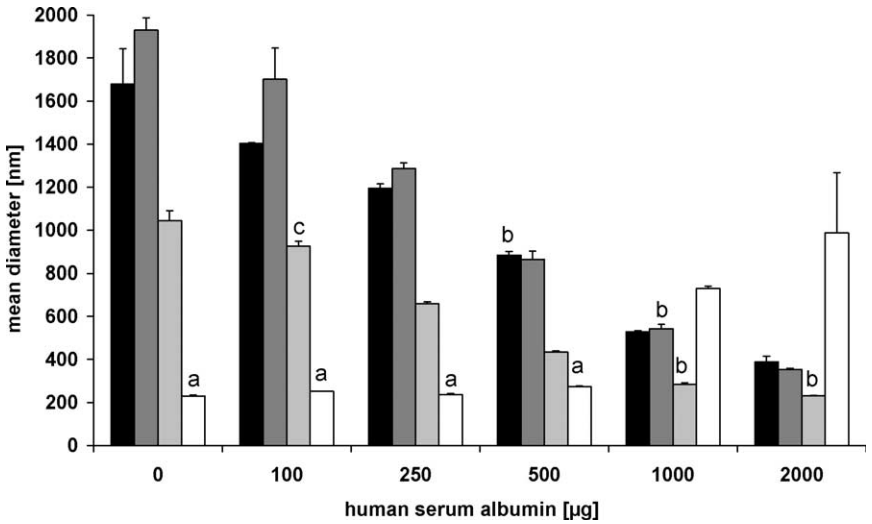

Fig. 4. PCS measurements of preparations including 30 μg oligonucleotides, 90 μg protamine sulphate and different amounts of albumin (HSA). The influence of different media on the mean diameter (d_h and the particle size distribution (PDI) was observed). The black columns representing isotonic NaCl solution, dark grey columns PBS buffer, grey columns cell medium (CM) and white columns MQ-water. Polydispersity (PDI) classes: (a) PDI ≤ 0.1, (b) PDI ≤ 0.15, (c) PDI ≤ 0.2. Without classification the preparation have a PDI > 0.2.

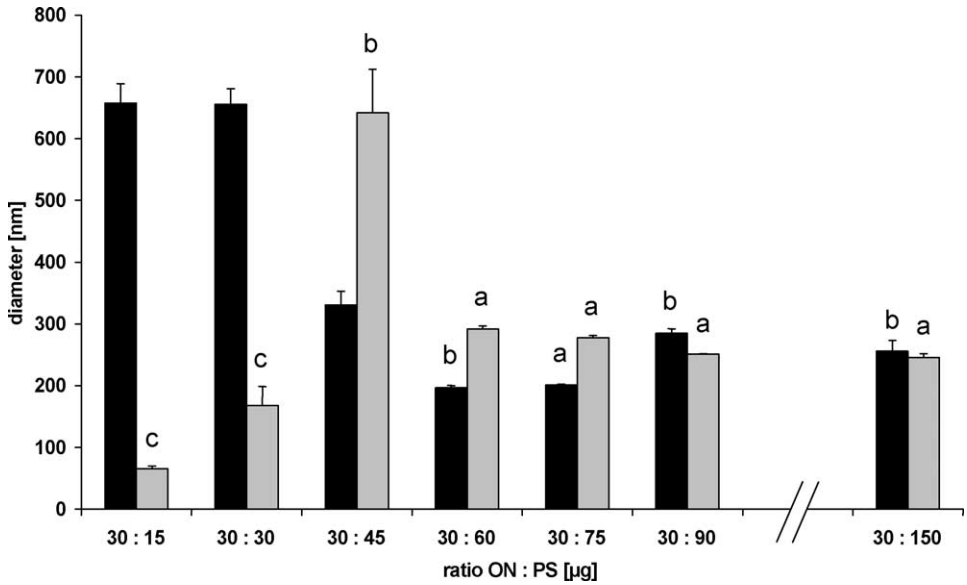

Fig. 5. PCS measurements of nanoparticles with different amounts of protamine sulphate in MQ-water (NP-MQ, grey columns) and in cell medium (NP-CM, black columns). Polydispersity (PDI) classes: (a) PDI ≤ 0.1, (b) PDI ≤ 0.15, (c) PDI ≤ 0.2. Without classification the preparation have a PDI > 0.2.

(PDI > 0.2) and a d_h of about 650 nm. At higher PS concentrations and an ON:PS ratio of about 30:60, the NP-CM preparations demonstrated comparable d_h and size distribution data to NP-MQ samples.

Concluding these effects of the PS concentration on the particle size, it was observed in our study that a minimal ON:PS ratio of 30:60, which indicated a minimal PS concentration of 60 μg/ml, has to be applied to prepare stable AlPrO-NP with a small monomodal size distribution.

3.3.3. Surface charge

The surface charge (zetapotential) is an important factor for the stability of the colloid [21] and the cellular uptake of NPs [10]. Furthermore, it gives information about the composition of the charged macromolecules in the NPs [35]. In our study, the zetapotential was characterised in order to identify preparations with a positive surface charge, which were described before to enhance cellular uptake of ON. However, NPs with a negative surface charge are known to be more biocompatible [10]. Therefore, we investigated the influence of HSA and protamine on the surface charge at a constant oligonucleotide concentration. NP without HSA demonstrated a positive surface charge at protamine concentrations higher than 45 μg/ml. Including HSA, a protamine concentration above 60 μg/ml was necessary to induce a positive surface charge under salt-free conditions. NPs prepared at a high HSA concentration (1000 μg/ml) under physiological conditions in CM possessed a slightly negative surface charge (−25 to −12 mV) for all protamine concentrations applied (Fig. 6). In addition, the influence of oligonucleotide backbone modifications on the surface charge was found to be negligible. NPs with phosphorothioate oligonucleotides (ON(20)) showed similar zetapotential profiles compared to ONs. This observation was expected because unmodified and phosphorothioate modified oligonucleotides are identical in terms of the number of negatively charged nucleic acid functions.

It is interesting, that NPs prepared at low HSA concentrations (100 μg/ml) demonstrated particle aggregation in water close to the charge neutralisation observed from the zetapotential profile (Fig. 5). Therefore, it seems to be desirable for stability reasons to prepare highly charged particles. NP-CM seems to be favourable in future studies, because these particles possess a negative charge with is independent of the protamine concentration.

3.3.4. Molecular composition

To get more detailed information about the chemical composition of the NP, the ON content was determined by HPLC in dependence of the protamine concentration. The protamine and HSA content was determined by fluorescence spectrometry after labelling the molecules with TRITC.

NP-MQ were assembled with 30 μg ON, 100 μg HSA and with various amounts of protamine in MQ-water. As shown in Fig. 7, at protamine concentrations higher than 45 μg/ml the total content of oligonucleotide was bound to the particles. Furthermore, the content of entrapped protamine increased with more protamine in the assembly mixture. HSA was entrapped into the particles only in low quantities (≤7%).

Additionally, the composition of NP-CM preparations was determined and similar amounts of ON and protamine were found in the particle matrix. About ≤10 μg of HSA

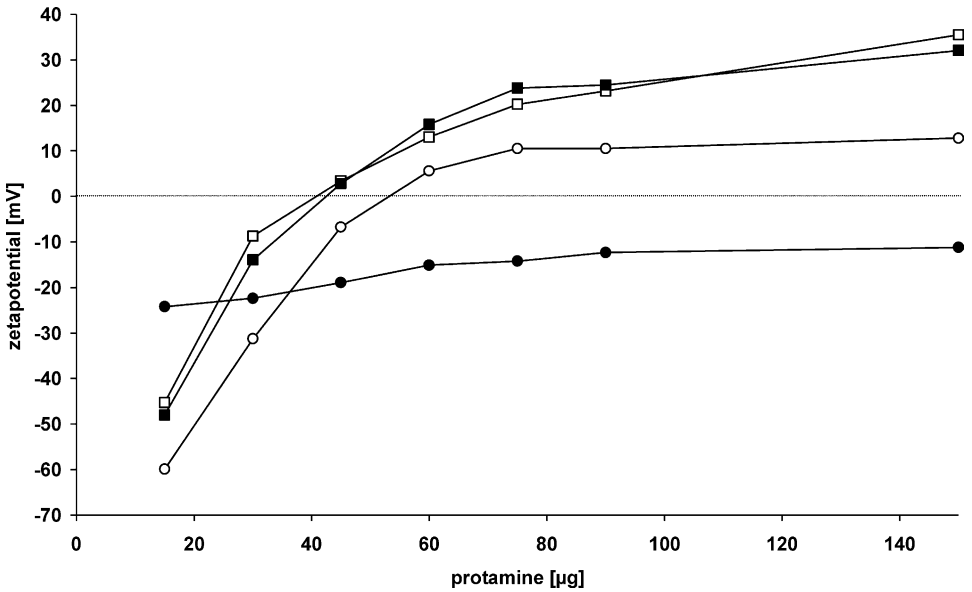

Fig. 6. Zetapotential measurements with different protamine–oligonucleotide nanoparticles. (■) NP with 30 μg ON, without HSA, prepared in MQ-water. (□) NP with 30 μg ON(20), without HSA, prepared in MQ-water. (○) NP with 30 μg ON, with 100 μg HSA, prepared in MQ-water (NP-MQ). (●) NP with 30 μg ON, with 1000 μg HSA, prepared in cell medium (NP-CM).

were entrapped into these particles which is in the same range of the NP-MQ preparation.

Summarising the data which are presented in this report, HSA could be described as a protective colloid [43,44]. These colloids are entrapped in the particle matrix only to a small amount. Most of the macromolecules are left in the surrounding medium, inhibiting the secondary aggregation of the particles visualised in Fig. 3. It can be assumed from the negative zetapotential that at high HSA concentrations (1000 μg/ml) the particles were totally coated with HSA. Thus, the stability of the NP dispersion was prolonged by this protective colloid. In our study, the HSA protects the preparation against fast aggregation under physiological conditions for more than 4 h. The d_h did not exceed 320 nm (PDI $\leq$ 0.15, data not shown) within this time range.

3.3.5. Molecular mass analysis of nanoparticles

The mean molecular masses of NP-MQ and NP-CM were estimated with fluorescence correlation spectroscopy (FCS). For the smaller NP-MQ particles ($d_h = 232$ nm) MW $= 2.7 \times 10^9$ Da and for the NP-CM ($d_h = 352$ nm) MW $= 1.1 \times 10^{10}$ Da was calculated (Table 2). In conclusion; the assembly of AlPrO-NP started with HSA (6.5×10^4 Da) and PS (5.4×10^3 Da) which formed an initial complex (approx. 6.5×10^5 Da). Adding ON (6.8×10^3 Da), a self-assembly of AlPrO-NP occurs.

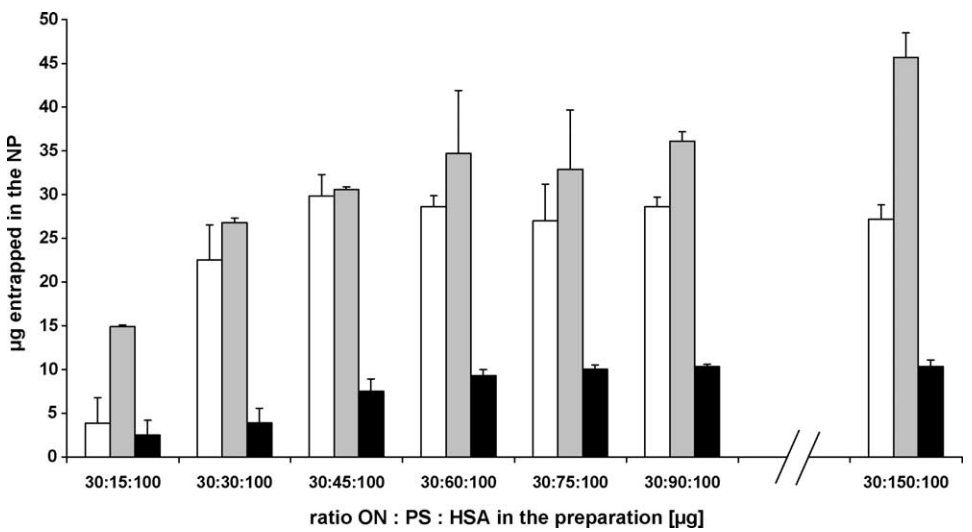

Fig. 7. Quantification of the components incorporated in the NP which were prepared in MQ-water (NP-MQ). White columns representing the oligonucleotides amount, grey columns the protamine sulphate, black columns the HSA amount incorporated in the NP matrix.

Table 3
Photon correlation spectroscopy (PCS) measurements with different nanoparticles preparations by using different numbers of phosphorothioate modifications in the backbone of the ON and siRNA

Oligonucleotides	Preparation	Hydrodynamic diameter (d_h)		Polydispersity	
		(nm)	SD (nm)	PDI	SD
ON (0)	NP-MQ	251	± 1	0.05	± 0.02
	NP-CM	285	± 7	0.12	± 0.02
ON (4)	NP-MQ	247	± 1	0.03	± 0.01
	NP-CM	304	± 3	0.08	± 0.03
ON (6)	NP-MQ	199	± 5	0.07	± 0.01
	NP-CM	248	± 3	0.09	± 0.01
ON (8)	NP-MQ	153	± 4	0.09	± 0.03
	NP-CM	218	± 2	0.18	± 0.01
ON (20)	NP-MQ	117	± 0	0.21	± 0.01
	NP-CM	109	± 1	0.26	± 0.01
siRNA	NP-MQ	202	± 7	0.03	± 0.01
	NP-CM	191	± 25	0.16	± 0.05

10 PCS subruns were performed for each of three independent preparations.

These NP have a MW of approximately 10^9–10^{10} Da depending on their particle size.

3.3.6. AlPrO-NP with modified DNA and RNA

Phosphorothioate ON are stable against nuclease degradation and are a promising drug candidates, entering clinical trials so far [11,45]. A new strategy in antisense research represents the use of siRNA for selective gene silencing [46,47]. With both types of antisense molecules, it was found in our study that AlPrO-NP could be prepared. As mention before (see Section 3.3, zetapotential) phosphorothioate modified oligonucleotides resulted in similar NP compared to unmodified ON. Depending on the phosphorothioate content a decrease in the mean particle size was observed (Table 3). Also siRNA, which has a higher molecular weight due to the double stranded configuration, formed AlPrO-NP at a 30:90 (siRNA:PS) ratio. Particle size and size distribution are in the same range as NPs prepared with phosphorothioate modified and ONs.

4. Conclusion

In this paper, a new preparation method [1] was characterised for oligonucleotide NPs consisting of HSA and protamine. The assembly process of these AlPrO-NP via an initial complex of HSA and PS was postulated and could be verified by SAXS and FCS measurements. Molecular weights of the macromolecules, initial complex and NPs were estimated. In addition the chemical content of the particles was investigated. HSA serves as a protective colloid in the NP suspension. Two different preparations could be identified as potential oligonucleotide carriers as well as for the application of siRNA. Further on, studies including the cellular uptake and the antisense effect of AlPrO-NP assembled in cell medium will be presented in the second part of this report [48].

Acknowledgements

This project was supported by the Bundesministerium für Bildung und Forschung (BMBF) FKZ 03C0308C, Germany and the Austrian–Nano–Initiative project Nano–Health. The authors would like to thank Mr. Manfred Ruppel from the Botanic Institute of the Johann Wolfgang Goethe-University, Frankfurt am Main, Germany, for kind advice concerning electron microscopy and Dr. Anke Friese for the helpful discussions.

References

[1] V. Vogel, D. Lochmann, J. Weyermann, G. Mayer, C. Tziatzios, J.A. van den Broek, W. Haase, D. Wouters, U.S. Schubert, J. Kreuter, A. Zimmer, D. Schubert, Oliganucleotide–protamin–albumin nanoparticles: preparation, physical properties and intracellular processing, J. Controlled Rel. (in press).
[2] T. Ando, M. Yamasaki, K. Suzuki, Protamines: Isolation, Characterisation, Structure and Function, vol. 1, Springer, Berlin, 1973.
[3] A. Kossel, Protamine und Histone, vol. 1, F. Deuticker Verlag, Leipzig, 1929.
[4] F.L. Sorgi, S. Bhattacharya, L. Huang, Protamine sulfate enhances lipid-mediated gene transfer, Gene Ther. 4 (1997) 961–968.
[5] T. Boulikas, F. Martin, Histones, protamine, and polylysine but not poly(E:K) enhance transfection efficiency, Int. J. Oncol. 10 (1997) 317–322.
[6] S. Li, L. Huang, Protamine sulfate provides enhanced and reproducible intravenous gene transfer by cationic liposome/DNA complex, J. Liposome Res. 7 (1997) 207–219.
[7] A.G. Sabelnikov, S.J. Ditjatkin, B.N. Iljashenko, About the role of protamine in transfection, Biochim. Biophys. Acta 299 (1973) 492–495.
[8] M.L. Stephenson, P.C. Zamecnik, Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide, Proc. Natl Acad. Sci. USA 75 (1978) 285–288.
[9] P.C. Zamecnik, M.L. Stephenson, Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide, Proc. Natl Acad. Sci. USA 75 (1978) 280–284.
[10] I. Lebedeva, L. Benimetskaya, C.A. Stein, M. Vilenchik, Cellular delivery of antisense oligonucleotides, Eur. J. Pharm. Biopharm. 50 (2000) 101–119.
[11] P. Couvreur, C. Malvy, Pharmaceutical Aspects of Oligonucleotides, vol. 1, Taylor and Francis, London, 2000.
[12] A.M. Gewirtz, D.L. Sokol, M.Z. Ratajczak, Nucleic acid therapeutics: state of the art and future prospects, Blood 92 (1998) 712–736.
[13] H.P. Zobel, J. Kreuter, D. Werner, C.R. Noe, G. Kumel, A. Zimmer, Cationic polyhexylcyanoacrylate nanoparticles as carriers for antisense oligonucleotides, Antisense Nucleic Acid Drug Dev. 7 (1997) 483–493.
[14] A. Zimmer, Antisense oligonucleotide delivery with polyhexylcyanoacrylate nanoparticles as carriers, Methods 18 (1999) 286–295. see also p. 322.
[15] E. Fattal, C. Vauthier, I. Aynie, Y. Nakada, G. Lambert, C. Malvy, P. Couvreur, Biodegradable polyalkylcyanoacrylate nanoparticles for the delivery of oligonucleotides, J. Controlled Release 53 (1998) 137–143.

[16] M. Gonzalez Ferreiro, L. Tillman, G. Hardee, R. Bodmeier, Characterization of complexes of an antisense oligonucleotide with protamine and poly-L-lysine salts, J. Controlled Release 73 (2001) 381–390.
[17] M. Junghans, J. Kreuter, A. Zimmer, Phosphodiester and phosphorothioate oligonucleotide condensation and preparation of antisense nanoparticles, Biochim. Biophys. Acta 1544 (2001) 177–188.
[18] H.P. Zobel, F. Stieneker, S. Atmaca-Abdel Aziz, M. Gilbert, D. Werner, C.R. Noe, J. Kreuter, A. Zimmer, Evaluation of aminoalkylmethacrylate nanoparticles as colloidal drug carrier systems. Part II. Characterization of antisense oligonucleotides loaded copolymer nanoparticles, Eur. J. Pharm. Biopharm. 48 (1999) 1–12.
[19] H.P. Zobel, M. Junghans, V. Maienschein, D. Werner, M. Gilbert, H. Zimmermann, C. Noe, J. Kreuter, A. Zimmer, Enhanced antisense efficacy of oligonucleotides adsorbed to monomethylaminoethylmethacrylate methylmethacrylate copolymer nanoparticles, Eur. J. Pharm. Biopharm. 49 (2000) 203–210.
[20] J. Kreuter, Evaluation of nanoparticles as drug-delivery systems. I. Preparation methods, Pharm. Acta Helv. 58 (1983) 196–209.
[21] J. Kreuter, Microcapsules and Nanoparticles in Medicine and Pharmacy, vol. 2, Marcel Dekker, New York, 1994.
[22] M. Junghans, J. Kreuter, A. Zimmer, Antisense delivery using protamine-oligonucleotide particles, Nucleic Acids Res. 28 (2000) e45.
[23] M. Gonzalez Ferreiro, R.M. Crooke, L. Tillman, G. Hardee, R. Bodmeier, Stability of polycationic complexes of an antisense oligonucleotide in rat small intestine homogenates, Eur. J. Pharm. Biopharm. 55 (2003) 19–26.
[24] D. Lochmann, V. Vogel, J. Weyermann, N. Dinauer, H. von Briesen, J. Kreuter, D. Schubert, A. Zimmer, Physicochemical characterisation of protamine–phosphorothioate nanoparticles, J. Microencapsulation (2004).
[25] N. Dinauer, D. Lochmann, I. Demirhan, A. Zimmer, A. Chandra, J. Kreuter, H. von Briesen, Intracellular tracking of protamine/antisense oligonucleotide nanoparticles and their inhibitory effect on HIV-1 transactivation, J. Control Release 96 (2004) 497–507.
[26] D.C. Carter, J.X. Ho, Structure of serum albumin, Adv. Protein Chem. 45 (1994) 153–203.
[27] X.M. He, D.C. Carter, Atomic structure and chemistry of human serum albumin, Nature 358 (1992) 209–215.
[28] D. Fischer, T. Bieber, S. Brusselbach, H. Elsasser, T. Kissel, Cationized human serum albumin as a non-viral vector system for gene delivery? Characterization of complex formation with plasmid DNA and transfection efficiency, Int. J. Pharm. 225 (2001) 97–111.
[29] E.J. Cohn, L.E. Strong, W.L. Hughes, D.J. Mulford, J.N. Ashworth, M. Melin, H.L. Taylor, Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids, J. Am. Chem. Soc. 68 (1946) 459–475.
[30] A. Bergmann, G. Fritz, O. Glatter, Solving the generalized indirect Fourier transformation (GIFT) by Boltzmann simplex simulated annealing (BSSA), J Appl. Crystallogr. 33 (2000) 1212–1216.
[31] W.A. Ausserer, M.L. Biros, High-resolution analysis and purification of synthetic oligonucleotides with strong anion-exchange HPLC, Biotechniques 19 (1995) 136–139.
[32] V. Metelev, S. Agrawal, Ion-exchange high-performance liquid chromatography analysis of oligodeoxyribonucleotide phosphorothioates, Anal. Biochem. 200 (1992) 342–346.
[33] B.J. Bergot, G. Zon, Separation of synthetic phosphorothioate oligonucleotides from phosphodiester-defect species by strong-anion exchange HPLC, Ann. N.Y. Acad. Sci. 660 (1992) 310–312.
[34] K. Wehling, S. Krauss, K.G. Wagner, Cooperative binding of fluorescein-labeled clupeine by DNA, Nucleic Acids Res. 3 (1976) 149–164.
[35] G. Barratt, Characterization of colloidal drug carrier systems with zeta potential measurements, Pharm. Technol. Eur. (1999) 25–32.
[36] R. Finsy, N. De Jaeger, Particle sizing by photon correlation spectroscopy. Part II. Average values, Part. Part. Syst. Charact. 8 (1991) 187–193.
[37] R. Finsy, N. De Jaeger, R. Sneyers, E. Gelade, Particle sizing by photon correlation spectroscopy. Part III. Mono and bimodal distribution and data analysis, Part. Part. Syst. Charact. 9 (1992) 125–137.
[38] R. Finsy, L. Deriemaeker, N. De Jaeger, R. Sneyers, J. Vanderdeelen, P. Van der Meeren, H. Demeyere, J. Stone-Masui, A. Haestier, J. Clauwaert, W. De Wispelaere, P. Gillioen, S. Steyfkens, E. Gelade, Particle sizing by photon correlation spectroscopy. Part IV. Resolution of bimodal and comparison with other particles sizing methods, Part. Part. Syst. Charact. 10 (1993) 118–128.
[39] B.J. Berne, R. Pecora, Dynamic Light Scattering—With Applications to Chemistry, Biology, and Physics, vol. 2, Dover Publications, Mineola, NY, 2000.
[40] R. Pecora, Dynamic light scattering measurement of nanometer particles in liquids, J. Nanoparticle Res. 2 (2000) 123–131.
[41] D.E. Koppel, Analysis of macromolecular polydispersity in intensity correlation spectroscopy, J. Chem. Phys. 57 (1972) 4814–4820.
[42] G.E. Moore, R.E. Gerner, H.A. Franklin, Culture of normal human leukocytes, J. Am. Med. Assoc. 199 (1967) 519–524.
[43] S.Y. Lin, K.S. Chen, H.S. Teng, Protective colloids and polylactic acid co-affecting the polymorphic crystal forms and crystallinity of indomethacin encapsulated in microspheres, J. Microencapsul. 16 (1999) 769–776.
[44] S.Y. Lin, K.S. Chen, H.S. Teng, Effect of protective colloids on the induction of polymorphic changes in indomethacin agglomerates after solvent evapora from o/w emulsions, J. Microencapsul. 16 (1999) 39–47.
[45] I. Lebedeva, C.A. Stein, Antisense oligonucleotides: promise and reality, Annu. Rev. Pharmacol. Toxicol. 41 (2001) 403–419.
[46] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.
[47] J. Harborth, S.M. Elbashir, K. Vandenburgh, H. Manninga, S.A. Scaringe, K. Weber, T. Tuschl, Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing, Antisense Nucleic Acid Drug Dev. 13 (2003) 83–105.
[48] J. Weyermann, D. Lochmann, C. Georgens, J. Kreuter, A. Zimmer, Albumin–protamine–oligonucleotide–nanoparticles as a new antisense delivery system. Part 2. Cellular uptake and effect, submitted for publication.