A simple improved desolvation method for the rapid preparation of albumin nanoparticles

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The current study tried to establish a simple and fast method for the preparation of BSA and HSA nanoparticles, based on an improved desolvation procedure under the aspect of a controllable particle size around 100 nm for drug delivery applications. The Procedure used for the nanoparticles preparation was simplified by using a designed apparatus for controlling the addition of ethanol and it was used instead of conventional tubing pump which enabled the preparation of nanoparticles under defined conditions. By using EDC as cross-linker instead of glutaraldehyde, the time of nanoparticles preparation procedure was reduced to 3 h. Several factors of the preparation process, such as the volume of the albumin solution, desolvating agent volume, the amount of cross-linker, the presence of salts and protein concentration were evaluated. Nanoparticles with smaller size were obtained under experimental conditions without the presence of salts or the use of buffers, 250 mg of protein/4 ml water, 5 mg cross-linker, the addition of 4 and 8 ml ethanol by using the designed apparatus to the HSA and BSA solution, respectively. By using this improved method, BSA and HSA nanoparticles of the size around 100 nm and polydispersity below 0.2 were obtained.

1. Introduction

In the recent decades, many investigations have focused on the preparation and the application of nanomaterials in the fields of nanobiotechnology and nanomedicine [1,2]. Different nanostructures with natural or synthetic origin are prepared by using various methods [3]. Albumin-based nanoparticles have received considerable interest due to their biological origin, biodegradability, lack of toxicity, non-immunogenicity, water solubility, easy availability and more importantly their ability to accumulate in the tumour sites [4–6]. Serum albumin as the main protein in the circulatory system, is a heart-shaped globular protein which is composed of \(~585\) amino acids and has the molecular weight of 66 kDa. Structurally, albumin consists of three main domains, namely as domain I, II and III and each domain includes two subdomains (A and B). There are two main binding sites on albumin for the substances located in subdomains IIA and IIIA and are known as Sudlow’s sites I and II, respectively (Fig. 1). Albumin enhances the solubility of hydrophobic molecules in the blood and acts as a transporter for different substances. Thus, it could deliver various elements to the specific tissues in the body and influences the fate of a molecule in the blood. Bovine and human serum albumins are the major available albumins extensively used in the protein binding studies and targeted drug delivery applications [7,8]. Abraxane is a famous albumin-based nanoparticle for the chemotherapeutic agent paclitaxel with the mean particle size of 130 nm approved by FDA in 2005 for the treatment of metastatic breast cancers [9,10]. Albumin nanoparticles can be prepared by three major methods including desolvation, emulsification, thermal gelation and recently nano spray drying, nab-technology and self-assembly techniques have also been used [6]. Desolvation or coacervation technique is known as a main and simple method for the fabrication of albumin nanoparticles. In this method, a desolvation agent such as ethanol or acetone is added to the aqueous solution of albumin under a constant stirring condition using a magnet and it continues until turbidity is observed in the solution. The flow rate and the volume of the added desolvating agent are an important parameter to obtain favourable size of albumin nanoparticles. After finishing the addition of desolvation agent, a cross-linker such as glutaraldehyde solution must be added in order to stabilize the unstable particles and the constant stirring condition for the pre-

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pared suspension continues over night to complete crosslinking of all amino acids residues in the protein [11,12]. The amino moieties in lysine residues and guanidino side chains in arginine residues of albumin are solidified by a condensation reaction with the aldehyde group of glutaraldehyde. Nanoparticles are purified using centrifugation to remove the unreacted albumins, ethanol and the excess cross-linking agent. The nanosuspension produced is freeze-dried using 5% mannitol as a cryoprotectant to obtain a fine powder of the nanoparticles. A tubing pump is an essential instrument for carefully controlling the flow rate of desolvation agent addition. The manual addition of ethanol by a syringe was also reported in the literature [13]. The use of pump is not easy and shows some disadvantages. In the manual addition, the addition of ethanol cannot be controlled carefully. Therefore, in the present investigation, a simple apparatus was designed for controlling the flow rate and the flow of ethanol addition instead of using any pump or syringe. Additionally, for cross-linking of produced albumin nanoparticles, EDC was used instead of glutaraldehyde and this resulted in reducing the time of preparation process from overnight to 3 h. Glutaraldehyde remains in the structure of the nanoparticles and shows some toxic effects. But EDC is a zero space cross-linker. It forms peptide bands between carboxyl and amide groups of amino acids in the stabilized nanoparticles and urea as the by-product of the reaction can easily be removed by centrifugation. The present investigation is expected to establish a simple and fast technique in accordance with the desolvation method for the production of protein-based nanoparticles with a smaller particle diameter in future.

2. Experimental

2.1. Reagents and chemicals

Bovine serum albumin (BSA) and human serum albumin (HSA) were obtained from Sigma-Aldrich (Steinheim, Germany). Sorbitol (cryo-protectant sugar) and N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) as cross linking agent with a purity of >99% were prepared from Merck (Schushardt OHG, Hohenbrunn, Germany). Double distilled deionized water was obtained from Milli-Q water purification system (Millipore, USA) and used in all experiments.

2.2. Methods

2.2.1. The preparation of nanoparticles

2.2.1.1. The preparation of BSA nanoparticles. The preparation of BSA nanoparticles was performed according to the desolvation technique with some modifications. Briefly, 250 mg of BSA powder was weighed and dissolved in 4 ml water. Then, 8 ml ethanol was added to BSA solution drop wise at a rate of 2 ml/min using the designed apparatus under constant stirring (1250 rpm and using a 1 cm magnet) at room temperature. The observation of turbidity in the solution indicated the formation of the nanoparticles. For the stabilization of the unstable particles, EDC (5 mg in 0.5 ml water) was added for cross linking. The stirring condition was continued for 3 h to ensure the cross linking of all amino acid residues.

2.2.1.2. The preparation of HSA nanoparticles. HSA nanoparticles were also prepared by desolvation technique as described above for the preparation of BSA nanoparticles. In principle, 250 mg of HSA in 4 ml of water was transformed into nanoparticles by the continuous addition of 4 ml of the desolvating agent ethanol under constant stirring (1250 rpm and using a 1 cm magnet) by designed apparatus at room temperature. After finishing the desolvation process, a freshly prepared EDC solution (5 mg in 0.5 ml water) was added to induce particle crosslinking. The crosslinking process was performed under constant stirring of the suspension at a time period of 3 h.

2.2.1.3. The purification of nanoparticles. The resulting BSA and HSA nanoparticles were purified by three cycles of ultra-centrifugation.
2.2.2. The characterization of the prepared nanoparticles

2.2.2.1. Particle size and zeta potential. The size of prepared BSA and HSA nanoparticles was measured by laser light scattering technique using particle size analyser (Wing SALD 2101, Japan). 0.2 ml of the Prepared BSA and HSA nanoparticles were diluted with 12 ml water and stirred continuously during the particle size measurement. Zeta potential of the both prepared BSA and HSA nanoparticles were measured by dynamic light scattering technique using Malvern Zetasizer Nano ZS (Malvern Instruments, UK). For zeta potential measurement, disposable folded capillary cuvette was used. Air bubbles were removed from the capillary before measurement.

2.2.2.2. The morphological analysis of nanoparticles. Scanning electron microscopy (SEM, Vega Tescan, Czech Republic) of the prepared BSA and HSA nanoparticles was carried out to study the morphological behaviours of the prepared nanoparticles like sphericity and aggregation. Lyophilized BSA or HSA nanoparticles in the form of powder were coated with gold under vacuum, and then the SEM images of samples were obtained.

3. Results and discussion

3.1. Ethanol addition system

In this study, in order to simplify the procedure of albumin nanoparticles preparation, an apparatus was carefully designed and it replaced the tubing or peristaltic pump and also the manual addition of desolvation agent using a syringe. Fig. 2A shows the different parts of this system in a cartoon image and Fig. 2B represents the position of this apparatus in the nanoparticle preparation system. Figs. 2C and D show the apparatus when its components are separated and connected together. This apparatus is composed of four main parts: the hollow cylinder of a 20 ml syringe without its piston as a container for ethanol, two constitutive facets for controlling the flow and also the flow rate of ethanol addition and a needle of syringe at the end of the apparatus. In our designed apparatus, the upper facet was used for opening and closing the flow of ethanol while another subordinate facet was used to control the speed of ethanol addition. The presence of a needle at the end of this apparatus created small and uniform drops of ethanol and it also prevented the sudden addition of final drops of ethanol within the desolvation procedure. By using this apparatus, the flow rate of ethanol was controlled easily and consequently nanoparticles with a small particle size were prepared.

3.2. Protein concentration

Different concentrations of BSA and HSA proteins (150, 200, 250 and 300 mg/4 ml water) were evaluated in order to determine the required amounts of BSA or HSA for the preparation of nanoparticles with favourite sizes. The influence of protein concentration on the particle diameter of the prepared nanoparticles is shown in Fig. 3. For both BSA and HSA proteins, the concentration of 250 mg/4 ml or 62.5 mg/ml was achieved as the required concentration of the albumin to obtain nanoparticles with a smaller size. The concentrations higher or lower than this determined value did not produce nanoparticles with the favourable size. In an investigation by Langer et al. [12], they observed a slight influence on the particle diameter in a concentration range of HSA between 25 and 100 mg/ml, with a shallow size minimum of 155 nm at 50 mg/ml HSA. In Galisteo-González and Molina-Bolívar [11], albumin nanoparticles with the size of 100–120 nm were obtained in
the presence of BSA concentration in the range of 40–60 mg/ml. The results of the present study and those previous investigations show that for the preparation of both HSA and BSA nanoparticles, the concentration of protein must be in the range of 50–60 mg/ml.

3.3. Solution volume

The volume of water for dissolving both BSA and HSA proteins was also considered for the first time in this study. For this purpose, different volumes of water were used for dissolving certain amounts of albumin proteins (250 mg). It can be observed from Fig. 3 that 4 ml water is a sufficient volume for dissolving 250 mg of BSA and HSA proteins powder in the preparation of albumin-based nanoparticles. It was revealed that in volumes higher or lower than 4 ml, the preparation of albumin nanoparticles for both BSA and HSA samples failed and this volume was the best choice for the successful preparation of both BSA and HSA nanoparticles (data are not shown). A fast review of the literature shows that various volumes of water were used for dissolving albumin protein. For example, in an investigation by Langer et al. [12], the amount of HSA was between 50 and 200 mg dissolved in 2.0 ml purified water. 10–50 mg of BSA was dissolved in 2 ml water for the preparation of albumin nanoparticles for the delivery of gabapentin [14]. In another investigation, BSA in the concentration between 10 and 30 mg/ml was used for the fabrication of metformin-loaded BSA nanoparticles [15]. Sripriyalakshmi et al. [16] prepared atorvastatin calcium-loaded BSA nanoparticles by dissolving 100 mg of albumin powder in 2 ml of water. In the preparation of vinblastine sulfate (VBLs)-loaded folate conjugated bovine serum albumin (BSA) nanoparticles, 3.21 mg of BSA was dissolved in 1 ml of deionized water [17]. In Rahimnejad et al. [18] study, they used 150 ml BSA (5 mg/l) to produce biological nanoparticles from BSA. In the
preparation of doxorubicin-loaded human serum albumin (HSA) nanoparticles, 200 mg of HSA was dissolved in 2 ml purified water [19]. These reported values are very confusing and in the current study, we decided to consider and determine the required volume of water in the preparation of both BSA and HSA nanoparticles.

3.4. Salts

In order to examine the effects of the addition of salts to the protein solution in the preparation of albumin nanoparticles with the favourite size, BSA and HSA proteins were dissolved in water containing sodium chloride (NaCl) with the concentration of 10 mM
and phosphate buffer (10 mM) with pH 7.4. After the preparation of albumin solutions in 10 mM NaCl, ethanol was added dropwise to them. Nanoparticles were formed upon finishing the addition of ethanol. Surprisingly, a solid yellow bulk was observed in both HSA and BSA prepared nanoparticles as shown in Fig. 4 and the size of these prepared particles was large. In the presence of phosphate buffer, particles with the size of below 150 nm were not formed. Fig. 4 shows the particle size of BSA and HSA nanoparticles prepared using phosphate buffer. It did not obtain nanoparticles with the diameter below 100 or even 200 nm and the size of the nanoparticles resulting from BSA and HSA proteins were almost higher than 300 nm. Nevertheless, numerous investigations were performed so as to develop a desolvation technique for the preparation of albumin nanoparticles under buffered conditions. In these cases, phosphate buffer (pH 7 and 8), and alkaline borate buffer (pH 8 and 9) were used in the concentrations between 20 and 50 mM. HEPES buffer (pH 7.5) and TRIS buffer (pH 8 and 9) were also used in the concentrations between 20 and 200 mM. All of the buffer salts applied interfered either with the desolvation process, leading to large albumin aggregates or precipitation of the buffer salts, or with the crosslinking process of the nanoparticles. With phosphate buffer, salt precipitation occurred during the desolvation procedure, whereas the alkaline borate buffer as well as the HEPES buffer led to a precipitation of the albumin in large agglomerates instead of the formation of nanoparticles. In the case of TRIS buffer, nanoparticles could be prepared due to their primary amino group and TRIS molecules interfered with the glutaraldehyde crosslinking of the particle matrix. [12]. For these reasons, in the current investigation, the use of buffer or the addition of any salt was abandoned and for the preparation of albumin nanoparticles, the powder of both BSA and HSA was dissolved directly in water. In accordance with our results, Langer et al. [12] reported that the production of HSA nanoparticles with a small size in the presence of salts or using buffers as a solution for albumin was not possible. They used distilled water in their study for the preparation of HSA nanoparticles. But in the studies of Jun et al. [20], Galisteo-González and Molina-Bolívar [11] and Taheri et al. [21], albumin nanoparticles have been prepared successfully in the presence of 10 mM NaCl and also phosphate buffer with the small particle size. Altogether, our findings show that phosphate buffer and sodium chloride are not suitable agents in the preparation of albumin nanoparticles with a smaller particle size.

3.5. Desolvation agent content

For the preparation of albumin nanoparticles, a desolvation agent such as ethanol or acetone is added drop-wise to the aqueous solution of protein. The volume and the speed of the addition of desolvation agent affect the size of obtained particles. Similar to the volume of water reported in the relevant literature, different amounts of desolvation agent mainly ethanol and also the speed of its addition have been used for the fabrication of albumin nanoparticles. In the present investigation, after testing various amounts of ethanol in the range of 6–8.5 ml, it was observed that the volume of 8 ml ethanol is a sufficient volume for the successful preparation of BSA nanoparticles. In the preparation of HSA-based albumin
nanoparticles, the volume of the added ethanol was different from the value observed in BSA nanoparticles and the turbidity was seen after the addition of 4 ml ethanol and continuing the addition of ethanol did not produce favourable particles. In accordance with these results, the use of this amount of ethanol is reported in many research papers [12,13,16,19,22,23]. In accordance with the studies of Langer et al. [12], the flow rate of the ethanol addition in the speed range of 1–2 ml/min produced nanoparticles with the size of around 100 nm. For this reason, in all experiments in the current investigation, ethanol was added with the flow rate of 2 ml/min using designed apparatus.

3.6. Cross-linker

Glutaraldehyde is a common cross-linker for the stabilization of albumin nanoparticles. After finishing the addition of desolvating agent, glutaraldehyde is added and the stirring condition continues for overnight in order to cross-link amino acid residues in the proteins to create albumin nanoparticles. Glutaraldehyde shows some disadvantages such as remaining in the body of nanomaterials and subsequently its toxicity, a long time for its reaction in the preparation of albumin nanoparticles. Thus, in this investigation, the cross-linker EDC was used for the stabilization of both HSA and BSA nanoparticles instead of glutaraldehyde. Different amounts of EDC were added in order to stabilize particles. The addition of 3–5 mg of EDC was sufficient for the preparation of both HSA and BSA nanoparticles with the size of around 100 nm while the addition of 10 mg of cross-linker produced particles with a larger diameter (Data are not displayed). In the study of Taheri et al. [21], they added 5 mg of cross-linker EDC to the formed HSA particles in order to stabilize them. In their study, the concentration of HSA was 250 mg in 2 ml phosphate buffer solution (PBS) pH 7.4.

3.7. The characterization of the prepared nanoparticles

After considering different parameters affecting the preparation of albumin-based nanoparticles, both BSA and HSA nanoparticles were prepared using the developed method and characterized for their size and morphology. The size and SEM images of the prepared BSA and HSA nanoparticles and also the photographic images of both BSA and HSA solution, prepared nanoparticles, redispersed nanoparticles and the lyophilized form of the nanoparticles are shown in Figs. 5 and 6. By using this improved desolvation technique, the size of prepared nanoparticles was around 100 nm and with the polydispersity of below 0.2 for both BSA and HSA nanoparticles. A minimum value of ± 20 mV for zeta potential of prepared nanoparticles is extremely recommended in the case of combined electrostatic and steric stabilization of the nanoparticles. The produced BSA and HSA nanoparticles showed a zeta potential of −35 and −29.6 mV respectively, which is desirable to a stable monosuspension. In accordance with the results of particle size analysis, SEM images indicate that the shape of produced nanoparticles are almost spherical and monodisperse without any aggregates.

4. Conclusion

In the present investigation, a simple and fast method was developed in order to improve the desolvation technique for the preparation of albumin nanoparticles. The preparation procedure was simplified by using a designed apparatus for carefully controlling the addition of ethanol. By using EDC as cross-linker instead of glutaraldehyde, the time for the preparation of both HSA and BSA nanoparticles was reduced from overnight to 3 h. After considering different parameters affecting the size of prepared nanoparticles, HSA and BSA nanoparticles were fabricated with the size of around 100 nm. SEM images confirmed the results of particle size analysis and the monodispersity of the prepared nanoparticles.

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References

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