

Recent Advances in Albumin Stabilization: Impact on Therapeutic Applications

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Abstract

Albumin, the most abundant protein in plasma, stabilizes and maintains blood osmotic pressure while transporting various endogenous and exogenous ligands. To ensure therapeutic and diagnostic uses of albumin, its structural integrity must be maintained. The manufacturing, storage, and use of albumin subject it to almost every type of stress (physical, thermal, chemical, and environmental). Denaturation, oxidation, glycation, aggregation, and conformational changes are the results of these stresses and affect its biological and clinical properties. Albumin's stability depends on its helical structure, disulfide bonds, hydrophobic core, and susceptibility to post-translational modifications. Temperature, pH, ionic strength, agitation, and storage conditions affect the relative instabilities of these factors. This review presents new technologies and recent advances in stabilizing albumin. Chemically modified albumin (e.g., PEGylated, site-specific mutagenesis), exogenous stabilizers (polyols, amino acid, surfactants, fatty acids), protein cross-linking (covalent and/or radiation), albumin fusion technologies, and nanoparticles can improve stress resistance and ligand binding. Several strategies can enhance drug delivery, pharmacokinetics, and efficacy in clinical settings. Albumin markers like Δ S-Cys-Albumin can monitor biological specimen quality and cumulative damage. Spectroscopic, calorimetric, light scattering, chromatography, and electrophoresis methods are available for mechanistically characterizing and optimizing albumin formulations. Stable and longer-acting albumin-based therapeutics are expected from advances in material innovation and computational and artificial intelligence-driven protein design.

Keywords: Albumin stabilization; drug delivery systems; excipients; nanoparticles; protein aggregation

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Introduction

Albumin is a protein found in the plasma of blood in mammals and is synthesized in the liver. There are many functions for albumin in the body. Albumin is the most abundant plasma protein and constitutes about 60% of the total protein content [1]. The primary function of albumin is to maintain the colloidal osmotic pressure, which is responsible for regulating the movement of fluid between the blood vessels and surrounding tissues. Albumin helps to maintain adequate volumes of fluid within the vascular compartment, thus preventing excess fluid being retained in the tissues causing oedema, and also providing a stabilizing force on the overall blood volume. The second most important function of albumin is that it is a major transport protein; it transports many endogenous and exogenous substances including fatty acids, hormones, drugs and toxins [2]. Albumin's ability to bind to hydrophobic molecules increases the solubility and transportability of those molecules. In addition to these major functions, albumin is a powerful antioxidant, neutralizing free radicals and reducing oxidative stress. By offering this protective quality, albumin is able to protect tissues from injury by reactive molecules. As such, there are many important clinical uses for albumin. These include its use as a carrier for certain medications, for the treatment of low albumin levels (hypoalbuminemia), and as a volume expander. Due to its relatively low toxicity, its biocompatibility and biodegradability, albumin is an excellent biomaterial for biomedical applications [3]. Stabilization of albumin is especially important for its therapeutic and diagnostic use due to the fact that albumin's structural integrity dictates its physiological activity. This is important for many different applications including drug delivery systems as well as treatments for patient's with hypoalbuminemia [2,4].

Any structural change made to albumin will significantly decrease its affinity for drugs, ligands, and other bioactive materials thereby decreasing its therapeutic efficacy in the delivery and pharmacokinetic aspects of drug delivery. Since it is now known that without a native conformation, albumin cannot function as an effective antioxidant [5], it must also retain the ability to maintain the colloidal osmotic pressure that is associated with these same therapeutic implications. Understanding and controlling factors that affect the stability of albumin (including temperature, pH, and ionic strength) are therefore of critical importance [6,7]. They render the clinical use of albumin while ensuring that the results of its application will be consistent in diagnostic and therapeutic settings.

The instability of albumin during manufacturing, storage and applications brings into question its integrity and efficacy [2]. During manufacturing, albumin undergoes a number of individual, technically demanding procedures to manufacture, purify and prepare virus-inactivated; thus causing issues of structural denaturation and aggregation [8,9]. During long-term storage, albumin has potential to develop a range of stability issues, thereby requiring specialized conditions to sustain the albumins quality for extended timeframes. Furthermore, when formulated as drugs and delivered as medicines, albumin is subjected to a wide variety of potential stresses, including temperature changes, changes in the pH level and mechanical overstress. All of these potentialities could negatively impact the stability and efficacy of the albumin [6,7,10]. Because it is possible for albumin to maintain its integrity, properties, and ability to be used for therapeutic purposes during its entire life cycle, including

manufacture and use in the clinic, it is critical that strategies be developed for the stabilization of albumin. This review focuses on novel methods developed to stabilize albumin and their effects on therapeutic and diagnostic applications. It then reviews challenges that occur in keeping the structure and function of albumin stable throughout its lifecycle from the production till the clinical use. Albumin stability is of great concern as purification and heat treatment can alter the native structure of albumin. Here in this review, certain technological improvements shall also be discussed that allow boosting production, storage, and application in albumin. Improved structural integrity leads to better native shape, binding, and hence, improved clinical use. This review discusses several stabilization strategies that have been developed to promote optimal clinical efficacy of albumin. From a diagnostic and therapeutic perspective, the stability of albumin applications is paramount for quality assurance regarding diagnosis and treatment.

2. Albumin structure and key stability determinants

Amino acid composition determines structure and stability of the protein to various forms of environmental stress [11,12]. Albumin consists of approximately 585 amino acid residues and its substantial α -helical structure creates a large degree of stability in general, along with conformational flexibility of structure and stability [13]. The albumin structure is maintained by 35 cysteines which create 17 disulfide bonds, stabilizing the structure under changes to heat, pH and other methods of denaturation [6,14]. The organization of both hydrophobic and hydrophilic amino acids contributes significantly to the stability and activity of albumin and its relative capacity for binding and transporting various ligands [5,11]. For these reasons, the structure of the amino acids in albumin is integral to the functional capabilities of the protein, and therefore, the structural integrity of the protein (Figure 1).

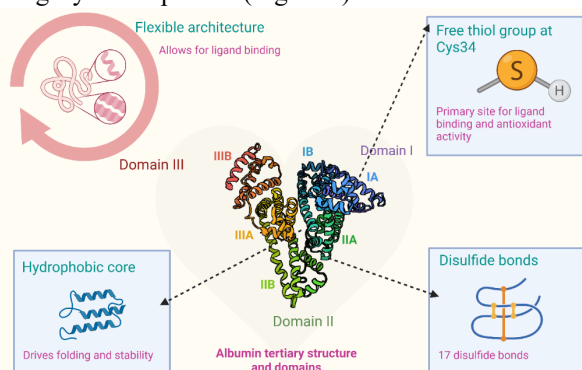


Figure 1: Albumin tertiary structure and key stability determinants. The protein is organized into three homologous domains (I, II, and III), and each domain has subdomains (A and B). The main factors that provide stability include a flexible alpha-helical structure for ligand binding, a hydrophobic core that helps in protein folding, 17 stabilizing disulfide bonds, and a free thiol group at Cys34, which acts as an important site for antioxidant activity and ligand binding.

Albumin has a considerable alpha-helical component present in the secondary structure of albumin. The majority of the structural components defining the

structure of albumin consist of alpha-helical regions located within the three homologous domains of albumin's (Domain I, Domain II, and Domain III) [15]. These alpha-helical regions provide the structural support and flexibility of albumin that enables it to maintain its conformation in a variety of environments [6,16,17]. To analyze the alteration of the albumin secondary structure (mainly alpha-helical components) as the result of their exposure to different environmental stimuli or their interactions with ligands, a variety of experimental techniques have been used, especially circular dichroism (CD) spectroscopy [13,18]. Altered content of alpha-helices within the structure of albumin will disrupt the stability of the protein and result in an inadequate ability of albumin to transport and bind ligands, making albumin ineffective as a therapeutic and diagnostic agent. Therefore, maintaining the integrity of the alpha-helical structure of albumin is critically important to sustaining the stability of albumin and inhibiting loss of functionality in the process of protecting ligands while in transport [19,20].

Both hydrophobic interactions and disulfide bonds are the major contributing factors to the maintenance of the tertiary structure of albumin, supporting the very compact globular structure of the protein and to its biological stability [11,21]. Disulfide bridges form between 34 of the 35 cysteines to create 17 different cross-links throughout the polypeptide chain, which further increase the ability of the protein to resist unfolding and denaturation when exposed to extreme environmental conditions, including high heat and high pH [6,10,15]. Hydrophobic interactions created among non-polar amino acids create a hydrophobic core inside the protein and provide additional three-dimensional stability while creating binding sites for many different ligands. Studies involving spectroscopy (spectroscopic) and molecular modeling have shown that the formation of disulfide bonds and the presence of hydrophobic interactions are critical for albumin's ability to bind and transport hydrophobic compounds, therefore affecting its overall stability and biological capabilities [11,15,22,23]. Thus, hydrophobic interactions and disulfide bonds are essential to maintaining albumin's structure and its efficacy as an effective transport protein.

Albumin is a monomeric protein and lacks a classical quaternary structure; but it may aggregate and significantly impact its stability and functionality [9,20]. Aggregation can result from several sources (e.g., heat, changes in pH, or increased levels of protein) and occurs when many albumins cluster together, producing insoluble aggregates that may change the protein's original conformation, diminish the protein's binding capacity, and reduce the therapeutic benefit of treatment [9,10,20]. It is indicated that thermal denaturation involves a conversion of albumin from an α -helix to intermolecular β -sheets, causing aggregation due to the presence of intermolecular interactions [20,24]. Stabilization of albumin in a manner that preserves its solubility, structure, and biological activity throughout the various stages of manufacturing, storage, and administration is necessary.

Both glycation and oxidation are post-translational modifications (PTMs) that may alter the structure and

function of albumin, which ultimately determines how stable and biologically active it is. Glycation occurs through non-enzymatic reaction where glucose combines with the amino groups of albumin (and this happens most often in situations of increased amounts of glucose). Glycated albumin can experience different types of structural change, unfolding and aggregation at significantly faster rates than non-glycated forms of albumin. Both will change how well albumin can bind substances, carry out transport functions and provide therapeutic benefit to patients [20,24]. Oxidative stress produces oxygen-containing radicals which can oxidatively modify amino acids in the albumin structure. Oxidation may occur at certain amino acids (e.g., cysteine, methionine) and will alter the ability of albumin as an antioxidant and the structure of albumin that provides structural stability [6,15]. Understanding and controlling glycation and oxidation of albumin is of utmost importance in order to maintain the quality of albumin and keep albumin performing consistently in both therapeutic and diagnostic settings.

3. Factors affecting albumin instability

Several environmental and chemical factors can affect the structural integrity of albumin, leading to denaturation and aggregation (Figure 2).

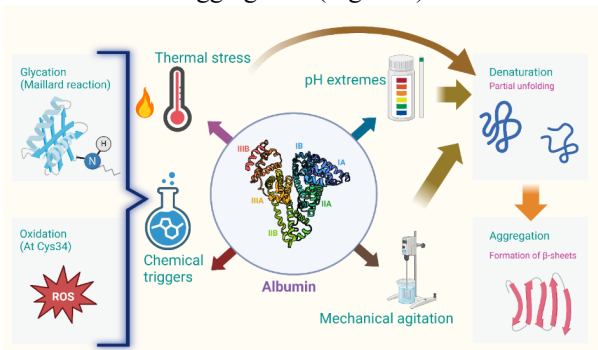


Figure 2: Pathways of albumin degradation and instability. Albumin is affected by different stresses such as thermal stress, extreme pH, and mechanical agitation. Chemical factors like oxidation at Cys34 by reactive oxygen species (ROS) and glycation through the Maillard reaction also reduce its stability. These stresses cause partial unfolding (denaturation) and lead to the formation of intermolecular aggregates rich in beta-sheet structure.

3.1. Thermal stress

Heating of albumin induce denaturation and aggregation of the protein. Increased heat causes the albumin molecule to denature; increasing temperature causes protein molecules to unfold exposing hydrophobic regions on the surface which promotes intermolecular interaction and thus aggregation between protein molecules [19,24]. Denaturation forms irreversible aggregates and therefore the protein is unable to be kept in solution and will no longer function. Evidence from differential scanning calorimetry (DSC) research demonstrates that unique thermal transitions are observed with albumin during denaturation and aggregation when heated. The level of denaturing and aggregating is dependent on a number of factors including temperature, the rate of heating, concentration of protein and presence of stabilizers, etc [9,12,15,20].

To preserve albumin stability and efficacy as a therapeutic, efforts must be made in order to minimize or eliminate the effects that heat has on albumin.

Heating modifies the structural and functional characteristics of albumin, including the secondary and tertiary structure, and its ability to bind and transport ligands. With increasing temperature, the amount of alpha-helical structure of albumin decreases, thus confirming the loss of the native form of the secondary structure of the protein [20]. The denaturation of the protein exposes the hydrophobic side chains of the amino acids causing the formation of aggregates and intermolecular beta-sheet [19,24]. Disruption of the tertiary structure occurs through the breaking of the covalent disulfide bonds and loss of hydrophobic interactions resulting in changing conformational stability within the albumin domains [11,21]. The temperature-induced changes to the structural integrity of albumin significantly decrease the ability of albumin to bind to a broad range of drugs, fatty acids, and other ligands. The knowledge of the thermal effects on albumin is essential for the development of methods to enhance the stability of albumin, so that its therapeutic and diagnostic capabilities can continue to be utilized.

3.2. Chemical degradation

Oxidation is a cause of the chemical degradation of albumin, as cysteine residues are vulnerable to oxidative damage. Albumin has 35 cysteine residues, of which 34 form disulfide bonds that enhance its structural stability, while Cys34 remains as a free thiol group. This free thiol is highly reactive and can undergo oxidation. This results in the formation of sulfenic, sulfinic, and sulfonic acids and subsequent change in the structure and function of albumin. Oxidative stress can also produce albumin dimers and higher-order aggregates via thiol/disulfide exchange reactions. Reduced binding and transport capacity of ligands due to oxidative damage to albumin may decrease its therapeutic effectiveness as well as its ability to act as a free radical scavenger. Minimizing oxidative modifications of Cys34 and other amino acid residues on albumin is a method for maintaining albumin's structural integrity and biological activity [6,10,15]. Glycation, as a result of non-enzymatic glycosylation, via the Maillard reaction, represents a significant modification of albumin resulting in decreased stability and increased propensity to form aggregates. The Maillard reaction is the result of the direct binding of a reducing sugar (e.g., glucose) to an amino group, and it occurs through the formation of advanced glycation end products (AGEs). Glycated proteins undergo subsequent changes in conformation and may undergo intermolecular cross-linking, resulting in accelerated aggregation. Glycated form of albumin may also lose its ability to bind and transport ligands due to structural disruption and, in some cases, form amyloid-like structures. Thus, it is critical that all procedures used to store and process albumin minimize the formation of the Maillard product via the use of glycation inhibitors and other approaches [6,24].

3.3. pH and ionic strength

The pH of a solution has a direct impact on the conformation of albumin and, therefore, on its structure/stability. At different pHs, albumin can adopt many unique conformational states. An acidic pH will

cause the formation of an expanded (partially unfolded) albumin molecule due to the acidification of the protein structure. However, an alkaline pH will cause the protein to adopt a more flexible, basic configuration [6,9]. The conformation of albumin at an alkaline or acidic pH will result in the alteration of secondary and tertiary structures of the protein molecule, resulting in a loss of alpha-helical content, altered domain interactions, and a less compact molecular structure. The extremes of the pH range will denature and/or aggregate the protein and will compromise the ability of albumin to solubilize and function biologically [6,9,22]. Maintaining an optimum pH is therefore required for the preservation of albumin's native conformation and functional properties.

Salt concentration and ionic strength affect protein hydration and electrostatic interactions. This in turn influences the stability of albumin. Albumin association can take place when the masking of the charged residues on the protein surface is masked. This occurs due to an increase in ionic strength and a lowering of electrostatic repulsion. Whether high salt levels stabilize or destabilize albumin depends on the type of salt and how it interacts with the protein. Some salts may cause salting-out effects resulting in aggregation and precipitation, and some others stabilize albumin by strengthening hydrophobic interactions. Strong resistance to variations in ionic strength in some formulations has been shown by albumin nanoparticles showing stability up to a 2M salt concentration. Maintaining the structural integrity of albumin and preventing aggregation thus depend on careful control of salt concentration and ionic strength [6,10].

3.4. Mechanical stress

Albumin aggregation and destabilization can be brought on by agitation or related mechanical stress. Its monomeric form may thus change as well as its soluble and insoluble aggregates change. Development of air-liquid interfaces and higher shear forces during agitation leads to aggregation by promotion of protein unfolding and intermolecular interactions. The degree of aggregation depends on factors including protein concentration, speed and duration of agitation, and the presence of stabilizers. Surfactants such as Tween 80 can prevent aggregation caused by agitation, although they may have either negligible or even negative effects under some circumstances, such as during heat treatment or storage free from agitation. Cysteine residue modification, such as Cys34, increases stability against mechanical loads [10]. Maintaining albumin's stability in liquid formulations requires control of agitation parameters and use of stabilizers.

3.5. Storage conditions

Extended storage influences the integrity of albumin. This results in different degradation processes that influence its structure and performance. Albumin will change in biophysical characteristics during storage by glycation, oxidation, and aggregation that build over time. Even short exposure to thawed conditions can start *ex vivo* biochemical reactions and damage the normal condition of the protein. Keeping albumin at temperatures of -80°C or below is needed to minimize these changes; freezer failures or shipment delays still carry a risk of degradation. Albumin integrity

monitoring during prolonged storage to guarantee its efficacy for therapeutic and research uses [10,25].

Although lyophilization (freeze-drying) with reconstitution is a common form of long-term storage enhancement, the process of freeze-drying and subsequent reconstitution introduces stresses to albumin that can negatively affect its overall stability. When freeze-drying, proteins need to first be frozen before the ice can be sublimed under vacuum; if care is not taken during these steps, denaturation or aggregation of protein molecules may occur. Therefore, it is critical to select the appropriate cryoprotectant (e.g., sugars such as trehalose or sucrose, and/or polyols such as mannitol) to help stabilize the structure of albumin during both the freezing and drying processes. The reconstitution process, which involves typically utilizing distilled water to rehydrate the lyophilized protein, also creates stress to the protein, especially if care is not taken when performing this process, e.g., reconstitution time, the temperature of the reconstituted liquid, and/or the use of protective agents to curtail the damaging effects of time or temperature. Optimizing both the freeze-drying and reconstitution processes is vital in order to maintain the structural integrity and function of albumin throughout the entire procedural cycle [26,27].

4. Recent advances in albumin stabilization strategies

4.1. Chemical modification

Site-directed mutagenesis is another technique that may be used for the stabilization of proteins by targeting specific amino acids in the protein. The purpose of site-directed mutagenesis is to generate improved proteins through specific alterations to the sequence of the gene to create improved versions of a specific protein with enhanced properties (e.g., higher thermal and/or chemical stability). A single residue substitution could result in a change of protein stability, thus by identifying important amino acids that contribute to instability, stable protein variants can be designed [12,21]. Several studies have investigated the stability of single and double residue mutants of human serum albumin (HSA) in order to ascertain how individual amino acid substitutions affect protein stability [28]. Different amino acid sequences in domain I allow dog albumin to show more resistance to GdnHCl-induced denaturation than HSA. This indicates that some amino acid residues are involved in the stabilization of the protein [29]. The absence of the free sulfhydryl group in the HSA component of the human interferon- $\alpha 2\text{b}$ and HSA fusion protein improves the stability of the protein in mechanical and thermal stresses [10]. By means of the targeted modification of the amino acid composition of the protein, site-directed mutagenesis is a successful approach of generating more stable functional proteins. PEGylation is the covalent binding of polyethylene glycol (PEG) chains to a protein, enhancing its stability and pharmacokinetic properties [30]. This alters the hydrodynamic size of the protein; reduces renal clearance and extends its circulation time in the bloodstream. Furthermore, lowering the immunogenicity of the protein and shielding it from proteolytic breakdown will help to increase the protein's overall therapeutic efficacy [31]. Through changes in size, charge, and surface chemistry, the attached proteins can affect the biodistribution and interaction of

therapeutics with biological entities including membrane receptors and enzymes [4,32]. Preformed ionic complexes with proteins can be formed. PEG-poly(histidine) diblock copolymer is an example that improves their stability and regulates their release rates [33]. PEGylation is thus a useful method for enhancing the pharmacological qualities of proteins, so making them more stable, longer-lasting, and less immunogenic.

4.2. Use of stabilizing excipients

Commonly used as excipients to stabilize proteins during freeze-drying and storage are polyols including mannitol and trehalose [34]. A high glass transition temperature distinguishes trehalose, a non-reducing disaccharide, in helping to preserve the amorphous state of the protein, so preventing crystallization and preserving structural integrity [35]. Effective stabilizers against recombinant human serum albumin's (rHSA) aggregation have been identified as both sucrose and trehalose [36]. Often used as a bulking agent, mannitol gives the lyophilized product mechanical strength and helps prevent collapse during the drying process. These polyols shield proteins from stresses including thermal denaturation and dehydration brought on by freeze-drying [27]. For freeze-dried HI-6-loaded rHSA nanoparticles, formulations including trehalose and sucrose showed better physicochemical characteristics and longer storage stability [26]. Moreover, trehalose has been demonstrated to improve the binding capacity of albumin-based nanoparticles by preserving their structure and functionality [37]. Thus, polyols are essential in preserving their natural conformation and stopping aggregation during freeze-drying and storage, so stabilizing proteins. Amino acids can improve proteins' thermal stability and gel strength. They can change the thermal stability of proteins in a non-specific fashion. Zwitterionic amino acids can increase the strength of junction zones as well as protein's participation in the gel network. Studies on bovine serum albumin (BSA) have found that some amino acid additions can raise the thermal denaturation temperature (Td) and boost gel strength. Amino acids affected Td elevation and gel strength in a predictable sequence. The net rise in Td of BSA was linearly correlated with the net increase in gel strength, suggesting that in both cases the fundamental molecular mechanism may be the same. Amino acids instead of polyols and sugars can help to improve the rheological qualities of protein gels. Thus, amino acids can be used to improve protein stability and gelation qualities, so making them useful excipients in many different uses [38–40].

Under mechanical stress, surfactants such as Tween 80 help to reduce protein aggregation. Induced by motions like shaking or stirring, mechanical stress can cause protein aggregation. By lowering surface tension and discouraging hydrophobic interactions that cause aggregation, surfactants help to stabilize proteins [10]. Tween 80 has been shown to prevent denaturation and aggregation linked with emulsification, so shielding BSA from aggregation. Consequently, surfactants are essential excipients for maintaining protein stability in formulations under mechanical stress [2,6,33]. Caprylate (octanoate, CA), among fatty acids, offers a stabilizing action against protein denaturation caused by heat. Intravenous human albumin products use caprylate and

acetyl-tryptophanate (AT) as stabilizers to reduce protein changes during heating at 60 °C for 10 hours, a process meant to inactivate hepatitis viruses. Albumin shows more thermal stability in a chloride medium. The stabilizing action of endogenous fatty acids is clearly visible even in the presence of 145 mM sodium chloride and 4 mM CA + 4 mM AT or 4 mM CA. Additionally capable of binding to the main medium-chain fatty acid binding site are these fatty acids. Thus, fatty acids such as caprylate can increase the thermal stability of albumin formulations, so guaranteeing their integrity during heat treatment [8,9].

4.3. Crosslinking techniques

Novel linkers used for conjugation of HSA to magnetic nanoparticles (MNPs) are glutardialdehyde (GDA) and cyclohexyl-diglycidyl ether (CDGE). Often used as a covalent cross-linker, GDA is well-known for forming Schiff bases from free amino groups of amino acids—especially lysine. Many high-performance liquid chromatography (HPLC) columns immobilize HSA onto a solid phase by using GDA. Covalent protein attachment involving lysine, serine, or cysteine side chains also favors epoxy linkers including epichlorohydrin. In a reported work, aminopropyltrimethoxysilane was used to modify core-shell-structured MNPs (Fe₃O₄ core with a SiO₂ shell) so introducing a primary amine group on the particles. These particles were subsequently reacted with either bisepoxide (CDGE) or GDA to produce active linkers capable of forming covalent bonds between the particle and HSA. Although the esterase-like enzymatic activity of HSA was much lowered, the HSA-MNP-CDGE complexes displayed a higher immobilization yield and retained API binding capability. Five different HSAs' covalent attachment was assessed using MNP-GDA and MNP-CDGE. GDA and CDGE are thus efficient linkers for albumin immobilization; CDGE shows better immobilizing yield and API binding retention [41].

Gamma irradiation is a method used to create stable nanoparticles by cross-linking albumin. When solutions of albumin are exposed to gamma radiation, covalent bonding occurs between albumin molecules, leading to nanoparticles that are stable. One of the major advantages of gamma irradiation is that it does not require any additional chemicals (cross-linkers). Therefore, the production of the nanoparticles using gamma irradiation is both clean and reproducible. Gamma-irradiated albumin nanoparticles are stable across a range of experimental conditions: pH, ionic strength, and denaturing agents like urea. When subjected to highly acidic conditions, BSA nanoparticles were found to denature similar to molecular BSA. When in a more alkaline environment, some denatured nanoparticles displayed the same type of denaturation that is typical of the molten-globule state. In addition to being used for drug delivery, gamma-irradiated albumin nanoparticles have also shown promise in terms of controlled release of therapeutic agents due to their pH responsiveness, which is similar to the pH of many tumors. This makes gamma irradiation an effective strategy for generating stable albumin nanoparticles for drug delivery [6,42,43]. In vitro studies show that these unloaded nanoparticles are highly biocompatible. At the same time, they are very effective in delivering

anticancer drugs such as emodin to induce tumor cell death [42].

4.4. Fusion proteins

Albumin fusion technology has gained popularity as a method for generating long-acting protein therapeutics, as it can improve the pharmacokinetic characteristics of these proteins. The process entails genetic fusion of the therapeutic protein with HSA in order to exploit the HSA's prolonged half-life in the circulation and increase the duration of the therapeutic effects of the drug [4,10]. Albglutide is a recombinant peptide with an albumin fusion that was approved for type 2 diabetes therapy and demonstrates the successful use of albumin fusion technology [2]. Similarly, interferon- α 2b-HSA is a long-acting form of interferon- α 2b used in hepatitis C treatment. This albumin fusion technology provides stability to the recombinant protein, decreased clearance through the kidneys, and increased accumulation in targeted areas (i.e., tumors). Structurally optimizing the fusion protein orientation was demonstrated to minimize the probability of disrupting the interferon- α 2b and HSA intermolecular structural interface and restore the normal disulfide bond formation of interferon- α 2b. Removing the free thiol group from the HSA component can further improve the stability of the fusion protein to mechanical and thermal stress, thus increasing the production yield and simplifying the formulation of these proteins. Therefore, albumin fusion technology not only helps to increase the half-life of therapeutic proteins, but also improves their stability and efficacy [2,4,10].

Pichia pastoris, *Saccharomyces cerevisiae*, or *Oryza sativa* are used to produce rHSA, which is an alternative to blood-derived HSA and lowers the possibility of spreading blood-borne infections. *Pichia pastoris* produces an rHSA solution that is sold under the brand name Medway in Japan to treat hemorrhagic shock and hypoalbuminemia. In terms of quality, low immunogenicity, and possibility of large-scale manufacturing, rHSA outperforms albumin derived from plasma. Through their prevention of rHSA aggregation during lyophilization and storage, sucrose and trehalose stabilize pharmaceutical formulations. In formulations including fusion proteins and nanoparticles, rHSA can function as a drug delivery carrier. It stabilized paclitaxel nanoparticles used with sodium octanoate and N-acetyl-L-tryptophanate. By ligand binding, domain-specific mutations, or co-lyophilization with surfactants, one can raise the stability of HSA fusion proteins. Apart from being a therapeutic agent, rHSA enhances the stability and efficiency of new pharmacological formulations [2,44–46].

4.5. Nanoparticle formulation

The use of albumin nanoparticles as drug delivery systems developed due to their ability to increase the stability of drugs and to provide greater bioavailability while having biocompatibility and biodegradability. Albumin nanoparticles can increase drug solubility, protect drugs from degradation prior to reaching the target site, and promote targeted delivery to specific cells or tissues. BSA nanoparticles have increased the bioavailability and efficacy of curcumin [47]. Similarly, glycosylated BSA can improve the solubility, stability, and emulsification characteristics of BSA nanoparticles.

Thus, more effective drug carriers are possible with such modifications [48]. Furthermore, albumin can be combined with other biopolymers and metal ions to form highly protective composite matrices. For example, when sensitive *Monascus* yellow pigments are encapsulated within a quercetin–Sn(II)–albumin–chitosan nanocomplex, their resistance to thermal and photodegradation increases significantly. This helps to maintain the cargo integrity much better than in unencapsulated form [49]. Meanwhile, albumin nanoparticles improve the stability of oligonucleotides and promote nuclear accumulation of certain oligonucleotides [50]. The conjugation of lipid components to nucleic acid therapeutics has increased their ability to induce cellular uptake by enhancing the interaction of the nucleic acid therapeutic with the cell membrane [4]. Albumin nanoparticles are stable under various physiological conditions (i.e., varying pH levels and ionic strengths), suggesting that they may be optimal drug delivery systems for both oral and intravenous administration.

The preparation methods of albumin-based nanoparticles have both advantages and uses [51]. The desolvation method involves dehydrating albumin using solvents like acetone or ethanol to cause the albumin to aggregate. Crosslinking is performed to stabilize the formed nanoparticles [52]. Crosslinking is done using chemical agents like glutaraldehyde or physical techniques like gamma irradiation [53]. Emulsification is another method and involves homogenizing an albumin emulsion in an oil phase and using heat to cause crosslinking [54]. Albumin nanoparticles are also made by the coacervation process followed by glutaraldehyde cross-linking [50]. The method of preparation of albumin nanoparticles affects their stability, drug loading efficiency, and the intended particle size. Desolvation with gamma irradiation is a reproducible method for BSA nanoparticles [6]. Freeze-drying is commonly used to improve the long-term storage stability of albumin nanoparticles. Cryoprotectants like trehalose and sucrose are used during this process to prevent aggregation [26].

Various strategies, including ligand binding, recombinant engineering, and the use of specific excipients, have been developed to improve the stability and functional performance of albumin-based systems (Table 1).

Table 1: Summary of albumin stabilization approaches, mechanisms, and therapeutic outcomes

Stabilization approach	Mechanism/Description	Outcomes	[Ref.]
Ligand-based stabilization	Ligands bind albumin and enhance stability via conformational stabilization	Sodium octanoate improves thermal stability; N acetyl L tryptophan improves	[55]

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		antioxidant activity; N acetyl L methionine enhances antioxidant and anti-photooxidation effects	
Chemical crosslinking of albumin nanoparticles	Aldehyde or carbodiimide crosslinkers create stable nanoparticle matrices	Glutaraldehyde most common but has toxicity limits; alternatives include carbodiimide derivatives and glucose with UV	[56]
Polymer coating of albumin nanoparticles	Polymers form protective shells preventing aggregation & improving circulation	PEG, PVA, and Gantrez coatings reduce aggregation and improve immune stealth	[56]
Alternative nanoparticle stabilization strategies	Avoiding toxic crosslinkers through novel chemistries	Eudragit S100 used as a stabilizing/crosslinking agent; thermal stabilization avoids chemical agents	[57]
Thermal and ethanol induced stabilization	Controlled gelation and denaturation–renaturation	Development of HSA hydrogels via combined thermal and ethanol approach with tunable properties and good structural stability	[58]
Excipient based stabilization (e.g.,	Sugars create a vitrified matrix to stabilize proteins	HSA and lactose strongly stabilizes biologics	[59]

lactose, sugars)		during drying and storage; prevents recrystallization at high RH	
Recombinant albumin engineering for enhanced stability	Algorithms introduce stabilizing mutations	PROSS-designed albumins with up to 40 °C higher thermal stability; maintain ligand binding	[60]
Self-assembly / reassembly driven stabilization	HSA fragments reorganize to encapsulate nanomaterials	Cleaved HSA drives formation of stable Se-containing nanoparticles, improving dispersibility and long-term stability	[61]
Surfactant-assisted stabilization	Controlled surfactant presence prevents aggregation during processing	Use of polysorbate 80 with HSA preserves monomer content during gentle drying	[62]

Recent advances have introduced a variety of modular and engineering-based approaches to improve the therapeutic profile and stability of albumin formulations (Figure 5).

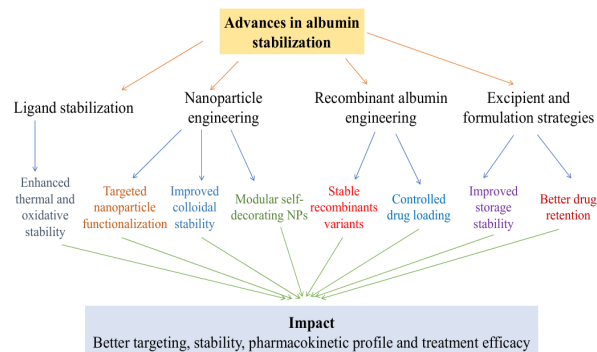


Figure 5: Overview of recent advances in albumin stabilization and their therapeutic impact.

5. Impact on therapeutic applications

5.1. Improved drug delivery

Improving the stability of drug-albumin complexes will increase their circulation lifetime and reduce side effects [63]. Albumin is essential for the movement of different drugs and compounds all around the body [64]. It can raise the half-life of drugs in the bloodstream by forming

complexes with them and prolong therapeutic effects [65]. Phloretin and HSA interact to produce a protein structure remarkably more resistant to oxidation and fibrillation [22]. Their bioavailability can be improved by stable albumin complexes that can stop drugs from being rapidly eliminated by the kidneys or their metabolism in the liver. By making sure the drug is mostly delivered to the intended site of action instead of being absorbed by healthy tissues, stabilizing drug-albumin complexes can also help to lower off-target effects. Chemically modified oligonucleotides with high-affinity binding to albumin have an unusually long half-life in serum [4]. Improving the stability of drug-albumin complexes is an approach for enhancing therapeutic results and lowering side effects. Targeted drug delivery can benefit from albumin's natural tendency to accumulate in tumor tissues. Albumin accumulates in solid tumor tissues due to increased endothelial transcytosis and the enhanced permeability and retention (EPR) effect [66]. This accumulation helps drugs especially targeted for cancer cells to be delivered more easily, so sparing healthy tissues from the harmful effects of chemotherapy. siRNAs designed to bind albumin, for instance, have been shown to gather in tumors and maintain gene silencing in mouse models [4,67]. HI-6-loaded albumin nanoparticles have been developed to improve transport across the blood-brain barrier (BBB) and reactivate organophosphate-(OP)-inactivated acetylcholinesterase (AChE) in an in vitro BBB model in cases of severe organophosphate intoxication [26,68]. Moreover, albumin nanoparticles can be made to release their drug payload, especially inside the tumor microenvironment, so improving the treatment accuracy. Tight-affinity produces reduced non-specific uptake while greatly increasing stability [4]. Therefore, albumin's natural targeting capacity makes it a perfect carrier for delivering drugs directly to tumor tissues, so improving therapeutic efficacy and lowering systemic toxicity.

5.2. Enhanced pharmacokinetics

Technologies for albumin binding and fusion provide efficient means to extend the half-life of medicinal drugs. Conjugating drugs to albumin increases the length of drug circulation, promoting less frequent dosage and continuous therapeutic effects. As the most plentiful protein in serum, albumin carries both synthetic and natural compounds [69,70]. Conjugation of ligand D1 to DNA strands allows strong and specific binding to albumin. Cys34 has no bearing on the extended half-life of albumin or albumin fusion proteins. Rather, this extension is ascribed to the naturally long half-life of albumin in the body, which protects the drug from quick degradation or elimination upon albumin binding. For example, albumin-binding oligonucleotides show clearly longer half-lives in serum, improving their therapeutic value. Moreover, albumin fusion technology helps to create protein drugs. The fusion protein produced by genetically fusing therapeutic proteins or peptides to albumin gains from albumin's longer half-life and favorable pharmacokinetic characteristics [4,10]. Thus, two essential instruments for maximizing drug pharmacokinetics are albumin binding and fusion technologies, which result in better efficacy and patient adherence.

By means of albumin-based approaches, improving drug release and distribution can greatly increase bioavailability. Serum protein binding results after attachment of lipid molecules to oligonucleotides, thereby changing the biodistribution profile. Strong affinity greatly improves stability but results in lower non-specific uptake. Reducing immune activation, enhancing stability, and controlling biodistribution all depend on controlling protein binding[4]. By means of engineering to release their drug payload, especially within the tumor microenvironment, albumin nanoparticles can improve treatment accuracy. Prodrugs can be made to bind to a different site on HSA than the parent drug, for instance, avoiding competition for HSA binding and improving the biopharmaceutical profile of the parent drug [23]. This focused approach guarantees the drug reaches the site of action, improving its therapeutic action and reducing systematic exposure. Maximizing drug bioavailability and therapeutic efficacy thus depends on optimizing drug release and distribution by means of albumin's special characteristics.

5.3. Clinical efficacy

Therapeutic results in many different clinical environments have been shown to be much influenced by stabilized albumin formulations. In an in vitro blood-brain barrier (BBB) model, researchers have created HI-6-loaded albumin nanoparticles to improve transport across the blood-brain barrier, so reactivating organophosphate-(OP)-inactivated acetylcholinesterase (AChE) [26]. Stabilized albumin formulations have great potential in cancer treatment, as shown by an analysis of albumin-binding small interfering RNAs (siRNAs) revealing tumor accumulation and extended gene silencing in mouse models. Moreover, it was found that although significantly increasing stability, stronger affinity causes lower non-specific absorption [4]. Achieving desired therapeutic effects and enhancing patient outcomes thus depend on stable albumin formulations. Treatment of hypoalbuminemia and hemorrhagic shock frequently calls for albumin. Managing hypovolemia and diseases including chronic renal failure and liver cirrhosis uses HSA [3]. The major protein in blood plasma, albumin, is essential for controlling colloidal osmotic pressure [71], thereby preserving fluid balance in the tissues and bloodstream. Albumin infusions can restore oncotic pressure and reduce related symptoms in patients with hypoalbuminemia, i.e., where albumin levels are low [71,72]. In cases of hemorrhagic shock, too, albumin can be given to boost hemodynamic stability and raise blood volume [3]. Therefore, the management of disorders marked by low albumin levels and blood volume shortages depends critically on the administration of albumin, which helps to enhance patient outcomes.

6. Diagnostic potential

Assessing the exposure of blood plasma and serum (P/S) to thawed conditions uses Δ S-Cys-Albumin as an intrinsic marker. It calculates P/S's exposure to temperatures above their freezing point of -30°C quantitatively. This is achieved using a 10- μl , dilute-and-shoot, intact-protein LC/MS-based assay looking at albumin proteoform relative abundances. The assay is based on the idea that, when samples are heated above

–30 °C, the relative abundance of S-cysteinylation (oxidized) albumin constantly increases and reaches a maximum value less than 100%. Δ S-Cys-Albumin is the variation in relative abundance of S-cysteinylation albumin (S-Cys-Albumin) before and after a controlled incubation that raises this proteoform to its peak level. Because of its well-known formation mechanism, this marker is special; a multi-reaction rate law controls albumin S-cysteinylation at 23°C, so enabling the estimate of exposure times for unknown samples. Δ S-Cys-Albumin is zero in totally expired samples [25]. Thus, Δ S-Cys-Albumin is a necessary marker for tracking and measuring the total exposure of P/S samples to thawed conditions, guaranteeing the integrity of biospecimens used in clinical environments and research projects.

In biomarker development, Δ S-Cys-Albumin is useful for evaluating P/S sample quality before proteomic study. Before starting a proteomic study, laboratories can assess P/S sample quality using Δ S-Cys-Albumin values in unidentified samples. It offers empirical molecular data, more precisely recording the integrity of archived P/S samples than paper traces. Reductions in plasma Δ S-Cys-Albumin below the range seen in pristine samples can act as a surrogate indicator to project the proportion of immunoassay-measured proteins that have become unstable in compromised specimens. Direct empirical link between clinically important proteins and Δ S-Cys-Albumin was found by concurrent measurements of Δ S-Cys-Albumin and the concentrations of 21 proteins of interest to (pre)clinical research [25,73,74]. Thus, Δ S-Cys-Albumin can be used as an estimator of cumulative damage to clinically important proteins present in plasma as well as as a marker of cumulative exposure to thawed-state conditions.

7. Methods for assessing albumin stability

7.1. Spectroscopic techniques

Circular dichroism (CD) spectroscopy is a used technique for evaluating changes in the secondary structure of proteins. This method is suitable for monitoring protein conformational alterations and analyzing their secondary structure. Far-UV CD spectra (190 to 260 nm) are sensitive to protein structures and play a crucial role in detecting modifications in protein secondary structures [75,76]. BSA exhibits two principal negative bands characteristic of its α -helix structure at approximately 208 nm (π - π^*) and 220 nm (n - π^*). The intensity and shape of these bands provide ideas into changes in the α -helix, β -sheet, β -turn, and random coil structures of albumin. The introduction of quercetin (Que) and myricetin (Myc) results in a decrease in the intensity of these negative peaks without significant alterations in their shape. Molecular dynamics simulations show that these polyphenols bind strongly to specific pockets in the BSA structure. This binding is stabilised by complex networks of hydrogen bonds and hydrophobic interactions. The α -helix content of BSA slightly decreased from 79.39% to 78.13% in the presence of Que. In contrast, the addition of Myc resulted in only minor intensity variations, with a similar slight change in α -helix content from 79.39 to 79.76% [18]. Software such as DichroWeb or CDNN is employed to calculate secondary structure compositions

from the spectra [5,15,75]. Near-UV CD (260–320 nm) is also utilized to investigate the asymmetry in a protein's aromatic amino acid environment and to evaluate the extent of conformational changes [76,77].

Examining polyphenol-protein interactions and structural changes is made very easy with fluorescence spectroscopy. The main endogenous protein components showing fluorescence excitation are tyrosine (Tyr) and tryptophan (Trp). Many times, interactions between protein-like macromolecules and small molecules are evaluated using changes in the microenvironmental polarity of certain amino acids [78]. For instance, the binding of phloretin to HSA induces changes in the environment surrounding Trp214[22]. Two proanthocyanidins (F1 and F2) with varying degrees of polymerization were obtained from Chinese quince fruits in a work. These were combined with BSA. The characteristic absorption peak of BSA was observed around 340 nm, where F1 (43.16–92.78 a.u.) and F2 (66.76–110.07 a.u.) displayed weak fluorescence intensity at various concentrations, significantly lower than BSA's intensity (649.72 a.u.) at 343 nm. As the concentrations of F1 and F2 increased, blue shifts in the λ_{max} of BSA were noted (F1-BSA from 343 nm to 338 nm, 345 nm to 338 nm, and 341 nm to 334 nm at 298 K, 303 K, and 310 K, respectively; F2-BSA from 343 nm to 340 nm, 345 nm to 341 nm, and 341 nm to 339 nm at 298 K, 303 K, and 310 K, respectively). These observations suggest that the polarity of the binding cavity near the Trp residue on BSA was altered. This resulted in increased hydrophobicity and changes in the secondary structure due to BSA's interactions with F1 and F2 [78].

Ultraviolet-visible (UV-Vis) spectroscopy can identify protein-ligand interactions. Both the UV-Vis absorption spectrum and fluorescence spectra help study the interaction between BSA and GBSA with kaempferol (Kae) [48]. Changes in absorption peaks or absorbance intensities are suggestive of structural modifications in albumin. This analysis provides an understanding of the effect of external factors on protein structure. Infrared (IR) spectroscopy, particularly Fourier Transform Infrared (FTIR) spectroscopy, is a good method for detecting changes in protein functional groups. BSA is characterized by three distinct absorption peaks that are sensitive to alterations in its secondary structure. They are 3340–3300 cm^{-1} (O-H stretching), 1700–1600 cm^{-1} (amide I band), and 1600–1500 cm^{-1} . The amide I band (1700–1600 cm^{-1}) is effective for studying protein secondary structure [18,48]. FTIR spectroscopy can reveal changes in albumin's secondary structure during thermal unfolding. A decrease in α -helical structure and the formation of intermolecular β -sheet structures at elevated temperatures have been identified using FTIR [20]. FTIR can also assess the effects of complexes on protein secondary structure [18,48]. The addition of F1 and F2 to BSA produces different spectral data. The intensity of the spectral peaks of the amide I and amide II bonds decreased after the complexation of F1 and F2 with BSA [78].

7.2. Calorimetry

Protein thermal stability and denaturation temperatures are evaluated using differential scanning calorimetry (DSC). It fits for investigating protein thermal stability

in a solution. It measures the heat flow associated with thermal transitions in a protein as temperature varies [79]. Characterization can be accomplished with reference to the thermal denaturation temperature (T_d). DSC studies evaluate protein stability. This is accomplished using the fundamental thermodynamic parameters enthalpy change (ΔH) and the transition temperature (T_m). DSC can investigate, with thermograms of excess heat capacity (C_p), the in vitro unfolding of proteins as a function of temperature [15]. Thermal denaturation of HSA in the presence and absence of fatty acids has been investigated using DSC. Variations in thermal unfolding are found depending on fatty acid content and sample quality [9]. Moreover, DSC can assess albumin's thermal stability under the effect of ligands such as ibuprofen [20]. This method offers understanding of the hydration behavior changes of polar and non-polar amino acids during unfolding based on thermodynamic analysis. Furthermore, given are conformational entropy and changes in internal interactions (including van der Waals forces and hydrogen bonding) [15]. DSC can detect multiple transition peaks of separate energetic domains within the protein that unfold in succession. The resultant data can be analyzed by models such as the two-state model or non-two-state models to comprehend the unfolding process [9,20]. This approach can investigate protein thermal stability under various additive conditions, including ionic liquids derived from amino acids. This method can help to find substances enhancing protein stability [15].

7.3. Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) is a technique to determine the size and polydispersity of particles. It can be used for aqueous dispersions of protein molecules like albumin and is effective for investigating protein aggregation. DLS measures the intensity of light scattered by particles as they undergo random motion and calculating of their size distribution and average size. The hydrodynamic diameter (DH) for BSA and HSA was determined in a study using DLS. The apparatus employed a 4 mW He/Ne laser as a light source, emitting vertically polarized light at a 90° scattering angle. DLS detects the Brownian motion of sample particles and correlates it to their size. The Stokes–Einstein equation was applied to establish a relationship between particle size and the velocity resulting from Brownian motion [15]. DLS can be used to evaluate particle size, polydispersity index (PDI), and zeta potential. DLS was employed to assess the size distribution of BSA nanoparticle (NP), BSA-Que NP, and BSA-Myc NP. The change in size was indicative of the incorporation of Que and Myc [18]. A high absolute potential indicates strong electrostatic repulsion between molecules and a stable solution. PDI measures the range of particle sizes in a sample with lower values identifying a more uniform population [80,81]. The PDI values for BSA-Que NP and BSA-Myc NP were 0.79 ± 0.21 and 0.32 ± 0.01 respectively. A lower value suggested an evenly dispersed population for BSA-Myc NP [18]. Stability of albumin formulations can be assessed by tracking changes in particle size and PDI through DLS. Such stability studies can be carried out under different conditions such as changes in pH,

temperature, or ionic strength. A recent study compared DLS and Tunable Resistive Pulse Sensing (TRPS) for particle sizing in three distinct vaccine formulations: a protein antigen, an outer membrane vesicle, and viral particles. Higher resolution for polydisperse and large particle samples was found by TRPS complementing DLS. Although both methods are successful their choice should rely on the type of formulation and the size range of interest [82].

Zeta potential measurements are crucial for assessing the stability of albumin and its formulations. This potential represents the surface charge of particles within a colloidal system and indicates the degree of repulsion between adjacent particles with similar charges. Typically, a high absolute zeta potential denotes strong electrostatic repulsion among molecules, thereby contributing to a stable solution system [83–85]. The stability of BSA-Liposomes is determined by their zeta potential. When the pH surpasses the isoelectric point of BSA ($pI \approx 4.6$), negatively charged groups such as carboxyl in proteins dissociate more than positively charged groups like amino, leading to an increased negative charge. The incorporation of liposomes introduces numerous negatively charged phosphate groups, further augmenting the system's negative charge [19]. Instruments such as the Zetasizer Nano ZSE can measure the zeta potential. For example, the zeta potentials of BSA-Que NP and BSA-Myc NP were -46.28 ± 15.57 and -36.06 ± 18.2 mV, respectively, indicating good physical stability [18]. Similarly, the zeta-potential values for BSA-Kae NPs and GBSA-Kae NPs were -17.6 mV and -21.9 mV, respectively, with the latter's higher absolute value suggesting greater stability for GBSA-Kae NPs [48]. By monitoring the zeta potential, one can predict the long-term stability of albumin formulations, as higher absolute values generally signify enhanced stability due to increased electrostatic repulsion.

7.4. Chromatographic techniques

Aggregation and fragmentation of albumin can be identified by size-exclusion chromatography (SEC). The method separates molecules according to their hydrodynamic volume during their pass through a column containing a porous matrix. Larger molecules that cannot pass through the pores elute fast. Meanwhile, smaller molecules that can pass through the pores follow a longer trajectory and elute later [86–88]. With the ability of SEC to check the size distribution of aggregated albumin, it has been utilized to examine metHb-albumin clusters in both solution and freeze-dried powder formulations [89]. It can estimate monomeric protein concentrations and soluble aggregates by checking UV absorbance at 280 nm [10,90]. The data from SEC indicates whether the protein is primarily monomeric or if aggregates or fragments exist. A comparison of the elution profiles of albumin samples under varying conditions (temperature, pH, or stabilizing agents) will facilitate SEC to elucidate the factors affecting albumin's aggregation and degradation [89].

Drug loading can be estimated using High-performance liquid chromatography (HPLC). The drug loading of nanoparticles is evaluated indirectly by determining the quantity of unbound drug via HPLC [26]. Protein

samples for HPLC analysis are prepared using a solid phase extraction (SPE) technique [23]. The mobile phase, flow rate, and detection wavelength can be adjusted as needed. An analytical column (25 cm × 2 mm i.d., 25 µm), Lichrospher 100 RP-18, was utilized in a reported study [17]. The reaction products can be characterized through ESI-MS analysis [78]. HPLC can provide details of the degree of polymerization and the stability of albumin by analyzing elution profiles and quantifying different species [78,91,92].

7.5. Electrophoresis

Native-Polyacrylamide Gel Electrophoresis (Native-PAGE), a technique for separating proteins under native-state conditions, provides information on protein charge, size, shape, hydrodynamic volume, and so forth [93]. In this technique, proteins are moved through a polyacrylamide gel under the influence of an electric field at a steady-state condition. Since proteins are maintained in their native conformations, Native-PAGE allows researchers to study the formation of protein complexes and the oligomeric state of proteins without altering such structures [89,94]. Proteins that have been loaded onto a polyacrylamide gel will migrate based on their native charge and hydrodynamic volume. The larger a protein aggregate is, the larger its effective size, and therefore, it moves slower than smaller aggregates. Therefore, distinct bands appear on the Native-PAGE gel due to the difference in aggregate size. An application of Native-PAGE is seen when determining the extent of metHb-albumin aggregation in either solution or as freeze-dried powders. In this case, Native-PAGE was used to examine how various conditions would affect the banding of albumin and reveal possible differences in both aggregation and stability [89]. Further, Native-PAGE was used to assess the interaction between phloretin and HAS [22].

8. Challenges and future directions

Though many obstacles and future directions still exist, recent developments in the stabilization of albumin have greatly affected its therapeutic uses. Problems in albumin stabilization center on protein folding, purification, stability, and immunogenicity. The efficacy of albumin-based drugs is seriously threatened by structural instability brought on by misfolding, unfolding, modifications, and aggregation [95]. Moreover, low delivery efficiency of external albumin formulations can raise the risk of systemic toxicity, so hindering their clinical relevance. Among the notable developments are the creation of endogenous albumin-binding prodrugs, which present different benefits for more effective and safer drug distribution [96]. Furthermore, helping to develop multifunctional proteins and new therapeutics is the combination of computational approaches with high-throughput experimental techniques [97].

Increasing physiological responsiveness and long-term stability in albumin stabilization would be possible by material innovation and integration of digital technologies [98]. To improve the stability, activity, and specificity of albumin-based proteins for biotechnological and medical uses, significant progress in computational techniques, including deep learning, reinforcement learning, and transfer learning, is indispensable. Furthermore, it is interesting to note that

initiatives should try to close the distance between experimental validation and computational forecasts. Moreover, ethical issues about artificial intelligence-driven protein design have to be resolved [97]. The development of albumin-based drug delivery systems still faces challenges related to purity, structural preservation, and clinical scalability (Table 2).

Table 2: Challenges and future prospects of albumin stabilization for drug delivery

Domain	Key challenges	Future prospects / Opportunities	[Ref.]
Protein source and purity	Variability and impurities in animal-derived albumin; risk of immunogenicity and contamination.	Development of recombinant albumin, standardized purification pipelines.	[99]
Structural stability during formulation	Organic solvents, heat, and pH shifts can denature albumin; drug binding may alter secondary structure.	Gentle solvent-free preparation, disulfide-based crosslinking, polysaccharide conjugation (steric stabilization), improved structural analytics.	[99–102]
Nanoparticle preparation issues	Desolvation agents damage protein; glutaraldehyde toxicity; instability near isoelectric point; scale-up variability.	Optimized ionic gelation, chitosan coating, engineered coronas (covalent albumin–NP linkers), automation and real-time quality monitoring.	[99,101,103–105]
Environmental sensitivity	Instability under heat, light, ionic strength shifts, and pH	Stabilizing excipients (sucrose/tris, trehalose, etc.)	[105–108]

	fluctuations; aggregation in storage.	polymers), lyophilization with optimized matrices; high-resolution monitoring of aggregation pathways.	
Premature drug release / weak binding	Drug–albumin interactions can be unstable; premature drug leakage limits efficacy.	Covalent linkers, engineered binding pockets, responsive release systems.	[104,106]
Biological interactions and corona effects	Endogenous proteins may replace albumin on NP surfaces (Vroman effect), altering biodistribution.	Covalent albumin coronas to enhance stability and targeting.	[104]
Scalability and reproducibility	Batch variability from pH, crosslinking, temperature; difficulty scaling solvent-based methods.	High-resolution analytics, automation, DoE-optimized nanoparticle synthesis.	[103,105]
Targeting limitations	Passive targeting leads to off-site accumulation; variable tumor uptake.	Ligand functionalization (RGD, SPARC targeting), computational design, receptor-mediated uptake strategies.	[105,109]
Stability of albumin–drug complexes	Multi-ligand binding alters conformation; destabilizing excipient interactions.	Mapping interaction sites; rational excipient selection; predictive binding models.	[110]
Storage and transport	Sensitivity to temperature and light; aggregation and	Controlled logistics, solid-state formulation	[105,108,111]

	denaturation during shipment.	ns, spray drying, hybrid sugar–protein matrices.	
Regulatory and characterization gaps	No standardized preparation/characterization methods for albumin drug delivery system.	Establishing regulatory frameworks, high-throughput biophysical characterization.	[99]

9. Conclusions

It is important that the native conformation of albumin is maintained for its clinical application. Many factors throughout the life cycle contribute to the instability of albumin, including thermal stress, shear, oxidative damage, etc. Therefore, stabilizing albumin will require a multi-faceted approach. Important excipients such as trehalose and sucrose have been shown to act as protective agents to prevent aggregation of albumin during lyophilization. Surfactants such as Tween 80 provide the necessary stabilization to albumin due to mechanical shearing forces. Other than stabilizing agents, there are alternative methods of stabilization of albumin that can be achieved through chemistry (PEGylation and fusion proteins), resulting in improved pharmacokinetics by increasing circulation half-lives and decreasing immunogenicity of the albumin molecule.

With the recent advances made in the area of targeted drug delivery, new opportunities will arise for stabilized albumin nanoparticles in the targeted delivery to the tumor microenvironment. In addition, through the use of markers such as Δ S-Cys-Albumin, we can directly assess the integrity of specimens with great accuracy. The use of calorimetric and spectroscopic methods will allow for good evidence supporting the validation of this type of system.

The future of this type of innovation will combine biotechnology with digital sciences. An interconnected network in the field will bridge the gap between computational predictions and experimental validations, enabling the potential to develop new and unique albumin variants with increased physiological stability through deep learning and artificial intelligence. By placing an emphasis on computational approaches, we can increase the speed of development for next-generation albumins.

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