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Human serum albumin (HSA) nanoparticles: Reproducibility of preparation process and kinetics of enzymatic degradation

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Abstract

Nanoparticles prepared from human serum albumin (HSA) are versatile carrier systems for drug delivery and can be prepared by an established desolvation process. A reproducible process with a low batch-to-batch variability is required for transfer from the lab to an industrial production. In the present study the batch-to-batch variability of the starting material HSA on the preparation of nanoparticles was investigated. HSA can build dimers and higher aggregates because of a free thiol group present in the molecule. Therefore, the quality of different HSA batches was analysed by size exclusion chromatography (SEC) and analytical ultracentrifugation (AUC). The amount of dimerised HSA detected by SEC did not affect particle preparation. Higher aggregates of the protein detected in two batches by AUC disturbed nanoparticle formation at pH values below 8.0. At pH 8.0 and above monodisperse particles between 200 and 300 nm could be prepared with all batches, with higher pH values leading to smaller particles. Besides human derived albumin a particle preparation was also feasible based on recombinant human serum albumin (rHSA). Under comparable preparation conditions monodisperse nanoparticles could be achieved and the same effects of protein aggregates on particle formation were observed.

For nanoparticulate drug delivery systems the enzymatic degradation is a crucial parameter for the release of an embedded drug. For this reason, besides the particle preparation process, particle degradation in the presence of different enzymes was studied. Under acidic conditions HSA as well as rHSA nanoparticles could be digested by pepsin and cathepsin B. At neutral pH trypsin, proteinase K, and protease were suitable for particle degradation. It could be shown that the kinetics of particle degradation was dependent on the degree of particle stabilisation. Therefore, the degree of particle stabilisation will influence drug release after cellular accumulation of HSA nanoparticles.

Keywords: Nanoparticles; Human serum albumin (HSA); Recombinant human serum albumin (rHSA); Particle size; Enzymatic degradation

1. Introduction

Nanoparticles have emerged as versatile carrier systems for the specific delivery of drugs to organs and tissues (Couvreur and Vauhtier, 2006). Various macromolecular substances such as synthetic and natural polymers can be used for nanoparticle preparation (Kreuter, 2004). Among these, human serum albumin (HSA) is a promising material and was used in a multitude of studies for particle preparation (Michaelis et al., 2006; Steinhauser et al., 2006). Human serum albumin (molecular weight of 65 kDa) belongs to a multigene family of proteins (He and Carter, 1992) and is the major soluble protein of the circulating system with a blood concentration of about 50 mg/ml. Human serum albumin consists of 585 amino acids containing 35 cysteine residues which build 17 disulfide bridges. One free thiol group, namely Cys34, remains unbound. In circulating plasma 30% of this free sulfhydryl Cys34 is oxidised by cysteine and glutathione (Carter and Ho, 1994). Oxidation can also occur by dimerisation during the isolation process. Some products contain up to 20% of dimerised albumin. This percentage increases with the age of the protein unless Cys34 has been blocked with cysteine or gluthathione. Since albumin dimers or higher aggregates may influence the preparation process of nanoparticles, the quality of the starting materials is of major importance.

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Besides oxidation leading to dimers, the human origin of HSA is another drawback of this material. Consequently, there is the potential risk of pathogen contamination (e.g. HIV, hepatitis, CJD) and the possibility of variability in quality. An answer to these problems could be seen in recombinant produced HSA (rHSA). Since HSA is a non-glycosylated protein a wide range of host organisms is appropriate for rHSA production (Kobayashi, 2006). Actually, the most frequently used expression system for rHSA production is *Pichia pastoris* (Chuang et al., 2002). By this organism, it is possible to produce proteins in a large scale and with identical primary, secondary and tertiary structure. Nevertheless, rHSA is still a very expensive alternative to human derived albumin.

Despite the problematic human origin, the first HSA-based nanoparticle formulation, ABI 007 (Abraxane®) was approved by the FDA in 2005. The 130 nm large nanoparticles contain the cytostatic drug paclitaxel. Due to the bad solubility of paclitaxel in water, the conventional drug preparation (Taxol®) contains polyethylated castor oil (Cremophor EL) and ethanol as vehicles. The drug incorporation in nanoparticles follows a new concept to improve drug solubility, with a variety of advantages conferred to the standard paclitaxel therapy (Gradishar et al., 2005; Desai et al., 2006).

Although HSA nanoparticles were described in a multitude of studies the variability of the preparation process was not in the focus of most of these investigations. For a scaling-up process of particle preparation and for industrial particle preparation an assessment of batch-to-batch variation is of major importance. In the present study nanoparticles based on human serum albumin were prepared by a well-defined desolvation process (Langer et al., 2003). Different HSA batches containing different amounts of albumin dimers and higher aggregates as well as recombinant HSA were used for this process. The influence of the starting materials' quality on particle size, size distribution, and yield of the resulting nanoparticles was investigated. On the other hand the biodegradability of the nanoparticles was assessed. By using a variety of different enzymes, physiological conditions were simulated in order to evaluate the enzymatic degradation of HSA as well as rHSA nanoparticles.

2. Materials and methods

2.1. Reagents and chemicals

HSA (fraction V, purity 96–99%), recombinant HSA (rHSA; ~95% SDS-PAGE, expressed in *P. pastoris*), and glutaraldehyde 8% solution were obtained from Sigma (Steinheim, Germany). Four different batches of human derived HSA (015K7535, 035K7566, 045K7535, 111K7614) as well as two different batches of recombinant HSA (095K1258, 116K1451) were used for particle preparation. For the enzymatic degradation of the nanoparticles proteinase K (batch 022K8620), protease (batch 010K7670), pepsin (batch 087H0163), pancreatin (batch 067H1090), and cathepsin B (batch 033K7685) were obtained from Sigma (Steinheim, Germany). Trypsin (batch 9019) was achieved from BDH Laboratory supplies (Poole, England). All other reagents were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade and used as received.

2.2. Composition of HSA batches by size exclusion chromatography (SEC) and analytical ultracentrifugation (AUC)

The molecular weight distribution of the four different HSA batches was analysed by size exclusion chromatography (SEC) on a HPLC system equipped with TSKgel G3000SW$_{XL}$ guard-column and a TSKgel G3000SW$_{XL}$ 7.8 mm × 30 cm column (Tosoh Bioscience, Stuttgart, Germany) using phosphate buffer pH 7.0 as mobile phase at a flow rate of 0.8 ml/min. The SEC system was calibrated for molecular weight with globular protein standards. Aqueous solutions of the proteins were prepared at a concentration of 1 mg/ml and an aliquot (20.0 μl) of each sample was injected into the SEC system. The eluent fraction was monitored by UV detection (280 nm). The amount of dimeric HSA was calculated relative to the total peak area in the respective chromatograms.

The same four different HSA batches and both rHSA samples were investigated by sedimentation velocity analysis in the analytical ultracentrifuge. Aqueous solutions of the proteins were prepared at a concentration of 1 mg/ml in 20 mM phosphate buffer containing 100 mM NaCl. The sedimentation velocity experiments were performed using a Beckman Optima XL-A ultracentrifuge at rotor speeds of 35,000 rpm at 20 °C. The absorbance versus radius data (collected at 280 nm) were modelled as c(s) and c(M) distribution of non-interactive species, used of the *sedfit* program by Schuck et al. (2002).

2.3. Preparation of HSA nanoparticles

HSA nanoparticles were prepared by a desolvation technique as previously described (Langer et al., 2003). In principle, HSA was dissolved at a concentration of 100 mg/ml in 10 mM sodium chloride solution and the pH of the solution was titrated to 7.5, 8.0, and 8.5, respectively. The resulting solutions were filtered through a 0.22 μm filtration unit (Schleicher und Schüll, Dassel, Germany). Aliquots (1.0 ml) of the HSA solutions were transformed into nanoparticles by the continuous addition of 4.0 ml of the desolvating agent ethanol under stirring (550 rpm) at room temperature. The ethanol addition was performed by a tubing pump (Ismatec IPN, Glattbrugg, Switzerland) which enabled nanoparticle preparation at a defined rate of ethanol addition of 1.0 ml/min. After the desolvation process 117.6 μl of 8% glutaraldehyde in water were added to induce particle crosslinking. This volume corresponds to 200% of the theoretic amount that is necessary for the quantitative crosslinking of the 60 amino groups present in the HSA molecules of the particle matrix. The crosslinking process was performed under stirring of the suspension over a time period of 24 h at room temperature.

The resulting nanoparticles were purified by three cycles of differential centrifugation (16,100 × *g*, 8 min) and redispersion of the pellet to the original volume in water. Each redispersion step was performed in an ultrasonication bath (Elma Transsonic

Digital T790/H) over 5 min. The resulting amount of HSA nanoparticles was determined gravimetrically.

For the enzymatic degradation study nanoparticles were prepared only from HSA batch 111K7614. Nanoparticles were prepared as outlined above except for the extent of particle stabilisation. Glutaraldehyde amounts of 23.5, 35.3, 47.0, and 58.8 μl of a 8% glutaraldehyde solution were employed resulting in crosslinking degrees of 40, 60, 80, and 100%, respectively.

For every batch of HSA the particle preparation was performed in three independent samples. The analytical results were given as mean value and standard deviation of these samples.

2.4. Preparation of rHSA nanoparticles

Recombinant human serum albumin (rHSA) was obtained as solution containing the following excipients: sodium chloride, sodium phosphate buffer pH 7.4, sodium caprylate, *N*-acetyltryptophan. Since additives can interfere with the desolvation process of albumin these substances were removed from the rHSA solution by dialysis using Slide-A-Lyzer dialysis cassettes (MWCO 3500, Pierce, Rockford, USA). Dialysis against purified water was performed according to the instruction of the supplier. After dialysis the rHSA solution was freeze-dried without addition of further excipients. For the freeze-drying process a labscale Lyovac GT2 (Leybold Heraeus, Hürth, Germany) freeze-dryer was used.

The resulting lyophilised rHSA was used to prepare nanoparticles by the desolvation technique described above. The preparation process was performed at a pH of 8.5. For the enzymatic degradation study nanoparticles were prepared only from rHSA batch 116K1451.

2.5. Determination of particle size and size distribution

After particle purification average particle size, polydispersity, and zetapotential were measured by photon correlation spectroscopy (PCS) and microelectrophoresis using a Malvern zetasizer 3000HSA (Malvern Instruments Ltd., Malvern, UK). The samples were diluted 1:400 with purified water and measured at a temperature of 25 °C and a scattering angle of 90°.

As an alternative method, size distribution was studied in some of the samples by sedimentation velocity analysis in the analytical ultracentrifuge (Vogel et al., 2002; Langer et al., 2003). In principle, the nanoparticle stock solution was brought, by addition of appropriately concentrated solutions and water, to 20 mM sodium phosphate (pH 7.0), 100 mM NaCl, 23.5% (w/v) sucrose, at a solute concentration giving turbidity between 0.6 and 0.7 at 420 nm in a cuvette with a 1 cm optical pathlength. The ultracentrifugation experiments were performed using a Beckman Optima XL-A ultracentrifuge at rotor speeds of 3000 rpm as described earlier (Vogel et al., 2002). The apparent absorbance (turbidity) versus radius data (collected at 420 nm) were modelled as a distribution of non-diffusing spherical particles, based on the results described (Vogel et al., 2002). The calculations used the ls-g*(s)-variant of the *sedfit* program by Schuck and Rossmanith (2000).

Table 1
Enzymes and conditions used for the degradation of HSA nanoparticles

Enzyme	Concentration (μg/ml)	Enzymatic activity (units/ml)	pH
Proteinase K from *Tritirachium album*	2	0.060	7.5
Protease from bovine pancreas, type I	10	0.073	7.5
Trypsin	50	Unknown	7.5
Pancreatin from porcine pancreas	50	1.25	7.5
Pepsin	5000	300	2.0
Cathepsin B	10	0.056	5.4
	10	0.056	6.4

2.6. Enzymatic degradation of HSA nanoparticles

For the enzymatic degradation of the nanoparticles the different enzymes were used as listed in Table 1. The pH values of 5.4, 6.4, and 7.5 were adjusted with phosphate buffer systems. In the case of pepsin the pH value was adjusted with 0.01N hydrochloric acid.

For the calibration of the photometric assay each nanoparticle batch was diluted with the respective buffers of the enzymes to nanoparticle concentrations of 0, 50, 100, 250, 500, and 1000 μg/ml, respectively. The turbidity of the nanoparticle suspensions was determined photometrically at a wavelength of 565 nm and was used for the calculation of calibration curves.

For the determination of the kinetics of enzymatic degradation an aliquot of the nanoparticle suspension containing 2.0 mg particles were diluted with the respective enzyme solution to a nanoparticle concentration of 1000 μg/ml. The mixture was incubated at 37 °C under shaking (Eppendorf Thermomixer 5436, Gerätebau Eppendorf, Engelsdorf, Germany). After various time intervals the turbidity of the samples was measured photometrically at 565 nm and the concentration of the remaining nanoparticles was calculated relative to the calibration curve.

3. Results and discussion

The objective of the present study was to take a more detailed look at the reproducibility of the preparation process of human serum albumin (HSA) nanoparticles by protein desolvation as well as at the kinetics of the enzymatic degradation of the resulting nanoparticles.

In the first part of the study the main focus was on reproducibility of the particle formation under the aspect of different batches of the starting material HSA. This part was based on our earlier work (Langer et al., 2003) describing the optimisation of the preparation process by the use of a pump-controlled system for the desolvation of HSA solutions. In the earlier study the pH value of the HSA solution prior to the desolvation procedure was found to be the major parameter to control the resulting particle size. It was shown, that the preparation method applying a pump-controlled system in combination with a defined pH adjustment in the presence of sodium chloride leads to well-defined mean particle sizes as well as to narrow particle size distributions. It

was a certain drawback of the earlier study that all of the experiments were performed with only one given batch of HSA and, therefore, the influence of different HSA batches was ignored. Under the future aspect of industrial particle preparation the establishment of a reproducible preparation method is of major importance and the influence of different batches of starting materials on the resulting physico-chemical characteristics has to be faced.

A further requirement for a pharmaceutical product intended for human use is the application of safe and well defined starting materials. Although human derived HSA is used for the FDA approved nanoparticle preparation Abraxane®, the remaining risk of pathogen contamination is often discussed. Therefore, additionally in the first part of the study the exchange of human derived HSA for recombinant HSA (rHSA) for particle preparation was evaluated.

In the second part of the study the kinetics of the enzymatic degradation of HSA as well as rHSA nanoparticles was focused on in the presence of different enzymes. This part was based on some of our preliminary results showing that the enzymes trypsin and proteinase K were well suited for the degradation of HSA nanoparticles (Wartlick et al., 2004). In this earlier study a clear dependency between glutaraldehyde crosslinking and enzymatic particle degradation was observed with higher amounts of crosslinking during the preparation process leading to a reduced ability for enzymatic particle degradation. This earlier study was done in order to evaluate suitable conditions for quantitative particle degradation under the aspect of a subsequent quantification of matrix-bound drugs. In the present study the enzymatic degradation was evaluated in more detail under the aspect of intracellular degradation after particle uptake. For this reason besides trypsin and proteinase K a range of further enzymes covering intracellular enzymes of the cathepsin family were focussed on.

3.1. Purity of different HSA batches

For the preparation of HSA nanoparticles by desolvation commercially available HSA isolated from human plasma was used. The purity of the starting material was declared by the supplier with values between 96 and 99%. On the other hand it is well known, that a certain amount of the protein is present in its dimeric or higher aggregated form. The dimeric form of HSA is due to a free sulfhydryl group (Cys34) in the primary structure of the protein which enables a covalent dimerisation of HSA molecules (Carter and Ho, 1994). In our earlier work with gelatin nanoparticles we have found that a broad molecular weight distribution of gelatin is responsible for problems with reproducible particle preparation. The problem was solved by establishing a double desolvation method which enables the separation of high molecular gelatin fractions (Coester et al., 2000; Balthasar et al., 2005). Therefore, in the case of HSA nanoparticles the amount of dimeric or higher molecular weight HSA impurities may as well have influence on the reproducibility of the preparation process. For this reason in the first part of the present study we have analysed the molecular weight distributions of the different commercial HSA batches by size exclusion chromatography (SEC) and by analytical ultracentrifugation (AUC).

Table 2
Amount of monomeric and dimeric protein in different HSA batches determined by peak area in size exclusion chromatography (SEC) and qualitative by analytical ultracentrifugation (AUC)

HSA batch	Amount HSA by SEC (%)		Higher molecular aggregates by AUC
	Monomeric form	Dimeric form	
015K7535	94.0	6.0	+
035K7566	93.2	6.8	+++
045K7535	94.9	5.1	+
111K7614	90.4	9.6	–

The SEC method revealed that the different HSA batches contained variable amounts of dimeric HSA (Table 2). By SEC an amount between 5 and 10% dimeric HSA was detected for all batches. The lowest content of 5.1% dimeric HSA was found for batch 045K7535, but AUC revealed a certain shift of the peak to lower molecular weights (Fig. 1). The highest amount was detected within batch 111K7614. The results were in good accordance with the data of AUC showing amounts between 8 and 11% for the respective batches. Therefore, the chromatographic results were supported by the data of the AUC method (Fig. 1). Moreover, the AUC method revealed higher HSA aggregates in the different batches. This was most pronounced within batch 035K7566 showing two distinct peaks for these compounds at molecular weights of about 240 and 360 kDa. On the other hand HSA batch 111K7614 only showed peaks for the monomeric and dimeric HSA at the expected molecular weights of about 65 and 130 kDa. Although this batch was characterised by the highest amount of dimeric protein no signs of higher aggregates were observed. In the case of batch 045K7535 a pronounced tailing of the dimer peak was observed. Taking all these observations together the amount of higher molecular HSA aggregates decreased in the following batch order: 035K7566 > 045K7535 = 015K7535 > 111K7614

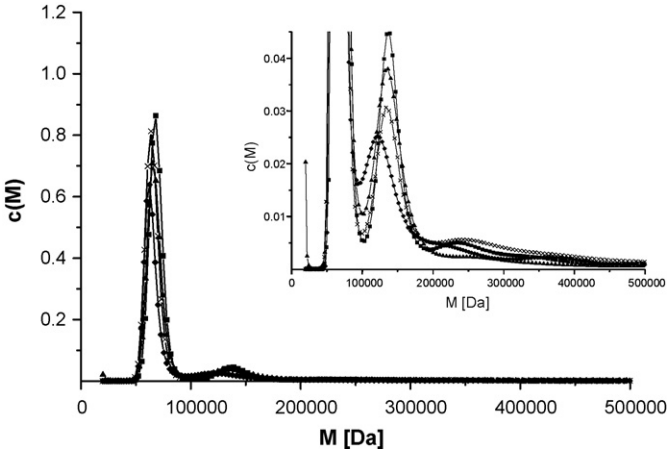

Fig. 1. Molecular weight distributions c(M) of HSA protein from different HSA batches. The protein was analysed at a concentration of 1 mg/ml in 20 mM phosphate buffer pH 7.0 containing 100 mM NaCl. Upper right: enlargement of the molecular weight distribution for detection of higher HSA aggregates. Symbols: (×) batch 035K7566; (■) batch 015K7535; (▲) batch 111K7614; (□) batch 045K7535.

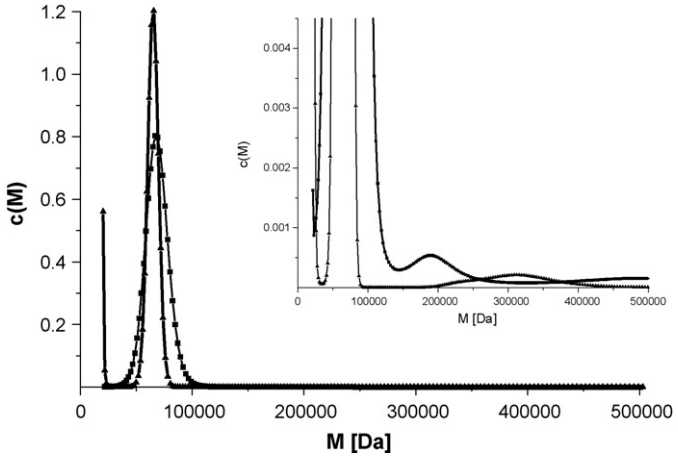

Fig. 2. Molecular weight distributions c(M) of recombinant HSA (rHSA) prior (▲) and after purification and freeze-drying (■). The protein was analysed at a concentration of 1 mg/ml in 20 mM phosphate buffer pH 7.0 containing 100 mM NaCl. Upper right: enlargement of the molecular weight distribution for detection of higher HSA aggregates.

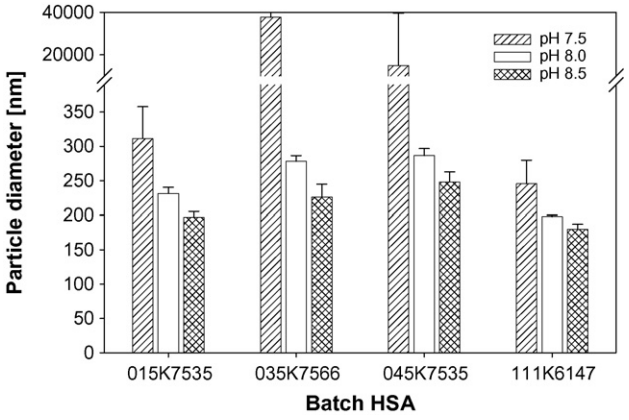

Fig. 3. Influence of the HSA batch on particle diameter of HSA nanoparticles prepared at different pH values in 10 mM sodium chloride solution (mean ± S.D.; $n = 3$). Rate of ethanol addition: 1.0 ml/min; initial HSA concentration: 100 mg/ml. Particle size measurement after particle purification.

(Table 2). Therefore, no correlation between the amount of dimeric HSA and amount of higher molecular HSA aggregates was observed within the batches.

Additionally, in this part of the study the influence of freeze-drying on the molecular weight distribution of rHSA was determined by analytical ultracentrifugation (AUC). As rHSA was provided in form of an aqueous solution containing different stabilising agents, protein purification by dialysis and freeze-drying of the purified protein was performed prior to nanoparticle preparation. Before freeze-drying the rHSA samples consisted of a high amount of monomeric rHSA with only traces of higher aggregates in the molecular weight range of about 300 kDa (Fig. 2). The situation was completely different after dialysis and freeze-drying of rHSA. A pronounced broadening of the monomer peak was observed, indicating change in the hydration state of the protein or aggregation of the rHSA molecules. Furthermore, an additional small peak at a molecular weight of about 190 kDa, representing the trimeric form of rHSA, occurred. In comparison to HSA of human origin, only minor traces of these higher aggregates were detected. However, rHSA purification was crucial with regard to the molecular weight distribution of the protein.

3.2. Reproducibility of HSA nanoparticle preparation

The different HSA batches were used for the preparation of nanoparticles by a well established desolvation method. The preparation was done under defined conditions. The HSA solution was prepared in the presence of 10 mM NaCl and the pH of the solutions was adjusted to 7.5, 8.0, and 8.5, respectively. The addition of the desolvating agent ethanol was performed by a pump-controlled system at a speed of 1.0 ml/min (Langer et al., 2003). After stabilisation and purification of the resulting nanoparticles the particle size and polydispersity of each sample were determined and the results were compared with the purity data of the starting material. Besides these size parameters the zetapotential of the HSA nanoparticles was determined. Independent of the pH and HSA batch used for protein desolvation the nanoparticles showed a zetapotential of -43.2 ± 3.0 mV ($n = 14$).

As previously described by our group the pH value of the HSA solution prior to the desolvation procedure was the major parameter to control the resulting particle size (Fig. 3). Within every HSA batch used the particle diameter was significantly reduced with higher pH values of the HSA solution (ANOVA, $p < 0.01$). Under the chosen conditions and a pH value of 7.5 nanoparticles were only formed with the HSA batches 015K7535 and 111K7614 whereas the other batches 035K7566 and 045K7535 led to the formation of aggregates in the micrometer scale instead of nanoparticles. At higher pH values of 8.0 and 8.5 the latter batches led to the formation of nanoparticles but even under these conditions their diameters were significantly increased compared to the preparations of batch 111K6147 (ANOVA, $p < 0.01$). For example at a pH value of 8.0 with the batches 035K7566 and 045K7535 nanoparticles with diameters of 278.1 ± 8.5 and 286.6 ± 10.2 nm were achieved, respectively. On the other hand under the same preparation conditions HSA batch 111K7614 led to significantly smaller nanoparticles with a diameter of 198.1 ± 2.3 nm.

The same is true for the batch dependency of the polydispersity index (Fig. 4). At a pH value of 7.5 both batches 035K7566 and 045K7535 led to aggregates with a high polydispersity index whereas with the other batches nanoparticles with a polydispersity index below 0.1, indicative for a monodisperse size distribution, were achieved. In contrast the yield of the nanoparticles seems to be independent of the respective HSA batch (Fig. 5). Although slight variations of the particle yield in the range between 18.1 and 23.1 mg/ml were observed, no clear correlation to pH value or batch was observed. But due to the method of analysis it has to be outlined that particle yield is not a parameter as sensitive as particle diameter or polydispersity. Particle yield is mainly controlled by the solubility of the protein in a respective solvent at a respective pH value and will hardly be influenced by the formation of protein aggregates. Additionally,

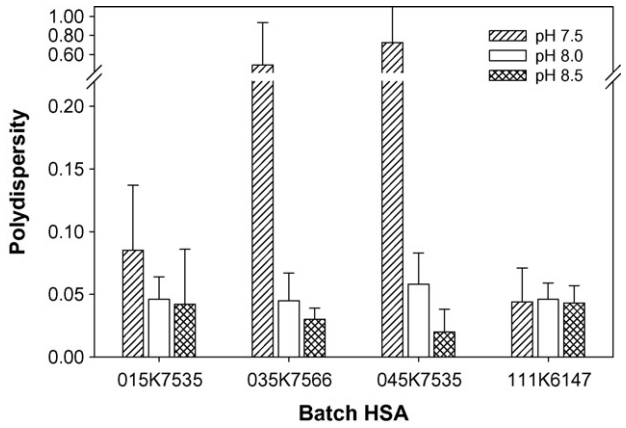

Fig. 4. Influence of the HSA batch on polydispersity of HSA nanoparticles prepared at different pH values in 10 mM sodium chloride solution (mean ± S.D.; $n = 3$). Rate of ethanol addition: 1.0 ml/min; initial HSA concentration: 100 mg/ml. Polydispersity measurement after particle purification.

due to the theory of PCS used for particle size analysis, already minor traces of larger particles will lead to a deviation of the correlation function and, therefore, to a significant increase of the polydispersity index.

Based on the idea that the amount of dimeric or higher molecular weight HSA impurities takes influence on the formation of nanoparticles during the desolvation process we have compared the amount of these impurities with the size and polydispersity data of the resulting nanoparticles. It becomes obvious that the amount of dimeric HSA has no influence on the resulting nanoparticles. The HSA batch 111K7614 with the highest amount of dimeric protein led to a reproducible particle preparation even at a pH value of 7.5 whereas the batch 045K7535 with the lowest amount of dimer led to aggregates and high polydispersity indices under the same preparation conditions. On the other hand a correlation between the amount of higher aggregates detected by AUC and the resulting particle size and polydispersity seems to exist. For example the batches 035K7566 and 045K7535 with high amounts of these impurities

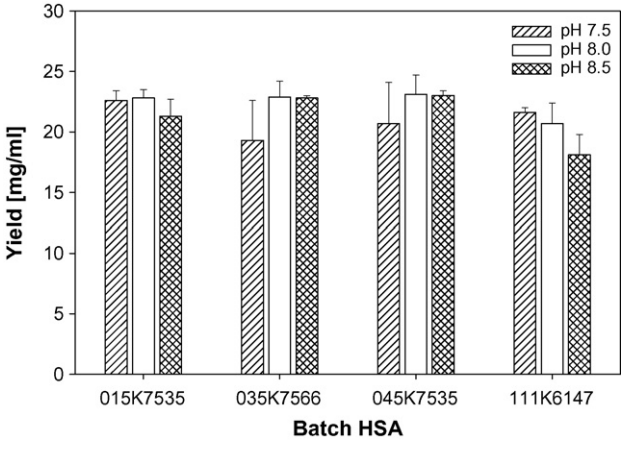

Fig. 5. Influence of the HSA batch on particle yield of HSA nanoparticles prepared at different pH values in 10 mM sodium chloride solution (mean ± S.D.; $n = 3$). Rate of ethanol addition: 1.0 ml/min; initial HSA concentration: 100 mg/ml. Yield determination after particle purification.

led to increased particle sizes at all pH values under evaluation. Furthermore at pH 7.5 both batches resulted in significantly increased polydispersity indices (ANOVA, $p < 0.01$). A certain exception can be observed for HSA batch 015K7535. Although this batch is characterised by higher aggregates in a proportion comparable to batch 045K7535, nanoparticles instead of microparticles were achieved at pH 7.5. Hence, other parameters such as low molecular weight impurities (i.e. salts) of HSA batches additionally may take influence on the formation of nanoparticles during the desolvation process. However, these particles prepared from batch 015K7535 were larger and showed an increased polydispersity compared to batch 111K6147.

Therefore, it has to be concluded that the purity of a HSA batch is a fundamental precondition for the reproducible preparation of HSA nanoparticles by our desolvation procedure and it is reasonable to verify the HSA quality under the aspect of protein aggregation and high molecular impurities prior to particle preparation.

3.3. Preparation of rHSA nanoparticles

The same desolvation process described for HSA nanoparticles was also used for the preparation of nanoparticles based on recombinant human serum albumin (rHSA). Since the human derived HSA bears the risk of pathogen contamination and variability in quality, rHSA can be taken as an alternative starting material for the preparation of drug carrier systems.

As described above the characterisation of the purified rHSA by analytical ultracentrifugation (AUC) showed a significant broadening of the monomer peak and some impurities in the molecular size range of the trimeric protein (Fig. 2). For particle preparation the lyophilised product was transferred to nanoparticles at pH 8.5 as previously outlined for HSA of human origin. After stabilisation and purification of the resulting nanoparticles the particle size, polydispersity, zetapotential, and yield was determined. The results of three independent particle samples are summarised in Table 3. In comparison to HSA nanoparticles prepared under the same conditions, the particles made of rHSA were somewhat larger and showed a higher variability in size than the HSA particles. Nanoparticles prepared of the four human derived HSA batches of human origin were received at particle sizes between 179.9 ± 7.2 and 248.2 ± 14.5 nm. In contrast, the rHSA nanoparticles showed a significantly increased size of 296.8 ± 27.0 nm (ANOVA, $p < 0.01$). This effect can probably be attributed to aggregation during protein purification

Table 3
Nanoparticles prepared based on recombinant human serum albumin (rHSA): results of physico-chemical characterisation of three independent nanoparticle batches

Parameter	Preparation			Mean ± S.D.
	#1	#2	#3	
Particle diameter (nm)	273.2	326.3	290.9	296.8 ± 27.0
Polydispersity	0.029	0.025	0.054	0.036 ± 0.016
Zetapotential (mV)	−52.8	−47.7	−47.6	−49.4 ± 3.0
Particle yield (mg/ml)	21.9	19.6	18.8	20.1 ± 1.6

and freeze-drying detected by AUC. The parameters polydispersity and particle yield were comparable to HSA nanoparticles. The zetapotential of the rHSA nanoparticles of −49.4 ± 3.0 mV indicated sufficient storage stability of the colloidal systems (Müller et al., 2000). In principle, preparation of rHSA nanoparticles under the chosen condition was feasible. Nevertheless, our investigations showed that the exchange of one starting material against a modified one requires systematic investigations in order to find optimal conditions for particle preparation.

3.4. Enzymatic degradation of HSA nanoparticles

Besides the reproducible preparation of nanoparticles the suitability of these systems for intracellular drug delivery is of major importance. As incorporative drug loading is mostly favoured to take advantage of the high drug carrier capacity of the particles, a controlled enzymatic degradation is a decisive prerequisite for intracellular drug release after cellular particle uptake. Therefore, in the second part of the study the kinetics of the degradation of HSA nanoparticles as well as rHSA nanoparticles in the presence of different enzymes was analysed.

The conditions for the enzymatic degradation in the presence of trypsin and pepsin were chosen in order to find conditions for a rapid degradation of the HSA nanoparticles. This could be useful for a fast determination of the incorporative drug loading after particle preparation. In the case of trypsin the enzymatic degradation of the particles was mainly influenced by the degree of particle crosslinking (Fig. 6A). Under these conditions HSA nanoparticles with a crosslinking degree of 40% were quantitatively degraded within 90 min whereas in the case of nanoparticles crosslinked with 100% glutaraldehyde only 9% of the particles were degraded within 24 h. Particles with a crosslinking degree of 80% showed a degraded fraction of 36.1% within 24 h and nanoparticles with a crosslinking degree of 60% were completely degraded within this time span. Comparable kinetics of nanoparticle degradation with a significant dependence on the crosslinking degree were observed in the presence of the enzymes proteinase K and protease (data not shown). In contrast particle degradation in the presence of pancreatin was not so pronounced. After an incubation period of 3 h only 30.6 and 11.2% of the HSA nanoparticles were degraded in the case of 40 and 100% crosslinked nanoparticles, respectively. Within 24 h between 81.6% (40% glutaraldehyde) and 52.8% (100% glutaraldehyde) of the nanoparticles were degraded. In contrast, in the presence of pepsin within 30 min a complete particle degradation was observed independent of the crosslinking degree of the particle matrix (Fig. 6B). In comparison to trypsin this rapid degradation was mainly due to the comparatively high enzymatic activity used for the assay. Therefore, the chosen pepsin conditions are convenient for the determination of a matrix-bound drug within a short time frame. But as the gastric enzyme pepsin shows the highest activity between pH 1.5 and pH 3.0 drug stability under these acidic conditions is required.

The results of HSA nanoparticles were supported by the enzymatic degradation of recombinant HSA (rHSA) nanoparticles. As could be expected all of the enzymatic conditions under

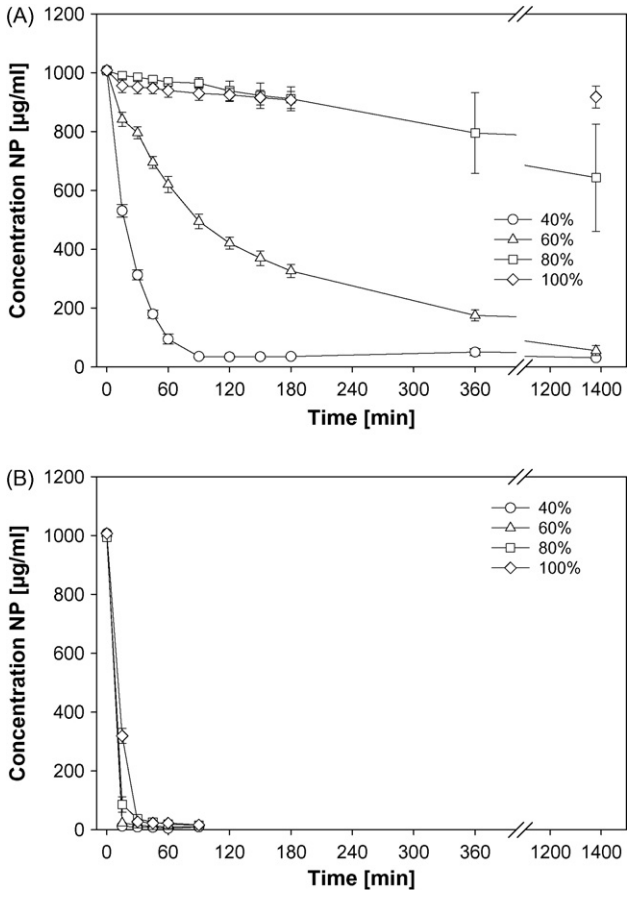

Fig. 6. Enzymatic degradation of HSA nanoparticles in the presence of (A) 50 μg/ml trypsin and (B) 5 mg/ml pepsin. Glutaraldehyde concentrations between 40 and 100% were used for particle crosslinking (mean ± S.D.; $n = 3$).

evaluation resulted in comparable degradation kinetics of the rHSA particles (Fig. 7A and B). Therefore, the exchange of HSA against rHSA will be feasible for nanoparticle preparation without changing the degradation behaviour of the drug carrier system.

Besides gastric and intestinal enzymes the intracellular enzyme cathepsin B was focussed on within the present study. Enzymes of the cathepsin family are mainly localised in the lysosomes of cells. These organelles are part of the endocytic compartment and are involved in the final breakdown of internalised material as well as the storage of indigestible material (Mukherjee et al., 1997). The pH of the lysosomes varies between 5.0 and 5.5. Nanoparticles are internalised into cells through endocytosis and, therefore, eventually are delivered to lysosomes. In order to enable an intracellular release of a drug entrapped within the nanoparticle matrix, the particles have to be degraded by lysosomal enzymes such as cathepsin. For the investigation of the biodegradability, cathepsin B was used at pH values of 6.4 and 5.4. The higher pH value only led to a negligible degradation of HSA nanoparticles (Fig. 8A). After 24 h there was almost no digestion of the HSA nanoparticles, independent of the glutaraldehyde amount used for particle stabilisation. In contrast, at pH 5.4 the degradation was more pronounced (Fig. 8B) because of the higher activity of cathep-

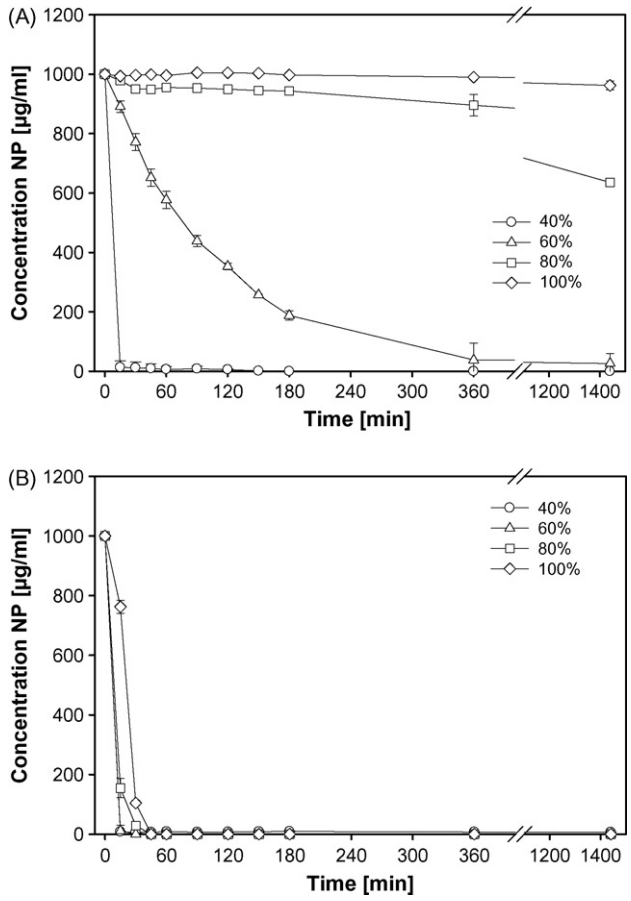

Fig. 7. Enzymatic degradation of recombinant HSA (rHSA) nanoparticles in the presence of (A) 50 μg/ml trypsin and (B) 5 mg/ml pepsin. Glutaraldehyde concentrations between 40 and 100% were used for particle crosslinking (mean ± S.D.; $n = 3$).

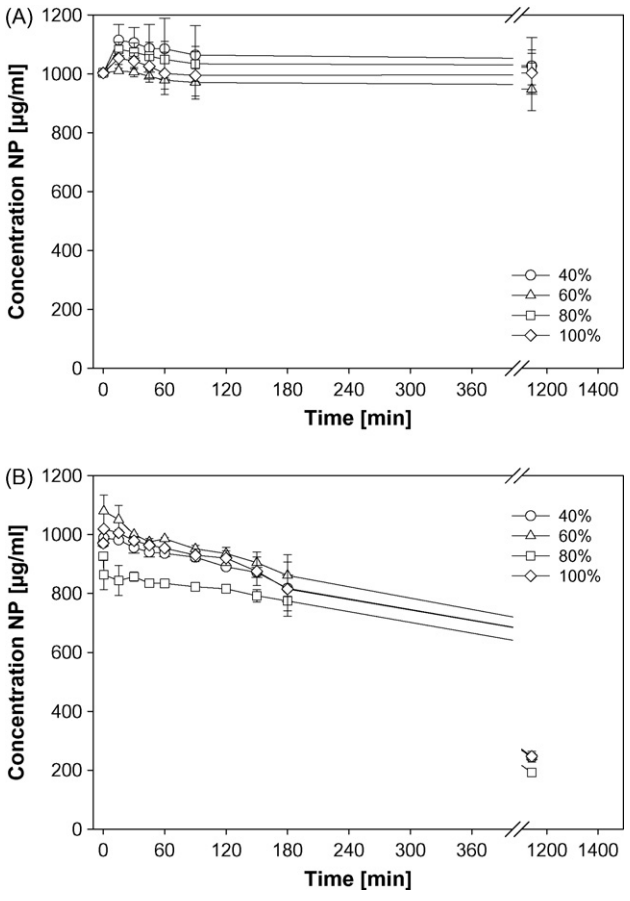

Fig. 8. Enzymatic degradation of HSA nanoparticles in the presence of 10 μg/ml cathepsin B at pH values of (A) 6.4 and (B) 5.4. Glutaraldehyde concentrations between 40 and 100% were used for particle crosslinking (mean ± S.D.; $n = 3$).

sin at this pH value. After 180 min about 16.0% of the particles with a crosslinking degree of 100% were degraded. This amount increased to 74.5% after 24 h. A difference between the distinct glutaraldehyde crosslinking degrees was not detectable. Particle degradation by enzymes of the cathepsin family at slightly acidic pH confirms the biodegradability of HSA nanoparticles after cellular uptake.

Taking the results of enzymatic degradation together, it has to be concluded that the enzymes trypsin, proteinase K, protease, and pepsin are well suited for a rapid degradation of HSA and rHSA nanoparticles. This could be useful for the analysis of drug compounds incorporated within the matrix of the nanoparticulate drug delivery system. In the presence of all of these enzymes the degradation kinetics was dependent on the crosslinking degree of the particles with higher degrees leading to longer time spans of degradation. Under the chosen conditions in the presence of trypsin, proteinase K, protease, and cathepsin B a quantitative degradation of 100% crosslinked particles was not possible over a time frame of 24 h. In contrast to these highly stabilised particles a lower crosslinking degree of 40% enabled particle degradation within 24 h by all enzymes except for cathepsin at pH 6.4. The choice of the respective enzyme is mainly affected by the pH dependent stability of an incorporated drug. If the drug is stable under acidic conditions, nanoparticle degradation in the presence of pepsin and cathepsin at pH 5.4 is a suitable method whereas if the respective drug is only stable under neutral conditions trypsin, proteinase K or protease are the enzymes of choice for particle degradation.

4. Conclusion

The present study shows that HSA nanoparticles can be prepared with predictable and reproducible size dependent on the batch of the starting material HSA used. The preparation is influenced by the amount of high molecular HSA components, with higher amounts leading to an increase of size and polydispersity of the nanoparticles. These parameters can also be influenced by the pH value of the HSA solution used for particle preparation. Higher pH values decrease particle size and polydispersity. Besides HSA of human origin monodisperse nanoparticles can also be prepared under comparable conditions with rHSA.

Enzymatic degradation of HSA and rHSA nanoparticles is possible with different enzymes. The kinetics of degradation depends on the crosslinking degree of the particles and the respective enzyme used. The fastest digestion can be achieved with pepsin at pH 2. Under neutral conditions trypsin, proteinase K, cathepsin B, and protease enable particle degradation over

24 h. Particle degradation in the presence of the intracellular enzyme cathepsin B confirms the biodegradability of HSA and rHSA nanoparticles as a prerequisite of drug release after cellular uptake.

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