European Journal of Pharmaceutics and Biopharmaceutics 72 (2009) 428–437

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Physico-chemical characterisation of PLGA nanoparticles after freeze-drying and storage

Melisande Holzer [a], Vitali Vogel [b], Werner Mäntele [b], Daniel Schwartz [c], Winfried Haase [d], Klaus Langer [a,*]

[a] Institut für Pharmazeutische Technologie, Goethe-Universität, Frankfurt am Main, Germany
[b] Institut für Biophysik, Goethe-Universität, Frankfurt am Main, Germany
[c] Merck Serono, Biotech Products Development, Darmstadt, Germany
[d] Max-Planck-Institut für Biophysik, Frankfurt am Main, Germany

ARTICLE INFO

Article history:
Received 10 July 2008
Accepted in revised form 2 February 2009
Available online 11 February 2009

Keywords:
Poly(D,L lactic-co-glycolic acid) (PLGA)
nanoparticles
Freeze-drying
Storage stability
Analytical ultracentrifugation
Dynamic light scattering
Electron microscopy
DSC
Moisture content

ABSTRACT

Nanoparticles represent promising carriers for controlled drug delivery. Particle size and size distribution of the particles are important parameters for the *in vivo* behaviour after intravenous injection and have to be characterised precisely. In the present study, the influence of lyophilisation on the storage stability of poly(D,L lactic-co-glycolic acid) (PLGA) nanoparticles, formulated with several cryoprotective agents, was evaluated. Nanoparticles were prepared by a high pressure solvent evaporation method and freeze-dried in the presence of 1%, 2%, and 3% (m/v) sucrose, trehalose, and mannitol, respectively. Additionally, to all samples containing 3% of the excipients, L-arginine hydrochloride was added in concentrations of 2.1% or 8.4% (m/V). Dynamic light scattering (DLS), analytical ultracentrifugation and transmission electron microscopy (TEM) were used for particle characterisation before and after freeze-drying and subsequent reconstitution. In addition, glass transition temperatures were determined by differential scanning calorimetry (DSC), and the residual moisture of the lyophilisates was analysed by Karl Fischer titration. It was demonstrated that 1% sucrose or 2% trehalose were suitable to maintain particle integrity after reconstitution of lyophilised PLGA nanoparticles. The storage stability study over 3 months showed notable changes in mean particle size, size distribution, and residual moisture content, depending on the composition of the formulation.

1. Introduction

The development of suitable nanoparticulate systems for sustained release of drugs is an actual problem. Incorporation of drug compounds in poly(D,L lactic-co-glycolic acid) (PLGA) nanoparticles (NP) offers a possibility to achieve this aim. Several advantages are ascribed to such a formulation: the prolongation of the dosage interval due to a sustained release of the incorporated drug. A controlled and passively targeted drug delivery, leading to preferred accumulation of the particles due to the enhanced permeability and retention effect and subsequently higher drug concentration in tumor tissues, thus minimizing unwanted side effects. For an intravenous application of nanoparticles, a mean particle size between 100 and 200 nm and a homogeneous size distribution are favourable to avoid the risk of embolism and to enable sterile filtration [1]. Therefore, the control of particle size and size distribution (known as polydispersity) is essential. To ensure and maintain these critical quality parameters up to the application, it is necessary to improve the NP stability during storage. A multitude of stability problems of NP in the pharmaceutical field have been previously reported by Sameti et al. [2]. In the case of PLGA nanoparticles, the hydrolytic instability of the polymer in aqueous suspension is the main problem. Lyophilisation is a promising approach for the stabilisation of PLGA nanoparticles, which otherwise stored in an aqueous environment would suffer instability by hydrolytic degradation in a short period of time. Few studies are reported in the literature, which discussed the lyophilisation of polymeric nanoparticles [3–6].

A variety of excipients for freeze-drying have been routinely employed to achieve suitable stability for biopharmaceuticals. For a stable lyophilisate, excipients serving as stabiliser and bulking agent were usually employed. In this study we used sucrose, trehalose, mannitol, and L-arginine hydrochloride as cryoprotective agents. Some of these excipients can fulfil several functions during the freeze-drying process. Sugars are favoured, because they also protect protein-based drugs from inactivation during processing and storage. They are chemically innocuous and can be easily vitrified during freezing. They are also attractive as excipients due to their influence on the glass transition temperature (T_g' and T_g)

* Corresponding author. WWU Münster, Institut für Pharmazeutische Technologie und Biopharmazie Correnstrasse 1 48149 Münster, Germany. Tel.: +49 (0) 69 798 29692; fax: +49 (0) 69 798 29694.
E-mail address: k.langer@uni-muenster.de (K. Langer).

0939-6411/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2009.02.002

[7]. T_g' and T_g values are of particular importance for the development of a robust and effective lyophilisation process, obtaining stable amorphous solids and consequently to achieve the desired properties, such as a high redispersion speed, an acceptable storage stability, a protein stabilisation, and also an appropriate residual moisture content [8].

Bulking agents are used to create physical stability and to generate a lyophilised cake with a pharmaceutically elegant structure. Often, bulking agents also fulfil stabilising properties [9]. This applies for the sugar sucrose and for some amino acids, e.g. arginine. Various L-arginine salts are of particular interest as stabilising agents in freeze-dried products. Therefore, we also tested L-arginine hydrochloride as excipient for the freeze-drying of nanoparticles. In a number of pharmaceutical freeze-dried formulations, mannitol is frequently used, even though it is well known that mannitol is prone to crystallisation [7].

The main objective of this work was the physical characterisation of PLGA-NP prior and after lyophilisation and after storage, respectively. The influence of freeze-drying and storage at 4 °C, 25 °C/60RH, and 40 °C/75RH over 1, 2, and 3 months in the presence of several cryoprotective agents on the resulting mean particle size and size distribution was evaluated. Within the study three different methods for size determination, before and after freeze-drying and subsequent reconstitution were compared: dynamic light scattering (DLS) [also called photon correlation spectroscopy (PCS)]; analytical ultracentrifugation, a state-of-the-art method but for this purpose uncommonly used; and transmission electron microscopy (TEM). While TEM allows the analysis of the morphological appearance of the particles, analytical ultracentrifugation and DLS enable an accurate description of the mean particle size as well as the size distribution of the particles.

2. Materials and methods

2.1. Reagents and chemicals

Poly(D,L lactic-co-glycolic acid) PLGA 50:50 (Resomer RG-502 S; intrinsic viscosity 0.2 dl/g) was purchased from Boehringer Ingelheim (Ingelheim, Germany), and polyvinylalcohol (PVA) (87–90% hydrolysed, average mol wt 30,000–70,000 Da), dichloromethane, and sucrose were purchased from Sigma–Aldrich (Steinheim, Germany). Trehalose and L-arginine hydrochloride were obtained from Merck (Darmstadt, Germany), and mannitol was obtained from Cerestar (Spain). All chemicals were of analytical grade and used as received.

2.2. Preparation of PLGA-NP

The PLGA-NPs were prepared by a high pressure solvent evaporation method [10]. An organic phase consisting of 2.0 g PLGA dissolved in 20 ml dichloromethane was dispersed into 100 ml of a 1% polyvinylalcohol (PVA) solution used as hydrophilic stabiliser. The mixture was homogenised with a two-step homogeniser (APV-1000, Invensys APV, Germany) for 3 min at 400 bar. The resulting o/w-emulsion was agitated under permanent stirring (20 h, 500 rpm) to allow the dichloromethane to evaporate and the PLGA to precipitate as nanoparticles. The particle suspension was purified three times by centrifugation (21,000 rcf, 4 °C, 10 min) and redispersion of the particles in water. Each redispersion step was performed in an ultrasonication bath (Elma Transsonic Digital T790/H) in combination with vortexing (Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY, USA). Directly after this process the nanoparticle suspension was freeze-dried.

2.3. Differential scanning calorimetry (DSC)

Measurements of the thermal behaviour of PLGA-NP during freezing and melting were performed with the Differential Scanning Calorimeter DSC821^e (Mettler-Toledo, Switzerland). The samples (40 μl) were loaded onto standard aluminium pans and cooled down from −10 °C to −90 °C with a cooling/heating rate of 5 °C/min and 1 °C/min, respectively. Data acquisition of the lyophilised samples (10 mg) was performed from 10 °C to 110 °C with a heating/cooling rate of 5 °C/min and 1 °C/min, respectively. A blank aluminium pan was used as reference in all the analyses. Glass transition temperatures (T_g', T_g) were measured for each cryoprotective agent alone in a concentration of 3%, and as excipient in combination with PLGA-NP. All T_g' and T_g values were reported as the onset of the transition.

2.4. Freeze–thaw experiments

The freeze–thaw testing was conducted with the Planer Kryo 10 Series III (Planer PLC, United Kingdom). The influence of controlled freezing on the cryoprotective behaviour of sucrose, trehalose, and mannitol at the concentrations of 1%, 2%, and 3% (m/v) was studied by freeze-thawing experiments. To all samples containing 3% of the sugar compounds, L-arginine hydrochloride was added in the concentrations of 2.1% (= 100 mM) and 8.4% (m/V; = 400 mM), respectively. NPs without cryoprotective agents were also prepared as reference samples. Aliquots of the formulations (1.0 ml) were placed in 2 ml lyovials and cooled from 25 °C room temperature to 5 °C with a cooling rate of 5 °C/min. After holding for 15 min at 5 °C, the formulations were frozen with a cooling rate of 1 °C/min to −40 °C and were held at this temperature for an additional 60 min. Thawing was performed with a heating rate of 1 °C/min up to 5 °C. The mean particle size and the size distribution of each sample were measured before and after the freeze-thawing procedure by dynamic light scattering (DLS) as described below, and the ratio of mean particle size (S_f) of the samples after the freeze-thawing cycle and the initial mean particle size (S_i) was calculated.

2.5. Freeze-drying of nanoparticles

The effect of the cryoprotective agents such as sucrose, trehalose, and mannitol on the stability of PLGA-NP during freeze-drying was determined. The cryoprotective agents were added to the NP suspension (20 mg/ml) at the concentrations of 1%, 2%, and 3% (m/v), respectively. In a further set of experiments to all samples containing 3% of the excipients, L-arginine hydrochloride was added in the concentrations of 2.1% or 8.4% (m/V). NPs without cryoprotective agents were also prepared as reference samples. Aliquots of the formulations (1.0 ml) were dispensed into 2 ml lyovials. Freeze-drying was performed with three independent samples of each formulation.

For lyophilisation, an industrial freeze-dryer (GT COM 6011; Fa. Hof Sonderanlagenbau GmbH, Lohra, Germany) was used and the freeze-drying cycle was performed according to an established generic protocol for unknown samples. In the first step, the samples were frozen at −50 °C for 3 h, in the second step primary drying was performed at a shelf temperature of −40 °C and a pressure of 5×10^{-2} mbar for 5 days followed by a secondary drying step at a shelf temperature of 20 °C and a pressure of 2.5×10^{-2} mbar for 2 days. After 7 days, the vials were sealed and removed.

2.6. Reconstitution of the freeze-dried samples

To avoid foam formation, each sample was reconstituted by slowly injecting 1 ml purified water onto the inside wall of the vial. Then the vial was allowed to sit for 5 min to ensure proper wetting

of the cake. Finally, the vial was gently shaken at vortex level 2 (Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY, USA) for another 2 min to ensure complete disintegration and dissolution of the cake. After reconstitution, the NP suspensions were used for physico-chemical characterisation.

2.7. Transmission electron microscopy (TEM)

Aliquots of the nanoparticle suspensions were applied to copper grids with a carbon-coated Formvar film. The film was glow-discharged for 30 s before use and the adsorbed sample was washed twice with purified water. Afterwards, the sample was negatively stained with 2% uranyl acetate. The morphology of the particles was investigated with a Philips EM 208S electron microscope, at nominal magnification of 13,000–40,000. The diameter of the nanoparticles was calculated based on a size distribution histogram. For this purpose, a defined number of particles (150–200) were measured using the software Paint Shop Pro5 (Jasc Software, Eden Prarie, USA).

2.8. Particle size measurement by dynamic light scattering (DLS)

The mean particle size and the size distribution of the nanoparticles were measured by DLS using a Malvern Zetasizer 3000HS$_A$ (Malvern Instruments Ltd., Malvern, UK) equipped with a 10 mW He–Ne laser (633 nm) and operating at an angle of 90° and at a temperature of 20 °C. The samples of PLGA-NP were diluted 1:150 with purified and filtered (0.22 μm) water. A sample volume of 3 ml was used in a 10-mm diameter cuvette (Sarstedt, Germany). The mean hydrodynamic diameter of the particles, d_h, was calculated from the intensity of the scattered light using the Malvern software package by Cumulants method (*Z*-average diameter, Z_{ave}, defined in ISO13321 Part 8), based on the theory of Brownian motion and the Stokes–Einstein equation:

$$D = \frac{kT}{3\pi\eta d_h}$$

where D is the diffusion coefficient; k, the Boltzmann constant; T, the temperature; and η, the solvent viscosity.

The mean particle size and the size distribution of each sample, before freeze-drying and after the reconstitution of the freeze-dried and stored sample, were measured threefold. Afterwards the average value of the measurements was calculated and used to determine the ratio of mean particle size (S_f) after the reconstitution of freeze-dried and stored samples and the initial mean particle size (S_i).

2.9. Particle size measurement by analytical ultracentrifugation

The same stock PLGA-NP sample preparation as used for DLS measurements was diluted 1:10 with 100 mM NaCl solution in distilled water. Optical turbidity was between 0.5 and 0.7 at 420 nm in a cuvette with an optical path length of 1.2 cm. The sedimentation velocity experiments were carried out as described earlier in [11], using a Beckman Optima XL-A ultracentrifuge (Beckman Coulter Inc., PaloAlto, USA), an An-50Ti rotor and double-sector charcoal-filled Epon centrepieces. The rotor speed was 3000 rpm and the rotor temperature was 20 °C. Apparent absorbance (turbidity) versus radius data $A(r,t)$ were collected at 420 nm, using a radial step size of 0.02 cm. The data were modelled as a distribution of non-diffusing particles using the $l - s\ g^*(s)$ variant of the program sedfit [12]. If advantageous, the sedimentation coefficients s_{20} were transformed to standard condition values, which would be observed, if the measurements had been performed at 20 °C in pure water and given as s_{20} [11,12].

2.10. Partial specific volume of nanoparticles

The partial specific volume (reciprocal density) of the NP, $\tilde{\upsilon}$, is an important parameter in their characterisation and also is required for the calculation of the particle mass. It was determined from the sedimentation of the NP in H_2O/D_2O mixtures (with 0%, 21%, 42%, 63% D_2O content and 20 mM NaCl) with different densities (densities of solutions were calculated with program Sednterp), by plotting the sedimentation coefficient s_m as a function of the density ρ of the medium and extrapolating the dependence $s_m(\rho)$ to $s_m = 0$ ("buoyant density method") [13,14]. The partial specific volume $\tilde{\upsilon}$ was calculated as the reciprocal of the corresponding solvent density.

2.11. Calculation of particle diameter based on analytical ultracentrifugation

For solid spherical particles, $g^*(s)$ curves obtained by the $l - s\ g^*(s)$ method can be converted into (relative) concentration-versus-diameter curves applying well-known relationships between $s_{20,w}$, molar particle mass M, diameter (d) and frictional coefficient of particles (f) of spherical particles applying Eqs. (1)–(3) [11]:

$$s = M(1 - \tilde{\upsilon}\rho)/N_A f \quad (1)$$

$$M = (1/6\tilde{\upsilon})\pi d^3 \quad (2)$$

$$f = 3\pi\eta d \quad (3)$$

where N_A is Avogadro's number; $\tilde{\upsilon}$, partial specific volume; ρ, solvent density; and η, solvent viscosity.

2.12. Storage stability study

The lyophilisates ($n = 3$) prepared with 3% (m/v) sucrose, trehalose, and mannitol were sealed directly after the freeze-drying process and stored at 4 °C, 25 °C/60RH, and 40 °C/75RH, respectively. After 1, 2, and 3 months, samples were drawn and analysed for mean particle size and size distribution by DLS and analytical ultracentrifugation. As described below the residual moisture was also determined directly after lyophilisation and after storage for 3 months.

2.13. Residual moisture content

The residual moisture content of the lyophilised formulations was measured by Karl Fischer titration using a Mettler DL 38 titrator (Mettler-Toledo, Switzerland). Samples containing 100.0 mg of each formulation in the presence of 3% (m/v) sucrose, trehalose, and mannitol were analysed directly after the freeze-drying process and after 3 months of storage at well-defined climatic conditions, such as 4 °C, 25 °C/60RH, and 40 °C/75RH. The measured moisture content was expressed in percentage as the result of the Karl Fischer titration.

3. Results and discussion

The objective of the present study was the size characterisation of PLGA nanoparticles (PLGA-NPs) prior and after freeze-drying, and after storage in the presence of different excipients such as stabilisers and bulking agents. Three different methods were used for mean particle size analysis, namely dynamic light scattering (DLS), analytical ultracentrifugation, and transmission electron microscopy (TEM). Besides the well-known DLS, the size distribution was studied by sedimentation velocity analysis in the analytical ultracentrifuge due to the superior resolving power of this technique. Especially, the high resolution of the latter technique enables the identification of small particle populations even when

the size distribution of the samples covers an extended size range. The detailed characterisation of the size distributions is of major importance for nanoparticle preparations in order to avoid risk of embolism after intravenous application. For such nanoparticulate dosage forms even small fractions of larger particles have to be excluded.

Nanoparticles based on poly(D,L lactic-co-glycolic acid) were prepared by high pressure solvent evaporation method followed by freeze-drying in an industrial freeze-dryer. In advance to lyophilisation, freeze-thawing experiments and differential scanning calorimetry were conducted in order to preselect and identify the best-suited excipients in their optimal concentration for lyophilisation of the established nanoparticle system. The decision on optimal excipient composition for freeze-drying was based on the ratio of mean particle size after the reconstitution of freeze-dried and stored samples (S_f) and initial mean particle size prior to the freeze-drying process (S_i) determined by DLS. To define suitable freeze-drying conditions and to assess the storage stability of the resulting nanoparticles formulations, criteria such as glass transition temperatures and residual moisture content of the formulations were determined. All lyophilised formulations were reconstituted with water, leading to preparations of different tonicities. Therefore, to adjust a tonicity suitable for an intravenous application, different reconstitution media other than water may be required.

3.1. Differential scanning calorimetry (DSC)

In order to design a robust lyophilisation cycle and to ensure the storage stability, the glass transition temperatures (T_g', T_g) of the preparations were determined prior to the lyophilisation process. The thermal analysis is the commonly used technique for this kind of characterisation and optimisation. Table 1 summarises the T_g' and T_g values obtained for each cryoprotective agent alone or in combination with PLGA-NP. The different cooling and heating rates used showed no influence on the results. In the DSC thermograms of the aqueous solutions, the inflexion point in the baseline thermogram for sucrose was observed at −33.5 °C, for trehalose at −30.5 °C, and for mannitol at −31.7 °C. For the PLGA-NP without cryoprotective agents, only a small endothermic peak at −25.4 °C was detected. The results of PLGA-NP in combination with the cryoprotective agents were almost similar as for the cryoprotective agents alone. This is in accordance with the observations of other groups, also describing that the changes during NP freeze-drying are independent of the polymer forming the particle system, and exclusively depend on the cryoprotective agent [4,15–18]. In the case of T_g, the results of previous studies are much more inconsistent [4,16–18]. Within our study, the DSC thermograms of the lyophilised samples indicated the inflexion point in the baseline for sucrose at 68.1 °C, for trehalose at 70.9 °C, and for mannitol at 40.1 °C. For the lyophilised PLGA-NP without cryoprotective agents, an inflexion point at 41.6 °C was observed. Lyophilised PLGA-NP in combination with cryoprotectives changed the T_g values. Thus, for PLGA-NP in combination with sucrose a T_g value at 65.6 °C, in combination with trehalose a T_g value at 60.9 °C, and in combination with mannitol a T_g value at 43.7 °C was measured.

To prevent product collapse during freeze-drying and storage, the product temperature must be kept below the glass transition temperature (T_g', T_g) of the formulation. Therefore, formulations with high glass transition temperatures (T_g', T_g) are favoured.

Table 1
Glass transition temperatures T_g' and T_g of PLGA-NP, cryoprotective agents, and combinations of both PLGA-NP and cryoprotective agents.

Formulations	T_g' (°C)	T_g (°C)
Sucrose 3%	−33.5	68.1
Trehalose 3%	−30.5	70.9
Mannitol 3%	−31.7	40.1
PLGA-NP	−25.4	41.6
PLGA-NP + 3% sucrose	−33.0	65.6
PLGA-NP + 3% trehalose	−33.3	60.9
PLGA-NP + 3% mannitol	−32.3	43.7

3.2. Freeze–thaw experiments

Freezing is a critical step in the lyophilisation process for NPs. Therefore, we analysed the ability of several cryoprotective agents to prevent the agglomeration of PLGA-NPs during the freeze-thawing process. The mean particle size and the size distribution of each sample were measured by DLS before and after a freeze-thawing procedure. The measurements were used to determine the ratio of mean particle size (S_f) of the samples after the freeze-thawing cycle and the initial mean particle size (S_i). The results in Table 2 indicate that no significant changes in mean particle size were detected either with or without cryoprotective agents. Therefore, the stability of particle samples was not affected by freeze-thawing procedure.

3.3. Freeze-drying and reconstitution of the freeze-dried samples

The PLGA-NPs were lyophilised in the presence of increasing concentrations of sucrose, trehalose, mannitol, and L-arginine hydrochloride, respectively, followed by sample reconstitution in water at defined conditions. The results show the most suitable concentration and combination of cryoprotective agents for the freeze-drying process of PLGA-NP. In the presence of any cryoprotective agent used, the visual inspection of the lyophilised products did not show any signs of collapse or shrinkage after the freeze-drying process. All the lyophilised cakes were brittle and white, easy and rapid to reconstitute. Only for the formulations without cryoprotective agents a macroscopic and irreversible aggregation was detected. Even after reconstitution of PLGA-NP containing only 1% of cryoprotective agent (sucrose, trehalose, or mannitol) visually acceptable suspensions with no aggregation were achieved.

3.4. Morphology and size distribution of the particles by TEM

The NP suspensions, containing 3% sucrose, trehalose, or mannitol, were analysed by TEM after particle preparation and purifica-

Table 2
Summary of the S_f/S_i ratio characteristics of the PLGA-NP after the freeze-thawing method, as well as the S_f/S_i ratio before and after freeze-drying in the presence of different cryoprotective agents.

Cryoprotective agents (m/v)	Ratio (S_f/S_i) Freeze-thawing	Ratio (S_f/S_i) ± SD Freeze-drying
Without cryoprotectants	0.994	389.9 ± 156.2
Sucrose 1%	0.999	1.007 ± 0.004
Sucrose 2%	0.992	1.004 ± 0.010
Sucrose 3%	0.968	1.005 ± 0.009
Sucrose 3% + 2.1% L-arginine	1.019	1.027 ± 0.009
Sucrose 3% + 8.4% L-arginine	0.998	1.023 ± 0.004
Trehalose 1%	0.957	1.041 ± 0.009
Trehalose 2%	0.997	1.014 ± 0.008
Trehalose 3%	1.014	1.015 ± 0.013
Trehalose 3% + 2.1% L-arginine	1.028	1.034 ± 0.005
Trehalose 3% + 8.4% L-arginine	0.992	1.023 ± 0.020
Mannitol 1%	0.981	1.104 ± 0.011
Mannitol 2%	1.021	1.131 ± 0.033
Mannitol 3%	1.063	1.149 ± 0.023
Mannitol 3% + 2.1% L-arginine	1.041	1.009 ± 0.003
Mannitol 3% + 8.4% L-arginine	1.032	1.007 ± 0.010

tion, before freeze-drying in the presence of the respective excipients, and after reconstitution of the freeze-dried sample without further purification. Typical micrographs of the NP in the presence of trehalose as cryoprotective agent are shown in Fig. 1. The most prominent property of the NP is their almost spherical shape with diameters in the range of 100–160 nm. Independent of the excipients used and the reconstitution after freeze-drying almost similar mean particle size and size distribution were observed.

3.5. Particle size and size distribution by DLS

In order to ensure the reproducibility of the preparation method and the maintenance of particle stability during the freeze-drying process in the presence of cryoprotective agents and after subsequent reconstitution, at each handling step the average particle diameter was measured by DLS. All formulations in the presence of cryoprotective agents could easily be redispersed and the size measurement revealed almost identical mean particle sizes before and after freeze-drying (Table 2). However, some of the preparations showed a slight increase in mean particle size after freeze-drying and reconstitution, which could be attributed to the formation of small populations of aggregated particles. In case of 1% sucrose after reconstitution, a mean particle size of 163.7 ± 0.3 nm was determined which was comparable to the initial size of the nanoparticles (162.5 ± 0.5 nm) (Fig. 2a). In the presence of 2% sucrose, the initial size (162.1 ± 0.8 nm) remained unchanged after freeze-drying (162.7 ± 1.1 nm). The same was true in the presence of 3% sucrose where mean particle sizes of 161.7 ± 0.5 nm (initial) and 162.4 ± 1.1 nm (after freeze-drying) were measured. At all employed sucrose concentrations no significant difference between the initial and final mean particle size and size distribution was observed. Therefore, a minimum amount of 1% sucrose was well suited to preserve particle stability of PLGA-NP during lyophilisation and subsequent reconstitution.

Almost comparable results were achieved in the presence of trehalose as cryoprotective agent (Table 2 and Fig. 2b). In the presence of 1% trehalose during freeze-drying the initial size (162.2 ± 0.9 nm) slightly changed to 168.9 ± 1.7 nm, whereas in case of 2% trehalose after reconstitution a mean particle size of 164.7 ± 1.5 nm was determined which was comparable to the initial size of the NP (162.5 ± 0.5 nm). In the presence of 3% trehalose it was slightly changed from 163.7 ± 0.7 to 166.2 ± 1.6 nm. Therefore, a minimum amount of 2% trehalose was required for suitable reconstitution of the prepared PLGA-NP.

Freeze-drying in the presence of combinations of sucrose and trehalose with L-arginine led to increased mean particle sizes after reconstitution (Table 2 and Fig. 2a and b). Depending on its salts L-arginine has the ability to interact with multivalent counter ions, which leads to an increase in hydrogen bonding network and eventually it reduces the mobility of the freeze-dried formulation [8]. In this context, the reason for the higher S_f/S_i ratio of these combinations with L-arginine could be seen in the interaction between the slightly negative surface charge (zeta potential) of PLGA nanoparticles and the positively charged protonated guanidyl and amino groups of arginine. This interaction will cause a charge neutralisation of the particles leading to a reduced interparticulate repulsion during redispersion and, therefore, to higher S_f/S_i ratios.

The freeze-drying in the presence of mannitol led to increased mean particle sizes after reconstitution independent of the mannitol concentrations used, whereas the combination of mannitol with L-arginine led to comparable mean particle sizes prior and after freeze-drying (Table 2 and Fig. 2c). A reason for this different behaviour of mannitol might be seen in its polyalcohol structure. Previously, it has been reported that the different stereochemical conformation of such polyalcohols may be the reason for a modified interaction between the excipient and the structure of the frozen mass [3,4]. Furthermore, it is well known that mannitol is the only compound under evaluation which tends to a different crystal morphology during freeze-drying and storage [7]. On the other hand, in the combination of amorphous (L-arginine) and crystallising (mannitol) stabilising excipients, the presence of L-arginine could serve to retard the crystallisation of mannitol. In conclusion of the experiments with mannitol it has to be stated that only combinations of the sugar alcohol and L-arginine were suitable for preserving particle stability during lyophilisation and subsequent reconstitution of PLGA-NP.

In summary, the results of our study in combination with an earlier study of Saez et al. [4] indicate that sucrose and trehalose are well suited as cryoprotective agents. The excipients form an amorphous mass and maintain the integrity of PLGA-NP suspension. When sucrose was employed, the smallest S_f/S_i ratios were received followed by mannitol in combination with L-arginine and trehalose as excipients.

3.6. Determination of the size distribution of the particles by sedimentation velocity analysis

In order to support the results of the DLS measurements and to get more detailed information about particle size distribution after freeze-drying, storage and reconstitution, the analytical ultracentrifugation with its significantly higher resolution was employed.

In contrast to protein-based NP intended for drug delivery, which were recently studied by Vogel et al. [19], the sedimentation of the produced PLGA-NP in water is slow enough to obtain sufficient data points for the sedimentation velocity analysis. Typical sedimentation velocity data on the latter particles, together with the curves fitted by the $l - s\ g^*(s)$ method, are shown in Fig. 3. According to Fig. 3a, the fits are of reasonable quality, thus con-

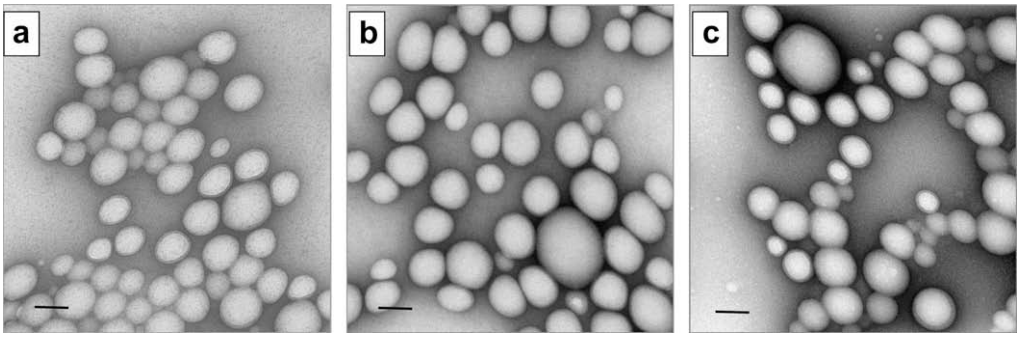

Fig. 1. Morphology of the PLGA nanoparticles analysed by TEM (a) after purification, (b) before freeze-drying in the presence of 3% trehalose, and (c) after freeze-drying in the presence of 3% trehalose and redispersion; bar: 100 nm.

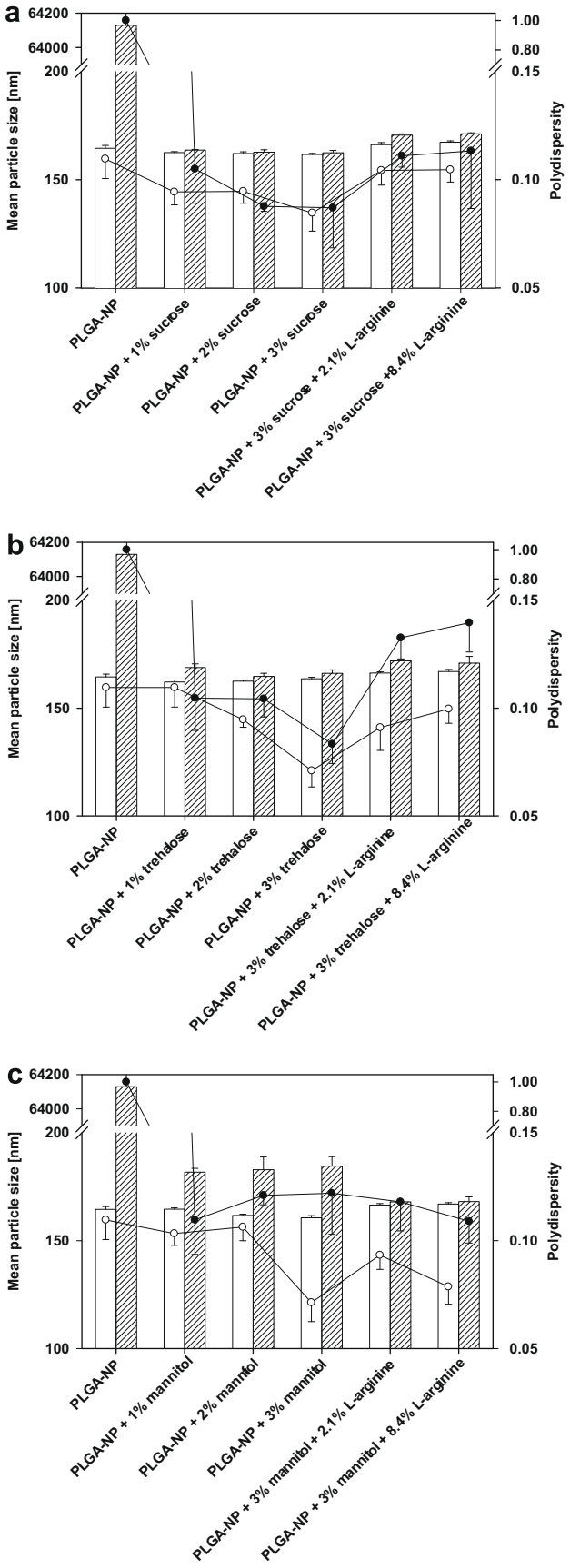

Fig. 2. Mean particle size (DLS) of PLGA-NP before (□) and after (▨) freeze-drying and polydispersity of PLGA-NP before (○) and after (●) freeze-drying in the presence of the excipients such as (a) sucrose, (b) trehalose, and (c) mannitol, and L-arginine, respectively (mean ± SD; $n = 3$).

firming the "ideal and non-diffusing" model [12]. The distributions of the normalised values for the sedimentation coefficient, $g^*(s_{20})$, for samples in 100 mM NaCl are given in Fig. 3b. Their shape clearly indicates that the particle size distribution is somewhat more complex than suggested by the DLS measurements: besides a main peak for nanoparticles sedimenting with s_{20}-values around 2000 S, minor amounts of faster sedimenting material were observed.

The data conversion from s_{20}-distributions to distributions of diameter described below requires knowledge of the partial specific volume of the prepared PLGA-NP. This was determined by the "buoyant density method" with D_2O as a densifier, plotting s_{20} of the peak of $g^*(s)$ versus solvent density ρ instead of M_{eff} from sedimentation equilibrium runs [20,21] (Fig. 4). The zero crossing of the straight line resulted in a partial specific volume of $\tilde{\upsilon} = 0.847 \pm 0.005$ ml/g.

Taking into account (i) the finding from TEM that the particles are spherical in shape and (ii) the value of the particles determined above the $g^*(s)$-curves of Fig. 3b, the sedimentation coefficient distribution data can be converted into (relative) concentration-versus-diameter curves. The transformation is shown in Fig. 5. The amplitudes of the curves do not represent true relative particle concentrations but are distorted by differences in light scattering for smaller and larger particles, respectively. However, as recently shown this effect is small for particles with narrow size distributions [21]. The diameter values corresponding to the maximum of the curve at 135 nm can thus safely be compared to the values derived from TEM (~130 nm) and DLS (162 and 165 nm). It is apparent that the values determined by analytical ultracentrifugation are in good agreement with the particle diameters determined by TEM and DLS. In this context, it should be recalled that the DLS method has relatively low resolving power, particularly when the size of particle population even in traces covers an extended size range.

In order to study the influence of lyophilisation and storage on particle size distribution, velocity sedimentation runs were performed for PLGA-NP formulations that are freeze-dried in the presence of different cryoprotective agents. The results are shown in Fig. 6. Comparable results for all formulations were observed before and after freeze-drying. For all the samples the size distribution of the characteristic main species of PLGA-NP at a maximum of ~130–140 nm remains the same after freeze-drying and reconstitution. In accordance with the results of DLS the formulations with sucrose and trehalose showed the best results, but some differences for species in the range of higher mean particle sizes (~250–400 nm) were observed (Fig. 6b and c). In contrast to these samples, in the case of mannitol the (relative) concentration-versus-diameter curve after sample reconstitution was characterised by a tailing to higher mean particle sizes (Fig. 6a). This is indicative for certain amounts of larger particles in the NP suspension after reconstitution of the samples. Therefore, the result is in accordance with the DLS data, which showed increased mean particle sizes after freeze-drying in the presence of mannitol (Table 2 and Fig. 2c). Based on these results it becomes obvious that directly after preparation nanoparticles with a unique size distribution were achieved, whereas higher mean particle sizes occurred accidentally as a secondary effect during the reconstitution process of freeze-dried samples.

3.7. Storage stability study

To ensure the NP stability during storage it is very important to maintain the critical parameters mean particle size and size distribution up to the application. Therefore, we performed a storage study at specific climatic conditions, such as 4 °C, 25 °C/60RH, and 40 °C/75RH. After 1, 2, and 3 months of storage, three samples

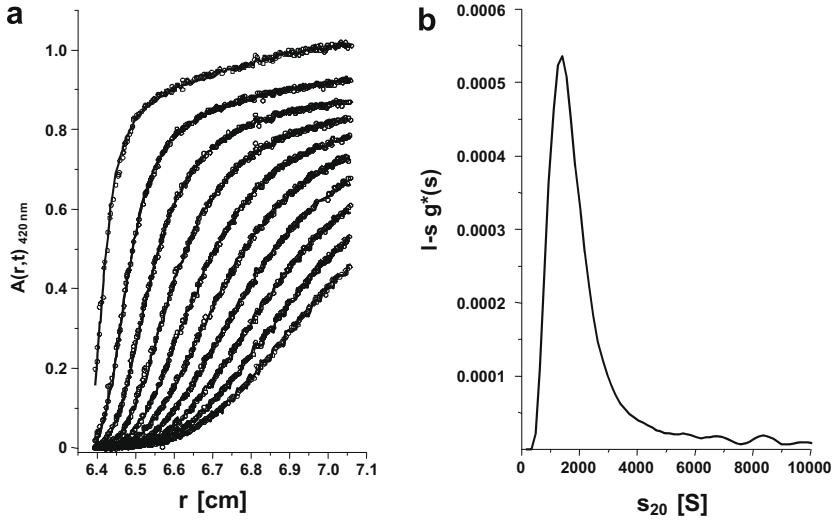

Fig. 3. Sedimentation velocity analysis of PLGA-NP. (a) Experimental absorbance versus radius data, $A(r,t)$, for sample containing PLGA-NP in H_2O and curve fitted to them by direct boundary modeling. Typically experimental absorbance scans in an analytical ultracentrifuge cell are shown. The scans represented by a line were measured in series with a time lag of ~5 min during the sedimentation of the nanoparticles. (b) Sedimentation coefficient distribution $g^*(s_{20})$ of the sample of (a). Particle concentration: approx. 0.25 mg/ml, H_2O + 100 mM NaCl.

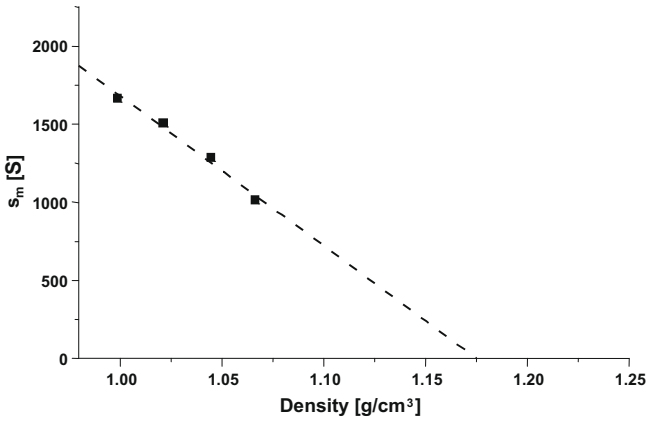

Fig. 4. Determination of $\bar{\upsilon}$-value for PLGA-NP in H_2O/D_2O mixtures (plus 20 mM NaCl): sedimentation coefficient s_m at the maximum of the $g^*(s)$ distribution versus solvent density ρ. Particle concentration: approx. 0.25 mg/ml.

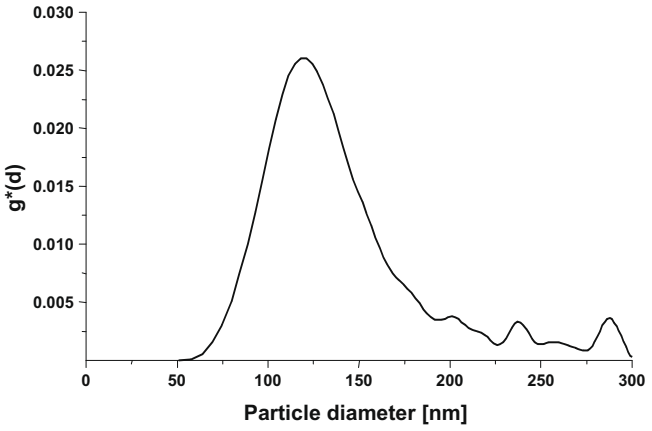

Fig. 5. Particle diameter distribution calculated from the data shown in Fig. 3b using a "solid sphere" model.

of each lyophilised NP preparation were reconstituted and were characterised by DLS and analytical ultracentrifugation. In Fig. 7a,

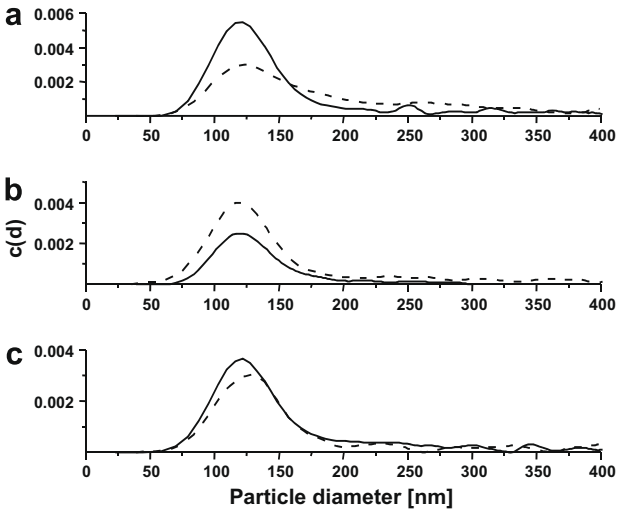

Fig. 6. Particle diameter distribution characteristics of the PLGA-NP before (solid line) and after (dashed line) freeze-drying in the presence of different cryoprotective agents calculated from velocity sedimentation data. (a) NP + 3% mannitol; (b) NP + 3% trehalose; and (c) NP + 3% sucrose.

the DLS results after a storage period of 1 month are presented. All formulations lyophilised in the presence of sucrose and trehalose and stored at 4 °C and at 25 °C/60RH could easily be redispersed and the size measurements revealed no changes in particle diameter. NP with trehalose showed a slight increase in polydispersity at 25 °C/60RH (0.140 ± 0.007) compared to the particle size distribution before storage (0.095 ± 0.007). At 40 °C/75RH the mean particle size increased slightly in the formulations with sucrose and trehalose. As described above, the formulations prepared in the presence of mannitol led to increased mean particle sizes and particle size distributions directly after freeze-drying and before storage. At 25 °C/60RH, notable changes in mean particle size (from 194.73 ± 0.13 to 228.53 ± 7.30 nm) and particle size distribution (from 0.135 ± 0.001 to 0.184 ± 0.036) were observed. After storage at 40 °C/75RH, the NPs in presence of mannitol were not redispersible.

After 2 months of storage (Fig. 7b), the NP formulations in the presence of trehalose and when stored at 40 °C/75RH showed a further increase in particle size distribution. Comparable results were also observed for formulations in the presence of mannitol under the climatic conditions of 25 °C/60RH. No redispersion of NP with mannitol was possible after storage over 2 months at 40 °C/75RH.

The results became much more pronounced with longer periods of sample storage. Notable changes were observed after 3 months storage (Fig. 7c). During storage at 4 °C, no major changes in all formulations were detected. In contrast, the formulations with sucrose and trehalose showed an increased mean particle size and size distribution when stored at 40 °C/75RH. In the case of sucrose as excipient, the particle diameter increased to 197.43 ± 15.13 nm and the polydispersity to 0.378 ± 0.193 (data not shown). In the presence of trehalose, a particle diameter of 192.96 ± 4.54 nm and a polydispersity of 0.216 ± 0.030 were measured. NPs lyophilised in the presence of mannitol were not redispersible after 3 months storage at 25 °C/60RH and 40 °C/75RH, respectively.

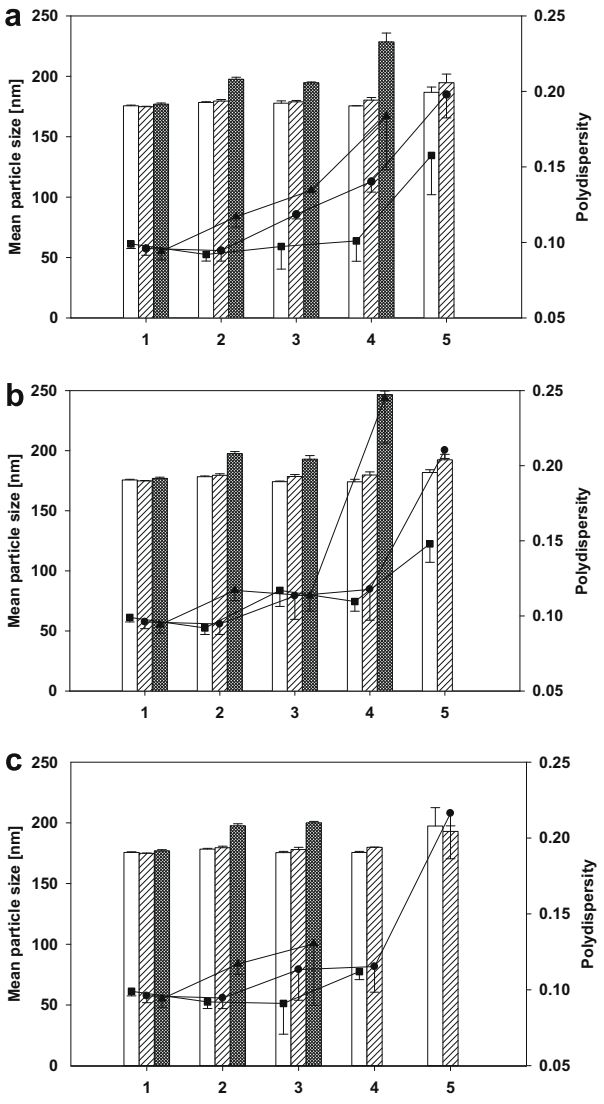

Fig. 7. Mean particle size (DLS) of PLGA-NP after (a) 1 month, (b) 2 months, and (c) 3 months of storage in the presence of sucrose (□), trehalose (▨), and mannitol (▩); and polydispersity of PLGA-NP in the presence of sucrose (■), trehalose (●), and mannitol (▲): 1. before freeze-drying, 2. after freeze-drying, 3. storage at 4 °C, 4. storage at 25 °C/60RH, 5. storage at 40 °C/75RH (mean ± SD; $n = 3$).

The effect of storage on particle diameter and polydispersity was confirmed by the results of analytical ultracentrifugation (Fig. 8).

The type of excipient used for lyophilisation as well as the storage conditions affected the storage stability of the NP. As already outlined, mannitol seems to be unsuitable for freeze-drying and storage of PLGA-NP at temperatures above 4 °C. Franks, 1994 recommended that the T_g value of a stable formulation should be at least 20 °C above ambient storage temperature [22]. The product T_g should also be higher than 40 °C to achieve long-term stability [23]. Both conditions are not guaranteed by the application of mannitol as an excipient for the freeze-drying of PLGA-NP formulations. As indicated by the DSC measurements, the T_g value of the PLGA-NP formulation in the presence of mannitol is at 43.7 °C, and therefore not suitable for a long-term storage of the preparations. On the other hand, sugar alcohol-based excipients such as mannitol have a tendency to crystallise during storage. As previously described, the rate of crystallisation increases with increasing temperature or relative humidity [24]. Taking both aspects into account, mannitol is not suitable as excipient for the freeze-drying of PLGA-NP.

In combination with proteins it was previously described that the higher glass transition temperatures of sucrose and trehalose are at least partially due to the formation of sucrose– or treha-

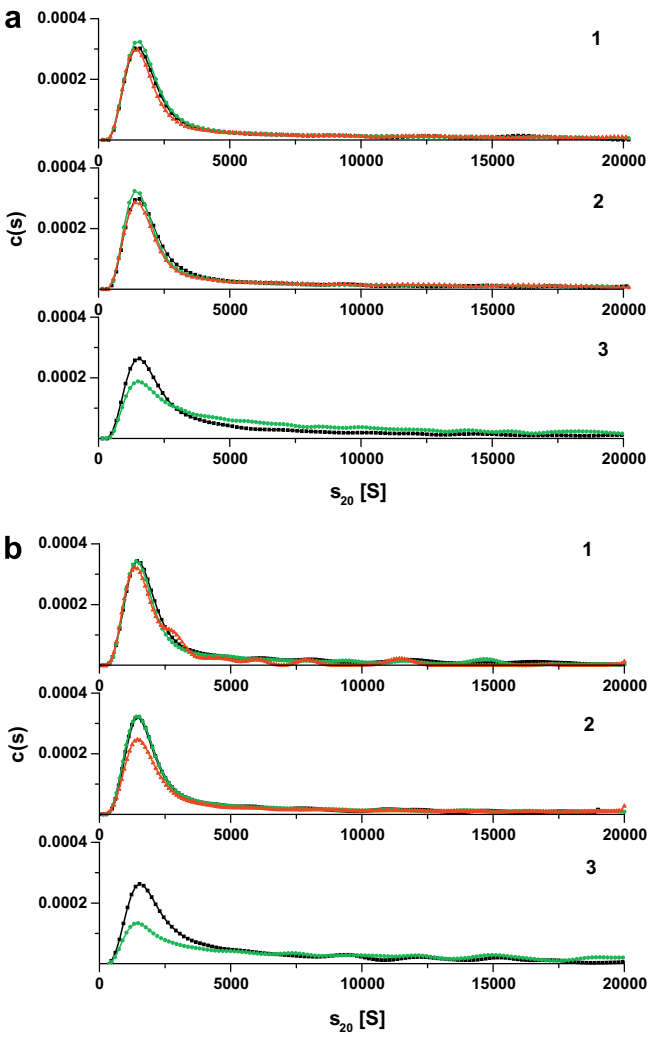

Fig. 8. Sedimentation coefficient s_{20} distribution of PLGA-NP after (a) 1 month and (b) 3 months of storage in the presence of sucrose (1) trehalose (2), and mannitol (3): storage at 4 °C (black line), storage at 25 °C (green line), and storage at 40 °C (red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

lose–protein–water micro crystals, thus preventing water from acting as plasticizer of the amorphous phase [25]. A comparable effect may be conceivable for the freeze-drying of PLGA-NP. Other properties of sucrose and trehalose are also considered to be advantageous, which include marginal hygroscopicity, an absence of internal hydrogen bonds, which allows more flexible formations, and very low chemical reactivity [18,26]. But in reality, the relative stabilisation effect of these two sugars seems to depend on the formulation and sugar concentration [27].

The unsatisfying results of the NP formulations in the presence of sucrose and trehalose after a storage period of 3 months at 40 °C/75RH were previously described in [28,29]. They reported that lyophilised sucrose is mainly amorphous but tends to crystallise with increasing residual water content or during heating and storage at elevated temperatures. Additionally, they described that trehalose can easily crystallise at 25 °C/52RH.

3.8. Residual moisture content

The residual moisture content of the lyophilised formulations was measured directly after the freeze-drying process and after 3 months of storage at well-defined climatic conditions, such as 4 °C, 25 °C/60RH, and 40 °C/75RH. Directly after the freeze-drying process all formulations showed a residual moisture content of less than 1% (Table 3). After 3 months of storage at 4 °C, just a slight moisture increase in the formulations with sucrose and trehalose was observed. The water content of PLGA-NP with mannitol exceeded 1%. In the case of sucrose and trehalose as excipients, the water content continued to rise from storage at 4 °C over 25 °C/60RH to 40 °C/75RH. However, for the formulations containing mannitol no clear tendency of the water content was observed.

As already described, the residual moisture content after lyophilisation often controls long-term pharmaceutical formulation stability, both physically and chemically. As described by Wang [27], the moisture content of a lyophilised formulation may change significantly during storage due to a variety of factors, such as stopper moisture release and leakage, crystallisation of an amorphous excipient, or moisture release from an excipient hydrate [30]. If water affects formulation stability as a plasticizer, it drastically decreases glass transition temperature of polymers, proteins or other excipients of the formulation [31–36]. Therefore, a lyophilised pharmaceutical formulation may easily adsorb sufficient amounts of moisture during storage leading to a reduction of its T_g below the storage temperature. This effect may accelerate formulations' instability and may cause product collapse [37]. High moisture content also facilitates the crystallisation of formulation of excipients such as various sugars.

Table 3
Residual moisture content of the PLGA-NP directly after freeze-drying in the presence of different cryoprotective agents and after 3 months of storage, determined by Karl Fischer titration (mean ± SD; $n = 3$).

Formulations	Residual moisture (%)			
	Directly after freeze-drying	After 3 months storage		
		4 °C	25 °C/60RH	40 °C/75RH
PLGA-NP + 3% sucrose	0.64	0.86 ± 0.14	1.03 ± 0.32	1.14 ± 0.01
PLGA-NP + 3% trehalose	0.80	0.96 ± 0.15	1.07 ± 0.23	1.51 ± 0.11
PLGA-NP + 3% mannitol	0.85	1.20 ± 0.06	1.33 ± 0.11	1.19 ± 0.02

4. Conclusion

In this paper, we have compared two frequently used techniques, DLS and TEM, and the less frequently used analytical ultracentrifugation, to characterise mean particle size and size distribution of unmodified and unloaded PLGA nanoparticles prior and after freeze-drying in the presence of different excipients as well as after storage.

The different analytical techniques used in the present study showed that unmodified and unloaded PLGA-NP can be prepared by a high pressure solvent evaporation method with predictable and reproducible size in a size range of 130–160 nm. Mean particle size analysis revealed that minimal amounts of 1% sucrose or 2% trehalose were necessary for an optimal reconstitution of lyophilised PLGA-NP. In the case of mannitol 3% of the sugar alcohol in combination with L-arginine hydrochloride was required for reconstitution. The storage stability study showed no changes in the behaviour of the NPs after storage for 3 months at 4 °C in all investigated formulations. The formulations with 3% sucrose and 3% trehalose were also stable after 3 months at 25 °C/60RH. Therefore, freeze-drying and storage in the presence of these excipients has proven to be an appropriate method for the preservation of such PLGA-NP formulations.

Acknowledgements

We are grateful to Matthias Winzer (Merck KGaA) for providing his skills in freeze-drying, and to Stefan Hindel and Katharina Ziegler for their support. This work was financially supported by the German "Bundesministerium für Bildung und Forschung (BMBF)" (Projects 13N8671 and 13N8672).

References

[1] K. Langer, S. Balthasar, V. Vogel, N. Dinauer, H. von Briesen, D. Schubert, Optimization of the preparation process for human serum albumin (HSA) nanoparticles, Int. J. Pharm. 257 (2003) 169–180.
[2] M. Sameti, G. Bohr, M.N.V. Ravi Kumar, C. Kneuer, U. Bakowsky, M. Nacken, H. Schmidt, C.-M. Lehr, Stabilisation by freeze-drying of cationically modified silica nanoparticles for gene delivery, Int. J. Pharm. 266 (2003) 51–60.
[3] S. De Chasteigner, G. Cavé, H. Fessi, J.-P. Devissaguet, J. Puisieux, Freeze-drying of itraconazole-loaded nanosphere suspension: a feasibility study, J. Drug Dev. Res. 38 (1996) 116–124.
[4] A. Saez, M. Guzmán, J. Molpeceres, M.R. Aberturas, Freeze-drying of polycaprolactone and poly(D,L-lactic-glycolic) nanoparticles induce minor particle size changes affecting the oral pharmacokinetics of loaded drugs, Eur. J. Pharm. Biopharm. 50 (2000) 379–387.
[5] Y.N. Konan, R. Gurny, E. Allémann, Preparation and characterisation of sterile and freeze-dried sub-200 nm nanoparticles, Int. J. Pharm. 233 (2002) 239–252.
[6] S. Bozdag, K. Dillen, J. Vandervoort, A. Ludwig, The effect of freeze-drying with different cryoprotectants and gamma-irradiation sterilization on the characteristics of ciprofloxacin HCL-loaded poly(D,L-lactide-glycolide) nanoparticles, J. Pharm. 57 (2005) 699–707.
[7] F. Franks, Freeze-drying of bioproducts: putting principles into practise, Eur. J. Pharm. Biopharm. 45 (1998) 221–229.
[8] K.I. Izutsu, Y. Fujimaki, A. Kuwabara, N. Aoyagi, Effect of counterions on the physical properties of L-arginine in frozen solutions and freeze-dried solids, Int. J. Pharm. 301 (2005) 161–169.
[9] H.R. Constantino, M.J. Pikal, Lyophilization of biopharmaceuticals, in: Pharmaceutical Aspects, AAPS Press, Arlington, USA, 2004, pp. 139–228.
[10] M. Ueda, J. Kreuter, Optimization of the preparation of loperamide-loaded poly (L-lactide) nanoparticles by high pressure emulsification-solvent evaporation, J. Microencapsul. 14 (1997) 593–605.
[11] V. Vogel, K. Langer, S. Balthasar, P. Schuck, W. Mächtle, W. Haase, J.A. van den Broek, C. Tziatzios, D. Schubert, Characterization of serum albumin nanoparticles by sedimentation velocity analysis and electron microscopy, Prog. Colloid Polym. Sci. 119 (2002) 31–36.
[12] P. Schuck, P. Rossmanith, Determination of the sedimentation coefficient distribution by least-squares boundary modeling, Biopolymers 54 (2000) 328–341.
[13] H.K. Schachman, Ultracentrifugation in Biochemistry, Academic Press, New York, USA, 1959, pp. 108–116.
[14] S.J. Edelstein, H.K. Schachman, The simultaneous determination of partial specific volumes and molecular weights with microgram quantities, J. Biol. Chem. 242 (1967) 306–311.

[15] P. Meredith, A.M. Donald, R.S. Payne, Freeze-drying: in situ observations using cryoenvironmental scanning electron microscopy and differential scanning calorimetry, J. Pharm. Sci. 85 (1996) 631–637.
[16] B. Lueckel, D. Bodmer, H. Leuenberger, Formulations of sugar with amino acids or mannitol-influence of concentration ratio on the properties of the freeze-concentrate and lyophilizate, Pharm. Dev. Technol. 3 (1998) 325–336.
[17] K. Kasraian, T.M. Spitznagel, J.A. Juneau, K. Yim, Characterisation of the sucrose/glycine/water system by differential scanning calorimetry and freeze-drying microscopy, Pharm. Dev. Technol. 3 (1998) 233–239.
[18] H. Levine, L. Slade, Another view of trehalose for drying and stabilizing biological materials, Biopharm 5 (1992) 36–40.
[19] 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, Oligonucleotide-protamine-albumin nanoparticles: preparation, physical properties, and intracellular distribution, J. Control. Release 103 (2005) 99–111.
[20] V. Vogel, J.F. Gohy, B.G.G. Lohmeijer, J.A. van den Broek, W. Haase, U.S. Schubert, D. Schubert, Metallo-supramolecular micelles: studies by analytical ultracentrifugation and electron microscopy, J. Polym. Sci. A 41 (2003) 3159–3168.
[21] A. Bootz, V. Vogel, D. Schubert, J. Kreuter, Comparison of scanning electron microscopy, dynamic light scattering and analytical ultracentrifugation for the sizing of poly(butyl cyanoacrylate) nanoparticles, Eur. J. Pharm. Biopharm. 57 (2004) 369–375.
[22] F. Franks, Long-term stabilization of biologicals, Biotechnology 12 (1994) 253–256.
[23] J.F. Carpenter, M.J. Pikal, B.S. Chang, T.W. Randolph, Rational design of stable lyophilized protein formulations: some practical advice, Pharm. Res. 14 (1997) 969–975.
[24] E.A. Schmitt, D. Law, G.G.Z. Zhang, Nucleation and crystallization kinetics of hydrated amorphous lactose above glass transition temperature, J. Pharm. Sci. 88 (1999) 291–296.
[25] F. Librizzi, E. Vitrano, L. Cordone, Dehydration and crystallization of trehalose and sucrose glasses containing carbonmonoxy-myoclobin, Biophys. J. 76 (1999) 2727–2734.
[26] B. Roser, Trehalose drying: a novel replacement for freeze-drying, Biopharm 4 (1991) 47–53.
[27] W. Wang, Lyophilization and development of solid protein pharmaceuticals, Int. J. Pharm. 203 (2000) 1–60.
[28] T. Moreira, J. Pendas, A. Gutierrez, R. Pomes, J. Duque, F. Franks, Effect of sucrose and raffinose on physical state and on lactate dehydrogenase activity of freeze-dried formulations, Cryo-Letters 19 (1998) 115–122.
[29] V. Naini, P.R. Byron, E.M. Phillips, Physicochemical stability of crystalline sugars and their spray-dried forms: dependence upon relative humidity and suitability for use in powder inhalers, Drug Dev. Ind. Pharm. 24 (1998) 895–909.
[30] L. Yu, N. Milton, E.G. Groleau, D.S. Mishra, R.E. Vansickle, Existence of a mannitol hydrate during freeze-drying and practical implications, J. Pharm. Sci. 88 (1999) 196–198.
[31] L. Slade, H. Levine, J.W. Finley, Protein–water interactions: water as a plasticizer of gluten and other protein polymers, in: R.D. Philips, J.W. Finley (Eds.), Protein Quality and the Effects of Processing, Marcel Dekker, New York, 1989, pp. 9–124.
[32] Y. Roos, M. Karel, Phase transitions of mixtures of amorphous polysaccharides and sugars, Biotechnol. Prog. 7 (1991) 49–53.
[33] M.P. Buera, G. Levi, M. Karel, Glass transition in polyvinylpyrrolidone: effect of molecular weight and diluents, Biotechnol. Prog. 8 (1992) 144–148.
[34] M.P. te Booy, R.A. de Ruiter, A.L. de Meere, Evaluation of the physical stability of freeze-dried sucrose-containing formulations by differential scanning calorimetry, Pharm. Res. 9 (1992) 109–114.
[35] Y. Roos, Melting and glass transitions of low molecular weight carbohydrates, Carbohydr. Res. 238 (1993) 39–48.
[36] W.F. Wolkers, H. Oldenhof, M. Alberda, F.A. Hoekstra, A Fourier transform infrared microspectroscopy study of sugar glasses: application to anhydrobiotic higher plant cells, Biochim. Biophys. Acta 1379 (1998) 83–96.
[37] C.A. Oksanen, G. Zografi, The relationship between the glass transition temperature and water vapor absorption by poly(vinylpyrrolidone), Pharm. Res. 7 (1990) 654–657.