

# Factors Affecting the Synthesis of Bovine Serum Albumin Nanoparticles Using the Desolvation Method

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**Abstract:** Currently, protein-based nanoparticles are in high demand as drug delivery systems due to their exceptional qualities, including nontoxicity, nonantigenicity, and biodegradability. Other qualities include high nutritional value, abundance of renewable resources, excellent drug binding capacity, greater stability during storage and in vivo, as well as ease of upgrading during manufacture. Examples of protein suitable for this purpose include ovalbumin (OVA) derived from egg white, human serum albumin (HSA), and bovine serum albumin (BSA). To create albumin nanoparticles, six different processes have been investigated in depth and are frequently used in drug delivery systems. These included desolvation, thermal gelation, emulsification, NAB technology, self-assembly, and nanospray drying. Several experimental conditions in the synthesis of albumin nanoparticles can affect the physicochemical characterization. Therefore, this study aimed to provide an overview of various experimental conditions capable of affecting the physicochemical characteristics of BSA nanoparticles formed using the desolvation method. By considering the variation in optimal experimental conditions, a delivery system of BSA nanoparticles with the best physicochemical characterization results could be developed.

**Keywords:** bovine serum albumin, BSA, desolvation, experimental conditions, physicochemical characteristics

## Introduction

In recent decades, nanotechnology, particularly, the use of nanoparticles is reported to have transformed the therapeutic process, leading to more potent medicines, diminished adverse effects, and intelligent therapeutics capable of targeting disease areas.<sup>1,2</sup> Nanoparticles, defined as solid or liquid colloidal particles with a size ranging from 10 to 1000 nm offer various benefits, including the administration route and the enhancement of the therapeutic impact. Therefore, this nanotechnology is more advanced and extensively explored by various studies.<sup>3</sup> Nanoparticles can be constructed from synthetic or natural macromolecules and must be biocompatible or preferably biodegradable.<sup>4</sup>

Due to their exceptional qualities, including nontoxicity, nonantigenicity, biodegradability, high nutritional value, abundant renewable sources, extraordinary drug binding capacity, greater stability during storage and in vivo, alongside ease of scaling up during manufacture, protein-based nanoparticles have attracted significant interest as drug delivery devices.<sup>5</sup> The main objective of designing protein-based nanoparticles as a drug delivery system is to regulate the particle size, surface area, and characteristics. This ensures that nanoparticles system carries the necessary amount of drug and releases the active substance to achieve part-specific action and the desired pharmacological activity.<sup>6</sup> Since 2008, numerous studies have focused on the use of protein as drug delivery in the form of nanoparticles.<sup>1</sup> One example of protein suitable for this purpose is albumin,<sup>4</sup> which has unique features making it an ideal choice as a drug transporter. For instance, given the abundance in the

body, injecting excessive albumin would have lower side effects compared to other carriers.<sup>7</sup> Transporting therapeutic drugs with albumin not only reduces costs but also decreases toxicity.<sup>8</sup> The bond between albumin and hydrophobic substances is reversible, facilitating drug transport in the body and the release to the cell surface.<sup>9</sup> Albumin has many functional groups, which result in a high capacity for binding drugs. Furthermore, the amino acid sequence and albumin structure allow the conjugation of drugs to nanoparticles through different mechanisms such as electrostatic power with negatively charged drugs (Ganciclovir), positively charged drugs, amphipathic compounds (Doxorubicin) and hydrophobic drugs (Paclitaxel).<sup>10</sup>

The macromolecular carrier known as albumin is biodegradable, non-toxic, and degraded *in vivo* to yield innocuous, non-immunogenic, easily purifiable, and water-soluble products. Given these properties, albumin is an excellent choice for nanoparticles formulations.<sup>11</sup> The high water solubility and easy purification process make albumin a good candidate for the manufacture of nanoparticles or as a versatile drug carrier that can be easily delivered by injection. Through covalent bonding, the medicine and other molecules adhere to nanoparticles by the amine and carboxyl groups.<sup>12</sup> Moreover, albumin is one of the most attractive materials for the development of novel medications, as evidenced by its successful use in the clinical nanomedicine application of Abraxane.<sup>13</sup> Ovalbumin (OVA), a protein generated from egg white, human serum albumin (HSA), and bovine serum albumin (BSA) are three different forms of albumin proteins that have been isolated and used for various biomedical applications.<sup>9</sup>

Nanoparticles can be created using two methods namely top-down and bottom-up methods. The top-down method entails milling, etching, photolithography, or ball milling to reduce a material with a large particle size into nanoparticles. The process normally takes a long time and produces large particles. On the other hand, the term “bottom-up method” describes the creation of nanoparticles one atom at a time, one molecule at a time, or one cluster at a time. The formation and growth of nanoparticles occur due to a chemical reaction that takes place under carefully regulated conditions in a liquid or gas phase. Albumin nanoparticles are prepared through bottom-up methods such as the desolvation, thermal gelation, emulsification, NAB technology, self-assembly, and nanospray drying.<sup>12</sup>

All these methods have been widely studied and the desolvation method is the most widely used in the synthesis of BSA nanoparticles.<sup>14,15</sup> The results of nanoparticles size, polydispersity index, zeta potential, and encapsulation efficiency (EE) in the desolvation method are influenced by several variables. These include variations in the pH and the amount of BSA present, as well as the rate at which the desolvating agent is added.<sup>6</sup>

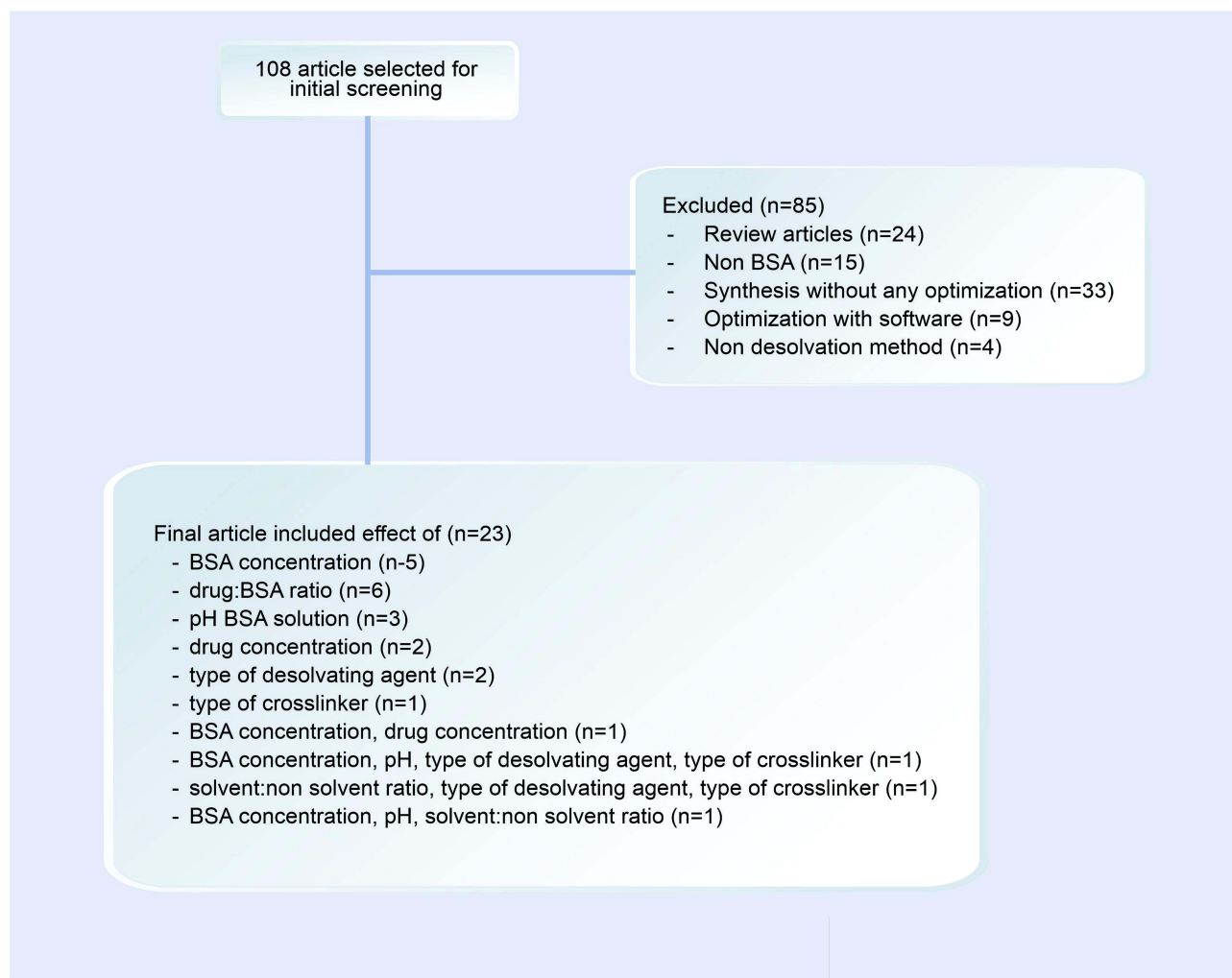
This study aimed to provide an overview about the types of albumin and the method of synthesizing nanoparticles as a drug delivery system, with a focus on the desolvation method. In addition, factors influencing the synthesis of BSA nanoparticles by the desolvation method were identified.

## Materials and Methods

All electronic databases were examined for this review, including PubMed, Scopus, and Google Scholar. The articles analyzed were those relevant to the production of BSA nanoparticles by the desolvation method and published in the last 10 years. The search was carried out using the keywords “BSA nanoparticles with the desolvation method”, “synthesis of BSA nanoparticles desolvation method”, and “production of BSA nanoparticles desolvation method”. The exclusion criteria applied were review articles, non-BSA studies, synthesis without any optimization, optimization with software, and non-desolvation method. The flowchart of the literature search flow is presented in [Figure 1](#).

## Type of Albumin

Albumin is easily obtained commercially with the main source being animal, vegetable, or human. BSA, HSA, and egg white (OVA) are a few examples of albumin frequently used. These three types of albumin have been widely adopted both in different studies and for the food industry.<sup>16</sup> BSA is commonly used in studies due to its low price and easy purification process.<sup>17</sup> HSA is frequently used to avoid losses from animal serum albumin, one of which is bovine spongiform encephalopathy.<sup>18</sup> Furthermore, the ability of OVA to create foam and gel networks has led to its widespread use in the food industry.<sup>11</sup> BSA and HSA are homologous in structure, conformation, and properties, sharing approximately 76% sequential identity, and the major difference lies in tryptophan (Trp) residue location. In HSA, only one Trp exists, located at position 214, while BSA has a Trp-212 at subdomain IIA, with an additional Trp-134 exposed in the solvent at subdomain IB.<sup>19</sup>



**Figure 1** Flowchart of the Methodology Research.

In general, albumin structure contains three homologous alpha-helical domains I, II, and III. Each domain comprises two subdomains A and B, which consist of four and six alpha helices, respectively. Additional important binding sites include the free thiol located at the cysteine-34 amino acid residue and Sudlow sites I and II, which bind a variety of hydrophobic drugs. The unique affinity and nature of each of these binding sites are of interest for the production of albumin-binding drugs.<sup>20</sup> Preformed albumin treatment and in situ binding are the two common binding methods. Drugs categorized as in situ binders have the ability to bind circulating (endogenous) albumin after being ingested. This in situ binding facilitates the transport, blood circulation, and medication dissolution of hydrophobic substances. Exogenous albumin is associated with the medicine in prepared formulations before ingestion by the patient.<sup>21</sup>

## Ovalbumin (OVA)

The dietary protein OVA, commonly known as egg albumin, is frequently used in the creation of food matrices,<sup>22</sup> and is the major globular protein in chicken egg whites.<sup>12</sup> OVA has an isoelectric point (PI) of 4.8 and a molecular weight of ~47 kDa. Furthermore, it is a 385-amino acid monomeric phosphoglycoprotein with four free sulfhydryl groups and an internal disulfide bond.<sup>11</sup> Due to its affordability and availability in comparison to other proteins, capacity to form emulsions, gels, and foams, as well as sensitivity to pH and temperature, OVA is used in drug delivery systems.<sup>9</sup>

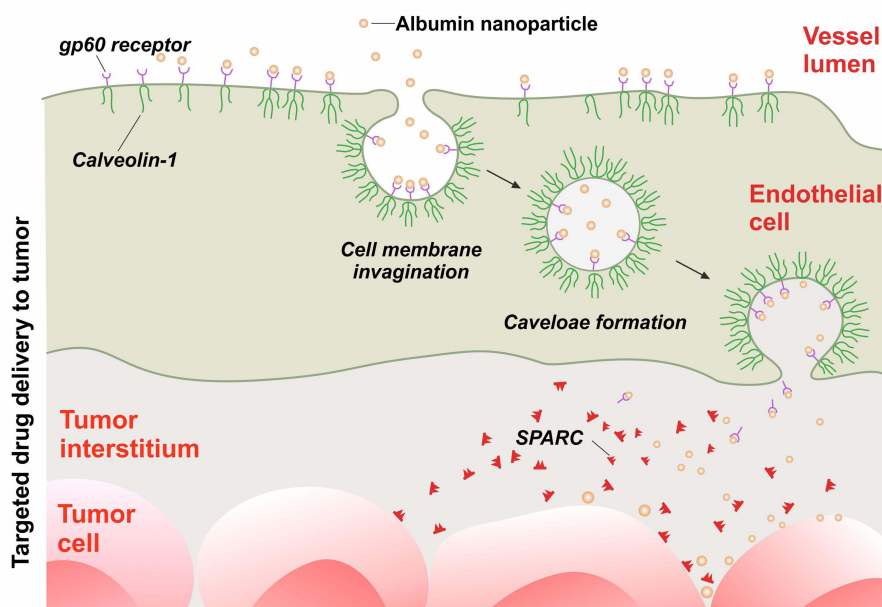
## Human Serum Albumin (HSA)

HSA is derived from human serum and represents a heart-shaped globular protein monomer.<sup>22</sup> With a molecular weight of ~67 kDa and a half-life of 14 days in the body, HSA is a blood protein that serves as a carrier for fatty acids, thyroid hormones, steroids, endogenous ligands, metabolites, and medications. Several other features include maintaining extracellular fluid volume,<sup>12</sup> stable in the pH range of 4–9, and heat resistance up to 60°C for 10 hours.<sup>23</sup> HSA is a single polypeptide of 585 amino acids produced in the liver. It has a high cysteine and a low tryptophan concentration.<sup>8</sup> Due to the benefits, such as high drug delivery capacity, targeting ability, strong stability, good biocompatibility, long half-life, and simple purification procedure, HSA has received significant attention recently as a drug carrier.<sup>24,25</sup>

## Bovine Serum Albumin (BSA)

BSA is derived from bovine serum as a globular protein with a molecular weight of ~66.43 kDa and an isoelectric point of 4.7.<sup>26</sup> The water-soluble monomeric protein BSA has 583 amino acid residues, 17 disulfide bonds, 9 loops created by bridges, one cysteine, and 8 pairs of disulfide bonds.<sup>22</sup> BSA is the most widely used type of protein in the pharmaceutical field,<sup>8</sup> specifically as a protein model to examine how proteins accumulate and fibrillate. One of the most crucial drug delivery systems used to treat illnesses, including cancer, is made from amyloid nanofibers generated from BSA.<sup>1,27</sup> Furthermore, this natural biomacromolecule has been extensively applied as a drug delivery system,<sup>28</sup> gene drugs, antibodies, and others.<sup>29</sup> The advantages compared to other carrier materials include biodegradability, non-toxicity, and non-immunogenicity.<sup>30</sup> BSA also has dual targeting capabilities for tumor tissue, binding to albumin gp60 receptor on the surface of vascular endothelial cells and interacting with secreted proteins acidic and rich in cysteine (SPARC) over-expressed on the surface of a variety of tumor cells. This efficient binding to albumin promotes the aggregation of nanoparticles loaded with drugs in the stroma of tumor cells (Figure 2).<sup>31,32</sup>

The three types of albumin (OVA, HSA, and BSA) have differences in terms of molecular weight, length (amino acid), organism, half-life, and main functions. The different characteristics are presented in Table 1 and the 3D structure is depicted in Figure 3.<sup>33,34</sup>



**Figure 2** Schematic Diagram of the Mechanism of Albumin Nanoparticles Targeting Tumor Tissue.

**Table I** Basic Information on the Three Types of Albumin

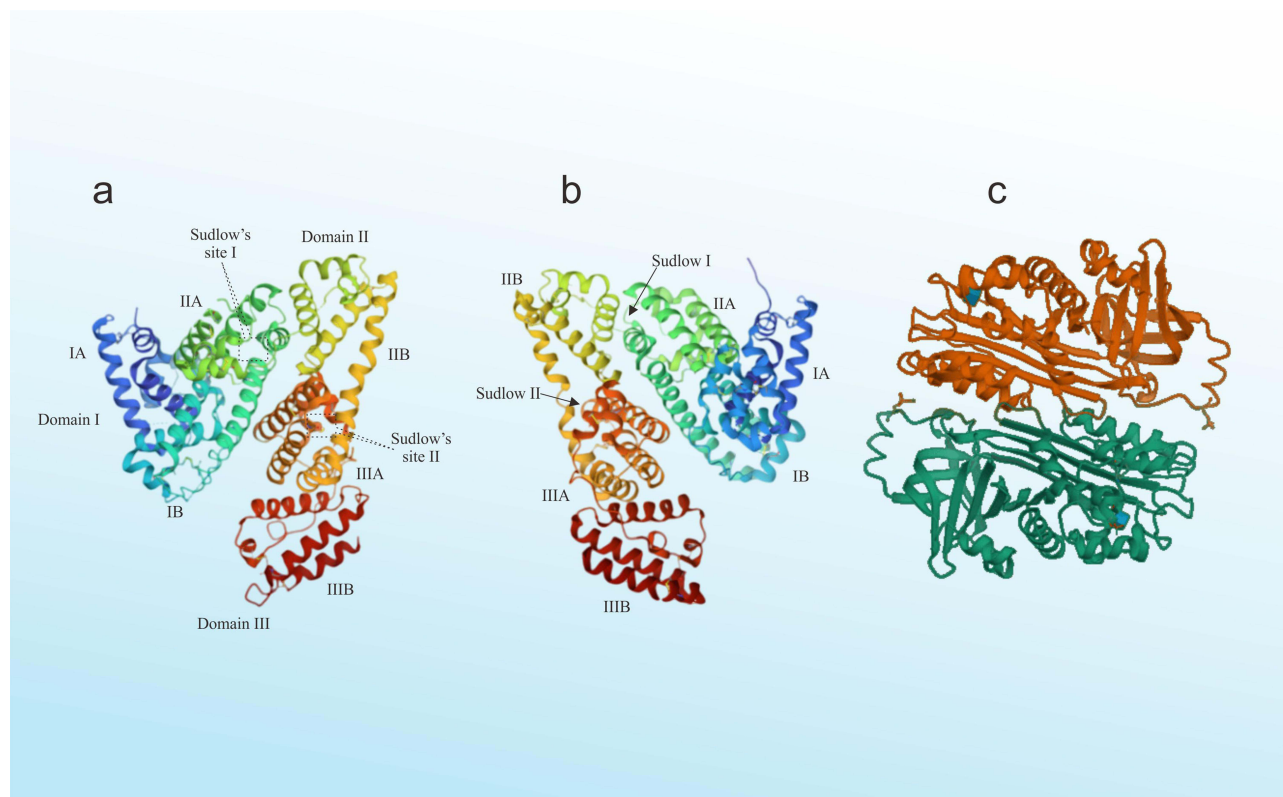
Type of Albumin	PI	Molecular Weight (kDa)	Length (Amino Acid)	Organism	Half-Life	Main Functions	Ref
OVA	4.8	~47	385	Egg white	19 days	Noninhibitory serpin, storage protein of egg white	[35–37]
HSA	4.7	~67	585	Human serum	14 days	Binds water, metal ions, fatty acids, hormones, bilirubin, and drugs and regulate the colloidal osmotic pressure of blood	[35,36,38]
BSA	4.7	~66.43	583	Bovine serum	19 days	Binds water, metal ions, fatty acids, hormones, bilirubin, and drugs and regulate the colloidal osmotic pressure of blood	[35,36,39]

## Method for Synthesizing Albumin Nanoparticles

Albumin nanoparticles synthesis method can be divided into two, namely physical and chemical.<sup>8</sup> The desolvation, emulsification, and self-assembly are included in the chemical method,<sup>1</sup> while the physical method consists of thermal gelation, nanoparticles albumin-bound (NAB) technology, and nanospray drying.<sup>40</sup> A total of six methods for synthesizing albumin nanoparticles have been extensively explored and used in drug delivery systems.<sup>5</sup> A schematic representation of the various processes is shown in Figure 4.

## The Desolvation

The desolvation method is the initial method in the synthesis of albumin nanoparticles.<sup>41</sup> This method entails the gradual addition of water-soluble organic solvents such as ethanol to dilute albumin solution with constant stirring. This leads to a reduction in the solubility of albumin, resulting in phase-based separation.<sup>35</sup> However, albumin particles are still not

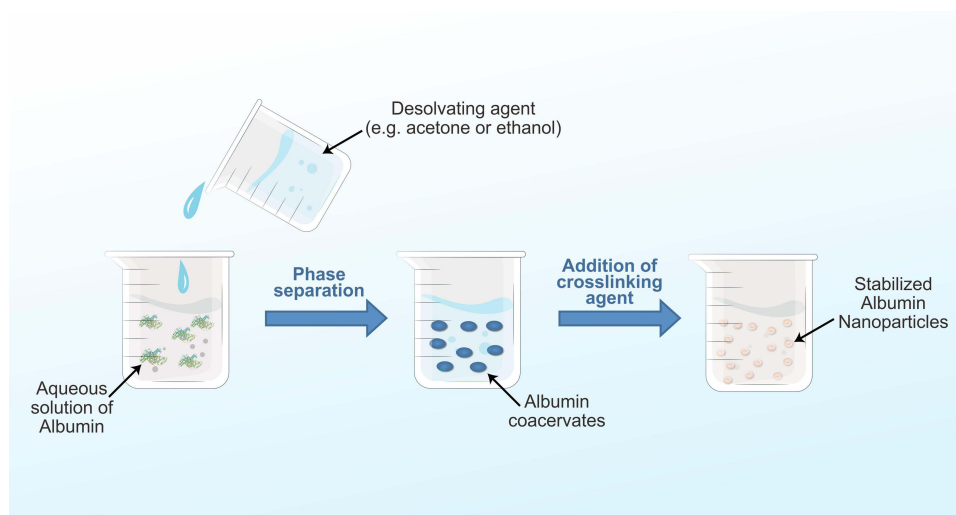


**Figure 3** The 3D Structure of Albumins: (a) Human Serum Albumin (b) Bovine Serum Albumin (c) Ovalbumin.



**Figure 4** Various Process for Synthesis Albumin Nanoparticle.

totally stable at this point and may return to the aqueous phase,<sup>12</sup> showing the need to add a crosslinker to increase the rigidity of nanoparticles formed. Crosslinking on desolvated particles is a critical step in the creation of albumin nanoparticles as it affects particle durability, biodegradability, and drug release from the carrier system.<sup>35</sup> The addition of glutaraldehyde as a crosslinker causes condensation of the amino group on the lysine residue and the arginine group on the guanidin albumin side chain through a condensation process with the aldehyde group.<sup>12</sup> The flow of preparing albumin nanoparticles using the desolvation method is illustrated in Figure 5.



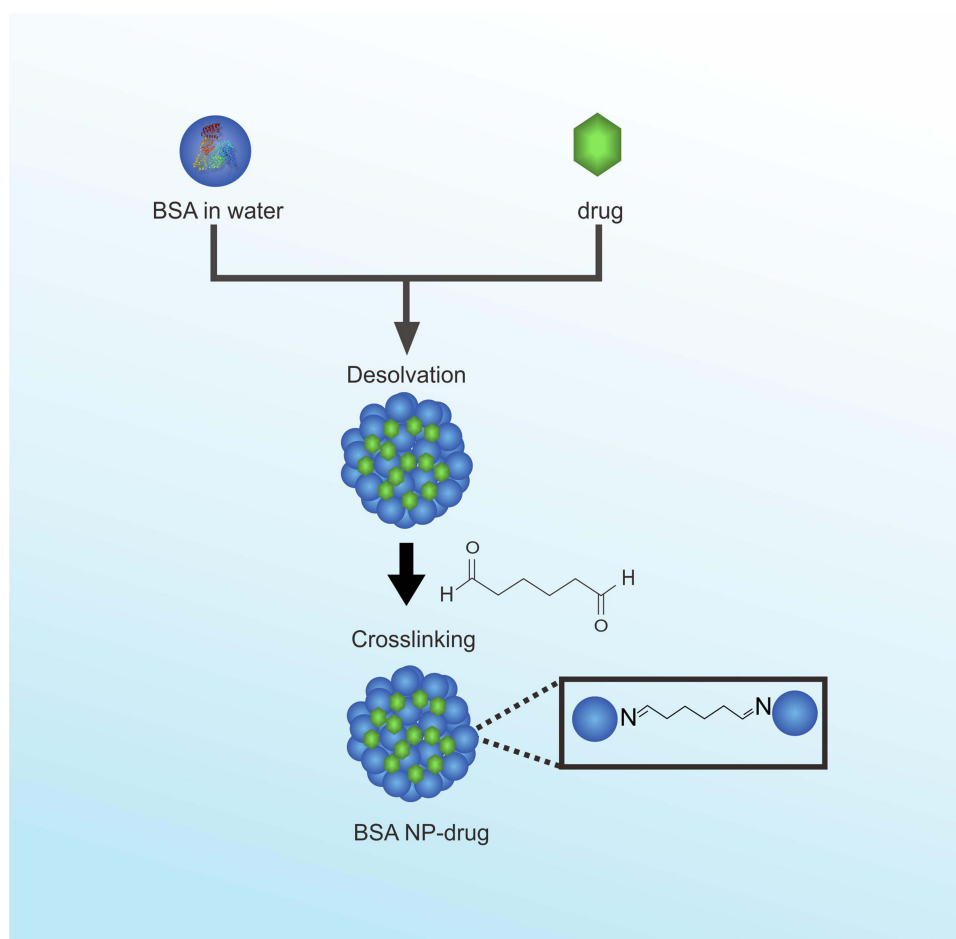
**Figure 5** Desolvation Process for Synthesis of Albumin Nanoparticles.

Albumin nanoparticles continue to maintain their shape and physical state even when subjected to several centrifugation and reconstitution cycles. These processes aim to remove ethanol and glutaraldehyde.<sup>41</sup> The interaction between BSA, crosslinker, and drug is illustrated in Figure 6.

## Emulsification

The emulsification method is the most established and effective method for producing albumin nanoparticles.<sup>12</sup> This method entails emulsifying a hydrophilic protein solution in a non-aqueous media, such as oil.<sup>42</sup> Two alternative emulsification processes, including high-energy and low-energy procedures, can be used to synthesize these emulsions. Specifically designed mechanical equipment, such as high-pressure homogenizers and ultrasonic generators, are used in the high-energy emulsification process. Due to the high efficiency of the device, emulsification only requires a small portion of the mechanical energy generated. The application of such particular mechanical devices will result in nanometric emulsions, with the formation of droplets, initial deformation, macrometric disruption, and the adsorption of surfactants at the interfaces to ensure steric stability.<sup>5</sup> Meanwhile, the creation of emulsion droplets in the nanometric range is achieved by the low-energy emulsification process, which transfers the intrinsic physicochemical characteristics of the surfactants, co-surfactants, and excipients in the formulation using the stored chemical energy of the system.<sup>43</sup>

Albumin nanoparticles formed by the emulsification method can be stabilized in two ways, namely chemically or physically.<sup>12</sup> Thermal heating for 10 minutes at a temperature of 175–180 °C is used to carry out the physical stabilization procedure. The viscosity of the oil is then reduced by adding ethyl ether to this combination after cooling,



**Figure 6** Synthesis of Albumin Nanoparticles.

facilitating centrifugation.<sup>5</sup> This thermal method only applies to drugs that are thermostable.<sup>35</sup> The chemical stabilization process is carried out by adding crosslinkers such as glutaraldehyde, formaldehyde, or diacid chloride.<sup>31</sup>

## Self-Assembly

The self-assembly method is a new method in the synthesis of albumin nanoparticles.<sup>44</sup> This method entails eliminating the primary amine group on the protein surface or enhancing the hydrophobicity of albumin by breaking the disulfide bonds. By introducing lipophilic drugs into the process, albumin nanoparticles can be formed through the self-assembly of molecules.<sup>12</sup> During the process, hydrophobic drugs or reducing agents are incorporated into the core to gradually accumulate hydrophobically modified albumin.<sup>41</sup> Commonly used reducing agents include  $\beta$ -mercaptoethanol, dithiothreitol, and cysteine. Several stages in the self-assembly method are similar to those in the desolvation. Albumin nanoparticles formed from this self-assembly method can better maintain protein function, resulting in active targeting properties.<sup>36</sup>

## Thermal Gelation

Albumin is degraded in the presence of heat in a method called thermal gelation, which leads to the aggregation of particles under optimum environmental conditions. This procedure, which is inexpensive and simple,<sup>45</sup> entails thermally mediated albumin opening and is induced by heating the solution at a temperature of 75–80 °C.<sup>46</sup> Interactions between hydrophobic proteins occur through electrostatic, hydrogen bond, and disulfide-sulfhydryl exchanges,<sup>11</sup> resulting in a stable three-dimensional protein molecular network. The addition of a denaturing agent, such as urea ( $\text{CO}(\text{NH}_2)_2$ ) accelerates the denaturation of albumin.<sup>35</sup> Therefore, the addition of 5 M urea in BSA solution can fasten the rate of gelation.<sup>45</sup> In general, thermal gelation is one of the methods used to avoid the potential for toxicity caused by the addition of organic solvents in the desolvation.<sup>46</sup>

## Nanoparticle Albumin-Bound (NAB) Technology

Currently, the NAB Technology method has been widely used, specifically in the development of anticancer drug delivery systems following the approval of the Nab-Paclitaxel (Abraxane<sup>TM</sup>) product by the US Food and Drug Administration.<sup>5</sup> This method is excellent for encapsulating hydrophobic anticancer drugs in a protein matrix for effective delivery.<sup>12</sup> NAB drug is produced by mixing an organic solution of chloroform/acetone+acetone with an aqueous albumin solution, which includes a hydrophobic drug with low rotation, resulting in a crude emulsion. Subsequently, a high-pressure homogenization is used to produce nanoemulsion. Rotary evaporation under vacuum is used to remove the organic solvent initially present in the colloid. After the production, nanoparticles are centrifuged or filtered (using a syringe filter), and the supernatant is lyophilized to produce nanoparticles powder.<sup>47,48</sup>

## Nanospray Drying

The pharmaceutical industry uses a method known as “nanospray drying” to convert liquid materials into powder nanoparticles.<sup>5</sup> This method has several benefits due to the narrow size distribution, high EE, and long shelf life of albumin nanoparticles produced. Additionally, this method may be applied at low temperatures and depends less on the solubility of albumin and drugs, minimizing the risk of degradation.<sup>49</sup> The spray drying method comprises four stages, namely atomization of the material, drying through gases, forming, and collecting particles.<sup>31</sup> This process is accomplished by means of a liquid sample sprayed into a chamber where heated nitrogen and carbon dioxide gases flow toward the spray from a nozzle. To stabilize the polymer particles, albumin liquid sample is added with surfactant. The electrodes at the bottom of the chamber are used to collect nanoparticles. The sprayed droplets are electrostatically charged due to these electrodes, causing movement toward the bottom of the chamber. This process is a fast and cost-effective way to produce small-scale protein particles.<sup>6</sup>

This review article focuses on the method of synthesizing albumin nanoparticles using the desolvation method, which has several advantages such as a simple preparation process, rapid reaction, and no need for surfactants. This method is suitable for the encapsulation of various hydrophobic drugs, and the preparation of albumin nanoparticles. By using the desolvation method, nanoparticles can be stored for a long time after freeze-drying. Moreover, the resuspended nanoparticles can be further treated for adsorbing drugs or covalently modifying targeted ligands on the particle surface.<sup>31</sup> The desolvation method is widely used in making albumin nanoparticles because the emulsification method has shortcomings including the difficulty



of removing organic solvents and the need for surfactants as stabilizers in the emulsion system.<sup>5</sup> Meanwhile, albumin nanoparticles derived using the Nab-Technology method have less stable colloidal stability and tend to separate into bound drug-albumin, albumin, and particles in systemic administration.<sup>35</sup> Thermal gelation and nano-spray drying methods are not suitable for hydrophobic drugs,<sup>5</sup> while the self-assembly method can cause potential toxicity due to the reducing agent used.<sup>35</sup> In recent years, nanoparticles prepared by the desolvation method have been widely studied in antitumor applications. For example, doxorubicin-loaded HSA nanoparticles prepared using the method showed good antitumor activity.<sup>50</sup> Ziaaddini et al constructed a type of BSA nanoparticles that enhanced the efficacy and reduced cytotoxicity of anticancer drugs.<sup>51</sup>

## Factors Affecting the Synthesis of BSA-NP Using the Desolvation Method

This review article focused on using polymeric BSA as a carrier, a choice well-received in the pharmaceutical industry and extensively used in fundamental investigations.<sup>4</sup> Nanoparticles based on BSA can be prepared by two methods, namely precipitation and emulsification.<sup>52</sup> The principal components of nanoparticles are dissolved in a solvent, and nanoprecipitation is based on the degradation of the solvent quality. Nanoprecipitation can be divided into several submethods, including non-solvent precipitation, desolvation, coacervation, salting, and albumin-bound technology, depending on how the solvent quality is reduced. In essence, this entails dissolving proteins in water while ethanol acts as a poor solvent. Combining these stages leads to saturation and development of a protein nucleus.<sup>53</sup> The free protein units condense around the core, creating nanoparticles. This method yields small particles with an unimodal distribution, and three phases make up the non-solvent-based deposition process namely supersaturation production, nucleation, and growth. Each phase is influenced by one or more experimental factors, and the deposition step in the process rate and formulation behavior impact the physicochemical characteristics of nanoparticles.<sup>54</sup> Therefore, this review article discussed several experimental conditions affecting the physicochemical characterization of BSA nanoparticles synthesized using the desolvation method (Table 1).

## Effect of BSA Concentration and Drugs: BSA Ratio

The classical nucleation theory can be used to explain why the concentration of BSA in the initial solution is a major variable in the formation of nanoparticles.<sup>55</sup> About eight studies have been conducted to optimize variations in BSA concentrations to obtain the best characterization results for particle size, isoelectric point (IP), and zeta potential. According to Table 2, there are six studies conducted by Tazhbayev et al<sup>56</sup> Fatemeh et al<sup>57</sup> Wilson et al<sup>58</sup> Aljabali et al<sup>59</sup> Diaz-Saldivar et al<sup>60</sup> and Noorani et al<sup>61</sup> the greater the concentration of BSA, the larger the particle size. BSA concentration affects the physicochemical characteristics of nanoparticles. This was supported by a report from Galisteo-González and Molina-Bolivar stating that increasing the concentration of BSA from 1.2% to 10% induced a rise in particle size from 75 to 135 nm.<sup>2</sup> Furthermore, as stated by Noorani et al, high concentrations of BSA increased the possibility of coagulation through electrostatic and hydrophobic interactions.<sup>61</sup>

Similar results were obtained by Fatemeh et al, stating that increasing the concentration of BSA could lead to a rise in particle size and polydispersity index. Nucleation theory helps explain the significance of BSA initial concentration in the synthesis of nanoparticles.<sup>57</sup> According to this theory, when the concentration of BSA increases, the viscosity rises and the frequency of protein transfer between water and the desolvating agent decreases, resulting in a slower nucleation rate and increased particle sizes.<sup>55</sup> A high BSA concentration also results in a greater degree of saturation, leading to smaller and more numerous nuclei. Concurrently, this high level of saturation accelerates agglomeration through collisions between larger particles to produce larger nanoparticles.<sup>62</sup>

The drug: ratio in the preparation of nanoparticles is also important because it affects particle formation, stability, and loading capacity.<sup>55</sup> In principle, the effect of this ratio on the physicochemical characteristics of nanoparticles drugs is the same as the impact of BSA concentration.<sup>68</sup> Based on the literature study (Table 2), six studies discussed optimization by varying the ratio of drug: BSA. According to Abd Ellah et al.<sup>69</sup> Jenita, Wilson, and Chocalingam,<sup>70</sup> Spada et al.<sup>71</sup> Jenita,<sup>72</sup> and Syed and Sailaja<sup>73</sup> the particle size and polydispersity index value increase with the amount of BSA used. This is in accordance with previous studies also stating that the higher the concentration of BSA, the larger the particle size and the greater the polydispersity index. This relationship can be explained through the classical nucleation theory.<sup>65</sup> On the other hand, several studies have reported that increasing BSA concentrations only had a slight influence on the size of BSA nanoparticles.<sup>55</sup> Radwan SES et al stated that an increase in BSA concentration from 2.5% to 10% resulted in a decrease in

**Table 2** Characterization of BSA Nanoparticles (NP) by Size, Polydispersity Index (PDI), Zeta Potential, and %EE of Encapsulation Efficiency in Different Experimental Conditions

Active Substances	Variation of							Optimal Conditions	Characterization Results				Ref
	%BSA	Drugs: BSA Ratio	pH	% drugs	Solvent: Non-Solvent Ratio	Type of Desolvating Agent	Type of Cross Linker		Particle Size (nm)	PDI*	ZP* (mV)	%EE* (%)	
NA	√ (2, 2.5, 5%)	-	-	-	-	-	-	2%	84 ± 4.3	0.268 ± 0.014	-	-	[46]
	-	-	-	-	-	√ (AC, ET)*	-	ET*	76 ± 1.2	0.245 ± 0.011	- 18.19 ± 0.47	-	
	-	-	√ (3,4,5,6,7,8,9,10)	-	-	-	-	9	156 ± 3.6	0.346 ± 0.011	-	-	
	-	-	-	-	-	-	√ (GT, GLU, SOR, AS, CA, TA)*	GT*	76 ± 1.2	0.245 ± 0.011	- 18.19 ± 0.47	-	
NA	√ (1,1.5,2, 2.5,3%)	-	-	-	-	-	-	3%	150	0.144	-27.2	-	[4]
	-	-	-	-	√ ((1:3),(2:5), (1:2),(2:3), (1:1))	-	-	1:2	165	0.116	-23.5	-	
	-	-	√ (3,5, 7,9,11)	-	-	-	-	7	82	0.29	-21.9	-	
Abacavir	√ (0.5–2.5%)	-	-	-	-	-	-	0.5%	418.2	0.191	40.8	-	[47]
Hydroxyurea	√ (1–3%)	-	-	-	-	-	-	2%	239±10	0.242±0.015	-20±3	78±1	[45]
Apatinib	√ (2.5–10%)	-	-	-	-	-	-	5%	198.9±3.52	0.05	29.2±1.3	-	[59]
Fruit extract <i>Cucumis prophetarum</i>	-	-	√ (5,7,9)	-	-	-	-	7	188.5	-	-4.86	82.3	[62]

Doxorubicin	-	√ ((1:1), (1:2), (1:4))	-	-	-	-	-	1:4	130.1	0.143	-	71.8	[52]
Piceatannol (PIC)	√ (0.5– 3.5%) -	-	-	-	-	-	-	0.5%	211	0.11	-19	-	[48]
								0.125%	210	0.11	-20	-	
ATPs	√ (1–4%)	-	-	-	-	-	-	1%	224.4±6.9	0.23±0.024	-	-	[49]
Docetaxel	-	-	-	-	-	-	√ (GT, GLU, UV, GLU+UV)*	(GT and GLU+UV)*	141.7 ± 22.9 (GT)* 140.2 ± 20.1 (GLU +UV)*	0.032 ±0.018 (GT)* 0.034 ± 0.022 (GLU+UV)*	32.5 ± 3.5 (GT)* -32.4 ± 2.8 (GLU +UV)*	-	[63]
Parvifloron D (PvD)	-	-	-	-	-	-	√ (DMSO, ET, AC, HX)* -	GLU*	225±60	<0.250	-14	-	[64]
								AC*	244±9	<0.260	-8	-	
								3:1	244±9	<0.260	-8	-	
Metoclopramide	-	√ ((1:1), (1:2), (1:4))	-	-	-	-	-	1:2	120	0.13±0.02	-31±9	63±2	[53]
Efavirenz	-	√ ((1:2), (1:3), (1:4), (1:5), (1:6))	-	-	-	-	-	1:2	106.3	0.112	-28.97	72.49	[54]
Sulfasalazine	-	-	-	√ (1–3%)	-	-	-	1%	229	<0.1	-24.8	28	[65]

(Continued)

Table 2 (Continued).

Active Substances	Variation of							Optimal Conditions	Characterization Results				Ref
	%BSA	Drugs: BSA Ratio	pH	% drugs	Solvent: Non-Solvent Ratio	Type of Desolvating Agent	Type of Cross Linker		Particle Size (nm)	PDI*	ZP* (mV)	%EE* (%)	
Colchicine (CCI-001)	–	√ ((1:4), (1:10), (1:20))	–	–	–	–	–	1:10	133.7 ± 25.5	0.08 ± 0.03	–27.6 ± 1.9	2.9 ± 1.3	[55]
Aspirin	–	–	–	–	–	√ (AC, ET, SS)*	–	ET*	209	–	–39.97	59.25	[66]
Salicylic acid	–	–	√ (5.4, 7.4, 9.0)	–	–	–	–	7.4	110±7	–	–33.2±1	57.5 ±2.5	[61]
Stavudine	–	√ ((1:1), (1:2), (1:3), (1:4), (1:5))	–	–	–	–	–	1:1	255.9 ±12.59	–	–16.5 ±0.84	73.70± 0.8	[56]
Naproxen Sodium	–	√ ((1:1), (1:2), (1:3))	–	–	–	–	–	1:2	385.1	–	–16.5	76.01	[57]
	–	–	–	–	–	(AC, ET, IP)*	–	AC*	556±3	–	–26.7 ± 3	60	[67]
Albendazole	√ (0.1 and 0.25%)	–	–	–	–	–	–	0.1	230	–	0.18	>80	[50]
Tenofovir	–	–	√ (7, 8, 9)	–	–	–	–	8	160.3±4.61	–	–37.4	–	[58]
5-fluorouracil	–	–	–	√ (0.05–0.25)%	–	–	–	0.25	142 ± 7	0.374	– 30.3 ± 0.9	–	[24]

**Abbreviations:** \*AC, acetone; ET, ethanol; HX, hexane; SS, sodium sulphate; GLU, glucose; SOR, sorbitol; AS, ascorbic acid; CA, citric acid; TA, tannic acid; GT, glutaraldehyde; IP, Isopropanol; PDI, polydispersity index; ZP, Zeta Potential; %EE, %encapsulation efficiency.

particle size from 260 nm to 100.5 nm.<sup>74</sup> Yang et al, also found that the greater the amount of BSA used, the smaller the particle size.<sup>68</sup>

The changes in particle size with increased BSA concentrations may be attributed to several factors such as solution pH, and salt concentration. Moreover, the volume and speed of adding desolvating agents affect the protein precipitation process, causing changes in the environment and altering its conformation.<sup>4</sup>

## Effect of pH in BSA Solution

The pH is the most influential factor in controlling the size of the resulting nanoparticles. As reported by Burns and Zydney, the surface charge had a strong influence on protein adsorption.<sup>75</sup> Based on the literature review in Table 2, five studies conducted by Fatemeh et al.<sup>57</sup> Bronze-Uhle et al.<sup>76</sup> Hemlata, Gupta, and Tejavath,<sup>77</sup> Jenita et al<sup>65</sup> and Tarhini et al<sup>4</sup> discussed the influence of BSA solution pH on the physicochemical characterization of the formed nanoparticles.

Jenita et al, underscored the importance of pH in the desolvation process, attributing changes in chemical characteristics and molecular structure of proteins to pH variations. This study showed that the particle size of BSA-Tenofovir nanoparticles decreased with increasing pH values. BSA solution with a pH of 8 produced a small particle size because the aggregation between molecules was hindered by electrostatic repulsion.<sup>65</sup>

In a study by Bronze-Uhle et al where salicylic acid was used as the active ingredient, modifying the pH of the solution significantly affected the net charge on the protein surface. This is because proteins are made up of amino acids and hydrogen, which interact with ions in the solution. The isoelectric point (IP) of BSA is 4.9, and at this pH, the protein has no surface charge. At pH 4.9, the electrostatic repulsion is lower, leading to the rapid formation of amorphous aggregates through non-specific interactions, specifically hydrophobic.<sup>76</sup> Positive charges can be produced at pH levels beyond BSA IP value by protonation of main amino groups, particularly lysine. Meanwhile, negative charges can be produced by deprotonation of carboxylic acid groups (glutamate and aspartate).<sup>78</sup> By increasing electrostatic repulsion and decreasing hydrophobic interactions, the resultant charge across the protein surface reduces aggregation and enhances structural rearrangement of the protein. Additionally, it significantly changes how pharmacological molecules interact or bind with one another. The hydrophobic interactions are dominated by the negative charge and electrostatic forces at pH levels above the IP value.<sup>79</sup>

The results obtained by Hemlata, Gupta, and Tejavath are similar to those of Bronze-Uhle et al in which the pH of the solution directly changed the particle size. At different pH values of the solution, the average particle size and zeta potential of BSA nanoparticles from Cucumis prophetarum fruit extract differ based on the principle of charge conductivity to ensure formulation stability. Furthermore, the particle diameter decreased as the pH of the solution approached the IP value of BSA. This implies that electrostatic contact is one of the critical aspects influencing the synthesis of BSA nanoparticles.<sup>77</sup> At a solution pH close to the IP value, a lower EE is produced because the charge on the protein surface inhibits the trapping of the active substance into nanoparticles products.<sup>2</sup>

Fatemeh et al and Tarhini et al conducted a study to determine the effect of pH in BSA solution on the physical characterization of nanoparticles without the addition of drug compounds. As showed by Tarhini et al, the zeta potential value of BSA nanoparticles was significantly impacted by variations in pH. The zeta potential value was positive at pH 3, while at 5 to 11, the value was negative.<sup>4</sup> The properties of the protein, which modify its conformation at various pH values, can be used to explain the outcomes of these fluctuating surface charges. Additionally, protein loading may be affected by pH, which potentially alters the formation profile and particle properties.<sup>80</sup> Fatemeh et al stated that the different pH of BSA solution significantly influenced the polydispersity index (PDI) value and the size of nanoparticles. The results showed that a pH value of more than 9 produced more uniform nanoparticles and smaller particle sizes through the desolvation process. The interaction between the amino acid hydrogens and the various ions in the protein solution affects the net surface charge of protein. This was further supported by Tarhini et al and Bronze-Uhle et al stating that the value of the isoelectric point (IP) could affect the solubility of the protein. Amorphous aggregates are formed by the non-specific interactions between protein molecules around the isoelectric point of the protein, where there are no electrostatic repulsions.<sup>57</sup> The high pH of the solution enhanced the solubility and surface charge of the protein, producing particles with a small size. When the pH of a solution is high, surface protein molecules have a negative charge and precipitation tends to occur. The secondary structure is impacted by this pH level, causing a rise in beta-sheet

production. Smaller particles are produced through hydrophobic interactions, such as thiol-disulfide. Furthermore, the pH value, a critical factor in the cross-linking process, determines the degree of protonation for the -amino group. By increasing the amount of protonated amino groups and decreasing pH, the possibility of cross-linking will reduce. At high pH, the amino group deprotonates, release in a free amino that can connect with another group.<sup>80</sup>

## Effect of Drug Concentration

The drug concentration in the initial solution plays a crucial role in the particle formation process,<sup>54</sup> and this impact is associated with two mechanisms. A higher concentration of dissolved drugs leads to the formation of more and smaller nuclei, increasing the degree of saturation. However, concurrently, the growth of the nuclei is enhanced due to an elevated frequency of particle collisions and a shortened diffusion length. The increase in drug concentration also raises viscosity, hindering drug diffusion between the solvent and the anti-solvent. This results in inconsistent saturation, a slower nucleation rate, and greater particle agglomeration, leading to bigger particles.<sup>66,81</sup> Furthermore, the increased viscosity reduces the number of collisions and mass transfer due to diffusion.<sup>64</sup>

Based on the literature review in Table 2, three studies conducted by Olaitan and Chaw,<sup>67</sup> Wilson et al<sup>30</sup> and Aljabali et al<sup>59</sup> discussed the effect of drug concentration on characteristics of BSA nanoparticles formed. Olaitan and Chaw obtained the best characterization results at a Sulfasalazine concentration of 1%, Aljabali et al at a Piceatannol concentration of 0.125%, and Wilson et al at a 5-fluorouracil concentration of 0.25%. Previous studies by Olaitan and Chaw and Aljabali et al showed that the higher the drug concentration, the larger the particle size. Therefore, a low drug concentration was considered the optimum formula for further testing. As stated by Aljabali et al, the particle size and zeta potential value increase with rising drug concentration. This was attributed to the higher viscosity and less dispersion of the organic into the aqueous phase.<sup>63,82</sup> On the other hand, Wilson et al, found that the formula with the highest drug concentration was considered optimal. This was based on the particle size required for tumor cell targeting (less than 400 nm). In this context, the higher the drug concentration, the greater the resulting drug loading.<sup>30</sup>

## Effect of Solvent and Non-Solvent Ratio

In the desolvation method, the addition of a desolvating agent (non-solvent), such as ethanol or acetone, reduces the hydration level and solubility of albumin in water (solvent), leading to the formation of nanoparticles.<sup>83</sup> The parameter of solvent and non-solvent ratio has been shown to dramatically affect the size and zeta potential of the particles formed. Based on the literature review in Table 2, two studies conducted by Tarhini et al, and Santos-Rebelo et al examined the effect of solvent and non-solvent ratio on characteristics of nanoparticles formed. Visual examination of the samples in the study by Tarhini et al clearly showed that characteristics of nanoparticles synthesized at different solvent and non-solvent ratios varied. When the ratio was adjusted to 1:2, the color (or opacity) of the samples changed from a faded, transparent white to a brighter, opaque white. Furthermore, when the ratio was increased to (2:3 and 1:1), the turbidity gradually returned before disappearing completely. The increased opacity shows an increase in particle size.<sup>4</sup>

The interfacial turbulence produced during solvent transfer is the foundation for the theory of nanoprecipitation. Due to the continuous mixing of the two solvents, the additional non-solvent disperses widely, enhancing the nucleation of protein nanoparticles when the proteins disperse beyond the thermodynamic solubility limitations. In other words, the amount of non-solvent injected affects the number of nanoparticles formed. Inadequate non-solvent in the mixture prevents precipitation, resulting in poor particles with a broad size distribution.<sup>84,85</sup>

Similar to particle size, the value of the polydispersity index decreased when the solvent and non-solvent ratio was increased from (1:3) to (1:2). This shows that the higher the ratio (up to 1:2), the lower the polydispersity index value. However, when the ratio was raised to more than 1:2, the polydispersity index value increased and the measurement became inaccurate. A similar conclusion was reached by Stainmesse et al who produced poly-ε-caprolactone nanoparticles using the nanoprecipitation method. When the solvent and non-solvent ratio was equal to or higher than 1, identifying the particle size became impossible.<sup>86</sup>

Concerning the zeta potential, the yield decreased with increasing solvent and non-solvent ratio from 1/3, 2/5 to 1/2, then increased to reach a maximum at S/NS of 1/1. According to a previous study by Jahanshasi et al, samples can be very polydisperse, leading to incorrect results at high solvent and non-solvent ratios. It is also well known that colloidal

nanoparticles show good stability when the absolute zeta potential is high.<sup>87</sup> As stated by Pal et al, a zeta potential value of less than  $-30$  mV or more than  $+30$  mV was considered stable, meaning that the particles would not flocculate during the storage time.<sup>88</sup>

## Effect of Desolvating Agent Type

The type of the desolvation agent has been clearly proven to affect the particle size and distribution of BSA nanoparticles formed. Due to the poor solubility in organic solvents such as acetone and ethanol, BSA can facilitate nucleation and deposition, leading to the spontaneous aggregation of nanoparticles.<sup>89</sup> Based on the literature review in Table 2, three studies conducted by, Fatemeh et al, Sailaja, Santos-Rebelo et al, and Krishna Sailaja and Nandini examined the effect of desolvating agent types on characteristics of the formed albumin nanoparticles. Fatemeh et al reported that the ethanol desolvating agent produced more nanoparticles characteristics compared to acetone.<sup>57</sup> Furthermore, the advantage of ethanol compared to acetone is that the characterization of the formed nanoparticles including particle size, PDI, and zeta potential is more stable even though there are variations in temperature during the manufacturing process. The use of ethanol desolvating agent also produces smaller nanoparticles sizes compared to acetone due to the difference in polarity of the two desolvating agents. The dielectric constants of acetone and ethanol are 21 and 24.5 respectively.<sup>65</sup>

Sailaja<sup>90</sup> also showed that ethanol is the best desolvation agent because it produces nanoparticles with smaller sizes and better entrapment efficiency compared to acetone and sodium sulphate. This study identified two important characteristics of the desolvation agents contributing to the production of large nanoparticles. These include the ability to form hydrogen bonds and low polarity which potentially generates additional hydrophobic interactions.<sup>91</sup> Two types of the desolvation agents were used in this study namely polar-protic (ethanol and sodium sulfate) and polar-aprotic (acetone). In this context, ethanol can act as a hydrogen donor and acceptor, producing nanoparticles better than acetone.<sup>90</sup>

Santos-Rebelo et al<sup>92</sup> and Krishna Sailaja and Nandini<sup>93</sup> showed that acetone desolvation agent produced nanoparticles with better characterization compared to ethanol, hexane, and DMSO. Acetone is an ICH Class 3 solvent, which is less toxic and poses less risk to human health at acceptable limits in pharmaceuticals.<sup>92</sup> These results were consistent with an earlier study by Arroyo-Maya et al, who produced uniform, smaller, and spherical  $\alpha$ -lactalbumin nanoparticles using acetone as the optimum desolvation agent. The differences in the results of these studies could be influenced by variations in nanoparticle preparations, protein configurations, and different measurement methods.<sup>94</sup>

## Effect of Crosslinker Type

The type of crosslinker added to BSA nanoparticles manufacturing process plays a crucial role in affecting the results of particle size, zeta potential, and PDI. However, the different types of crosslinkers used do not have a significant effect on entrapment efficiency (EE). The surface charge of nanoparticles is modified by the crosslinker. Additionally, this feature affects the electrostatic potential and colloidal stability of albumin nanoparticles in solution.<sup>76</sup> The literature review showed that three studies conducted by Fatemeh et al.<sup>57</sup> Niknejad and Mahmoudzadeh,<sup>95</sup> and Santos-Rebelo et al<sup>92</sup> examined the effect of the crosslinker type used on the characterization results of BSA nanoparticles (Table 2).

Fatemeh et al, and Niknejad and Mahmoudzadeh found that glutaraldehyde was the best type of crosslinker, producing BSA nanoparticles with low size and PDI characteristics. Furthermore, Fatemeh et al reported that different types of crosslinkers had varying effects on particle size and PDI nanoparticles, but did not significantly impact efficiency. Compared to other crosslinkers, glutaraldehyde, sorbitol, and glucose produced more stable BSA nanoparticles as showed by the measurement results of the zeta potential value. Glutaraldehyde, in particular, formed a structure encapsulating the drug molecules within BSA nanoparticles.<sup>57</sup> Varying amounts of glutaraldehyde significantly changed the surface charge of the resulting nanoparticles but did not alter the size. This phenomenon was attributed to the constant quantity of glutaraldehyde associated with free surface amino groups of proteins during nanoparticles production. Glutaraldehyde interacts with all free amine groups in albumin. According to a study, protein cross-linking occurs when two carbonyl groups attack the amino groups of lysine and arginine residues in proteins, forming Schiff bases in solution. When the pH is alkaline, Schiff base is very stable, but when the pH is acidic, it is unstable.<sup>76</sup>

Other chemical cross-linking agents, such as glucose, have comparable abilities to glutaraldehyde.<sup>96,97</sup> A study conducted by Santos-Rebelo et al reported glucose as the best type of crosslinker. Niknejad and Mahmoudzadeh also confirmed glucose

as a reliable type of crosslinker in the manufacture of BSA nanoparticles. This was supported by the particle size and PDI characterization results, which were not significantly different compared to those of glutaraldehyde. Physical cross-linking can be achieved in several ways, including drying, heating, or UV irradiation. Among these methods, UV irradiation has proven to be a better and more potent way to crosslink materials.<sup>98,99</sup> According to Niknejad and Mahmoudzadeh, UV exposure changed the electrical characteristics of albumin nanoparticles, with significantly different zeta potential values compared to that of the glutaraldehyde group. The results also showed that UV-crosslinked nanoparticles were more toxic than glutaraldehyde. This could be attributed to the fact that the core of aromatic residues such as tyrosine and phenylalanine contains free radicals produced by UV exposure.<sup>99</sup> Furthermore, when glucose + UV was used as the crosslinker, the resulting particle size, PDI, and zeta potential were not significantly different from those of glutaraldehyde. The addition of glucose caused an increase in the matrix strength and stability. This was attributed to UV-generated free radicals, which could speed up crosslinking with glucose by forming reactive linear glucose molecules. In other words, immediately UV radiation produced harmful free radicals, linearly cross-linked complexes were formed with free glucose molecules. Since each substance (in free form) is poisonous to cells, using UV and glucose as cross-linkers “simultaneously” may explain the reduction in cellular toxicity. Furthermore, crosslinked glucose+UV nanoparticles were found to be less harmful to cells compared to those crosslinked with glutaraldehyde. This suggests that glucose+UV is a better option for cross-linking albumin nanoparticles than glutaraldehyde.<sup>95</sup>

## Conclusion

In conclusion, the desolvation method was identified as the most frequently used method for the synthesis of BSA nanoparticles. In the synthesis process, several experimental conditions may affect the physicochemical characteristics of the formed nanoparticles. These included BSA concentration and drugs: BSA ratio, pH of BSA solution, drug concentration, solvent: non-solvent ratio, as well as type of desolvating agent, and crosslinker. The best experimental conditions from previous studies could be used as a reference in the process of synthesizing BSA nanoparticles using the desolvation method. However, other influencing parameters need to be further studied to optimize the production of nanoparticles with the best physicochemical characteristics.

## Abbreviation

AC, Acetone; AS, Ascorbic acid; BSA, Bovine serum albumin; BSA-NP: Bovine serum albumin-nanoparticles; CO (NH<sub>2</sub>)<sub>2</sub>: Carbonyl diamide; CA, Citric acid; DMSO, Dimethyl sulfoxide; EE, Encapsulation efficiency; ET, Ethanol; gp60, *Glycoprotein 60*; GLU, Glucose; GT, Glutaraldehyde; HAS, Human serum albumin; HX, Hexane; ICH, International Council for Harmonisation; IP, Isoelectric point; IP, Isopropanol; kDa, Kilodalton; OVA, Ovalbumin; NAB, Nanoparticle Albumin Bound; nm, Nanometer; pH, Potential of hydrogen; PDI, Polydispersity index; SOR, Sorbitol; SPARC: Secretory cysteine-rich acid protein; SS, Sodium sulphate; TA, Tannic acid; US, United States; UV, Ultraviolet.

## Acknowledgments

The authors are grateful to FiNder U CoE.

## Disclosure

The authors declare that there are no conflicts of interest in this work.

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