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A toolbox for the upscaling of ethanolic human serum albumin (HSA) desolvation

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ABSTRACT

Nanoparticles consisting of human serum albumin (HSA) play an emerging role in the development of new drug delivery systems. Many of these protein-based colloidal carriers are prepared by the well-known desolvation technique, which has shown to be a robust and reproducible method for the laboratory-scale production of HSA nanoparticles.

The aim of the present study was to upscale the ethanolic desolvation process utilizing the paddle stirring systems *Nanopaddle I and II* in combination with a HPLC pump in order to find the optimal conditions for the controlled desolvation of up to 2000 mg of the protein.

For characterization of the HSA nanoparticles particle size, zeta potential as a function of the pH, polydispersity index and particle content were investigated. The particle content was determined by microgravimetry and by a turbidimetry to allow optimized in-process control for the novel desolvation technique. Furthermore the sedimentation coefficient was measured by analytical ultracentrifugation (AUC) to gain deeper insight into the size distribution of the nanoparticles.

The formed nanocarriers were freeze dryed to achieve a solid preparation for long-term storage and further processing. Particles ranging in size between 251.2 ± 27.0 and 234.1 ± 1.5 nm and with a polydispersity index below 0.2 were achieved.

1. Introduction

Research over the last decade has found human serum albumin (HSA) to be a promising material for the preparation of modern drug carrier systems. These nanocarriers can easily be prepared by ethanolic desolvation and allow the binding of a variety of different molecules either by incorporation, adsorption or through covalent modification of the nanoparticle surface (Wartlick et al., 2004b; Anhorn et al., 2008b; Kufleitner et al., 2010a,b; Wacker et al., 2010; Wagner et al., 2010; Low et al., 2011). Other technological approaches such as the one used by nab technology™ are of advantage especially for poorly soluble drugs (Henderson and Bhatia, 2007). However, a covalent stabilization or surface functionalization of these nanocarriers is difficult because of the residues of dissolved HSA in the resulting suspension. Nanoparticles obtained by the ethanolic desolvation of human serum albumin (HSA) allow these modifications (Wartlick et al., 2004a; Michaelis et al., 2006; Zensi et al., 2009, 2010) to be carried out.

In principle, HSA precipitates due to the controlled addition of ethanol to a solution of the protein followed by the stabilization of albumin nanoparticles with a chemical crosslinker. The lab-scale preparation of these nanoparticles has become a standard method but an industrial production based on ethanolic desolvation has not yet been established.

Whilst an increasing number of new chemical and biological entities exhibit poor water solubility, insufficient bioavailability, and/or stability, a high availability of the nanosized carrier material will be of advantage for the investigation of novel drug delivery systems. A cost-effective and reproducible large-scale preparation technique can provide high amounts of these HSA nanoparticles for the drug formulation which can be used for preclinical and/or clinical research.

The ethanolic desolvation process has been proposed to be a robust method which allows the particle size and polydispersity index to be controlled by varying the ion strength, pH, HSA concentration, and stirring speed (Langer et al., 2003). However, one disadvantage of the existing preparation methods is the choice of magnetic stirring bars which have little suitability for the upscaling of the production method because of the variable stirring speed, decreased stirring efficiency for larger volumes, irregular hydrodynamics, and friction between the bottom of the vessel and the stirrer inside the reaction vial.

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Furthermore, since a variability in the solvent flow was previously observed during the desolvation process because of the peristaltic pump used, this may also have a negative influence on the achieved product outcome especially when a greater desolvation volume is chosen.

To overcome these disadvantages, two paddle stirring systems were developed in order to ensure a homogeneous distribution of HSA molecules during the desolvation process for increased volumes of protein solutions. These paddle stirring systems show good suitability for their application in an industrial large-scale process. A constant solvent flow was achieved by utilizing a HPLC pump for the addition of ethanol to the protein solution.

The characterization of HSA nanoparticles prepared by the large-scale desolvation is one major challenge in the upscaling process. Different analytical methods were combined and a number of key parameters were identified for the preparation of a well-defined product which shows characteristics comparable to the nanoparticles achieved by lab-scale preparation.

With regard to the need for easy-to-use methods for the in-process control in the pharmaceutical industry, the particle content was determined by microgravimetry and turbidimetry. The particle diameter, polydispersity index, and zeta potential were measured by dynamic light scattering (DLS) in order to provide information on the size distribution and the surface charge of the nanocarriers prepared by this novel desolvation technique.

A deeper insight into the size distribution of the major fraction of the colloid system was gained by performing an analytical ultracentrifugation (AUC) analysis before and after the purification of the colloid system. This method which was validated earlier for nanoparticles consisting of human serum albumin provides information on the sedimentation coefficient distribution (Vogel et al., 2002) of the colloid system.

The purification steps of centrifugation and redispersion which are part of the standard lab-scale preparation of HSA nanoparticles are of minor suitability for an industrial application and were assumed to have low influence on the particle size distribution.

After the desolvation process, the organic solvent must be removed from the particle formulation in order to assure compatibility with the physiological environment. Although the particle system may show aggregation during the heat-induced drying process, freeze drying of the hydroethanolic suspension leads to a solid formulation which can be resuspended either in water or isotonic sodium chloride solution for parenteral application (Tang and Pikal, 2004). It has been shown that freeze drying is known to be a suitable method to transfer HSA nanoparticles from the aqueous suspension to the solid state (Anhorn et al., 2008a) but a freeze drying process for the hydroethanolic medium has not yet been established. Due to the risk of aggregate formation, it is therefore important to show the absence of large amounts of dissolved HSA prior to the drying process. As previously described (Wacker et al., 2010), these residues can easily be quantified by size exclusion chromatography (SEC).

Before and after the freeze drying process, particle size and size distribution were investigated. After resuspension, these HSA nanoparticles will allow a further processing by adsorption of drugs or covalent modification of the nanoparticle surface with drug targeting ligands (Dreis et al., 2007; Kufleitner et al., 2010b; Wacker et al., 2010).

2. Materials and methods

2.1. Chemicals and reagents

HSA (fraction V, purity 96–99%, batches 028K7550 and 114H9314) and glutaraldehyde, 8% solution, were obtained from Sigma (Steinheim, Germany). All other reagents were purchased from Merck (Darmstadt, Germany) and were of analytical grade and used as received.

2.2. Upscaling of ethanolic desolvation process

HSA nanoparticles were prepared by the well-known desolvation technique as described previously (Langer et al., 2003). Using this method, approximately 200–2000 mg of HSA was dissolved in different volumes of 10 mM NaCl solution at a concentration of 10% [m/V]. These solutions were then titrated to a pH of 7–10 with 1 N sodium hydroxide solution using a Callimatic 766 pH meter (Knick, Berlin, Germany).

For the upscaling of ethanolic desolvation process, volumes of 2 mL, 4 mL and 20 mL of the HSA solution were desolvated by addition of 8, 16, and 80 mL of ethanol, respectively. For a final volume of 10 mL of the hydroethanolic suspension, a magnetic stirring system was used, for 20 mL either the *Nanopaddle I* and *II* stirring systems (see Fig. 1) were used whilst for a final volume of 100 mL only the *Nanopaddle II* stirring system was utilized. The stirring speed of the magnetic stirring device was set to 550 rpm. The speed of all Nanopaddle systems was monitored using the photo tachometer system ebro D1238L (ebro GmbH & Co. KG, Ingolstadt, Germany) and adjusted to 550 rpm before and after addition of ethanol. At all times the immersion depth was maintained at 1 cm.

The constant addition of ethanol to the protein solution was achieved using an automatic peristaltic pump (ISMATEC IPN, Glattbrugg, Switzerland). According to the standard method developed by Langer et al. (2003) ethanol was added at a rate of 1 mL/min for a final nanoparticle suspension volume of 10 mL. In these studies at the greater volumes, the pumping speed was increased to 2 mL/min and 4 mL/min for the final volumes of 20 and 100 mL, respectively.

For the different batch sizes of 200, 400 and 2000 mg of HSA, 115, 231 and 1155 μL of aqueous glutaraldehyde solution 8% [V/V] was carefully added. The hydroethanolic suspensions were subsequently stirred for 12 h in presence of the crosslinker to stabilize the colloid system.

2.3. pH dependence of upscaled desolvation process

The pH dependence of the upscaled ethanolic desolvation process was determined for the final batch volume of 100 mL of HSA nanoparticles in hydroethanolic suspension. This was examined by dissolving 2000 mg of HSA in 20 mL sodium chloride solution (10 mM). The pH value was titrated to 8.0, 8.25, and 8.5 with 1 M sodium hydroxide solution using a Callimatic 766 pH meter (Knick, Berlin, Germany). The solution was filled into a 100 mL glass bottle (Schott Duran, Wertheim, Germany) and the *Nanopaddle II* stirring device (see Fig. 1) was adjusted to a stirring speed of 800 rpm with a photo tachometer system ebro D1238L (ebro GmbH & Co. KG, Ingolstadt, Germany). The immersion depth was maintained at 1 cm. A volume of 80 mL of ethanol 96% [V/V] was added using a Merck Hitachi HPLC pumping device L-6220 at a flow rate of 5.0 mL/min. The stirring speed was adjusted to 800 rpm again. For stabilization of the colloid system, 1155 μL of glutaraldehyde solution 8% [V/V] was added. This aldehyde concentration corresponds to a 100% crosslinkage of the aminogroups of the HSA molecule.

2.4. Purification of HSA nanoparticles and determination of nanoparticle content

The HSA nanoparticles were purified by threefold centrifugation (20817 g, 10 min) and redispersion in 1.0 mL of purified water using Bandelin Sonorex ultrasonic bath (Bandelin electronics, Berlin, Germany). The particle content of the purified samples

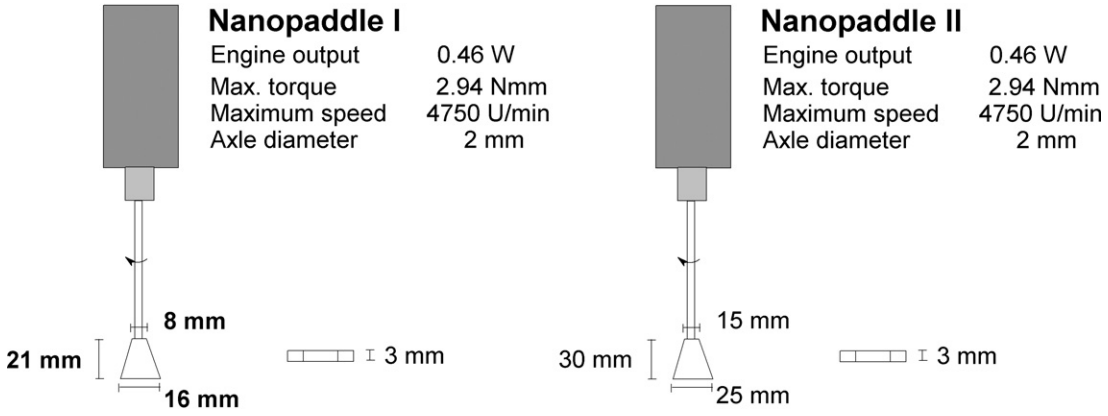

Fig. 1. Schematic of the paddle stirring devices *Nanopaddle I* and *II*.

was measured by microgravimetry. For the hydroethanolic suspension, the particle content was determined turbidimetrically without purification. Linearity of the method was shown at the observed wavelength (see Section 2.7).

2.5. Determination of particle size and size distribution

The particle size and the size distribution of the nanoparticles were measured by photon correlation spectroscopy (PCS) using a Malvern Zetasizer© 3000 HS_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 were diluted 1:50 with purified water and placed into single-use PCS cuvettes (10 mm × 10 mm × 48 mm) (Sarstedt, Nürnbrecht, Germany). The mean hydrodynamic diameter of the particles was calculated from the intensity of the scattered light using the Malvern software package.

To determine the sedimentation coefficient distribution of the colloidal carriers, analytical ultracentrifugation (AUC) measurements were performed according to a method described for HSA nanoparticles in the literature (Vogel et al., 2002). The HSA nanoparticles were prepared by desolvation at three different pH values (see Section 2.3) and analyzed before and after purification of the colloid system. The purified samples were taken from the aqueous and the unpurified samples from the hydroethanolic suspension. The particle content of the unpurified samples was measured by turbidimetry whilst microgravimetry was used to measure the particle content of the purified samples.

In principle, the nanoparticle suspension was diluted with an aqueous stock solution of 20 mM sodium phosphate (pH 7.0), 100 mM sodium chloride, and 23.5% [m/V] sucrose resulting in a turbidity between 0.6 and 1.0 at 420 nm in a cuvette with a 1 cm optical pathlength. The ultracentrifugation experiments were performed using a Beckman Optima XL-A ultracentrifuge, an An-50-Ti rotor, and double-sector charcoal-filled Epon centrepieces of 12-mm optical pathlength at a rotor speed of 3000 or 5000 rpm and a rotor temperature of 20 °C. The sample and the reference volumes were 300 and 310 μL, respectively. Apparent absorbance (turbidity) versus radius data were collected at 420 nm, using a radial step-size of 0.03 mm. For the analysis of the particle size distribution, the sedimentation velocity data were modelled as a distribution of nondiffusing particles based on the observations of large sedimentation coefficients and of large particle diameters from PCS measurments. This in turn allowed application of the recently introduced method for calculating apparent sedimentation coefficient distributions, g*(s), by least-squares boundary modelling, the "ls–g*(s) method" using the program sedfit by P. Schuck (see www.analyticalultracentrifugation.com).

2.6. Statistical analysis of data

To compare particle size distribution of nanoparticles prepared by small-scale and large-scale preparation and at different pH values, ANOVA at a significance level of $\alpha = 0.05$ was applied using Sigmaplot 11 (Sysstat Software, San José, USA). All other calculations were carried out using Excel© 2003 (Microsoft, Redmond, WA, USA).

2.7. Turbidimetric quantification of HSA nanoparticles

For tubidimetric quantification of HSA nanoparticles in suspension, the purified colloid system was used as a standard, diluted to concentrations of 0.05, 0.25, 0.50, 0.75 and 1.0 mg/mL and the absorbance measured at 420 nm using a Hitachi U-3000 photometer (Hitachi, Tokio, Japan). Linearity was observed over this concentration range. To exclude any non-specific absorption effects by residues of either the dissolved HSA or the two solvents used, wavelength spectra of HSA nanoparticles were recorded in ethanol, purified water, and an aqueous solution of 3% [m/V] of dissolved HSA.

The control measurements were performed using three different particle batches with particle sizes ranging between 231.9 and 278.6 nm and a polydispersity index below 0.2. The samples were taken from hydroethanolic suspension directly after desolvation without a purification step followed by dilution (1:20) with purified water and were measured as described above. There was no significant difference in the apparent absorbance (turbidity) of the different particle size species detected (ANOVA).

2.8. Freeze drying of HSA nanoparticles

After turbidimetric quantification of nanoparticle content, the volume corresponding to a particle mass of 10 mg was filled into a freeze drying vial (Machery Nagel, Düren, Germany) followed by the addition of 300 μL of a 10% [m/V] mannitol solution and 100 μL of trehalose solution. The volume was filled up to 1000 μL with purified water and the drying process was started. Freeze drying was performed using a Christ Epsilon 1-4 freeze dryer (Martin Christ GmbH, Osterorde, Germany).

In order to freeze the samples, initially the shelf temperature was reduced to −65 °C and maintained for 4 h. The primary drying process was initiated by decreasing the pressure to 2.170 mbar over 10 min. The temperature was slowly increased to −35 °C and the pressure decreased to 0.2 mbar over a time period of 10 h. At a constant 0.2 mbar the temperature was increased to −25 °C at a rate of 0.7 °C/h.

For the secondary drying, the temperature was increased to −5 °C and the pressure decreased to 0.1 mbar over a 10 min period. Thereafter the pressure was decreased to a minimum of 0.001 mbar over 5 h. Finally, the temperature was slowly increased to 20 °C over a time period of 5 h whilst at 0.001 mbar.

After freeze drying, each sample was stored for 3 days at room temperature and resuspended in purified water. To ensure proper wetting of the lyophilised cake, the vial was allowed to sit for 5 min and then vortexed (Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY, USA) for 2 min at vortex level 2.5. This was followed by particle size analysis.

2.9. Quantification of HSA residues via SEC

Residues of dissolved HSA were determined from the supernatant of the desolvated protein after crosslinking of the amino groups with glutaraldehyde.

A 60 μL volume of the hydroethanolic solution was directly injected into a Merck Hitachi size exclusion chromatography (SEC) system (Merck-Hitachi, Darmstadt, Germany) using an L-7120 automatic pumping device, an L-7200 autosampler system and a Phenomenex BioSep® SEC S-3000 column (300 mm × 7.80 mm, 5 μm). The protein was detected using a L-7455 diode array detector at a wavelength of 280 nm. The absorbance was monitored between 200 and 500 nm. The concentration was analyzed by comparing the absorbance to standards solutions of HSA prepared in purified water in the 100–400 μg/mL concentration range. There were no other peaks observed (see Fig. 6).

2.10. Surface properties of HSA nanoparticles

For determination of the surface properties of the colloid systems, standard samples were prepared according to a lab-scale desolvation method described earlier (see Section 2.2). In principle, a volume of 2 mL of a 10% [m/V] HSA solution was desolvated by the addition of 8.0 mL of ethanol at pH 8.0, 8.25, and 8.5. These samples were crosslinked with glutaraldehyde.

Titration experiments on the HSA nanoparticles prepared by large-scale and lab-scale desolvation were performed over a pH range between 3 and 10 using the Malvern Zetasizer© 3000 HS_A coupled with an autotitrator unit MPT-1 (Malvern Instruments Ltd., Malvern, UK). For the analysis, 150 μL of the unpurified nanoparticle suspension was filled up to a volume of 50 mL with purified water and the pH value of the suspension was automatically adjusted by the titration unit by addition of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide solution. At 8 predefined pH values between 3 and 10, the zetapotential of the nanoparticles was measured by microelectrophoresis. All calculations were performed using the Malvern software package.

3. Results and discussion

Over the past few years a variety of different methods for the preparation of nanoparticular drug delivery systems have been established some of which have been extensively used and characterized.

Many of these particle systems have shown to be efficient tools for the drug delivery. These include anticancer agents (Wartlick et al., 2004b; Dreis et al., 2007; Anhorn et al., 2008b; Wacker et al., 2010), life-saving medication (Kufleitner et al., 2010a,b) or antiviral drugs (Schafer et al., 1992). In 2009 Abraxane® a first drug formulation containing albumin as a nanoparticular carrier material has been approved by the EMA.

Due to limitations in either the particle preparation and/or the particle characterization that assures the quality and safety of these nanotechnological products, to date there are only few methods that have been established to allow the upscaling of the production process (Vauthier and Bouchemal, 2009).

It is widely known that the ethanolic desolvation process is a robust and reproducible method for the denaturation of albumin with increased stability of the colloid system after crosslinking the amino groups on the surface of HSA nanoparticles with a bifunctional aldehyde (Langer et al., 2003).

The purpose of the current study was to transfer the scientific knowledge into a practical application and to provide a toolbox for the conversion into a modern production process. In order to achieve this, the nanoparticle preparation was upscaled to a maximum volume of 100 mL of the hydroethanolic suspension and the process parameters were varied in order to find the optimal desolvation conditions.

Furthermore the most important characteristics (e.g. particle size, size distribution, surface charge) of the nanosized drug carrier material were identified and determined to allow the large-scale production of HSA nanoparticles comparable to those prepared by lab-scale desolvation.

3.1. Upscaling of ethanolic desolvation process

As the desolvation process at pH values of 8 or higher leads to a nearly monodisperse size distribution, this pH was chosen for the upscaling of ethanolic desolvation. Under these conditions the surface charge of the HSA molecules leads to a high stability of the drug carrier system (Langer et al., 2003).

The upscaling was performed in two steps. Firstly 400 mg of dissolved HSA was desolvated using a magnetic stirring bar and the *Nanopaddle I* stirring system (see Fig. 1) which has a limited suitability for higher volumes because of the small paddle size and the inefficient homogenization of the solution during the denaturation of the protein.

In the second step *Nanopaddle II* was used for the desolvation of 400 and 2000 mg of the protein. From the literature it is known that not only is the rate of ethanol addition of major importance for the preparation of the drug carrier system but also flow rates above 1 mL/min are optimal for the nanoparticular desolvation (Langer et al., 2003). As a consequence, a higher flow rate was chosen for larger volumes in order to ensure the controlled precipitation of the protein under the modified conditions. For the tenfold greater volume of HSA solution the flow rate was increased four- to fivefold compared to the standard procedure. Generally speaking, an increase of the flow rate needs to be undertaken in conjunction with an increase in stirring speed.

For the different batch sizes between 10 and 100 mL of the hydroethanolic suspension no significant difference could be observed for either particle size or polydispersity index (see Fig. 2).

The batches prepared with a magnetic stirrer showed greater variability in particle yield at larger volumes due to the comparably unfavorable homogenization efficiency (see Table 1). For all paddle stirring devices particle yield, particle size and polydispersity index were comparable to the magnetic stirring system at lower volumes.

Aside from the commonly monitored parameters like flow rate and pH (see Section 3.2) the glass vial used and immersion depth of the paddle stirrer influenced the desolvation process in terms of reproducibility. For open glass vials and beakers a lower reproducibility of the process was observed due to the unfavorable hydrodynamic conditions within the reaction vial. In these cases the protein accumulated on the edges of the reaction vial adjacent the stirrer which led to a loss of the hydroethanolic suspension. To prevent the formation of any foam, the paddle was immersed 1 cm below the surface of the hydroethanolic suspension.

For the investigated batch size of 100 mL glass bottles were optimal. For the industrial production of nanoparticles utilizing

Table 1
Particle yield achieved for different batch sizes and stirring devices (Average ± S.D.; $n = 3$ except no. 5 which is $n = 6$).

No.	Albumin amount (mg)	Ethanol volume (mL)	Particle yield (%)	Stirring device
1	200	8	87.7% ± 5.7	Magnetic stirrer
2	400	16	92.2% ± 16.5	Magnetic stirrer
3	400	16	88.5% ± 5.7	Nanopaddle I
4	400	16	90.5% ± 2.0	Nanopaddle II
5	2000	80	80.2% ± 8.5	Nanopaddle II

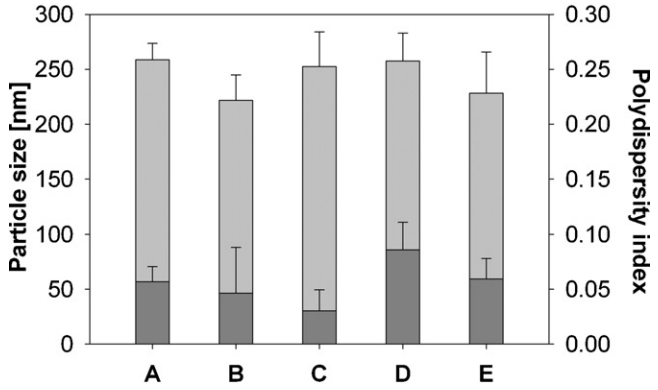

Fig. 2. Particle size ( ) and polydispersity index ( ) of HSA nanoparticles prepared by ethanolic desolvation with a magnetic stirring device and a volume of 10 mL (A), 20 mL (B), Nanopaddle I and a volume of 20 mL (C), Nanopaddle II and a volume of 20 mL (D), Nanopaddle II and a volume of 100 mL (E), (Average ± S.D., $n = 3$ except E which is $n = 6$).

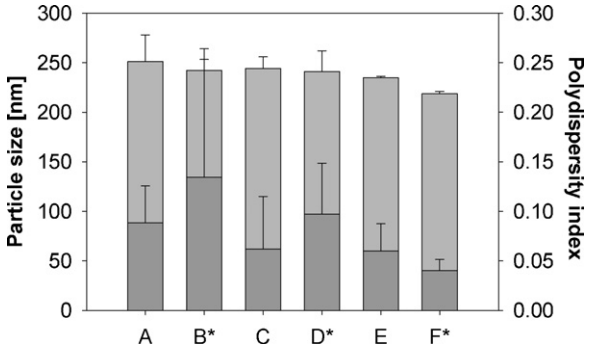

Fig. 3. Particle size ( ) and polydispersity index ( ) of HSA nanoparticles prepared by ethanolic desolvation with the Nanopaddle II stirring device at a final volume of 100 mL at pH values of 8.0 (A), 8.25 (C), and 8.5 (E) and after the freeze drying process (*) from the hydroethanolic suspension, (Average ± S.D., $n = 3$, except A which is $n = 6$).

ethanolic desolvation careful consideration needs to be made regarding the reaction vial.

3.2. pH dependence of upscaled desolvation process

The pH dependence of the upscaled desolvation process was investigated in the pH range between 8.0 and 8.5. Within this pH range a monodisperse size distribution was expected due to the negative charge of the HSA molecules. To optimize the upscaled desolvation method the solvent flow rate and the stirring speed of the *Nanopaddle II* system were increased and a glass bottle with favorable hydrodynamics was chosen for the reaction. Neither precipitation nor the accumulation of the protein was observed during or even 48 h after the desolvation process. A HPLC pumping device was utilized to guarantee a constant flow rate.

As shown in Fig. 3 there was no significant influence observed on either particle size or polydispersity index over the pH range between 8.0 and 8.5. Higher pH values were shown earlier (Langer et al., 2003) to result in an inefficient desolvation process which is not desirable because the residues of HSA in the suspension may aggregate during the crosslink reaction and/or freeze drying process.

3.3. Determination of particle size and size distribution

The HSA nanoparticles were characterized investigating the particle size and the polydispersity index using dynamic light scattering (DLS). The greater batch volume and modified desolvation conditions had no influence on these parameters (ANOVA). Furthermore the particle size was not significantly influenced by pH in the 8.0–8.5 range. Using these conditions particles ranging in size between 251.2 ± 27.0 and 234.1 ± 1.5 nm and with a polydispersity index below 0.2 were prepared.

The size distribution of the particle systems was also measured by analytical ultracentrifugation (AUC). These measurements were performed according to a method developed and validated for human serum albumin nanoparticles published previously (Vogel et al., 2002) and provides information on the size distribution of the major fraction of HSA nanoparticles. Sedimentation coefficient distributions of different batches, as shown by ls–g*(s) profiles from sedimentation velocity runs are given in Fig. 4.

For pH values between 8.0 and 8.5 a narrow homogeneous size distribution was observed for all particle preparations with maxima of apparent s-value approximately 1600-1400 S. Surprisingly the purification steps had minor influence to the polydispersity of the colloid systems (see Fig. 4) but changes in the s-values were observed. The maxima shifted to 2300-1600 S and it can be assumed that a loss of small particles occurred during the purification process.

Whilst the PCS is a useful tool for the measurement of size and size distribution of only a small amount of particles, the AUC velocity run experiment determines an apparent sedimentation coefficient distribution of the suspension and provides information on the homogeneity and the particle size distribution of the major fraction of the nanoparticles. This method can therefore be seen as a complementary technique for the characterization of the colloid system. In summary a nearly monodisperse size distribution of the HSA nanoparticles obtained by ethanolic large-scale desolvation can be assumed with and without purification of the colloid system.

3.4. Turbidimetric quantification of HSA nanoparticles

The quantification of HSA nanoparticles after the desolvation process was performed by two different methods. Microgravimetry is known to be an effective standard technique for the determination of the HSA nanoparticle content.

Furthermore a turbidimetric method was utilized to quantify the nanoparticle content in the presence of non-desolvated protein in the hydroethanolic suspension. One major aim of the study was to provide a toolbox for the in-process control regarding the industrial production of HSA nanoparticles in a monitored process.

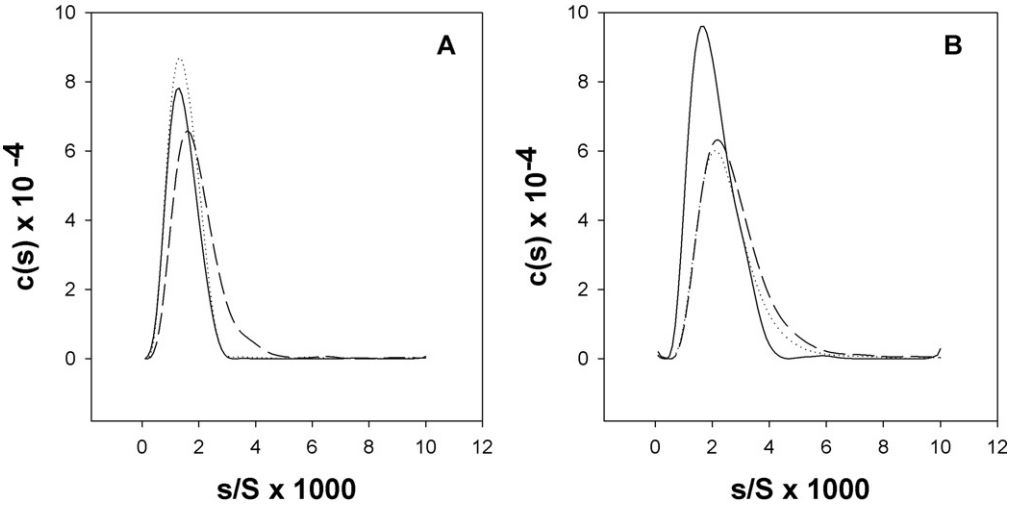

Fig. 4. Sedimentation coefficients of HSA nanoparticles prepared by ethanolic desolvation at pH 8.0 (—), 8.25 (. . .) and 8.5 (- -) with (A) and without purification (B) of the colloidal system.

Whilst turbidimetry is less precise with regards to quantifying the particle mass, it is useful in the *in situ* determination of the particle content, e.g. using a flow-through cell. There was no significant difference (ANOVA) in the apparent absorption (turbidity) measured for particles ranging in size between 231.9 and 278.6 nm. The solvent composition of the ethanol–water mixture and dissolved HSA at a concentration of 3% [m/V] had no influence on the detected particle amount.

3.5. Freeze drying of HSA nanoparticles

Freeze drying was shown to be a suitable technique for increasing the long-term stability of many different parenteral drug formulations (Anhorn et al., 2008a; Holzer et al., 2009). For HSA nanoparticles trehalose and mannitol were previously found to be excellent cryoprotectants and freeze drying experiments from the aqueous suspension have been performed (Anhorn et al., 2008a). In contrast to earlier studies the colloid system was freeze dryed from the hydroethanolic suspension without further purification. These centrifugation and redispersion purification techniques are critical for an industrial application and have no major impact on the size distribution of the colloid system (see Fig. 3). The freeze dryed nanocarriers show a relatively good monodisperse size distribution before and after the drying process. Using these conditions a solid formulation suitable for long-term storage and further processing of the nanoparticles could be achieved (see Fig. 3).

3.6. Surface properties of HSA nanoparticles

The zeta potential of HSA nanoparticles prepared by the small-scale and the large-scale preparation process was investigated at different pH values (see Fig. 5). These measurements provide information on the surface charge of the colloid system and indicate comparable surface properties of the different HSA nanoparticle batches.

Although the number of free aminogroups on the particle surface decreases with an increasing degree of chemical crosslinkage, the zeta potential measurements as a function of the pH indicates similarities in the surface properties of the colloid systems. A nearly identical rate of crosslinkage can be assumed for HSA nanoparticles prepared by lab-scale and large-scale desolvation.

3.7. Quantification of HSA residues via SEC

The desolvation efficiency of HSA was investigated previously by Langer et al. (2003) and residues of the protein were determined using a μBCA assay.

In the present study size exclusion chromatography (SEC) was used to quantify the dissolved protein after desolvation and crosslinkage of the nanoparticles. Both steps were able to reduce the protein concentration in the supernatant of the nanoparticles due to the precipitation of the protein with increasing concentrations of the organic solvent and the chemical modification of the functional groups of HSA. At pH values 8.25 and 8.50 a complete

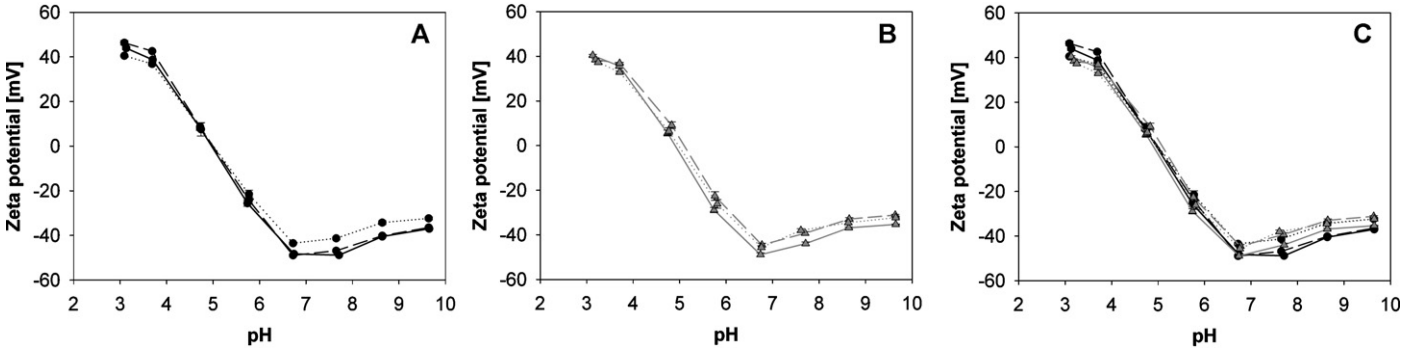

Fig. 5. Zeta potential of HSA nanoparticles which were prepared at pH 8.0 (—), 8.25 (. . .) and 8.5 (- -) in a large-scale (A, ●) and a small-scale (B, ▲) preparation process and overlay (C). (Average ± S.D., $n = 3$).

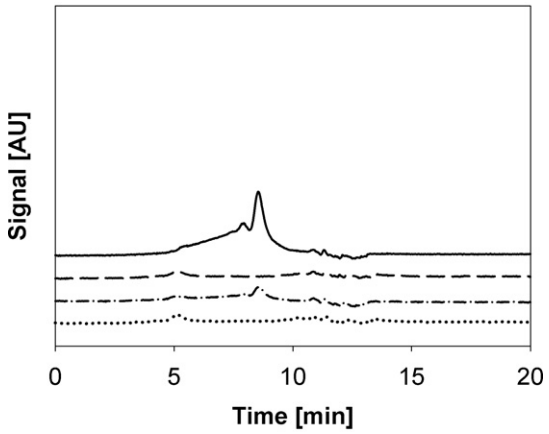

Fig. 6. Size exclusion chromatography (SEC) analysis of the supernatant of HSA nanoparticles directly after desolvation at pH 8 (---), 8.25 (—) and 8.5 (. . .) and the standard sample (HSA 400 μg/mL, —) in the overlay.

desolvation of the protein was determined whilst at pH 8 residues of HSA were found (see Fig. 6).

4. Conclusion

Nanocarriers for the delivery of drugs can be prepared by the ethanolic desolvation of human serum albumin (HSA). These colloid systems allow the binding of a variety of different drug molecules (Dreis et al., 2007; Kufleitner et al., 2010a; Wacker et al., 2010) or targeting ligands (Wartlick et al., 2004a; Michaelis et al., 2006; Anhorn et al., 2008b; Wagner et al., 2010; Low et al., 2011) and are well-suited for *in vivo* application due to their biodegradability and good stability in the physiological environment. The aim of the present study was to prepare HSA nanoparticles in a large-scale process and to monitor the important characteristics of these nanocarriers in order to obtain a product comparable to the colloid systems prepared by lab-scale desolvation. The nanoparticles were characterized with regards to particle content, average particle diameter, size distribution, zeta potential as a function of pH value, and concentration of undesolvated HSA.

Initially experiments indicated that there were no negative effects on particle size or polydispersity index when the desolvation volume was increased to a maximum of 100 mL (see Fig. 2) of the hydroethanolic suspension. The paddle stirring devices *Nanopaddle I* and *II* were utilized to guarantee a homogeneous distribution of the HSA molecules during the desolvation process. For small volumes both devices showed a similar desolvation efficiency. By increasing the flow rate of ethanol from 1 mL/min to 5 mL/min this resulted in an efficient and fast preparation process. By selecting a favorable reaction vial and by using an adequate immersion depth of the paddle stirrer accumulation of the protein at the glass surface of the reaction vessel was prevented.

Particle sizes between 251.2 ± 27.0 and 234.1 ± 1.5 nm with polydispersity indices below 0.2 could be achieved and transferred to the solid state by freeze drying the hydroethanolic suspension.

A nearly monodisperse size distribution of the colloid system was shown by DLS measurements and confirmed by analytical ultracentrifugation (AUC). Further the purification process was shown to have low or no influence on polydispersity index of the particles. Surprisingly, a shift in the particle size was observed and this can be assumed to be due to the loss of small nanoparticles during the purification process.

The surface properties of HSA nanoparticles prepared by lab-scale and large-scale desolvation were investigated. There was no significant difference in the zeta potential profiles detected. This suggests that the upscaled large-scale desolvation process led to a product comparable to the HSA nanoparticles prepared by the lab-scale standard method. Furthermore there were no or low amounts of HSA found in the supernatant of the nanoparticles after the desolvation process. A benefit of this method is the high desolvation efficiency and the absence of dissolved HSA that may have negative influence on the freeze drying of the colloid system.

It can therefore be concluded that the preparation of HSA nanoparticles by ethanolic desolvation is a suitable method for the large-scale production of drug delivery systems and a removal of the solvents by freeze drying without any further purification of the colloid system is possible. In further investigations a modification of these nanoparticles with different model drugs will be performed.

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