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Desolvation process and surface characterisation of protein nanoparticles

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Abstract

The objective of the present study was to characterise and optimise the desolvation process of human serum albumin (HSA) for the preparation of nanoparticles and to characterise the resulting colloidal system. Following the desolvation of the protein, the resulting nanoparticles were stabilised by the addition of varying amounts of glutaraldehyde or by heat denaturation. The particle size, zeta potential, and the number of available amino groups on the surface of the nanoparticles were determined. The amino groups were quantified by a spectrophotometric method using 2,4,6-trinitrobenzenesulfonic acid (TNBS). The results indicated that the particle size depended mainly on the amount of desolvating agent added, but not on the amount of cross-linker or the kind of cross-linking procedure. Increasing amounts of glutaraldehyde reduced the number of amino groups on the surface of HSA nanoparticles and also decreased the zeta potential of the carrier system. The temperature and heat denaturation time only had an influence on the stability of the nanoparticles but not on the amount of amino groups or the particle size. It was shown that heat denatured HSA nanoparticles possessed the greatest number of amino groups on their surface. Additional experiments for the characterisation of gelatin A and B nanoparticles were performed.

Keywords: Nanoparticles; Human serum albumin (HSA); Gelatin; Surface characterisation; Desolvation procedure; Amino group determination

1. Introduction

The major advantage of colloidal drug carrier systems is the possibility of drug targeting by a modified body distribution (Kreuter, 1983) as well as the enhancement of the cellular uptake (Schäfer et al., 1992) of a number of substances. As a result undesired toxic side effects of the free drug can be avoided, for instance with methotrexate (Narayani and Rao, 1993).

Among these colloidal systems those based on proteins may be very promising, since they are biodegradable and non-antigenic (Rubino et al., 1993), relatively easy to prepare and their size

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distribution can be monitored easily (MacAdam et al., 1997). Because of the defined primary structure of proteins the protein-based nanoparticles may offer various possibilities for surface modification and covalent drug attachment. A rational development of a protein based colloidal drug carrier systems requires a systematic characterisation of the particles.

In the field of microparticles a great deal of work has already been performed. The extend of reaction of functional groups with glutaraldehyde in model compounds and proteins was already studied by Habeeb and Hiramoto (1968). Similar studies were performed with human serum albumin (HSA) microparticles prepared in biphasic systems. A complete and systematic study regarding the influence of HSA concentration, emulsification time and power, stirring rate, heat stabilisation temperature, and the type of the non-aqueous phase was carried out by Gallo et al. (1984). The influence of polycondensation pH (Edwards-Levy et al., 1993), cross-linking reaction time (Edwards-Levy et al., 1994), and cross-linker concentration (Andry et al., 1996) on the content of free amino groups on the capsule surface of HSA microcapsules cross-linked using terephthaloyl chloride was analysed by reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS). The number of modified carboxylic and hydroxylic groups was investigated using Fourier Transform Infrared Spectroscopic (FTIR) studies (Levy et al., 1994, 1995). MacAdam et al., (1997) determined the surface carboxylic and amino groups of albumin microspheres by covalent binding of radiolabelled markers. Several turbidity ratio tests were performed to evaluate the efficiency of cross-linking with glutaraldehyde (Rubino et al., 1993; Chen et al., 1994; Lin et al., 1994).

However, to date a systematic characterisation of protein nanoparticles is lacking. The objective of the present study is to evaluate the desolvation of HSA for the preparation of nanoparticles by investigation of particle size and the percentage of protein still dissolved in the reaction mixture as a function of the amount of added desolvating agent. Further investigations were focused on the influence of various glutaraldehyde concentrations and heat denaturing conditions on the number of available amino groups at the surface of HSA and gelatin nanoparticles. The amount of free amino groups also may be considered as a measure for the degree of cross-linking since lysyl is the only residue in the albumin molecule which is modified in the presence of glutaraldehyde (Rubino et al., 1993). Cross-linking in turn affects biodegradability and drug release from the carrier system (Lee et al., 1981).

2. Materials and methods

2.1. Reagents and chemicals

Human serum albumin (Fraction V), glutaraldehyde 8%, 2,4,6-trinitrobenzenesulfonic acid (TNBS) 5% aqueous solution, gelatin type A from porcine skin (175 Bloom), and gelatin type B from bovine skin (75 Bloom) were obtained from Sigma (Steinheim, Germany). Formaldehyde solution 35% was purchased from Merck (Darmstadt, Germany), and the BCA protein assay reagent was obtained from Pierce (Rockford, Illinois, USA). All reagents were of analytical grade and used as received.

2.2. Instruments

An Eppendorf Thermomixer 5436 (Gerätebau Eppendorf, Engelsdorf, Germany) was used for the incubation of the nanoparticles at various temperatures. Centrifugation was performed using an Eppendorf Centrifuge 5417 (Gerätebau Eppendorf, Engelsdorf, Germany) or a Beckman L-80 Ultracentrifuge (Beckman Instruments, Palo Alto, USA). Spectrophotometric analysis was performed employing a Hitachi U3000 spectrophotometer (Berkshire, UK). The zeta potential was measured using the Laser Zee Meter, Model 501 (PenKem, Bedford Hills, New York, USA) and the particle size measurements were performed by photoncorrelation spectroscopy (PCS) using a BI-200 SM Goniometer Version 2 (Brookhaven Instruments Corp., Holtsville, New York, USA). For ultrafiltration microcon® 10 microconcentrators (Amicon, Witten, Germany) were used.

2.3. Preparation of HSA nanoparticles with different glutaraldehyde concentrations

The albumin nanoparticles were prepared by a desolvation technique as described previously by Marty et al. (1978). HSA (200 mg) was dissolved in 2.0 ml purified water. Under constant stirring desolvation of the 10% HSA solution was achieved by dropwise addition of 8.0 ml ethanol. Volumes of 0.75 ml desolvated particles were transferred into Eppendorf tubes, and aliquots of 2.64, 5.28, 10.50, 15.84, 21.12, 26.40, 31.70, and 52.80 μl of a 2.7 % glutaraldehyde solution were added gradually to achieve particle cross-linking. The volume was adjusted with purified water. After 24 h incubation at 20°C under constant shaking the nanoparticles were analysed for particle size and zeta potential, dissolved HSA in the dispersion medium and the number of available amino groups at the particle surface.

2.4. Preparation of heat stabilised HSA nanoparticles

The desolvation of the particles was performed as described previously. In order to avoid the addition of a cross-linker, the particles were stabilised by incubating at different temperatures (50, 60 and 70°C) for 2, 4, 6, 24, and 48 h under constant shaking in the thermomixer.

2.5. Evaluation of the desolvation process of HSA nanoparticles using different volumes of ethanol

Under constant stirring aliquots of 0.25 or 0.1 ml ethanol were added dropwise to 2.0 ml of a 10% aqueous HSA solution at temperatures of 4, 20, 30 and 40°C, respectively. After each desolvation step, aliquots of 0.05 ml were taken from the samples, and to each aliquot 5 μl of a 1.6% glutaraldehyde solution were added for cross-linking. The cross-linked particle samples were incubated for 24 h at the same temperature that was employed for the desolvation process. After the addition of 8.0 ml ethanol, desolvation was complete. For the evaluation of the desolvation process, the particle size and the percentage of dissolved protein were determined.

2.6. Preparation of gelatin A nanoparticles with different glutaraldehyde concentrations

Gelatin A nanoparticles were prepared by a two step desolvation method, previously described by Coester et al. (1999). Gelatin type A (1.25 g) was dissolved in 25 ml purified water under constant heating. By addition of 30 ml acetone the protein was desolvated and sedimented after a short time. The supernatant was discarded and the remaining sediment redissolved in water under heating. After adjusting the pH to 2.5, the gelatin was desolvated again by dropwise addition of a three-fold volume of acetone. As described previously 1.5 ml aliquots of the particle dispersion were transferred into Eppendorf tubes for cross-linking with different glutaraldehyde concentrations. For this purpose, volumes of 3, 6, 9, 12, 15, 20, 30, and 40 μl of a 4% aqueous glutaraldehyde solution were added, the lacking volume was supplemented with purified water, and the preparation for 24 h incubated at 20°C in the Thermomixer.

2.7. Preparation of gelatin B nanoparticles with different glutaraldeyde concentrations

The procedure for preparing gelatin B nanoparticles was similar to the method described previously for gelatin A particles except that the pH that was adjusted to 12. Volumes of 10, 15, 20, 30, and 40 μl of a 2% aqueous glutaraldehyde solution were added to the desolvated particle preparation. For the characterisation of the gelatin nanoparticles the same parameters were investigated as described previously for the HSA nanoparticles.

2.8. Preparation of gelatin nanoparticles with different formaldehyde concentrations

For the preparation of the nanoparticles gelatin was desolvated as described above. Instead of glutaraldehyde, equal volumes of formaldehyde 35% were used for cross-linking.

2.9. Determination of the non desolvated percentage of protein after desolvation

Following homogenisation of the cross-linked or heat stabilised nanoparticle samples by ultrasonication, aliquots of the samples were diluted 1:10 and the particles separated from the supernatant by ultracentrifugation at 105.000 × g for 40 min at room temperature. The amount of protein dissolved in the supernatant was determined using a standard BCA protein assay. To 30 μl of the supernatant 800 μl of the BCA working reagent were added. After incubating the mixture at 40°C for 30 min the samples were analysed spectrophotometrically at 562 nm. The protein content of the samples was calculated relative to reference samples, which contained different volumes of a HSA standard and were treated as described previously.

2.10. Quantification of amino groups at the surface of protein nanoparticles

The determination of amino groups was performed using the reaction of TNBS with free amino groups. The nanoparticles were washed four times with water by centrifugation at 20 000 × g for 10 min, followed by redispersion. The nanoparticle content of the dispersion after the washing process was determined gravimetrically (100°C; 4 h).

Approximately 75–300 μl of the nanoparticle dispersion was diluted to a final volume of 400 μl with water and 400 μl of 4% sodium hydrogen carbonate solution (pH 8.5) and 400 μl aqueous 0.1% TNBS solution were added. The reaction mixture was shaken at 500 rpm for 2 h at 40°C. In order to separate the nanoparticles from the supernatant, the samples were centrifuged (20 000 × g/25 min) and 125 μl of the supernatant diluted with 500 μl water. These dilutions were assayed at 349 nm for unreacted TNBS. The content of amino groups on the particle surface was calculated relative to a TNBS reference that was treated in the same manner as described above, using water instead of the particle dispersion. In some cases ultracentrifugation of the samples with microcon® 10 microconcentrators was necessary to separate the unreacted TNBS from dissolved protein or dissolved TNBS–protein–conjugate, respectively.

2.11. Determination of the particle size and the zeta potential

For both determinations the samples were diluted 1:400. The PCS samples were diluted with purified water and measured at a temperature of 25°C and a scattering angle of 90°. The total countrate of each sample and the particle size were evaluated. However, for the zeta potential determination 0.02 M phosphate buffered saline pH 7.0 was used for the dilution as the zeta potential depended on the pH value and on the ion strength of the solvent. The employed voltage for particle movement was 150 V.

3. Results and discussion

The desolvation of HSA or gelatin with organic solvents followed by cross-linking with glutaraldehyde is a commonly used method for the preparation of protein nanoparticles (Marty et al., 1978). However, up to now a systematic characterisation of the desolvation process for the preparation of nanoparticles is still lacking.

The desolvation process of HSA nanoparticles was evaluated concerning the particle size and the quantity of protein still dissolved in the aqueous phase after desolvation in correlation to the amount of ethanol added as the desolvating agent. The particle size as well as the light intensity counts of the samples were determined by photoncorrelation spectroscopy (PCS). The light intensity counts represent the cumulative light pulses of the sample over the duration of the photoncorrelation experiment. These pulses depend on the particle concentration in the test tube as well as on the particle size in that the countrate increases with the size. The results of the PCS measurements are shown in Fig. 1. Up to addition of a 1.5-fold volume of ethanol relative to the volume of the initial HSA solution, the particle size increased significantly. Consequently, up to this volume the diameter of the resulting nanopar-

ticles was controlled by the amount of desolvating agent. Further addition of ethanol apparently caused no changes in particle size. However, the number of light intensity counts still increased up to the 2.5-fold ethanol volume. This can be taken as an indication that the desolvation process was not completed. Since the number of counts depends on the particle size as well as the particle concentration and since the particle size did not increase after addition of the 1.5-fold ethanol volume the increasing number of counts must be due to higher particle concentrations in the sample. This conclusion was supported by the results of the determination of dissolved HSA in the dispersion medium as shown in Fig. 2. The percentage of dissolved HSA was quantified using the BCA protein assay. The protein content was calculated relative to reference samples containing different volumes of a HSA standard solution. Only a slight interference of free glutaraldehyde in the supernatant of the nanoparticles with the BCA assay was observed. The maximal contribution of glutaraldehyde added to the aliquots of the nanoparticle suspension was about 1.3% of the total absorption in the BCA assay and was therefore neglected for the calculation of the percentage of dissolved HSA. The experiments showed that the percentage of dissolved HSA continued to decrease significantly with addition of the desolvating agent even above the 1.5-fold

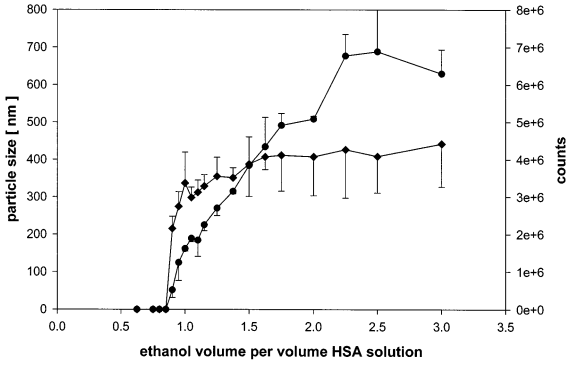

Fig. 1. HSA nanoparticles prepared by desolvation process: Particle size (— ◆ —) and light intensity counts in the PCS measurement (— ● —) in correlation to the amount of ethanol added to the HSA solution during the desolvation procedure (mean ± S.D.; $n = 3$).

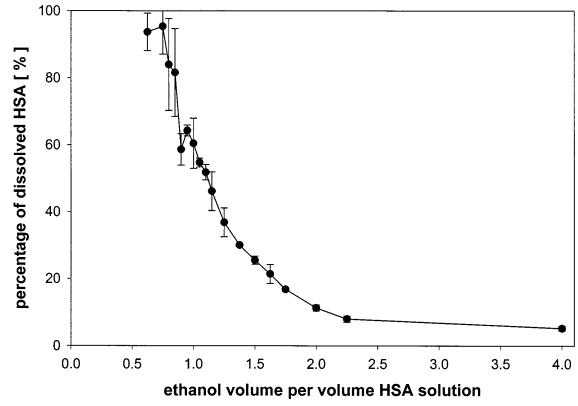

Fig. 2. HSA nanoparticles prepared by desolvation process: percentage of dissolved HSA in the supernatant of the nanoparticles in correlation to the amount of ethanol added to the HSA solution during the desolvation procedure (mean ± S.D.; $n = 3$).

ethanol addition. Therefore the desolvation process can be divided into two parts; a first part where an increase in desolvating agent leads to an increase in particle size and a second part above a 1.5-fold ethanol volume addition where the particle size remains constant but the particle concentration is still increasing.

It could be conceivable that the particle formation and hence the particle size was thermodynamically controlled. For this reason additional

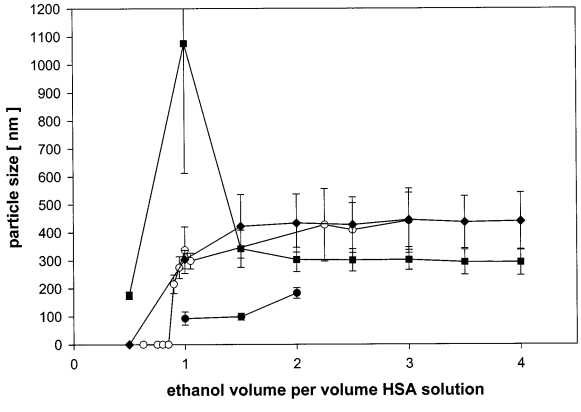

Fig. 3. HSA nanoparticles prepared by desolvation process: Particle size in correlation to the amount of ethanol added to the HSA solution during the desolvation procedure at temperatures of 4°C (— ● —), 20°C (— ○ —), 30°C (— ◆ —) and 40°C (— ■ —), respectively (mean ± S.D.; $n = 3$).

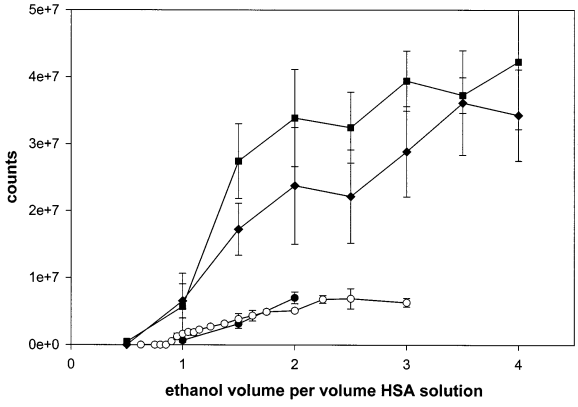

Fig. 4. HSA nanoparticles prepared by desolvation process: Light intensity counts in correlation to the amount of ethanol added to the HSA solution during the desolvation procedure at temperatures of 4°C (— ● —), 20°C (— ○ —), 30°C (— ◆ —) and 40°C (— ■ —), respectively (mean ± S.D.; $n = 3$).

experiments were carried out at different temperatures (Figs. 3 and 4). Although some temperature influence can be seen no clear trend is visible. Preparation at 4°C produced significantly smaller particles, but no difference occured between production at 20 and 30°C and smaller particles again resulted at 40°C. At 30 and 40°C clearly more particles were produced than at lower temperatures (Fig. 4) due to the lower solubility of HSA at higher temperatures under these conditions.

Further investigations were focused on the influence of the cross-linker on the properties of nanoparticles concerning particle size, zeta potential, percentage of dissolved HSA, and the number of available amino groups on the surface of HSA nanoparticles after cross-linking. The content of amino groups on the surface of the particles may also be considered as a measure of the degree of cross-linking by the aldehyde (Rubino et al., 1993). Alternatively to the stabilisation of the HSA nanoparticles using the cross-linker glutaraldehyde, the particles were stabilised by storage at elevated temperatures for several hours.

For the cross-linking experiments with glutaraldehyde different amounts of aldehyde were added to the desolvated HSA nanoparticles. Aldehyde concentrations ranging between 0 and 200% of the theoretic amount that is necessary for the quantitative cross-linking of the 59 amino groups in the HSA molecule were employed (Carter and Ho, 1994). After intensive washing of the nanoparticles by centrifugation and redispersion, the amino groups at the surface of the particles were determined using TNBS. The terminal amino group of the peptide chain as well as the ε-amino groups of lysyl residues can be considered to represent the reactive groups for the TNBS reaction with proteins. In 1966 Habeeb (1966) described a photometric assay for the quantitative determination of free amino groups in proteins. TNBS reacts with these groups at a pH of 8.5 under formation of a coloured TNBS–protein conjugate. At a wavelength of 349 nm the TNBS–protein conjugate showed a significantly higher absorption coefficient than TNBS itself. A generalisation of their results in turns of amino acid contribution was not possible, since different proteins or peptides showed different molar absorption coefficients. For the improvement of the solubility of this conjugate, Habeeb employed sodium laurylsulfate (SDS), but since the TNBS-HSA or TNBS-gelatin conjugates were soluble in aqueous solutions without any problem, in our study the reaction was performed without this additive. Moreover, the aim of the present work was to determine the amount of available amino groups at the surface of a solid system, namely the nanoparticles. Consequently a direct spectrophotometric analysis of the samples containing the colloidal system was impossible after the TNBS reaction. Therefore, after incubation of the particulate system with TNBS the nanoparticles were separated from unreacted TNBS by centrifugation, and the amount of unreacted TNBS was determined spectrophotometrically at 349 nm. Investigations regarding the reaction time revealed that the TNBS protein coupling reaction reached the maximal level after 6 h. However, the increase between 2 and 6 h was rather small. As a result, the reaction time was fixed to 2 h in the present study.

The results of the TNBS reaction with HSA nanoparticles are shown in Fig. 5. The content of available amino groups at the surface of HSA nanoparticles decreased significantly after the ad-

dition of increasing amounts of aldehyde ranging between 40 and 200%. This decrease also indicated a progressive degree of cross-linking as already reported by Lin et al. (1994) for modified HSA nanospheres. The unexpectedly low number of amino groups on the nanoparticle surface at low cross-linker concentrations was probably caused by soluble (non-particulate) TNBS–HSA conjugates that increased the absorption at 349 nm in the supernatant after centrifugation of the nanoparticles. This means that the addition of 10% glutaraldehyde and, to a much smaller extent, the addition of 20% glutaraldehyde led to unstable nanoparticles and therefore after washing of the nanoparticles this redissolved HSA reacted with TNBS. This conclusion was supported by the quantitation of the percentage of redissolved HSA in the supernatant of the purified nanoparticles. The amount of redissolved HSA mainly depended on the amount of glutaraldehyde added: about 30% of the desolvated HSA redissolved again after dilution of the nanoparticles with water in the 10% glutaraldehyde samples. Consequently these particles were not sufficiently stabilised by cross-linking. The addition of 20% glutaraldehyde led to more stable nanoparticles, but even here about 5% HSA redissolved again. The lowest required glutaraldehyde concentration for the production of stable nanoparticles appeared to be about 40% which is in agreement with earlier results of Roser and Kissel (1993).

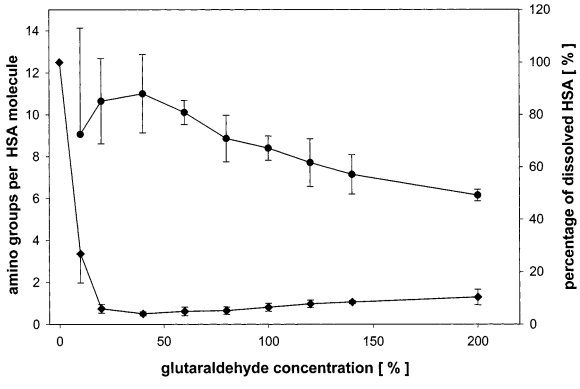

Fig. 5. Stabilisation of HSA nanoparticles with different amounts of glutaraldehyde: amino group content (— ● —) and percentage of dissolved HSA in the supernatant of the nanoparticles (— ◆ —) in correlation to the amount of glutaraldehyde added (mean ± S.D.; $n = 3$). Aldehyde concentrations between 0 and 200% of the theoretic amount for quantitative cross-linking of the 59 amino groups in the HSA molecule were employed.

Table 1
Storage of glutaraldehyde cross-linked HSA nanoparticles[a]

Storage duration (h)	Amino groups per HSA molecule			
	40% glutaraldehyde		100% glutaraldehyde	
	Mean	S.D.	Mean	S.D.
6	8.309	2.041	6.773	1.118
24	11.861	1.815	8.125	0.558
48	9.988	0.820	7.505	0.435
336	12.885	1.200	8.441	0.095

[a] The number of amino groups on the surface of the nanoparticles was determined after 6, 24, 48 h and 14 days, respectively ($n = 3$). Aldehyde concentrations of 40 and 100% of the theoretic amount for quantitative cross-linking of the 59 amino groups in the HSA molecule were employed.

The storage of nanoparticles cross-linked with 40 and 100% of glutaraldehyde relative to the theoretic amount required for the cross-linking of all amino groups over several days at room temperature had no significant influence on the number of these groups, even if residues of unreacted aldehyde still were not removed directly after cross-linking (Table 1). Basically this agrees with the work of Lin et al. (1993) who had already demonstrated an increasing degree of cross-linking over the first 2 h by monitoring the turbidity ratio of nanoparticle colloids, although we detected a very slight instability of the nanoparticles after 6 h but not at later times. The determination of the dissolved HSA in the supernatant of the nanoparticles by the BCA reaction revealed nearly 5% HSA after 6 h but only 1.3% after 24 h for the 40% glutaraldehyde containing samples and 6.9% after 6 h to 1.0% after 24 h for the 100% glutaraldehyde containing samples. The reason for the lower amount of amino groups after 6 h of cross-linking can obviously be attributed to the instability of the particles or the amount of redissolved HSA. Therefore a reaction time of 24 h for sufficient cross-linking is imparative.

An alternative commonly used method for particle stabilisation is thermal reaction. However, most of the published heat denaturation methods concern nanoparticle preparation in biphasic systems (Lee et al., 1981; Gallo et al., 1984; Rubino et al., 1993; Lin et al., 1994; Esposito et al., 1996; MacAdam et al., 1997). Nevertheless, Chen et al. (1994) described the stabilisation of HSA microspheres prepared by desolvation in acetone-water mixtures at 75°C for 15 to 30 min. The thermal cross-linking mechanism was shown to represent a condensation reaction between carboxylic groups and amino groups of adjacent gelatin chains (Esposito et al., 1996). In the present study heat denaturation was performed at 50, 60 and 70°C for 2, 4, 6, 24 and 48 h, respectively. The results are shown in Table 2. Again, similarly to cross-linking with glutaraldehyde, dissolved HSA in the surrounding phase obtained using low temperatures and short reaction times interfered with the amino group determination on the particle surface. The experiments indicated that denaturation at 50°C for less than 48 h and denaturation at 60°C for <24 h did not stabilise the colloidal system sufficiently, confirming that albumin survives heat pasteurisation at temperatures of 60°C for 10 h without deleterious effects (Carter and Ho, 1994). Things were quite different at 70°C. At this temperature even after 2 h stabilised particles were formed and further heating did not change the percentage of cross-linked amino groups any more. This is basically consistent with the results of Chen et al. (1994) who showed, that increasing the heat denaturation time or temperature will lead to a higher degree of cross-linking of HSA and will therefore reduce the rate of dissolution of HSA in water.

The results of the TNBS determination after ultrafiltration showed that even the unstable HSA particles prepared at 50°C possessed approximately the same number of available amino groups as the stable nanoparticles (70°C) of about 13–14 amino groups per HSA molecule. The denaturation time only effected the stability of the particulate system, but not the surface characteristics, in this case the number of available amino groups. For the heat stabilisation procedure this may be interpreted as an intraparticulate interaction during which mainly the internal functional groups of the protein were involved in the cross-

Table 2
Stabilisation of HSA nanoparticles by constant heating at different temperatures[a]

Denaturation time (h)	Amino groups per HSA molecule					
	50°C		60°C		70°C	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
After centrifugation						
2	−7.447	0.357	3.906	0.718	13.580	1.530
4	0.183	1.443	7.519	0.611	13.191	0.524
6	3.131	0.571	8.756	1.049	12.748	1.295
24	11.363	0.309	10.761	0.279	12.814	0.221
48	11.648	0.696				
After ultrafiltration						
2	14.099	0.779				
4	14.790	3.742				
6	15.111	1.224				
24	14.451	1.418				
48	13.606	0.871				

[a] The number of amino groups on the surface of the nanoparticles was determined after heating times of 2, 4, 6, 24, 48 h and particle separation by centrifugation. Moreover, the number of the amino groups of the 50°C samples was determined after ultrafiltration ($n=3$).

linking reactions and consequently in particle stabilisation, whereas functional groups at the surface of the particles remained almost uneffected. This was supported by the fact that after a similar desolvation procedure of the protein the stabilisation with glutaraldehyde, that only reacts with amino groups at the particle surface, led to significantly lower amino group contents (7–11 amino groups per HSA molecule) in the TNBS assay (Fig. 5). In addition, cross-linking reaction with glutaraldehyde leads to the coupling of two amino groups by the aldehyde whereas heat denaturation leads to the formation of amide groups between neighbouring amino and carboxylic groups of the protein. The different contribution of the amino groups in the cross-linking reaction may be a further reason for the lower number of unreacted amino groups at the surface of glutaraldehyde-stabilised nanoparticles.

The number of available amino groups of native HSA determined with TNBS was about 9.05 ± 1.02 (mean $\pm$ S.D., $n = 4$) amino groups per HSA molecule. The HSA molecule contains 58 lysyl residues, therefore the number of available amino groups was drastically decreased compared to the theoretic content. This may not be surprising since the structure of albumin is predominantly (67%) α-helical. The helical structure is stabilised by 17 disulfide bonds, the majority of them is well protected and is not readily accessible to the solvent (Carter and Ho, 1994). Compared to the native HSA an average of 14.43 ± 1.71 amino groups per HSA molecule in the heat denatured particle samples was unexpectedly high. However, due to the preparation process, especially the addition of ethanol, partial denaturation of the protein including an increased exposure of amino groups by unfolding the HSA structure appeares to be probable. This phenomenon has already been described by Steven and Tristram (1958), who observed that the amount of reacting amino groups of ovalbumin increased with growing amounts of ethanol added to the reaction mixture.

Besides the number of available amino groups, other parameters such as particle size and zeta potential were determined for the characterisation of HSA nanoparticles. The PCS measurements

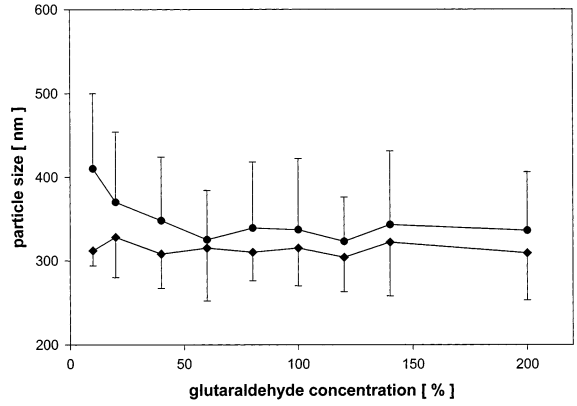

Fig. 6. Stabilisation of HSA nanoparticles with different amounts of glutaraldehyde: Particle size of purified (— ● —) and unpurified (— ◆ —) nanoparticles in correlation to the amount of glutaraldehyde added (mean $\pm$ S.D.; $n = 3$). The purification was achieved by 4-fold zentrifugation and redispersion of the nanoparticles in purified water.

were performed with unpurified and purified glutaraldehyde stabilised HSA nanoparticles as well as with heat denatured HSA nanoparticles. The different glutaraldehyde concentrations used for cross-linking appeared to have no significant effect on the particle size (Fig. 6). Even the unstable, partly redissolving particles prepared with low glutaraldehyde concentrations did not differ from the stable particles. Purification of the particulate system by washing four times with water slightly increased the average diameter of the particles. This may be due to swelling of the protein matrix of the particles after removal of the desolvating agent ethanol. Storage of the glutaraldehyde-cross-linked nanoparticles over 14 days had no influence on the particle size as shown in Fig. 7. The particle size of heat denatured HSA nanoparticles was independent on the denaturation time as well as the denaturation temperature (Table 3).

The zeta potential of the HSA showed a clear tendency to decrease with increasing glutaraldehyde concentrations (Fig. 8). The number of positively charged amino groups decreased with a raising degree of cross-linking. Consequently, the contribution of the negatively charged carboxylic groups became stronger and the zeta potential decreased. Nevertheless, the decrease of the zeta

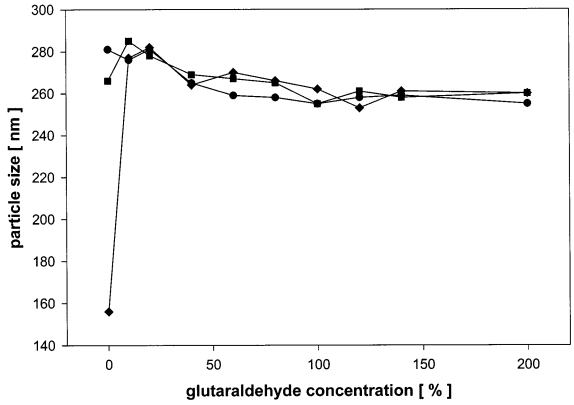

Fig. 7. Storage of glutaraldehyde cross-linked HSA nanoparticles: the particle size was determined after 6 h (— ◆ —), 24 h (— ■ —) and 14 days (— ● —), respectively.

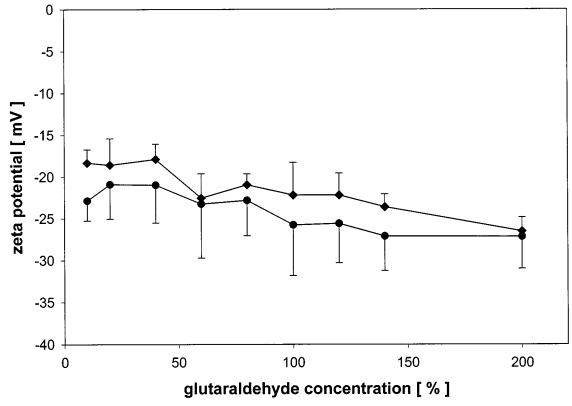

Fig. 8. Stabilisation of HSA nanoparticles with different amounts of glutaraldehyde: zeta potential of purified (— ● —) and unpurified (— ◆ —) nanoparticles in correlation to the amount of glutaraldehyde added (mean ± S.D.; $n = 3$). The purification was achieved by 4-fold centrifugation and redispersion of the nanoparticles in purified water.

potential was statistically non-significant. In order to avoid interferences caused by fluctuation of the pH or the ionic strength, the measurements were performed using phosphate buffered saline, pH 7.0. All zeta potential measurements were in the range between −17 and −25 mV which is in accordance with earlier results of Lin et al. (1994) who described values of about −19 mV at pH 7.0.

The results of the determination of available amino groups of gelatin type A and B nanoparticles are shown in Fig. 9. The decrease of these amino groups with increasing glutaraldehyde concentration was statistically significant for the gelatin A and B particles. Surprisingly, the number of available amino groups of the gelatin B nanoparticles was significantly lower than the number found for the gelatin A nanoparticles. After addition of about 80 μg glutaraldehyde per mg gelatin B, nearly no unreacted amino group could be detected on the particle surface. For this reason higher aldehyde concentrations were not employed. With gelatin A nanoparticles concentrations up to 230 μg glutaraldehyde per mg gelatin still led to higher numbers of amino groups than with gelatin B nanoparticles prepared with 80 μg glutaraldehyde per mg gelatin. Usually the amino aldehyde reaction is carried out at neutral or mildly alkaline pH for protein cross-linking (Lin et al., 1994) since the formation of

Table 3
Stabilisation of HSA nanoparticles by constant heating at different temperatures[a]

Denaturation time (h)	Particle size (nm)					
	50°C		60°C		70°C	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
2	315.67	30.07	279.00	10.58	309.33	10.69
4	328.00	31.32	289.67	15.28	355.67	38.08
6	326.33	30.02	305.33	29.19	330.33	25.50
24	344.33	37.31	327.33	29.02	358.00	43.00
48	333.67	15.01				

[a] The particle size of the nanoparticles was determined after heating times of 2, 4, 6, 24 and 48 h ($n = 3$).

Schiff's bases occurs considerably faster and to a higher degree at alkaline pH. For this reason the employed glutaraldehyde concentrations had to be higher for the cross-linking of gelatin A in the acidic than for the cross-linking of gelatin B in the alkaline medium. The protein gelatin has no defined primary structure, but values of 33 ε-amino groups per gelatin molecule of 1.000 amino acids were published (Digenis et al., 1994). Consequently, in the present experiment ≈30% of the gelatin A amino groups and 13% of the gelatin B amino groups were available on the particle surface, respectively.

Besides glutaraldehyde another commonly used cross-linker for the preparation of gelatin nanoparticles is formaldehyde. Therefore, some additional experiments were performed using formaldehyde with gelatin A nanoparticles. The mechanism of gelatin cross-linking with formaldehyde was extensively described by Digenis et al. (1994). During the treatment of gelatin with the monofunctional aldehyde several chemical reactions may occur, involving two ε-amino groups forming pyridinium rings, methylene links or imines that result finally in the formation of aminals. The stabilisation of gelatin A nanoparticles with corresponding concentrations of formaldehyde instead of the bifunctional aldehyde did not result in significantly different numbers of available amino groups at the surface of the nanoparticles, even though the amount of amino groups determined was slightly increased compared to glutaraldehyde (data not shown). Further increase of the formaldehyde concentration had no remarkable influence.

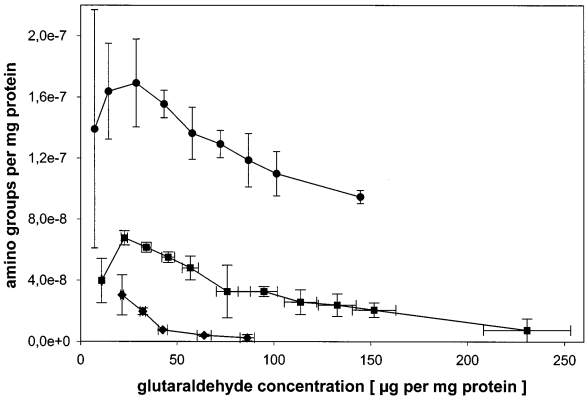

Fig. 9. Stabilisation of gelatin nanoparticles with different amounts of glutaraldehyde in comparison to HSA nanoparticles: Amino groups at the surface of HSA nanoparticles (— ● —), gelatin A nanoparticles (— ■ —) and gelatin B nanoparticles (— ◆ —) in correlation to the amount of glutaraldehyde added (mean ± S.D.; $n = 3$).

In conclusion the particle size as well as the number of available amino groups depends on the amount of desolvating agent or cross-linker. The higher the cross-linker concentration the lower was the number of amino group content on the particle surface. Heat denaturation also led to stable particles with an even higher number of amino groups compared to aldehyde stabilised nanoparticles. In contrast to aldehyde stabilisation this number was not influenced by a prolongation of reaction time. The cross-linker itself did not affect the mean diameter of the colloidal system. However, the zeta potential showed a slight tendency to decrease with growing glutaraldehyde concentrations.

In order to prepare a drug carrier system by covalent attachment of the drug to the colloidal system, the amount of available amino groups on the particle surface is one of the important parameters. A comparison between the different proteins and the various cross-linking procedures showed that the HSA nanoparticles possessed more lysyl residues on the particle surface than the gelatin nanoparticles. Heat stabilised HSA nanoparticles exposed the highest amount of available amino groups, followed by the glutaraldehyde cross-linked HSA nanoparticles. Gelatin A nanoparticles had more than double of the number of available amino groups compared to gelatin B nanoparticles.

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