International Journal of Pharmaceutics 363 (2008) 162–169

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Freeze drying of human serum albumin (HSA) nanoparticles with different excipients

Marion G. Anhorn, Hanns-Christian Mahler, Klaus Langer*

Institute of Pharmaceutical Technology, Biocenter of Johann Wolfgang Goethe-University, Max-von-Laue-Strasse 9, D-60438 Frankfurt, Germany

ARTICLE INFO

Article history:
Received 30 April 2008
Received in revised form 3 July 2008
Accepted 5 July 2008
Available online 15 July 2008

Keywords:
Nanoparticles
Human serum albumin (HSA)
Lyophilisation
Doxorubicin
Poly(ethylene glycol)
Freeze drying

ABSTRACT

Freeze drying is a suitable technique to improve the long-term storage stability of colloidal drug carrier systems such as nanoparticles. Aim of this study was to systematically evaluate excipients for the freeze drying and long-term stability of albumin-based nanoparticles. In our study, nanoparticles made of human serum albumin (HSA) were freeze dried in the presence of different cryoprotective agents and after reconstitution were evaluated with regard to their physico-chemical characteristics. Empty, doxorubicin-loaded, and PEGylated nanoparticles were prepared and were freeze dried in the presence of different concentrations of sucrose, trehalose, and mannitol, respectively. The samples were physicochemically characterised with regard to lyophilisate appearance, particle size, and polydispersity using photon correlation spectroscopy. For evaluation of long-term stability, the samples were stored at 2–8, 25, and 40 °C over predetermined time intervals. In the absence of cryoprotectants, particle growth was observed in all freeze-dried formulations. In the presence of sucrose, mannitol, and trehalose aggregation of HSA nanoparticles during the freeze-drying procedure was prevented. Although all of the excipients were identified to be suitable stabilisers for freeze drying of HSA nanoparticles, sucrose and trehalose were superior to mannitol, especially with regard to the long-term storage stability results.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Nanoparticles are solid colloidal drug delivery systems in a size range between 10 and 1000 nm. Nanoparticulate carrier systems are developed to enhance the efficiency of drugs and to achieve drug targeting to specific cell types (Moghimi et al., 2005; Torchilin, 2007). For the preparation of nanoparticles, different macromolecular materials such as synthetic or natural polymers can be used (Kreuter, 1983; Allémann et al., 1993; Langer, 2006). In comparison to other polymers the preparation of nanoparticles based on human serum albumin (HSA) shows a variety of advantages: Biodegradable nanoparticles can be prepared by an easy and well-defined desolvation procedure (Liu and Leong, 1997; Weber et al., 2000; Langer et al., 2003). Due to the primary structure of the albumin molecule, functional groups are present on the particle surface, which can easily be used, for covalent surface modification by attachment of drugs or drug targeting ligands (Akasaka et al., 1988; Langer et al., 2000; Wartlick et al., 2004; Steinhauser et al., 2006). On the other hand due to the high protein binding of various drugs the matrix of HSA nanoparticles can be used for an effective incorporation of these compounds. In a second step these nanoparticles can be surface-modified by the introduction of hydrophilic, sterical barriers such as poly(ethylene glycol) chains. With this modification, it is possible to prepare nanoparticles with a longer *in vivo* circulation time, i.e. to improve pharmacokinetic profile. This property is one important prerequisite to circumvent opsonisation and elimination by the reticuloendothelial system (RES) and to enable active drug targeting (Barratt, 2003). Such nanoparticles can take advantage of the enhanced permeability and retention (EPR) effect in tumour tissues (Maeda et al., 2000). Therefore, they are predestined for tumour targeting. Incorporated cytostatic drugs can be delivered selectively to tumour tissues with the consequence of reduced adverse drug reactions.

Anthracyclins such as doxorubicin and daunorubicin are an important group of cytostatic drugs to fight against cancer diseases. On the one hand they are very effective tools for the treatment of many neoplastic diseases, but on the other those substances and products can have extreme side effects, such as their high cardiotoxicity. Therefore, entrapping anthracyclins in nanoparticles to obtain a delivery system for specific tumour targeting is a promising approach to reduce adverse drug effects while maintaining the therapeutic efficacy.

One important challenge in the development of nanoparticles is their long-term stability upon storage. For stabilisation of drugs for parenteral administration, freeze drying is a commonly used process. Previous studies have reported freeze drying of PLGA or PLA

* Corresponding author. Tel.: +49 69 798 29692; fax: +49 69 798 29694.
E-mail address: k.langer@em.uni-frankfurt.de (K. Langer).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.07.004

nanoparticles (Konan et al., 2002), because of their hydrolytically unstable polyester structure. However, there is so far no systematic study about freeze drying of albumin-based nanoparticles available. Therefore, the goal of the present study was to use freeze drying in combination with HSA nanoparticles and to systematically analyse the influence of various excipients on the freeze drying process itself as well as on the long-term storage of the freeze-dried products. The storage of lyophilised samples was performed at both refrigerated as well as elevated temperatures. Although it is possible to store HSA nanoparticles in aqueous suspension even at 4 °C over a time period of 6 weeks without essential changes of their physico-chemical characteristics (Steinhauser et al., 2006), it is difficult to preserve their chemical and physical stability for a longer time period in this way (Carpenter et al., 1997). Therefore, freeze drying of HSA nanoparticles is considered to be an attractive way to achieve long-term storage stability with the additional advantage of easy handling (shipping, storage) (Tang and Pikal, 2004).

In order to stabilise products during the freeze-drying process as well as to ensure adequate tonicity and reconstitution, suitable excipients are required (Carpenter et al., 1997; Johnson et al., 2002; Pyne et al., 2003; Sameti et al., 2003; Passot et al., 2005). Depending on their function in the product, excipients can be essentially subdivided in bulking agents and stabilisers (Liu et al., 2005; Passot et al., 2005): Bulking agents are used to achieve a stable and pharmaceutical elegant non-collapsed cake (Costantino, 2004). Additionally, bulking agents could serve as tonicifier. Typically used bulking agents are sugars or sugar alcohols such as lactose, sucrose, mannitol, and sorbitol (Costantino, 2004). Especially for protein-based formulations, non-reducing compounds such as trehalose, mannitol, and sucrose are preferred to avoid potential Maillard reaction of the excipient with the protein. Furthermore, amino acids, such as arginine, glycine, and histidine (Costantino, 2004) can serve as bulking agents. Both mannitol and glycine show the tendency to crystallise during the lyophilisation process (Costantino, 2004; Chatterjee et al., 2005). Mannitol may crystallise only partially during freeze drying and crystallisation may continue after lyophilisation as a result of moisture and heat (Kim et al., 1998; Yu et al., 1999). Another disadvantage of mannitol is the fact that it exists in three different polymorph structures (α, β, and γ). Nevertheless, mannitol is an often used excipient for freeze-dried formulations.

Excipients used for the formulation of lyophilisates can also serve as stabilisers. Different theories exist about how these compounds actually stabilise protein-based formulations in freeze-dried products (Crowe et al., 1996; Chang et al., 2005a,b): although HSA nanoparticles cannot be compared to protein-based drugs, these theories may be useful for the freeze drying of the nanoparticulate systems. Stabilisers are able to form hydrogen bonds at specific sites on the surface of proteins. Water, which is lost during drying, is substituted by these additives. In the literature this mechanism is called “water replacement”. A different theory states that stabilisation occurs by immobilisation of the protein molecules in an inert and rigid glass matrix. For example, sugars (sucrose, trehalose), dextran, and hydroxyethyl starch are able to form glasses with high viscosity. Consequently, embedded proteins are preserved in their original state. Probably both mechanisms are involved in the stabilisation simultaneously and these mechanisms may be important during the drying of HSA nanoparticles.

In our study mannitol, sucrose, and trehalose were investigated in concentrations of 1%, 2%, and 3% (w/v) as potential excipients and stabilisers during freeze drying of aqueous 10 mg/ml HSA nanoparticle suspensions. Mannitol mainly serves as a bulking agent, whereas sucrose and trehalose are known as stabilisers in freeze-dried protein formulations. Different ratios of excipient to nanoparticles where tested, as not only the qualitative choice of excipient but also the ratio of stabiliser to drug often can determine long-term stability. Since the residual moisture content of the freeze-dried formulations may also have a potential impact on stability, this parameter was also determined. In order to determine the robustness of the formulations, a fixed concentration of the respective excipient was combined with variable amounts of HSA nanoparticles in an additional experiment. Finally, the stabilising effect of the selected excipients was not only evaluated in combination with unmodified HSA nanoparticles but also with PEGylated and doxorubicin-loaded HSA nanoparticles.

2. Materials and methods

Human serum albumin (HSA, fraction V, purity 96–99%), glutaraldehyde 8% aqueous solution, mannitol and sucrose were obtained from Sigma (Steinheim, Germany). Trehalose and apura® solvent was purchased from Merck (Darmstadt, Germany). The succinimidyl ester of methoxy poly(ethylene glycol) propionic acid with an average molecular weight of 5.0 kDa (mPEG5000-SPA) was purchased from Nektar (Huntsville, USA). Doxorubicin was obtained from Sicor (Milan, Italy). All other reagents were of analytical grade and used as received.

2.1. Preparation of HSA nanoparticles

HSA nanoparticles were prepared by a well established desolvation process (Weber et al., 2000; Langer et al., 2003): 200 mg HSA in 2.0 ml 10 mM NaCl solution, adjusted to pH 8.5 were transformed into nanoparticles by continuous (1 ml/min) addition of 8.0 ml desolvating agent ethanol under constant stirring at room temperature. After protein desolvation 235 μl of an 8% aqueous glutaraldehyde solution was added to achieve particle crosslinking. The resulting nanoparticles were purified by two cycles of differential centrifugation (16,100 × g, 10 min) with subsequent redispersion of the pellet to the original volume in water. The nanoparticle content of the suspension was determined by microgravimetry (see below).

2.2. Preparation of PEGylated HSA nanoparticles

HSA nanoparticles were prepared as described above and were modified as follows: 2 ml of HSA nanoparticle suspension (content 20 mg/ml) was incubated with 500 μl of mPEG5000-SPA solution (60 mg/ml in phosphate buffer pH 8.0) for 1 h at 20 °C under constant shaking (Eppendorf thermomixer, 600 rpm). The nanoparticles were purified by centrifugation and redispersion as described above. The content of the nanoparticles was determined by microgravimetry. A PEGylation efficiency of about 15 μg mPEG per milligram nanoparticle was achieved (determined by size exclusion chromatography (SEC), data not shown).

2.3. Preparation of doxorubicin-loaded HSA nanoparticles

An amount of 160 mg HSA was dissolved in 4 ml of MilliQ water. This solution was filtered through a 0.22 μm cellulose acetate membrane filter (Schleicher & Schuell, Dassel, Germany). An aliquot (500 μl) of this solution was added to 200 μl of a 0.5% (w/v) aqueous stock solution of doxorubicin. 300 μl of MilliQ water was added to this mixture. In order to adsorb doxorubicin to HSA in solution, the mixture was incubated under stirring for 2 h. For the preparation of nanoparticles by desolvation, 3 ml ethanol (96%, v/v) was added continuously (1 ml/min). After protein desolvation, an aliquot of 11.75 μl 8% glutaraldehyde solution was added to induce particle crosslinking (corresponding to 100% stochiometric protein crosslinking). The crosslinking was performed for 24 h under

constant stirring at ambient temperature. The resulting nanoparticles were purified by two cycles of differential centrifugation (16,100 × *g*, 12 min). The particle content of the doxorubicin-loaded nanoparticles was determined by microgravimetry.

The collected supernatants were used to determine the non-entrapped doxorubicin by HPLC. The content of entrapped doxorubicin was then calculated from the difference of total doxorubicin and unbound drug. For the quantification of doxorubicin, a Merck Hitachi D7000 HPLC system equipped with a Lichrospher-100 RP-18 column was used. Separation was obtained with a mobile phase consisting of water and acetonitrile (70:30) containing 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. Aliquots of 20.0 μl were injected and doxorubicin was quantified by UV detection at a wavelength of 250 nm. Drug concentrations were calculated relative to a calibration curve in the concentration range between 6.75 and 100 μg/ml.

2.4. *Preparation of the samples for the freeze-drying process*

Trehalose, sucrose, and mannitol were employed as excipients for the formulation to be freeze dried. First, aqueous stock solutions of these compounds were prepared. The final products were obtained by mixing the nanoparticle suspension with excipient stock solutions.

In a first set of experiments 1%, 2%, and 3% (w/v) sucrose, trehalose, and mannitol were used as excipients and were combined with a fixed concentration of 10 mg/ml of unmodified HSA nanoparticles. In a second set of experiments fixed concentrations of excipients (2% sucrose or 2% mannitol, respectively) were combined with variable amounts of HSA nanoparticles (10, 15, 20, 25 mg/ml). Finally PEGylated nanoparticles as well as doxorubicin-loaded nanoparticles were combined with sucrose, trehalose, and mannitol, respectively, and were tested for stability before and after freeze drying at the nanoparticle concentration of 10 mg/ml.

All nanoparticle samples were divided into two parts: (A) 1.0 ml of these suspensions were manually filled in 2 ml glass vials (Macherey–Nagel, Düren, Germany), partially stoppered using lyo rubber stoppers (Macherey–Nagel, Düren, Germany). Before use, the vials were washed with MilliQ water and dried for 1 h at 180 °C. (B) A smaller aliquot (200 μl) was analysed for particle size and polydispersity before freeze drying.

2.5. *Freeze-drying process*

For the freeze-drying process a Lyostar II freeze dryer model LYOACC2E (FTS Systems, New York, USA) was used. First, the shelf temperature was reduced from 5 to −40 °C at a rate of 1 °C/min. The pressure was 60 mT (=0.08 mbar). These parameters were held for 6 h. By increasing the temperature from −40 to −25 °C at 0.5 °C/min the primary drying was achieved. The pressure remained unchanged. At the end of the primary drying heat ramp, a pressure rise test (PRT) was performed. With termination of the primary drying the secondary drying followed by increasing the temperature at a rate of 0.2 °C/min to 25 °C. This temperature was held for 6 h at a pressure of 60 mT (=0.08 mbar).

2.6. *Determination of the glass transition temperature*

The glass transition temperature was determined by differential scanning calorimetry (DSC) using a Mettler Toledo system. For these purposes 20 μl of the samples were put in a pan for DSC measurement. The following temperature profile was used: starting from 25 °C the samples were cooled in 10 °C/min steps to −60 °C. This temperature was held for 15 min. Subsequently the temperature was raised in 5 °C/min steps to 25 °C. The values of the glass transition temperatures were determined relative to a control sample.

2.7. *Long-term stability*

To investigate the long-term stability of the freeze-dried products the samples were stored for 4, 8, and 13 weeks at 2–8 °C, 25 °C/60 RH, and 40 °C/75 RH. At each time point, the reconstituted samples were characterised with regard to particle size and polydispersity. Directly after freeze drying and after 13 weeks of sample storage the residual moisture content was determined.

2.8. *Determination of the residual moisture*

To determine the residual moisture of the freeze-dried samples a Karl Fischer titrator (Mettler DL18) was used. For that purpose about 100 mg of the lyophilised product was analysed.

2.9. *Reconstitution of lyophilised samples*

Each sample was reconstituted with 1.0 ml MilliQ water while putting the solution flow onto the inside of the vial. To ensure proper wetting of the lyophilised cake the vial was allowed to sit for 5 min. Then the vial was vortexed (Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY, USA) for 2 min at vortex level 2.5 followed by particle size analysis.

2.10. *Nanoparticle characterisation*

Nanoparticles were analysed with regard to particle diameter and polydispersity by photon correlation spectroscopy (PCS) using a Malvern Zetasizer $3000HS_A$ (Malvern Instruments Ltd., Malvern, UK) before freeze drying and after reconstitution (Langer et al., 2003). Prior to both measurements the samples were diluted with purified water.

2.11. *Determination of nanoparticle yield*

Particle yield was determined by microgravimetry. An aliquot (50.0 μl) of the respective nanoparticle sample was put in a pan made of aluminium (Lüdi AG, Flawil, Switzerland). After a drying time of 2 h at 80 °C these pans were put in an exccikator for 30 min to cool off. Subsequently, the pans were weighted with a microbalance (Sartorius, Göttingen, Germany). The content of the nanoparticles was calculated form the difference of the empty and nanoparticle filled pan (Langer et al., 2008).

3. Results and discussion

The objective of the present study was to use freeze drying as an approach to stabilise HSA nanoparticles. Different excipients were studied in different concentrations to obtain a stable freeze-dried formulation of HSA nanoparticles. Therefore, the influence of different excipients in different concentrations on the physicochemical characteristics of the nanoparticles prior and after freeze drying was evaluated. Furthermore, the long-term storage stability of these formulations under real-time and accelerated conditions was also studied. Two disaccharides, namely sucrose and trehalose, used as potential stabilisers and one sugar alcohol as bulking agent (mannitol) were tested in our study.

HSA nanoparticles were prepared by a well established desolvation procedure. Some of the particles were further surface-modified to PEGylated nanoparticles by the reaction of amino groups being located on the particle surface with the succinimidyl ester of methoxy poly(ethylene glycol) propionic acid (mPEG5000-SPA).

Table 1
Glass transition temperature of the solutions of the excipients sucrose, trehalose, and mannitol and their mixtures with HSA nanoparticles

Preparation	Onset (°C)	Midpoint (°C)
2% trehalose solution	−31.91	−31.13
HSA NP + 2%trehalose solution	−38.05	−36.84
3% trehalose solution	−32.11	−30.78
HSA NP + 3% trehalose solution	−36.80	−35.64
2% sucrose solution	−35.97	−34.62
HSA NP + 2% sucrose solution	−41.66	−40.27
3% sucrose solution	−35.14	−34.42
HSA NP + 3% sucrose solution	−40.27	−38.87
2% mannitol solution	−30.71	−29.68
HSA NP + 2%mannitol solution	−34.56	−32.95
3% mannitol solution	−30.69	−29.69
HSA NP + 3% mannitol solution	−33.84	−32.46

Unmodified as well as PEGylated nanoparticles were obtained with particle diameter between 157 and 213 nm with a polydispersity index between 0.011 and 0.047 indicating a monodisperse size distribution. In further experiments, the cytostatic drug doxorubicin was incorporated within the particle matrix by protein desolvation in the presence of the drug. Doxorubicin-loaded nanoparticles were achieved and found to be much larger with a particle diameter between 353 and 373 nm.

3.1. Glass transition temperature

In the first step the glass transition temperatures of the excipient solutions as well as of the final excipient/nanoparticle mixtures were determined. Substances, which do not show crystallisation behaviour, are characterised by a so-called glass transition. The glass transition temperature is the temperature at which the rubber-like mass is transformed into a rigid glass matrix. The glass transition temperature, also called Tg', is an important parameter for the freeze-drying process.

In Table 1 the results of the glass transition temperatures are summarised: the Tg' values of the excipient solutions are in accordance with the literature (Tang and Pikal, 2004). In principle the preparations containing either 2% or 3% of the respective excipient show comparable results. This is true for both preparations, the excipient solutions but also for the mixtures of excipients and nanoparticle suspension. However, it could be observed that the mixtures of sugars and nanoparticles tend to have a lower glass transition temperature in comparison to the excipient solutions alone. Additionally, in accordance with previous studies, the sugar alcohol mannitol shows the phenomenon that not only a glass transition, but also a partial crystallisation with building a eutecticum can be observed (Kim et al., 1998; Yu et al., 1999).

3.2. Freeze drying at fixed nanoparticle concentration

In a first set of experiments unmodified nanoparticles (10 mg/ml) were freeze dried either without further excipients or in the presence of sucrose or trehalose at concentrations of 1%, 2%, and 3% (w/v) as well as in the presence of 1%, 2%, and 3% (w/v) mannitol as bulking agent. In the absence of any excipient, directly after reconstitution of the freeze-dried nanoparticles large aggregates in the micrometer size range (Fig. 1A) and a drastic increase in polydispersity index (Fig. 1B) were observed. This suggested, that the process of freeze drying in the absence of any stabilisers was not sufficient to stabilise the HSA nanoparticles.

In the presence of the excipients sucrose, trehalose and mannitol, this particle aggregation obtained directly after the lyophilisation was avoided. However, for trehalose, sucrose as well as for mannitol at least 2% of the excipients were required to sufficiently stabilise the nanoparticles with regard to maintaining both particle diameter (Fig. 1A) and polydispersity (Fig. 1B). In the presence of lower concentrations of the excipients (1%) a significant increase in particle diameter and polydispersity was observed after freeze drying and sample reconstitution. Therefore, a minimum concentration of 2% sucrose, trehalose or mannitol is considered to be needed for a suitable reconstitution of HSA nanoparticles lyophilised at a particle content of 10 mg/ml. As shown in Fig. 1A the reconstituted samples with mannitol (2% and 3%) show a small increase in particle diameter after freeze drying in comparison to the samples before freeze drying, but the reconstituted nanoparticles are still in the nanometer size range. The polydispersity index (PI, Fig. 1B) confirms that mannitol could not completely prevent particle aggregation after reconstitution of the samples. This phenomenon was not observed with formulations containing sucrose or trehalose, where the nanoparticle size as well as the PI suggests that the process did not have a negative impact on the product quality.

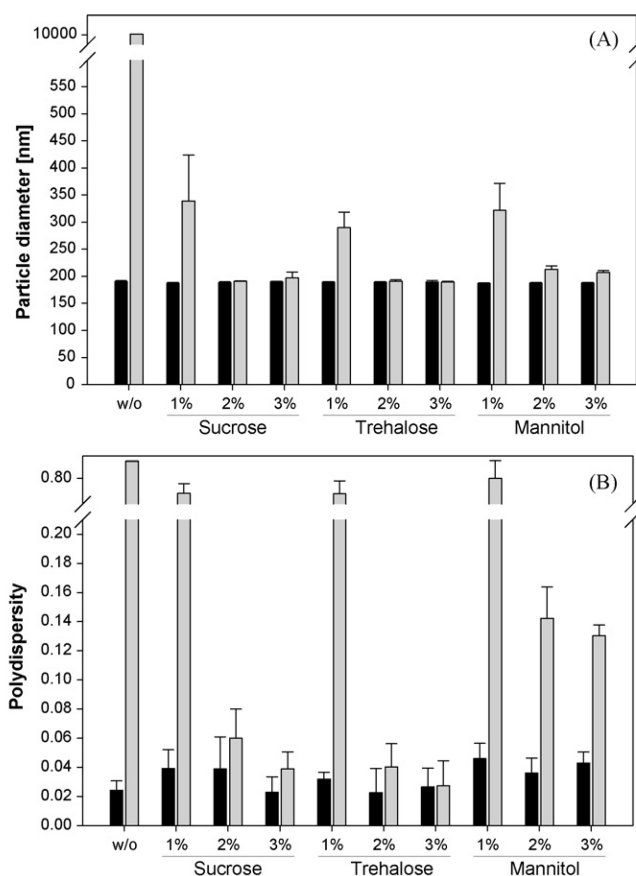

Fig. 1. Influence of the freeze-drying process of HSA nanoparticles (10 mg/ml) in the presence of different excipients on (A) particle diameter and (B) polydispersity before freeze drying (black bars) and after reconstitution of the samples in water (grey bars) (mean ± S.D.; $n = 3$).

In conclusion, sucrose and trehalose were better suited for lyophilisation of HSA nanoparticles than the bulking agent mannitol. A reason for the observation that trehalose and sucrose preserved the nanoparticles during freeze drying, while mannitol was not capable to do so, could be that sucrose and trehalose both form amorphous glasses, which were reported to interact with amorphous protein in the freeze-dried cake (Chang et al., 2005b).

3.3. Long-term storage of freeze-dried nanoparticles

To further evaluate stability not only in connection to the process but also with regard to long-term stability, formulation samples

Table 2
Residual moisture content of freeze-dried nanoparticle samples in the presence of different excipients

HSA nanoparticles in the presence of	Residual moisture content (%) after			
	Freeze drying	Storage 13 weeks 2–8 °C	Storage 13 weeks 25 °C, 60 RH	Storage 13 weeks 40 °C, 75 RH
2% trehalose	3.1	3.3	4.4	5.3
3% trehalose	3.3	3.0	4.3	4.9
2% sucrose	3.1	3.2	4.1	5.0
3% sucrose	3.0	3.3	3.9	4.9
2% mannitol	3.1	3.1	4.2	5.1
3% mannitol	3.0	3.4	4.3	5.0

after freeze drying (either with mannitol, sucrose or trehalose) were also included in a 3 months stability study at 2–8 °C (refrigerated conditions) as well as 25 and 40 °C (accelerated conditions). The particles were stored over a time period of 13 weeks at different temperatures and humidity conditions (2–8 °C, 25 °C/60 RH and 40 °C/75 RH). Directly after the freeze-drying process and after time periods of 4, 8, and 13 weeks samples were analysed with regard to particle diameter and polydispersity. Residual moisture content of the formulations was determined directly after freeze drying and after a storage time of 13 weeks. The results of the study are summarised in Table 2. Directly after the freeze-drying process and independent of the excipient used, the formulations showed a residual water content of about 3% (w/w). After a storage period of 13 weeks the samples stored at 2–8 °C showed no increase of the residual water content. Samples stored at 25 °C/60 RH and 40 °C/75 RH showed an increase of the water content up to 5% most likely due to moisture from the stoppers.

Although the residual water content of the samples was significantly enhanced under accelerated conditions, the nanoparticles still showed acceptable long-term storage stability with regard to particle diameter, and polydispersity after reconstitution (Fig. 2). The results of the long-term storage stability confirmed the previous data that trehalose and sucrose are superior to mannitol. Regardless of the concentration of mannitol used, the particle diameter and polydispersity increased over the storage time. When using 2% mannitol, the polydispersity indices rose up to 1, indicating a very heterogeneous particle size distribution (data not shown). Although samples with 3% mannitol showed a smaller increase of particle diameter (Fig. 2A) and polydispersity (Fig. 2B) in comparison to the 2% formulations, storage stability was a problem in the presence of the sugar alcohol. The higher the temperature of the storage condition and the longer the storage time, the larger and more heterogeneous the particles became. As already observed for the influence of the different excipients on freeze drying the best results for long-term storage were achieved with trehalose (Fig. 2A and B) and sucrose (data not shown), respectively. In the presence of 3% trehalose even after a storage period of 13 weeks at elevated temperature of 40 °C, no aggregation of the particles after reconstitution was observed. The storage revealed no influence either on particle diameter or polydispersity. The same was true for samples, which were lyophilised in the presence of sucrose. For both compounds it was observed that an excipient concentration of 3% showed slightly better results than formulations with only 2% of trehalose and sucrose, respectively. Therefore, a 3% concentration of trehalose or sucrose represents an optimum formulation for the long-term storage of HSA nanoparticles in freeze-dried state. This further suggests that the nanoparticles can be stored at 2–8 °C and potentially even at 25 °C for periods of at least several months.

3.4. *Freeze drying at fixed excipient concentration*

In a further step of the study various nanoparticle concentrations were studied, which were protected from aggregation by 2% formulations of sucrose and mannitol, respectively. For this reason a fixed concentration of 2% sucrose or mannitol was mixed with increasing amounts of HSA nanoparticles in the range between 10 and 25 mg/ml. This excipient concentration showed in the first trials to be sufficient to stabilise nanoparticles with particle content of 10 mg/ml from aggregation, although a 3% concentration led to a further improvement in the case of long-term sample storage.

In order to compare nanoparticles from different batches, the relative particle diameter and standard deviation were calculated. In order to easier compare the effect of the freeze-drying process step on the particle diameter, the particle sizes of different batches were normalized to 100% (prior to the freeze-drying step) and the sizes after freeze drying were calculated relative to this value.

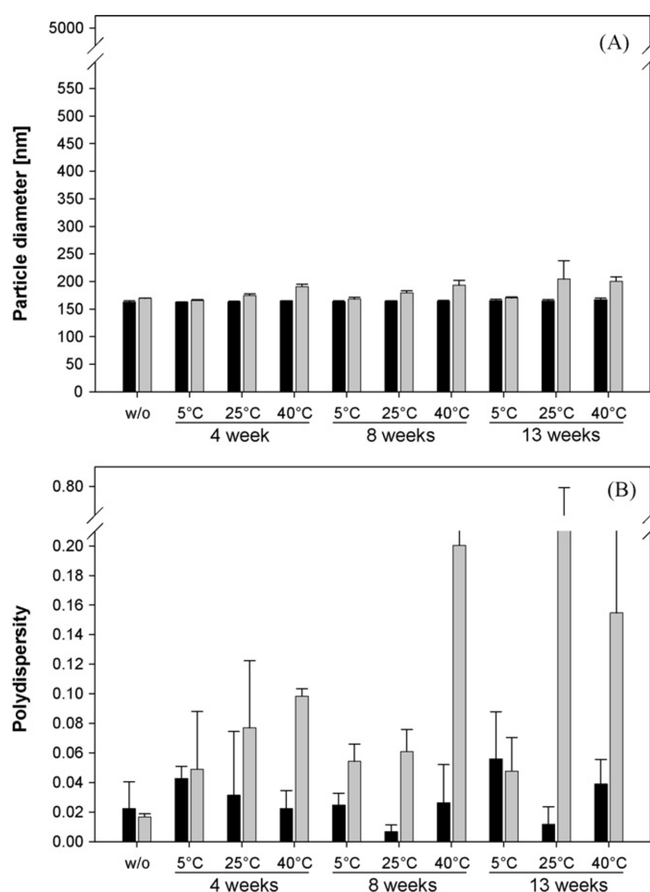

Fig. 2. Long-term storage study of freeze-dried HSA nanoparticles (10 mg/ml). The samples were lyophilised in the presence of 3% trehalose (black bars) or 3% mannitol (grey bars). Samples were analysed for particle diameter (A) and polydispersity (B) after storage at 2–8 °C, 25 °C/60 RH, and 40 °C/75 RH for time intervals of 4, 8, and 13 weeks, respectively. Freeze-dried samples without (w/o) storage were used as control (mean ± S.D.; $n = 3$).

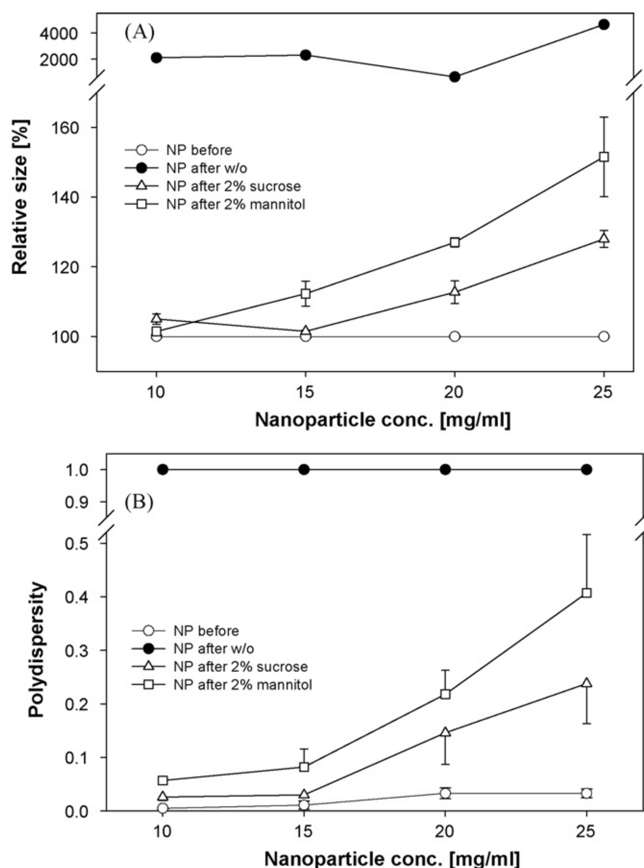

Fig. 3. Influence of the freeze-drying process of different concentrations HSA nanoparticles in the presence of fixed concentrations of excipients on the (A) relative particle size and (B) polydispersity before freeze drying (○) and after reconstitution of samples freeze dried in the presence of 2% sucrose (△), 2% mannitol (□), or without cryoprotector (●) (mean ± S.D.; $n = 3$).

At each nanoparticle concentration, no aggregation was observed by PCS prior to the freeze-drying process in all formulations tested. Particle diameter (Fig. 3A) and polydispersity index (Fig. 3B) remained constant over the whole nanoparticle concentration range. Therefore, the excipients itself did not change the size distribution of the nanoparticles with regard to particle diameter and polydispersity, suggesting that neither excipient tested interacted with the nanoparticles with regard to a potential impact on attractive or repulsive forces. As could be expected from the first part of the study, in the absence of any excipient a strong particle aggregation was observed after the freeze-drying process. Again, sucrose proved to be superior to mannitol: Formulations with 2% sucrose resulted in adequate stabilisation of nanoparticle concentration of 10 and 15 mg/ml, respectively. Even at particle concentrations of 20 mg/ml only a minor increase in relative particle size and polydispersity index was observed. The polydispersity index was only increased to about 0.14, indicating an almost monodisperse size distribution. At even higher particle contents (25 mg/ml) the sucrose concentration used, however, seemed to be too low. In formulations with 2% mannitol, the particle diameter increased already at nanoparticle concentrations of 15 mg/ml. Generally, it was observed that using sucrose or mannitol as excipients, increasing particle diameters were observed with increasing nanoparticle concentrations in the formulation. From these results we can conclude, that 2% sucrose was able to stabilise nanoparticles up to a concentration limit of about 15 mg/ml whereas mannitol did only show slight improvements up to about 10 mg/ml.

3.5. *Freeze drying of PEGylated nanoparticles*

In order to compare the freeze-drying behaviour of unloaded nanoparticles with surface-modified or drug loaded nanoparticles, further experiments were conducted with PEGylated and doxorubicin-loaded systems. Similar to the experiments of unmodified nanoparticles a standard concentration of 10 mg/ml nanoparticles was chosen. In principle, PEGylation of the particles is performed in order to achieve a prolongation of the circulation time in the body and therefore to increase tumour targeting capability under *in vivo* conditions (Greenwald, 2001; Barratt, 2003; Brannon-Peppas and Blanchette, 2004). In our study, the PEGylated nanoparticles were freeze dried in the presence of the excipients sucrose, trehalose, and mannitol at concentration levels of 1%, 2%, or 3% (w/v), respectively, in aqueous solution. As a result, sucrose and trehalose at concentrations of 2% and higher were suitable excipients for freeze drying of PEGylated HSA nanoparticles (Fig. 4). At concentrations of 2% and 3% neither a significant change in particle size (Fig. 4A) nor in polydispersity index (Fig. 4B) was observed. This was comparable to the results obtained for unmodified nanoparticles. Additionally, mannitol was less useful as an excipient, because particle diameter and polydispersity of the samples after lyophilisation and reconstitution were significantly increased in comparison to the samples before drying.

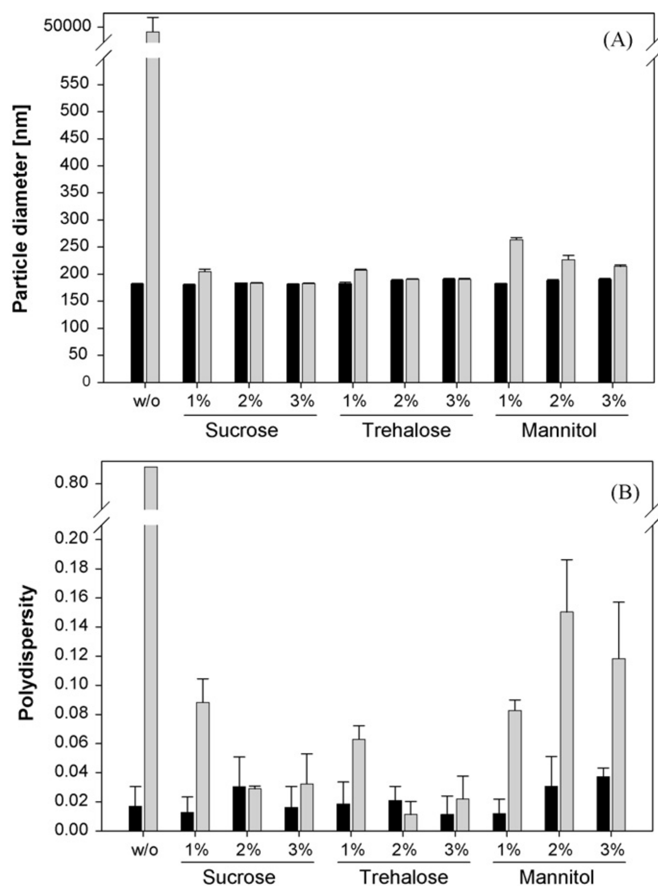

Fig. 4. Influence of the freeze-drying process of PEGylated HSA nanoparticles (10 mg/ml) in the presence of different excipients on (A) particle diameter and (B) polydispersity before freeze drying (black bars) and after reconstitution of the samples in water (grey bars) (mean ± S.D.; $n = 3$).

3.6. Freeze drying of doxorubicin-loaded nanoparticles

Nanoparticles are a promising vehicle for drug targeting in cancer therapy. Because of the EPR effect an accumulation of particles in solid tumours could be expected (Maeda et al., 2000). Due to this mechanism drugs entrapped in nanoparticles can selectively be accumulated in tumour tissues and therefore, adverse drug effects in healthy tissues can be reduced (Torchilin, 2006). Doxorubicin is a widely used cytostatic drug used in the therapy of different tumours such as, i.e. epithelial ovarian cancer (Stebbing and Gaya, 2002). Therefore, in the present study doxorubicin was used as a model drug for the incorporation in the matrix of HSA nanoparticles.

Doxorubicin-loaded nanoparticles were prepared by protein desolvation in the presence of the drug as previously described by Dreis et al. (2007). After particle crosslinking, the nanoparticles were purified by 2 cycles of differential centrifugation and the collected supernatants were used for quantification of non-entrapped doxorubicin. HPLC analysis revealed that the main part of unbound doxorubicin was found in the first supernatant whereas the second step of purification showed that less than 1% of the entrapped doxorubicin can be washed out from the particle matrix. This observation suggests the stable entrapment of the drug within the HSA nanoparticles matrix. The preparation yielded doxorubicin-loaded HSA nanoparticles with a particle content of 10 mg/ml in aqueous suspension, loaded with $583.3 \pm 15.0\ \mu g/ml$ doxorubicin. Therefore, a drug loading efficiency of $58.3 \pm 1.5\ \mu g$ doxorubicin per milligram nanoparticle was achieved.

Particle size and polydispersity were determined before freeze drying and after reconstitution of the freshly prepared lyophilised samples in water. Directly after preparation and purification, the drug-loaded particles showed a particle diameter ranging from 352 to 373 nm and, therefore, were found to be significantly larger than unmodified or PEGylated nanoparticles. The increase in particle size of doxorubicin-loaded nanoparticles could be a result of the lower pH of about 6.5 during protein desolvation compared to a pH value of 8.5 for the preparation of empty and PEGylated nanoparticles. The lower pH value was chosen for the incorporation of doxorubicin because of preliminary experiments, which showed a higher drug loading efficiency under these conditions (Dreis et al., 2007). The observed particle size increase is in accordance with a previous study, in which the pH value of the HSA solution prior to the desolvation procedure was identified as the major factor determining particle size (Langer et al., 2003).

The doxorubicin-loaded nanoparticles were also freeze dried in presence of the three excipients sucrose, trehalose, and mannitol at concentrations of 1%, 2%, and 3% (w/v). In accordance with the results of unmodified or PEGylated nanoparticles, freeze drying of doxorubicin-loaded nanoparticles without any excipient resulted in unacceptably high amounts of particle aggregation. After the freeze-drying process, only particles in the micrometer size range with a high polydispersity were obtained (Fig. 5).

In accordance with the results of unloaded nanoparticles, it was observed that for the doxorubicin-loaded nanoparticles an amount of 1% excipient was not sufficient to protect the particles from aggregation. By the addition of 2% and 3% of the excipients sucrose, trehalose or mannitol the aggregation of the nanoparticles could be avoided. Nevertheless, in comparison to the nanoparticles before freeze drying a small increase of particle size could be observed directly after freeze drying and reconstitution using sucrose, trehalose or mannitol at the concentrations of 2% and 3%.

In the case of drug loaded nanoparticles a further aspect, which needs consideration, is the loading stability after freeze drying. In principle, it may be conceivable that the freeze-drying process may influence the drug entrapment of the particles, i.e. may result in drug loss due to change or loss of the particle structures. Therefore, the samples were also investigated for potentially released doxorubicin directly after reconstitution. After redispersion the resulting particle suspensions were centrifuged for 20 min followed by HPLC analysis of the supernatant. In the supernatants of all samples only very limited amounts ($0.05 \pm 0.01\ \mu g/mg$) of free doxorubicin were detected, whereas total particle loading was determined to be 65.56 μg doxorubicin per mg nanoparticle. Therefore, it has to be concluded that the freeze-drying process exerts no influence on drug loading stability of HSA nanoparticles.

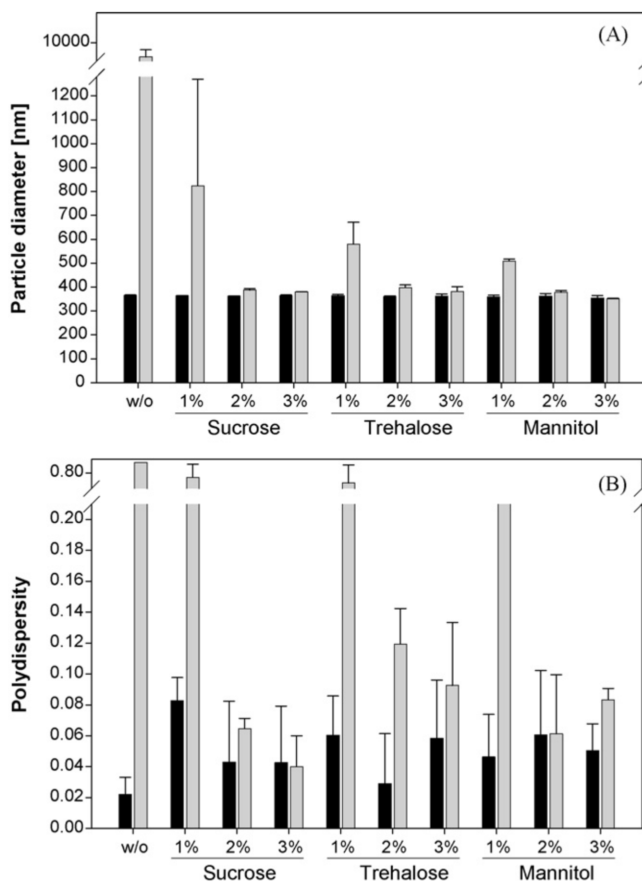

Fig. 5. Influence of the freeze-drying process of doxorubicin-loaded HSA nanoparticles (10 mg/ml) in the presence of different excipients on (A) particle diameter and (B) polydispersity before freeze drying (black bars) and after reconstitution of the samples in water (grey bars) (mean ± S.D.; $n = 3$).

4. Conclusion

The present study shows that HSA nanoparticles can be stabilised both during the lyophilisation process as well as for long-term storage after freeze drying when using adequate concentrations of suitable excipients. The study also showed, that the freeze-drying process has the potential to negatively influence stability if not using adequate excipients during the process.

Stability was assessed by aggregation state (particle diameter and polydispersity via PCS), residual moisture and in case of doxorubicin-loaded nanoparticles for drug loss. Three different types of nanoparticles, namely unmodified, PEGylated, and doxorubicin-loaded HSA nanoparticles were evaluated in the presence of the three different excipients mannitol, sucrose or trehalose. The choice of a suitable excipient was mainly dependent on the nature of the particle system. Stabilisers such as trehalose and sucrose in concentrations of 2–3% were well suited to preserve

10 mg/ml nanoparticles from aggregation during freeze drying and subsequent reconstitution. On the other hand bulking agents such as mannitol resulted in elegant lyophilisation cakes, but led to a significant increase in particle diameter and polydispersity after freeze drying and reconstitution.

It has to be concluded that freeze drying of doxorubicin-loaded nanoparticles with sucrose, trehalose or mannitol, respectively led to comparable results. On the other hand freeze drying of unmodified and PEGylated nanoparticles showed that sucrose and trehalose were superior to mannitol. This fact was confirmed by the long-term stability. In the latter case, the best results were obtained in the presence of 3% trehalose and sucrose, respectively.

Although for most preparations both sugars were well suited for freeze drying, the study showed that optimal conditions depend mainly on the nature of the particle systems. For each variation of the nanoparticles such as surface modification or drug loading a new evaluation of the most suitable formulation is encouraged.

Acknowledgments

This work was financially supported by the German Bundesministerium für Bildung und Forschung (BMBF) (Project 13N8671).

The authors would also like to thank Pierre Goldbach for support with freeze drying, Sylvia Kiese for logistical support and Giorgio Cirelli for support with DSC experiments.

References

Akasaka, Y., Ueda, H., Takayama, K., Machida, Y., Nagai, T., 1988. Preparation and evaluation of bovine serum albumin nanospheres coated with monoclonal antibodies. Drug Des. Deliv. 3, 85–97.

Allémann, E., Gurny, R., Doelker, E., 1993. Drug-loaded nanoparticles—preparation methods and drug targeting issues. Eur. J. Pharm. Biopharm. 39, 173–191.

Barratt, G., 2003. Colloidal drug carriers: achievements and perspectives. Cell Mol. Life Sci. 60, 21–37.

Brannon-Peppas, L., Blanchette, J.O., 2004. Nanoparticle and targeted systems for cancer therapy. Adv. Drug Deliv. Rev. 56, 1649–1659.

Carpenter, J.F., Pikal, M.J., Chang, B.S., Randolph, T.W., 1997. Rational design of stable lyophilized protein formulations: some practical advice. Pharm. Res. 14, 969–975.

Chang, L.L., Shepherd, D., Sun, J., Ouellette, D., Grant, K.L., Tang, X.C., Pikal, M.J., 2005a. Mechanism of protein stabilization by sugars during freeze-drying and storage: native structure preservation, specific interaction, and/or immobilization in a glassy matrix? J. Pharm. Sci. 94, 1427–1444.

Chang, L.L., Shepherd, D., Sun, J., Tang, X.C., Pikal, M.J., 2005b. Effect of sorbitol and residual moisture on the stability of lyophilized antibodies: implications for the mechanism of protein stabilization in the solid state. J. Pharm. Sci. 94, 1445–1455.

Chatterjee, K., Shalaev, E.Y., Suryanarayanan, R., 2005. Partially crystalline systems in lyophilization. I. Use of ternary state diagrams to determine extent of crystallization of bulking agent. J. Pharm. Sci. 94, 798–808.

Costantino, H.R., 2004. Excipients for use in lyophilized pharmaceutical peptide, protein, and other bioproducts. In: Costantino, H.R., Pikal, M.J. (Eds.), Lyophilization of Biopharmaceuticals, vol. 2. American Association of Pharmaceutical Scientists, Arlington, pp. 139–229.

Crowe, L.M., Reid, D.S., Crowe, J.H., 1996. Is trehalose special for preserving dry biomaterials? Biophys. J. 71, 2087–2093.

Dreis, S., Rothweiler, F., Michaelis, M., Cinatl Jr., J., Kreuter, J., Langer, K., 2007. Preparation, characterisation and maintenance of drug efficacy of doxorubicin-loaded human serum albumin (HSA) nanoparticles. Int. J. Pharm. 341, 207–214.

Greenwald, R.B., 2001. PEG drugs: an overview. J. Control. Release 74, 159–171.

Johnson, R.E., Kirchhoff, C.F., Gaud, H.T., 2002. Mannitol–sucrose mixtures-versatile formulations for protein lyophilization. J. Pharm. Sci. 91, 914–922.

Kim, A.I., Akers, M.J., Nail, S.L., 1998. The physical state of mannitol after freeze-drying: effects of mannitol concentration, freezing rate, and a noncrystallizing cosolute. J. Pharm. Sci. 87, 931–935.

Konan, Y.N., Gurny, R., Allémann, E., 2002. Preparation and characterization of sterile and freeze-dried sub-200 nm nanoparticles. Int. J. Pharm. 233, 239–252.

Kreuter, J., 1983. Evaluation of nanoparticles as drug-delivery systems. I. Preparation methods. Pharm. Acta Helv. 58, 196–209.

Langer, K., 2006. Peptide nanoparticles. In: Kumar, C.S.S.R. (Ed.), Biological and Pharmaceutical Nanomaterials, vol. 6. Wiley–VCH Verlag GmbH, pp. 145–184.

Langer, K., Anhorn, M.G., Steinhauser, I., Dreis, S., Celebi, D., Schrickel, N., Faust, S., Vogel, V., 2008. Human serum albumin (HSA) nanoparticles: reproducibility of preparation process and kinetics of enzymatic degradation. Int. J. Pharm. 347, 109–117.

Langer, K., Balthasar, S., Vogel, V., Dinauer, N., von Briesen, H., Schubert, D., 2003. Optimization of the preparation process for human serum albumin (HSA) nanoparticles. Int. J. Pharm. 257, 169–180.

Langer, K., Coester, C., Weber, C., von Briesen, H., Kreuter, J., 2000. Preparation of avidin-labeled protein nanoparticles as carriers for biotinylated peptide nucleic acid. Eur. J. Pharm. Biopharm. 49, 303–307.

Liu, S.Q., Leong, K.W., 1997. Delivery of protein and low-molecular weight drug by coacervate human serum albumin and heparin. Proc. Int. Symp. Control. Release Bioact. Mater., 911–912.

Liu, W., Wang, D.Q., Nail, S.L., 2005. Freeze-drying of proteins from a sucrose-glycine excipient system: effect of formulation composition on the initial recovery of protein activity. AAPS Pharm. Sci. Tech. 6, E150–E157.

Maeda, H., Wu, J., Sawa, T., Matsumura, Y., Hori, K., 2000. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J. Control. Release 65, 271–284.

Moghimi, S.M., Hunter, A.C., Murray, J.C., 2005. Nanomedicine: current status and future prospects. FASEB J. 19, 311–330.

Passot, S., Fonseca, F., Alarcon-Lorca, M., Rolland, D., Marin, M., 2005. Physical characterisation of formulations for the development of two stable freeze-dried proteins during both dried and liquid storage. Eur. J. Pharm. Biopharm. 60, 335–348.

Pyne, A., Chatterjee, K., Suryanarayanan, R., 2003. Solute crystallization in mannitol-glycine systems—implications on protein stabilization in freeze-dried formulations. J. Pharm. Sci. 92, 2272–2283.

Sameti, M., Bohr, G., Ravi Kumar, M.N., Kneuer, C., Bakowsky, U., Nacken, M., Schmidt, H., Lehr, C.M., 2003. Stabilisation by freeze-drying of cationically modified silica nanoparticles for gene delivery. Int. J. Pharm. 266, 51–60.

Stebbing, J., Gaya, A., 2002. Pegylated liposomal doxorubicin (Caelyx) in recurrent ovarian cancer. Cancer Treat. Rev. 28, 121–125.

Steinhauser, I., Spänkuch, B., Strebhardt, K., Langer, K., 2006. Trastuzumab-modified nanoparticles: optimisation of preparation and uptake in cancer cells. Biomaterials 27, 4975–4983.

Tang, X., Pikal, M.J., 2004. Design of freeze-drying processes for pharmaceuticals: practical advice. Pharm. Res. 21, 191–200.

Torchilin, V.P., 2006. Multifunctional nanocarriers. Adv. Drug Deliv. Rev. 58, 1532–1555.

Torchilin, V.P., 2007. Targeted pharmaceutical nanocarriers for cancer therapy and imaging. AAPS J. 9, E128–E147.

Wartlick, H., Michaelis, K., Balthasar, S., Strebhardt, K., Kreuter, J., Langer, K., 2004. Highly specific HER2-mediated cellular uptake of antibody-modified nanoparticles in tumour cells. J. Drug Target. 12, 461–471.

Weber, C., Coester, C., Kreuter, J., Langer, K., 2000. Desolvation process and surface characterisation of protein nanoparticles. Int. J. Pharm. 194, 91–102.

Yu, L., Milton, N., Groleau, E.G., Mishra, D.S., Vansickle, R.E., 1999. Existence of a mannitol hydrate during freeze-drying and practical implications. J. Pharm. Sci. 88, 196–198.