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Genipin Crosslinked Human Serum Albumin Nanoparticles

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Abstract
The use of human serum albumin (HSA) nanoparticles as drug delivery systems in controlled drug release studies has gained importance today. Albumin nanoparticles are biocompatible, biodegradable and provide sustained release. To maintain long-term drug delivery, HSA nanoparticles need to be cross-linked. A chemical crosslinker, glutaraldehyde is generally used in the literature and has some toxic effects on the cells. In this study, a biological crosslinker, genipin, was used for the production of HSA nanoparticles by desolvation technique. Two different temperatures and genipin concentrations were studied in order to decrease crosslinking time. The nanoparticles were characterized by Scanning Electron Microscopy (SEM) and Dynamic Light Scattering (DLS). The crosslinking time was reduced from 8-24 hours to 2 hours by raising the temperature to 37°C from room temperature. HSA nanoparticles which are crosslinked by genipin may have potential use in drug delivery system and may be applied in personalized medicine applications.

1. Introduction

Nanoparticles are structures with sizes ranging from 1 to 100 nm, according to the description of NNI (National Nanotechnology Initiative). The small size of nanoparticles provides them unique physicochemical and biological features; thus, they are more readily taken up by cells which gives them the ability of delivering drugs to the desired target organ [1]. Drug delivery systems play an important role for the controlled release of a drug molecule or its transport to the targeted organ [2]. Particle size and surface properties are main factors that affect the potential of drug carrier system [3]. Toxicity, distribution in living organism and targeting ability of nanoparticle heavily depend on particle size [4]. Nanoparticles are removed from the body by renal, lymphatic or reticuloendothelial system according to their sizes [5]. In a study, accumulation of nanoparticles in the spleen with diameters higher than 230 nm was shown [6]. Studies have shown that nanoparticles smaller than 100 nm is expected not to activate the lymphatic system however, particles with 200 nm and larger size tend to activate the lymphatic system [7]. The surface charge properties of the nanoparticles are described by zeta potential that represents the electrical potential of particles. Zeta potential is affected by parameters such as the particle composition and the medium in which it is dispersed. Nanoparticles with a zeta potential greater than (±) 30 mV form more stable suspensions, which means prevention of aggregate formation [4].

Different types of materials such as synthetic and natural polymers can be used for nanoparticle formation. Human serum albumin (HSA) is a remarkably soluble protein abundant in human blood plasma and has a molecular weight of 66.5 kDa and shows an average half-life of 19 days [8], [9]. The HSA protein acts as a carrier for endogenous and exogenous compounds with a high ligand binding capacity. Thanks to this feature, it carries out various functions in human body such as carrying hormones and fatty acids with low water solubility, making some toxins harmless and regulating pharmacokinetic

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properties of many drugs such as sulfonamides, penicillin etc. [10]. In addition to these compounds, HSA binds to various heavy metals to control their concentration in blood. Also, the major function of HSA is to regulate the colloid osmotic pressure of blood which facilitates water and water-soluble substances to pass from capillary vessels to tissues [11]. It is stable in the 4-9 pH range and at 60°C for 10h. It is primarily uptake by inflamed tissues and tumor cells that makes it a good candidate to be used as a directed drug carrier. When it is degraded, amino acids as degradation products supply nutrition to peripheral tissue [9]. In brief, nanoparticles manufactured using HSA for drug delivery systems exhibit many advantages: having functional groups such as amino and carboxylic groups on the nanoparticle surface which allow binding of albumin nanoparticles with drug targeting ligands, providing sustained release, biodegradability and prevention of toxicity [8], [9], [12].

Desolvation, emulsion, coacervation, nanospray drying, self-assembly, and nanoparticle albumin bound technology are some of the techniques which can be applied to obtain albumin nanoparticles. Desolvation and emulsification are the most preferred methods in the production of protein nanoparticles [13]. In desolvation technique, proteins in aqueous solutions are dehydrated by addition of desolvating agent, such as ethanol, and gain spherical shape to produce nanoparticles. The desolvation technique has been improved as an alternative to emulsification because it is a more robust and reproducible technique which minimizes possible denaturation and triggering self-assembly of proteins in the presence of desolvating agent [12], [14]. After nanoparticle production by desolvation technique, the protein nanoparticles are generally crosslinked with chemical cross-linking agents like glutaraldehyde, however this agent may lead to some toxic effects both *in vitro* and *in vivo* studies [15].

Genipin is a natural crosslinking agent that can be used to crosslink various polymeric materials including primary amine groups, such as chitosan, collagen, gelatin, and proteins. The mechanism of genipin crosslinking is based on crosslinking of free amino groups which are present in lysine, hydroxylysine, and arginine amino acids [16]. The biocompatibility of materials crosslinked with genipin is higher than that of materials crosslinked with glutaraldehyde or any other epoxy compound [17]–[19]. In the literature, Shahgholian et al. conducted studies on crosslinking of bovine serum albumin (BSA) nanoparticles with genipin instead of glutaraldehyde crosslinker by desolvation technique, which is the main method of albumin nanoparticle production. They have shown that genipin could crosslink BSA nanoparticles after 24 h incubation at 25ºC. In another experimental study [20], Luo et al. worked on tannic acid coating of genipin-crosslinked HSA nanoparticles and they showed that genipin was able to crosslink HSA nanoparticles at room temperature (RT) after 16 h, keep them stable and enhance their encapsulation efficiency [21]. Lin et al. have shown that sugar beet pectin-BSA nanoparticles with diameter ~180 nm was obtained by ultrasonication in the presence of genipin after 24 h at RT [22].

In this study, it was aimed to obtain genipin crosslinked HSA nanoparticles and to decrease crosslinking time with increasing crosslinking temperature. Nanoparticles obtained by desolvation technique were crosslinked with genipin. Optimization studies were carried out by changing the genipin concentration and temperature during crosslinking.

2. Material and Method

Albumin, Human Fraction V Powder was purchased from Sigma-Aldrich, USA. Genipin with 98% purity was obtained from Challenge Bioproducts, Taiwan. Ethanol Absolute with 99.9 % purity was purchased from Isolab, Germany.

2.1. Synthesis of genipin crosslinked HSA nanoparticles

HSA nanoparticles were synthesized by desolvation method [3] as illustrated in Fig 1. 10 mg HSA was dissolved in 500 µl ultra-pure water. For nanoparticle formation, 2 ml of desolvating agent, ethanol, was added (1 ml/min) dropwise by using a syringe-pump under constant stirring (600 rpm) at RT. After desolvation process, nanoparticles were crosslinked by addition of genipin with 0.2% and 2.2% w/w of HSA solution under constant stirring in an incubator set at 37°C and at RT. After crosslinking process, HSA nanoparticles suspension was recovered by centrifugation at 15000 rpm, 4°C for 20 min. Nanoparticles were washed with ultra-pure water three times. Finally, nanoparticles were resuspended in ultra-pure water.

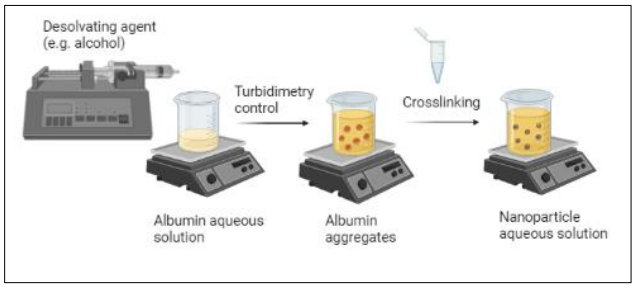


Figure 1. The schematic illustration of the HSA nanoparticle production via desolvation method

2.2. Characterization of HSA nanoparticles

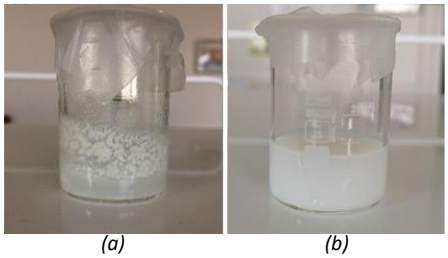
The morphology of nanoparticles and particle size were investigated by Scanning Electron Microscopy SEM (FEI – Quanta 650 Field Emission SEM, Çukurova University Central Research Laboratory) analysis. One droplet of diluted suspension was deposited on carbon tapes and dried at RT. Then the sample was sputter-coated with gold and then investigated by SEM. To compare the change in particle size and their distribution, the diameter of nanoparticles was measured by ImageJ (NIH, Bethesda, MD) software.

Dynamic Light Scattering (DLS) analysis was carried out to determine the average particle size, size distribution and zeta-potential of the nanoparticles. The sample was taken from the nanoparticle suspension after homogenization by sonication, and the final nanoparticle concentration was adjusted to 0.1 mg/ml by adding ultra-pure water at different pH values (7, 8 and 9) and PBS. Diluted and homogenized nanoparticle suspension was added into the device-specific cuvette and the measurement was done by a Malvern Zetasizer (Nano-ZS, Malvern, Mersin University (MEITAM)) at RT.

3. Results and Discussion

3.1. The effect of temperature and genipin concentration on the nanoparticle properties

In this study, we aimed to decrease the crosslinking time with changing genipin concentration and temperature. Favorable crosslinking condition of genipin is in the range of temperature between 25-45°C [17]. In this study, to decrease crosslinking time of the HSA nanoparticles, they were immediately placed into 37ºC-incubator soon after genipin addition and stirred at 600 rpm for different durations. Crosslinking of HSA nanoparticles at 0.2 w/w genipin concentration at RT for 24 h resulted significant aggregation of the nanoparticles on the interface of the solution and glass beaker as seen in Fig. 2a. However, there were not any aggregation in the nanoparticle suspension when they were crosslinked at 37ºC for 2 h at the same genipin concentration. Further studies were carried out at 37ºC. In the literature, HSA or BSA nanoparticles which were obtained by desolvation technique were generally crosslinked with glutaraldehyde for 24 hours at 25°C [3], [12]. In a study conducted by Shahgolian et al., glutaraldehyde as covalent crosslinker was used to obtain BSA nanoparticles. Genipin solution (0.1, 0.2, 0.3% w/w of BSA) is used to crosslink BSA nanoparticles for 24h at 25°C [20]. Here, genipin crosslinking time was reduced to 2 h with increased temperature.



(a) *(b)*

Figure 2. a) Genipin crosslinked HSA nanoparticles at RT for 24h **b)** Genipin crosslinked HSA nanoparticles at 37°C for 2 hours

3.2. Morphology of the nanoparticles

To determine the effect of genipin concentration and crosslinking time on HSA nanoparticles they were crosslinked with genipin concentrations (0.2% w/w and 2.2% w/w of HSA) for 2, 3 and 4 h at 37°C at 600 rpm. SEM images of these nanoparticles were given in Fig. 3. It was observed that uniform, spherical and nanometer range nanoparticles were synthesized successfully.

The particle size distribution of the nanoparticles was determined by ImageJ software (NIH, USA) from the SEM images (Fig. 4). The average diameter of the nanoparticles was not affected by increasing crosslinking time for 0.2% w/w genipin crosslinked HSA nanoparticles (Fig. 4a, b and c). On the other hand, there was a slight increase in the diameters of HSA nanoparticles which were crosslinked at 2.2% w/w genipin concentration (Fig. 4d, e and f). The average diameter of the nanoparticles slightly increased from ≈20 nm to ≈22 nm for 2 and 4 h crosslinking time, respectively. The diameter of the nanoparticle is important for drug delivery studies. Nanoparticles up to 200 nm are simultaneously taken into the cell by multiple endocytosis pathways [23]. In the literature, the size of nanoparticles crosslinked with glutaraldehyde is ranging from 150-300 nm [3], [12]. Here, genipin crosslinked HSA nanoparticles with very small diameter were obtained. Crosslinking

of HSA nanoparticles with 0.2 w/w genipin at 37ºC for 2 h resulted in a significant decrease in nanoparticle size. For further studies, 2h of crosslinking time was chosen.

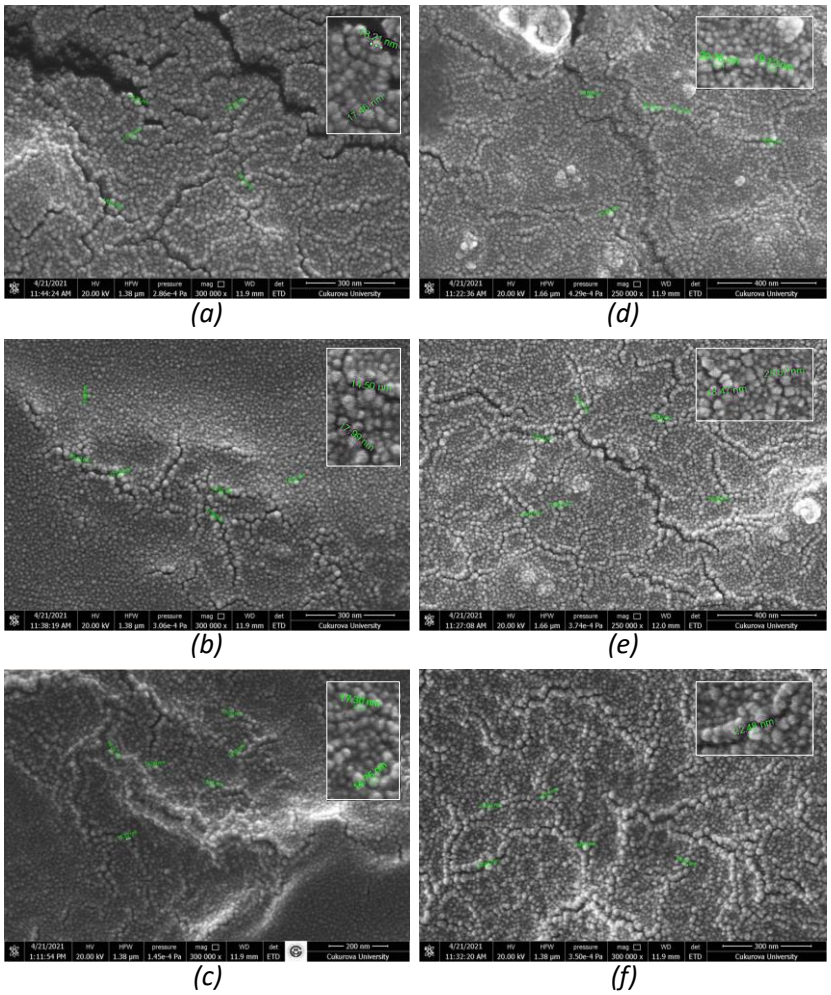


Figure 3. SEM images of 0.2% w/w genipin crosslinked HSA nanoparticles for **a)** 2 h, **b)** 3 h and **c)** 4 h; 2.2% w/w genipin crosslinked HSA nanoparticles **d)** 2 h; **e)** 3 h and **f)** 4 h

3.3. Particle size and size distribution of the nanoparticles by DLS

Particle sizes and size distributions of genipin crosslinked HSA nanoparticles by DLS analysis are summarized in Table 1. According to our results, the Z-average values were not correlated with the diameters of the nanoparticles as seen in SEM images. Due to the accumulation problem of the nanoparticles which were directly dispersed in ultra-pure water, it was difficult to measure the exact Z-average values of nanoparticles. However, it was clearly seen that, this accumulation problem was much more significant for 2.2% w/w genipin crosslinked HSA nanoparticles which may be due to high crosslinker concentration.

Zeta-potential of the nanoparticles is a very important parameter which describes the colloidal stability of the nanoparticles. Zeta-potential is a measure of repulsion between nanoparticles. Nanoparticles with charge other than -30 mV to +30 mV form more stable suspensions, which means prevention of aggregate formation [6]. The ideal formulation of nanoparticles has the smallest size and the zeta potential value other than -30 mV to +30 mV zeta potential, and the lowest polydispersity [24]. Due to the aggregation behavior of the nanoparticles here, zeta-potential values were out of this range.

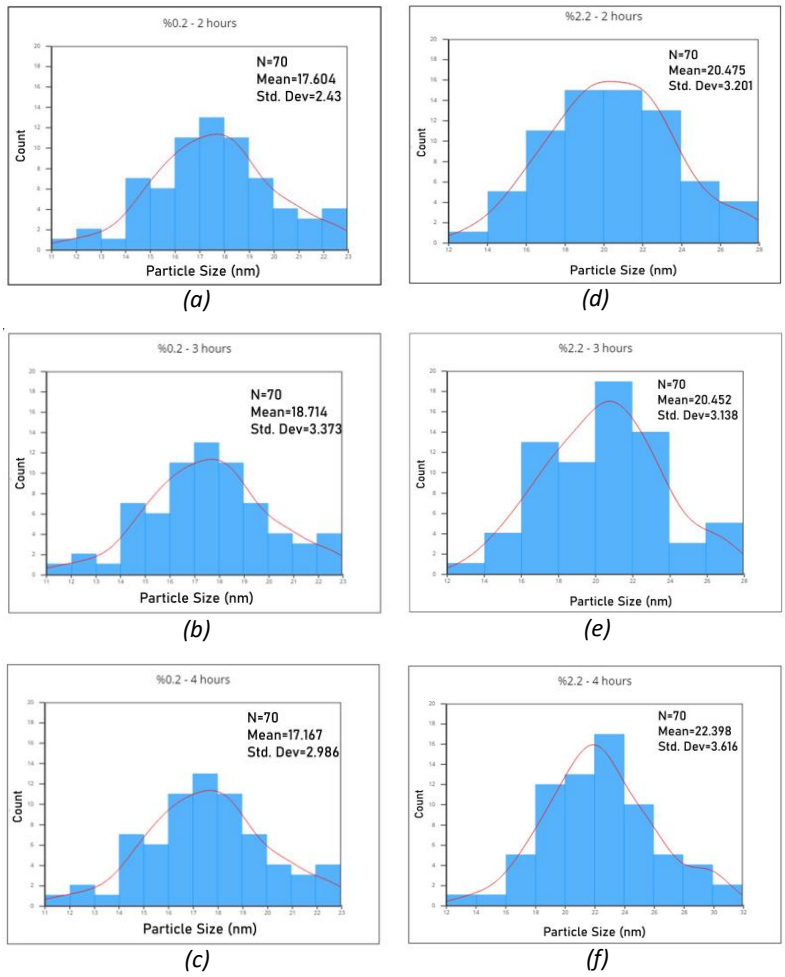


Figure 4. Size distribution curves of 0.2% w/w genipin crosslinked HSA nanoparticles for **a)** 2 h, **b)** 3 h and **c)** 4h; 2.2% w/w genipin crosslinked HSA nanoparticles **d)** 2 h; **e)** 3 h and **f)** 4 h.

Table 1. Z-average, zeta potential and PDI values of HSA nanoparticles

Genipin concentration (w/w)	Z-average (intensity, d.nm)	PDI	Zeta-potential (mV)
0.2%	323.5	0.668	-18.2
2.2%	1700	0.396	-1.21

To decrease the accumulation behavior the nanoparticles (0.2% w/w genipin crosslinked for 2 h) were diluted in PBS and in ultra-pure water with different pH values such as 8 and 9. The results are shown in Table 2. The accumulation tendency of the nanoparticles decreased when they were diluted in PBS environment and in ultra-pure water with increased pH. Z-average was decreased from 323.5 to 50.72 (intensity, d.nm) by increasing the pH from 7 to 9. PDI also was decreased from 0.668 to 0.227.

Protonation of carboxyl and amino groups of HSA protein below its isoelectronic point which is pH 4.7, results net positive charge and above its isoelectronic point results net negative charge. These differences in charges can alter protein electrostatic repulsion and trigger aggregation, which results in larger nanoparticles. This finding reveals that it is possible to modify the colloidal characteristics of HSA nanoparticles by varying the experimental parameters [24].

Table 2. Z-average and PDI values of HSA nanoparticles at different pH values.

Crosslinking condition	pH	Z-average (intensity, d.nm)	PDI
0.2% (w/w) genipin – 2h at 37°C	PBS (7.4)	144.6	0.278
	8	56.14	0.473
	9	50.72	0.227

4. Conclusion and Suggestions

Desolvation method is an efficient and simple method to produce human serum albumin nanoparticles. In this study, genipin crosslinked HSA nanoparticles with diameters smaller than 50 nm were successfully produced after 2 hours incubation at 37°C under stirring by desolvation technique. There was no significant morphological change between nanoparticles crosslinked at 0.2 or 2.2 w/w genipin concentrations. However, it was just observed that tendency of nanoparticles to coagulate increase as the genipin concentration increase. In conclusion, these genipin crosslinked HSA nanoparticles with diameters smaller than 50 nm may have potential to be used as drug carriers in drug delivery systems.

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Contributions of the authors

This study was conducted as MSc thesis of Emine Dila Kurtul and she contributed to the literature review, experiments, evaluation of data and article writing, while Merve Çapkın Yurtsever contributed to the formation of ideas, evaluation of the data, article writing and editing.

Conflict of Interest Statement

There is no conflict of interest between the authors.

Statement of Research and Publication Ethics

The study is complied with research and publication ethics.

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