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Review

The Uniqueness of Albumin as a Carrier in Nanodrug Delivery

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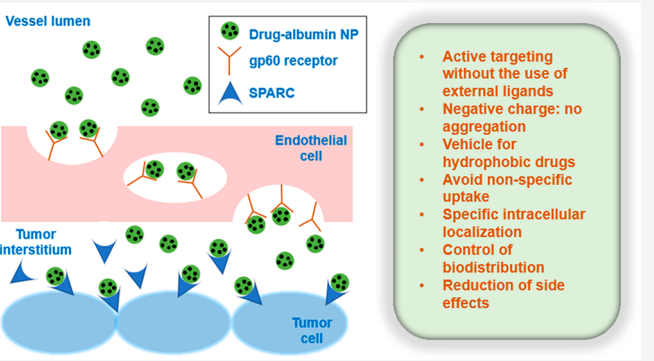
ABSTRACT: Albumin is an appealing carrier in nanomedicine because of its unique features. First, it is the most abundant protein in plasma, endowing high biocompatibility, biodegradability, nonimmunogenicity, and safety for its clinical application. Second, albumin chemical structure and conformation allows interaction with many different drugs, potentially protecting them from elimination and metabolism *in vivo*, thus improving their pharmacokinetic properties. Finally, albumin can interact with receptors overexpressed in many diseased tissues and cells, providing a unique feature for active targeting of the disease site without the addition of specific ligands to the nanocarrier. For this reason, albumin, characterized by an extended serum half-life of around 19 days, has the potential of promoting half-life extension and targeted delivery of drugs. Therefore, this article focuses on the importance of albumin as a nanodrug delivery carrier for hydrophobic drugs, taking advantage of the passive as well as active targeting potential of this nanocarrier. Particular attention is paid to the breakthrough NAB-Technology, with emphasis on the advantages of Nab-Paclitaxel (Abraxane), compared to the solvent-based formulations of Paclitaxel, i.e., CrEL-paclitaxel (Taxol) in a clinical setting. Finally, the role of albumin in carrying anticancer compounds is depicted, with a particular focus on the albumin-based formulations that are currently undergoing clinical trials. The article sheds light on the power of an endogenous substance, such as albumin, as a drug delivery system, signifies the importance of the drug vehicle in drug performance in the biological systems, and highlights the possible future trends in the use of this drug delivery system.



KEYWORDS: *albumin, drug delivery, Abraxane, Taxol, active targeting, clinical trials*

1. INTRODUCTION

Nanotechnology has shown great potential in pharmaceutical applications, especially in the area of drug delivery.[1] In particular, nanomaterials allowed the development of platforms for the efficient administration, protection, transport, and specific delivery of challenging therapeutic or diagnostic cargos, such as poorly soluble drugs, proteins, and gene therapeutics, in biological fluids toward cellular and intracellular targets.[2] Nanoparticles have been designed to overcome the limitations of conventional delivery and navigation through biological barriers.[3,4] In fact, in several instances, nanoparticles of various chemical structures, including lipid, polymer, and inorganic nanocarriers, have shown to effectively offer control on the biodistribution and/or release of single or multitherapeutic agents and the possibility to overcome biological barriers against targeted drug delivery to the diseased site. However, depending on their structure, such nanocarriers have also presented drawbacks restricting their success in targeted drug delivery, including nonspecific uptake by phagocytic cells, off-target distribution, nonspecific immune activation, inadequate control over drug release in biological systems, and poor intracellular internalization.[5] The plasma protein albumin has attracted attention as a natural, yet versatile, nanodelivery system due to its characteristics, including high binding capacities for both hydrophobic and hydrophilic drugs, relatively long half-life, specific targeting of inflammation sites, as well as virtually minimal toxicity and immunogenicity.[6] Albumin is one of the most abundant and important proteins in the body because of its role in the maintenance of intravascular colloid osmotic pressure, neutralization of toxins, and transport of therapeutic agents. In addition to the natural affinity of many drugs for binding to albumin, the possibility for chemical conjugation of drugs to albumin nanoparticles has also been explored for several entities. Moreover, the surface of the albumin-based nanoparticle can also be functionalized with ligands due to the presence of functional groups to which different types of

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linkers or spacers can be attached. Being recognized by the gp60 receptors and secreted protein, acidic and rich in cysteine (SPARC) pathways, it potentially can provide active targeting without the use of external ligands. Albumin nanoparticles have been the subject of excellent several reviews, to date.[6−9] The purpose of the current manuscript is to provide an update on the status of albumin in the nanomedical field, emphasizing the unique features of this delivery system responsible for its successful preclinical and clinical application for different therapeutic entities and in various disease conditions.

2. CHARACTERISTICS OF ALBUMIN FROM DIFFERENT SPECIES

Albumin is the most abundant protein in blood plasma, constituting approximately 60% of all proteins in the blood. It is a highly water-soluble small globular protein and has a molecular weight of 67 kDa and an average half-life of 19 days. It shows stability at a pH range of 4−9 and can be heated for 10 h at 60 °C. It can be extracted from many sources including human serum (human serum albumin, HSA) (Figure 1), bovine serum (bovine serum albumin, BSA), rat serum (rat serum albumin, RSA), and egg white (ovalbumin, OVA), but the two types most used for drug delivery are HSA[10] and BSA.

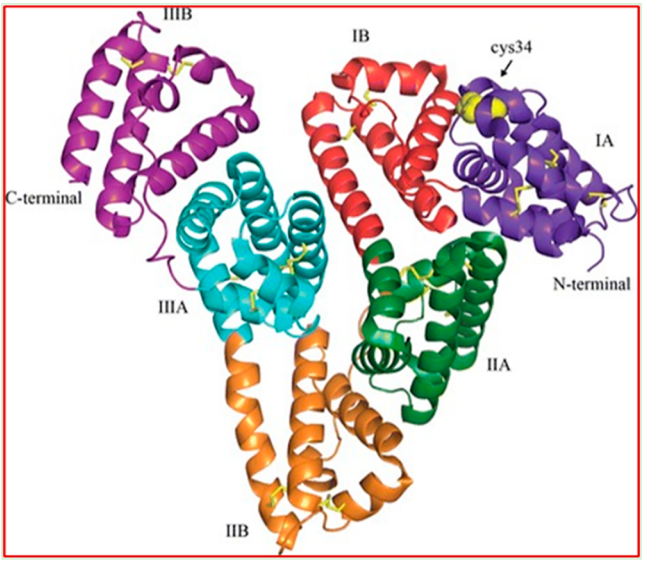

Figure 1. Structure of human serum albumin, a single polypeptide containing 585 amino acids. The three homologous domains [(I) residues 1−197, (II) residues 189−385, (III) residues 381−585] assemble to form a heart-shaped protein. Each of the domains is composed of two subdomains (A, B) with common structural motifs. The domains of albumin are shown in purple (IA), red (IB), green (IIA), orange (IIB), blue (IIIA), and violet (IIIB). The yellow sticks indicate the disulfide bridges, and the yellow spheres represent the free cysteine residue at position 34 (cys34) in domain IA. From ref 6 with permission.

The main role of this protein is to maintain the osmotic pressure in the blood and to act as the main carrier of hydrophobic molecules (such as fatty acids and hormones), making it a perfect candidate for drug delivery.[11] The lipophilicity of many existing drugs represents one of the main limitations of their applications. Hence, finding a nontoxic, widely available, and efficient vehicle that is able to improve the solubilization and the transport of therapeutics is of fundamental importance. As shown in Figure 1, albumin is characterized by three domains (I, II, III). Each domain is comprised of two subdomains (A, B), which contain 4 and 6 α-helices, respectively. The most important human serum albumin binding sites for hydrophobic compounds (especially neutrally and negatively charged hydrophobic drugs) are named Sudlow site I and Sudlow site II,[12] placed, respectively, in domains IIA and IIIA, highly elongated hydrophobic pockets with positively charged lysine and arginine residues.[13] In particular, site I is also known as the warfarin site since drugs such as azapropazone, phenylbutazone, and warfarin bind to it. Site II is also known as a benzodiazepine site since compounds such as diazepam, ibuprofen, and tryptophan bind to it. In this way, various therapeutics, such as paclitaxel and docetaxel, can be bound and subsequently delivered effectively to the tumor site.

2.1. Human Serum Albumin. Human serum albumin is made up of a single chain of 585 amino acids. Its secondary structure is highly flexible, characterized by 67% α helix and 17 disulfide bridges with 6 turns that act as cross-linkers for the three homologous domains.[14] Human serum albumin is a protein produced by hepatocytes in the liver, at a rate of 9−12 g/day, and is one of the most abundant (levels of plasma albumin in the range from 3.5 to 5 g/dL[15]) and important proteins in blood plasma. Although albumin is the most abundant plasma protein, the majority of albumin is not in blood circulation. As much as 60% of albumin is stored in the interstitial space. Even though its biological half-life is 19 days, it only lasts 16−18 h in circulation.[16] The transcapillary movement of albumin is reversible, as it can return inside the plasma through the lymphatics to maintain constant plasma protein concentrations. Its production is modulated by the body's needs. In particular, the synthesis is stimulated by insulin, thyroxine, and cortisol or conditions like hypoalbuminemia, whereas it is hindered by potassium and exposition of hepatocytes to excessive osmotic pressure. Furthermore, an adequate supply of nutrients is fundamental to trigger albumin production. In fact, poor adsorption of nutrients reduces the liver's ability to produce protein. Degradation of albumin can take place in any tissue, but it occurs mainly in the liver and kidney. The balance between albumin production, degradation, and movement between intravascular and interstitial spaces determines the effective plasma albumin concentration. Albumin is responsible for the maintenance of the blood osmotic pressure, nourishes tissues, transports hormones, vitamins, drugs, and divalent cations, like calcium and zinc, throughout the body.[17] It acts as a free radical scavenger in inflammatory conditions, and it is involved in processes like coagulation and wound healing.[18] Furthermore, it possesses antioxidant properties and has a heparin-like activity by enhancing antithrombin III activity. In fact, hypoalbuminemia can cause coagulopathy.[19] The compounds that bind to albumin can be classified as endogenous and exogenous. The first class includes all the substances that are already found in the body, such as bilirubin, fatty acids, cations, free radicals, vitamins, and hormones. The second category consists of drugs that are introduced to the body from outside, such as anticoagulants, anti-inflammatory drugs, antibiotics, anticonvulsants, cardiovascular and renal drugs, central nervous system drugs, and hypoglycaemic agents.[20] Albumin molecules carry many charged amino acid residues and have a net charge of −17 mV at physiological pH. For this reason, sodium ions, and other cations, are strongly attracted by albumin. As a

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consequence of sodium attraction to albumin, water is dragged into the blood vessels (1 g of albumin can retain 18 mL of water within the blood vessels). When conditions affect its production by the liver, levels of albumin may decrease, protein breakdown may increase, the protein loss via the kidneys may be augmented and the volume of the liquid portion of blood (plasma) may be expanded, diluting the blood. Examples of the conditions that can alter albumin concentration are severe liver disease and kidney disease.[21] Since albumin is produced by the liver, a loss of liver function can provoke a decrease in the levels of albumin. One of the main roles of the kidneys is to maintain the plasma proteins, such as albumin, preventing them from being eliminated in urine with waste products. If the kidneys are damaged, for example in diabetes, hypertension, or nephrotic syndrome, they lose their ability to maintain the concentrations of the plasma proteins and levels of albumin decrease.

2.2. Serum Albumin from Other Species. Bovine serum albumin is derived from bovine serum, and it is very similar to the HSA. Its molecular weight is 69.323 kDa, and it has an isoelectric point (pI) of 4.7 in water (25 °C), which makes it negatively charged at a neutral pH and positively charged under acidic conditions.[22] The presence of both negatively charged amino acids and positively charged ones in BSA can result in the binding of both positively and negatively charged substances. Because it is widely available at low cost and easy to purify and control, it has been widely used as a carrier for drug delivery in the literature. Furthermore, it has high loading capability, and it is water-soluble and can bind both hydrophilic and hydrophobic drugs, making it a very versatile carrier. The only downside could be a possible immunogenic response *in vivo* in humans[23] but also in mice.[24] Human serum albumin and BSA share 80% sequence homology; their molecular weights differ by less than 1%, and their isoelectric points are identical. One important difference is the number of tryptophan (Trp) residues. Human serum albumin has one Trp, whereas BSA has two. Tryptophan is responsible for the fluorescence of the protein, so human serum albumin and bovine serum albumin can be distinguished by spectrofluorimetry. The antibody response to BSA exposure has been explored in animal models and this protein has been used as a model protein to investigate the immune response to proteins. Human exposure to BSA begins early in life via consumption of bovine milk and meat, and also via vaccinations and medications. In 2005, a quantitative radioimmunoassay was developed to measure anti-BSA IgG antibodies in healthy subjects and cancer patients.[25] Western blot analysis confirmed the presence of anti-BSA antibodies after human exposure to BSA, but increased levels of anti-BSA antibodies were not accompanied by any clinical events in either healthy or cancer patients. In another study, BSA was administered to three strains of rabbits.[26] The results highlighted the fact that the immunogenicity of BSA varied considerably in the different strains of rabbits and that some rabbits even failed to respond to the stimulation. This lack of response may be genetic or related to the nature of the antigen. Furthermore, it was demonstrated that the immunogenicity of BSA is linked to its molecular state. BSA can be present partly as a monomer and partly in aggregated form, which is able to induce an antibody response. The nonimmunogenicity of BSA in some rabbits may be due to the close similarity with rabbit serum albumin, which presents some structural homologies with BSA. Ovalbumin (OVA) is the main protein found in the egg white, representing the 55% of the total protein contents.[27] Its function is still unknown, but it is thought to be a storage protein. It belongs to the serpin family and is a globular, acidic glycoprotein, characterized by a molecular weight of 42−47 kDa composed of a single polypeptide chain of 386 amino acids.[28] Its isoelectric point is 4.8, similar to that of HSA and BSA. However, OVA is often chosen as one of the most popular model antigens due to its immunogenicity.[29,30] Rat serum albumin (RSA) possesses 608 amino acids, and it has a molecular weight of 69 kDa. Furthermore, its isoelectric point is 5.7. Comparing the amino acid composition of RSA with that of HSA, it is evident that the RSA presents more tyrosine and less lysine, cystine, and leucine.[31] After HSA and BSA, it is frequently used as a carrier protein for several anticancer agents, and the resulting albumin conjugates have been tested in heterologous tumor models.[32] It is not known whether the choice of the species might affect the pharmacokinetics or the tumor uptake of the developed delivery systems *in vivo*. Considering that albumins from different species are highly homologous in terms of amino acid sequencing, it had been anticipated that all of the serum albumins had binding sites analogous to the ones identified on HSA. However, different hypotheses have been reported over the years. Panjehshahnin et al.[33] conducted a comparative study on six mammalian serum albumins, using warfarin and dansylarcosine as fluorescent markers of sites I and II, respectively, to evaluate binding in the presence of various displacers (phenylbutazone, diazepam, and fatty acids). The results showed that all the serum albumins exhibit binding sites similar to those of HSA, except for RSA. In particular, the displacement of warfarin by phenylbutazone was lower on rat serum albumin than on all the others, providing evidence that the warfarin binding site was structurally different on this type of albumin. On the other hand, only some minor differences were found on binding site II. In another study, Schmidt and Janchen[34] reported differences in ligand binding sites of other acidic drugs on RSA, compared with HSA. Massolini et al.[35] analyzed the differences between HSA, RSA, and rabbit serum albumin and demonstrated that stereoselective binding of subprofen and ketoprofen was observed on HSA and rabbit serum albumin, but not on RSA. Also, Aubry et al. reported several differences in the binding characteristics of RSA and rabbit serum albumin with HSA.[36] Rabbit serum albumin showed two binding sites for warfarin, one of which presented many similarities with the warfarin site on HSA (site I). Some years later, Kosa et al.[37] suggested that the previous studies should have carried out a more detailed characterization of the drug binding sites, such as the location and drug binding properties, in order to allow more valid comparisons of drug−drug interactions to be made between animal and human albumins. They attempted to identify drug binding sites on different albumin species using typical site I binding drugs (warfarin and phenylbutazone) and typical site II binding drugs (ibuprofen and diazepam), using rigorous competition experiments. Their study showed that binding affinities of site I drugs to rabbit serum albumin, RSA and BSA were actually quite similar to HSA. However, those to dog albumin were significantly smaller than human albumin. On the other hand, binding parameters of diazepam to BSA, rabbit serum albumin and RSA appeared different from those of HSA. In their opinion, these diversities could be due to the microenvironmental changes in the binding sites due to a change of size and/or hydrophobicity of the binding pockets, rather than a variation in amino acid residues. In particular, in

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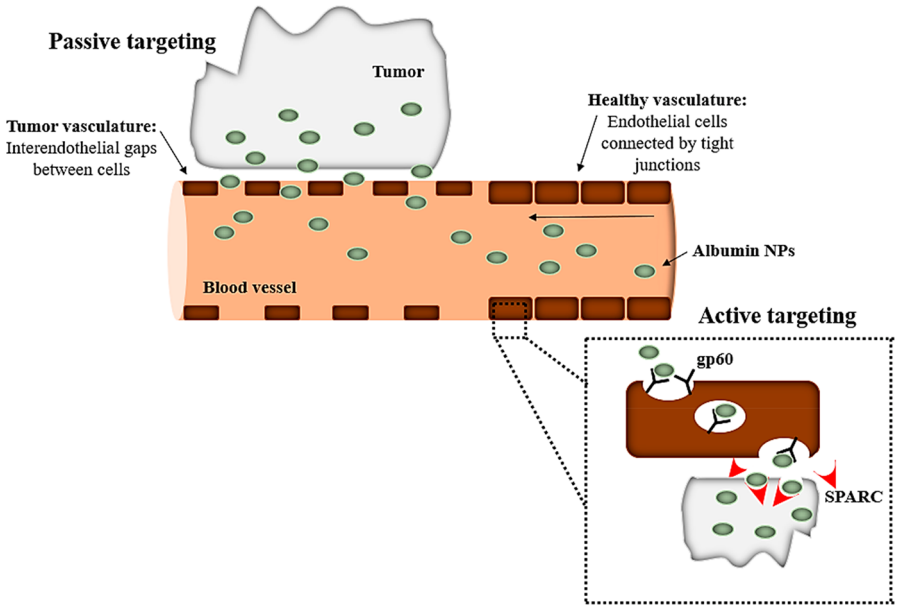

Figure 2. Schematic representation of passive versus active targeting.

the case of dog albumin, a defect might exist at the location corresponding to site I. The hydrophobic interactions between DZ and BSA, rabbit serum albumin and RSA are weakened as a result of the tertiary structure change in cavity size, rather than the loss of hydrophobic residues. In conclusion, it can be stated that RSA and rabbit serum albumin contain a drug binding site, corresponding to site I on HSA, and dog albumin contains a specific drug binding site corresponding to site II on HSA.

3. THE UNIQUE PROPERTIES OF ALBUMIN NANOCARRIER IN DRUG TARGETING

3.1. The Natural Ability of Albumin in Targeting Cancer and Other Proangiogenic Environments. The hyper-permeability of blood vessels and the impaired lymph drainage, the well-known enhanced permeation and retention (EPR) effect in solid tumors has been proposed as a responsible mechanism for passive targeting of many nanocarriers in solid tumors[7] (Figure 2). However, the defining role of the EPR effect as a responsible mechanism for passive targeting of nanocarriers in solid tumors, even in preclinical animal models, has been questioned recently.[38] Although it constitutes a paradigm in cancer nanomedicine, Chan et al. demonstrated that 97% of the nanoparticles under their study enter into solid tumors by endothelial cells through an active process of transcytosis and that the interendothelial gaps, which characterize the EPR effect, are not responsible for the transport of nanoparticles into solid tumors, since the frequency of gaps is too low to significantly account for the accumulation of nanoparticles into tumors.[39] Several studies[40−42] suggested the use of vascular dilators, such as nitric oxide (NO), which could augment vascular permeability via vasodilation, increasing the EPR effect, to overcome problems of insufficient accumulation of drugs inside the tumor site. Nitric oxide has a very short blood residence time, and this creates a limitation for its usage. For this reason, Kinoshita et al.[40] developed a NO drug delivery system, S-nitrosated HSA dimer (SNO-HSA dimer), which was able to deliver NO efficiently to the tumor site. The combination of an albumin-based drug delivery system (nab-paclitaxel) with SNO-HSA dimer, enhancing the passive targeting, showed significantly higher suppression of the tumor growth (even the ones with low vascular permeability) than the drug delivery system used alone. One of the unique features that make albumin such a powerful and effective drug carrier is that it binds to receptors, which are overexpressed by the tumor. The main pathway that albumin relies on for the internalization inside the tumors is receptor-mediated endothelial transcytosis (Figure 2). Albumin binds with high affinity to the gp60 receptor, a 60 kDa glycoprotein (albondin).[43] This receptor is found on the surfaces of endothelial cells of the tumors, and after the connection with albumin, it binds to caveolin-1, an intracellular protein that gives rise to an invagination of the cell membrane, leading to the formation of transcytosis vesicles (caveolae) transporting albumin inside the tumor. Moreover, SPARC (secreted protein acidic rich in cysteine), also known as antiadhesin, osteonectin, BM-40, and 43K protein, which is overexpressed by many types of tumors and absent in normal tissues, attracts albumin and contributes to its accumulation inside the tumor. These two main mechanisms allow the protein to be actively internalized by the tumor. Among the albumin receptors, apart from gp60, there are also gp18 and gp30, which are cell surface glycoproteins characterized by molecular weights of 18 and 30 kDa, respectively.[44] They are expressed in endothelial cell membranes of the liver and peritoneal macrophages. They are scavenger receptors that have a high affinity for damaged albumin. In fact, modified BSA shows a 1000-fold higher tendency of interactions for gp18 and gp30, compared to native BSA.[10] These two receptors are involved in the endolysosomal sequestration and catabolism of this protein, probably as a safety mechanism to degrade old, damaged, or altered albumin (for example, generated by oxidation from inflammation or hyperglycation in diabetes). On the other hand, native albumin does not have a high affinity for gp18 and gp30 receptors but binds mainly to the previously described gp60, which is involved in the transcytosis of albumin through the endothelium. Another receptor that takes part in albumin homeostasis is FcRn, the neonatal Fc receptor, also known as the Brambell receptor. It is a transmembrane heterodimeric protein, consisting of an

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MHC-class I-like heavy chain, characterized by three extracellular domains (α1, α2, α3), which is noncovalently associated with a β_2-microglobulin light chain, necessary for the function of the receptor.[45] It is found in many tissues including vascular endothelium, gut, lungs and kidney.[46] This receptor can determine a different fate for albumin-based nanoparticles after internalization, playing a vital role in maintaining high levels of albumin. Usually, as already pointed out, HSA is nonimmunogenic and it is not recognized as a foreign element, but, if altered or damaged, it is immediately targeted by the immune system and degraded. However, the protein is well-known for its prolonged half-time, which makes it a useful carrier in drug delivery. The long half-life of albumin is due to the escape from intracellular degradation by FcRn receptor, which recycles internalized albumin back to the bloodstream through a pH-dependent mechanism.[47] In conclusion, in drug delivery, it is gp60 that allows albumin to specifically target the cancer cells, since it mediates albumin-based drug delivery systems transport over two biological barriers, epithelial and endothelial, and it is overexpressed on cancer cells, functioning as a targetable molecule. Furthermore, SPARC, whose expression is associated with pathophysiological conditions that involve extracellular matrix remodelling, such as cancer and neo-angiogenesis processes, is used as a prognostic tool, and its high levels are linked to the efficacy of the albumin-based drug delivery systems in inhibiting cancer proliferation.

3.1.1. Albumin Modification for Active Drug Targeting. In addition to natural receptors for albumin interaction, an additional step has been taken to enhance the targeting capability of albumin even further through covalent or noncovalent bonding modification of albumin nanoparticle surface with specific targeting agents.[48] These molecules could include antibodies or fragments of them, peptides, folic acid,[49] and also aptamers, which are short DNA or RNA structures characterized by better site-specificity and less immunogenicity than antibodies.[50] For example, aptamer-modified doxorubicin-loaded albumin-based nanoparticles for targeted drug delivery were constructed.[51] DOX@Apt-BSA resulted to have bigger sizes (163 ± 2.5 nm for DOX@Apt-BSA nanoparticles, compared to 145 ± 2.5 nm for DOX@BSA nanoparticles) and more negative ζ potential (−20.7 ± 0.3 mV for DOX@Apt-BSA nanoparticles, compared to −18.5 ± 0.3 mV for DOX@BSA nanoparticles). Through the specific interaction between aptamer AS1411 and its receptor overexpressed on tumor cells, DOX@Apt-BSA nanoparticles demonstrated higher cellular uptake and cytotoxicity compared to non-functionalized nanoparticles. Flow cytometry was used to analyze the cellular uptake of the nanoparticles, and the mean fluorescence intensity (MFI) of the MCF-7 cells treated with the DOX@Apt-BSA nanoparticles was higher than that of free DOX and DOX@BSA nanoparticles (800000 MFI for DOX@Apt-BSA, compared to 500000 and 400000 MFI for DOX@BSA and free DOX, respectively). The cell toxicity studies in MCF-7 cells, evaluated at 24 h for free DOX, DOX@BSA nanoparticles, and DOX@Apt-BSA nanoparticles, showed a decrease in cell viability, with an almost 10% improved efficacy for the DOX@Apt-BSA nanoparticles compared to free DOX. Cabazitaxel (CTX)-loaded HSA nanoparticles via self-assembly method had been previously developed and shown efficacy in prostate cancer therapy. Due to the overexpression of folic acid (FA) receptor on the surface of tumor cells, Sun et al. improved the pre-existing formulation producing FA-modified BSA-CTX nanoparticles, functionalizing the surface of the albumin nanoparticles with folic acid.[52] After the modification, the nanoparticles showed a size of 152 ± 5 nm, slightly larger than the unmodified nanoparticles (143 ± 6 nm) and sustained release (similar to the unmodified nanoparticles, with a total release after 100 h). The cytotoxicity of FA-NPs-CTX, NPs-CTX, and free CTX was assessed in FR-overexpressing HeLa and FR-negative A549 cells at 24, 48, and 72 h. The two NP formulations showed improved cytotoxicity at longer time points (48 and 72 h), which suggested that CTX was effectively released from the nanoparticles. In FR-overexpressing HeLa cells, the cellular uptake of FA-FITC-NPs-CTX was 1.87 times that of FITC-NPs-CTX at 4 h. On the other hand, there was no significant difference in the uptake of the two formulations in FR-negative A549 cells. At the same drug concentration, in FR-overexpressing HeLa FA-NPs-CTX showed more cytotoxicity than the unmodified nanoparticles ($P < 0.05$), due to the increased FR-mediated uptake. Also, in FR-negative A549 cells the cytotoxicity was slightly augmented, this time due to the nonspecific endocytosis, instead of FR-mediated endocytosis. A dual-peptide-functionalized BSA-based formulation, cRGD-BSA/KALA/DOX nanoparticles, was developed for tumor-targeted delivery to glioblastoma cells. It is a multifunctionalized nanosized albumin-based drug delivery system with tumor-targeting, cell-penetrating and pH-responsive properties.[53] Glioblastoma multiforme (GBM) is one of the most aggressive brain tumors and it is a challenging one because of the difficulty in the delivery of therapeutics caused by the blood-brain barrier. To overcome this limitation, the cyclic Arg-Gly-Asp (cRGD) peptide, which has selective affinity for integrins overexpressed on the endothelial cells of tumor blood vessels and GBM cells, was conjugated to BSA. The antineoplastic drug doxorubicin was loaded into the obtained nanoparticles via hydrophobic and hydrophilic interactions and later stabilized by hydrogen bonding. In the end, positively charged cell-penetrating peptide KALA and the negatively charged nanoparticles (cRGD-BSA/DOX) self-assembled through electrostatic interactions, producing the final formulation. The nanoparticles showed enhanced cellular uptake in U87-MG cells, evaluated by confocal laser scanning microscopy. However, for NIH 3T3 cells with a low integrin expression, coincubation with cRGD-BSA did not lead to increased cell internalization. Furthermore, the intracellular accumulation of the delivery system in this cell line could be inhibited by excess free cRGD, which provided evidence that enhanced cell uptake was due to the specific interaction between cRGD and integrin overexpressed on the glioblastoma cells. Moreover, it was shown that accumulation of cRGD-BSA could be reduced at 4 °C, suggesting that adenosine-triphosphate was involved in the uptake process. The presence of KALA showed enhanced green fluorescence (FITC-labeled cRGD-BSA), confirming the increased cellular accumulation of internalized cRGD-BSA/KAlA nanoparticles. Furthermore, the pH-sensitivity triggered the disassembly of the nanoparticle and the release of the therapeutic when needed in the tumor environment. In fact, cRGD-BSA/KALA/DOX complexes formed at neutral pH and disassembled at acidic pH. Another example is constituted by monoclonal antibody-modified BSA nanoparticles. 1F2, an anti-HER2 monoclonal antibody (mAb), was covalently added to the surface of 5-Fluorouracil (5-FU)-loaded BSA-based nanoparticles.[54] 5-Fluorouracil is an antimetabolite that has been used against various types of

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Table 1. Characteristics of Different Methods of Albumin Nanoparticle Preparation

technique	advantages	disadvantages	size (nm)	morphology	ref
desolvation	robustness, reproducibility, absence of toxic organic solvents, simplicity, possibility to obtain smaller size nanoparticles	use of toxic cross-linkers, demand for strict purification steps and removal of unreacted cross-linker, not appropriate for highly water-soluble drugs	150−300	spherical shape with a smooth surface	62, 63
emulsification	higher drug entrapment efficiency	use of toxic chlorinated solvents, use of toxic cross-linkers or thermal stabilization, demand for removal of both the surfactants and oily residues, harmful to heat-sensitive drugs (if thermal stabilization is used), high energy requirement in homogenization, larger size nanoparticles, difficulty of controlling the albumin particles' size	100−800	albumin nanospheres	64, 65
self-assembly	high loading of poorly water-soluble drugs	use of only lipophilic drugs, difficulties in scaling-up the technology, insufficient storage stability, different solubility protocols for different drugs	130−160	spherical shape and core−shell structures	66
thermal gelation	possible fabrication of nanoscale hydrogels	encapsulation only of drugs that are not heat sensitive	100−200	spherical core−shell structure nanogels	67
nanospray drying	single-step continuous and scalable process, versatile technique, useful for heat-sensitive samples, control for particle size	production of larger particles	500−3000	smooth spherical nanoparticles	68−71
microfluidic mixing	tunable size, structure, and surface, narrow size distribution, controlled release profile, high versatility and reproducibility, smaller size nanoparticle, ow reagent consumption, better mixing, better drug loading capability	risk of fouling and channel clogging, complex device design, not fully automated, labor-intensive, sometimes requiring special equipment, such as cleanroom facilities	100−160	versatile shape, from nanofibers to spherical core−shell nanoparticles	72, 74−76
NAB-technology	ideal for encapsulating lipophilic drugs, safe and suitable for intravenous usage of poorly soluble drugs, no requirements for surfactants or polymeric materials for preparation, disulfide formation induced by homogenization does not substantially denature HAS, higher drug content, smaller size	demand for high pressure, use of hydrophobic drugs only	100−200	spherical nanoparticles with a smooth surface	77−80

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tumor, such as colon, ovarian, breast, and skin cancers. It interferes with RNA expression, limits cell division, and causes the tumor to shrink. However, this compound has many side effects, such as cardiotoxicity. For this reason, it has been coupled with albumin-generating nanoparticles. To make the formulation even more efficient, the surface of the nanoparticles has been functionalized with monoclonal antibodies, thanks to the available amino and carboxylic groups on the surface of albumin. The mAb used (anti-HER2 mAb, 1F2) targets the epidermal growth factor receptor-2 (HER2), overexpressed by many types of cancer, allowing the formulation to selectively recognize the target site and enter via receptor-mediated endocytosis. The formulation was tested on HER2-positive SKBR3 cells and HER2-negative MCF7 cells. As expected, no cellular uptake was observed for HER2-negative MCF7 cells, whereas the 1F2-coupled 5-FU-loaded BSA nanoparticles interacted with almost all HER2-positive SKBR3 cells. The *in vitro* cytotoxicity, evaluated by MTT assay, showed lower SKBR3 viability (50.7 ± 9%) after 5 h treatment with 1F2-coupled-5-FU-loaded BSA NPs, in comparison with the controls (free 5-FU and 5-FU-loaded BSA NPs, which showed a cell viability of around 91 ± 8% and 89 ± 9%, respectively), due to the cell attachment and internalization. Furthermore, no significant enhancement of toxicity was observed on MCF7 cells. The results obtained provided evidence that the system could efficiently be exploited for targeted delivery of 5-FU to HER2-positive cancerous cells.

3.1.2. Stealth Albumin Nanoparticles. Attempts have been made to decorate the nanoparticle with poly(ethylene oxide) (PEO) or polyethylene glycol (PEG), which are antifouling polymers able to make the particle "invisible" to the immune cells, such as macrophages.[55] PEGylation is one of the most promising and investigated strategies for the improvement of the pharmacokinetic of therapeutic agents. The main effects of PEGylation in albumin nanoparticles are favorable consequences in overall circulation life-span, tissue distribution, and elimination pathway. In particular, as demonstrated by Fujita et al.,[56] the PEGylation of three derivatives of BSA (lactosylated BSA, mannosylated BSA, and cationized BSA) caused a 7-fold, 45-fold, and 130-fold decrease in their systemic clearance, respectively. Preventing the metabolization and the recognition by the immune defense mechanism, the PEGylation led to longer residence time in systemic circulation. BSA offers various target sites for covalent modification, such as the free amino groups. Although the quantification of the PEGylation is still challenging, modification with PEG has been widely performed on human serum albumin nanoparticles for the prolongation of plasma half-life of colloidal carrier system. The proof for the efficacy of PEGylation was demonstrated *in vivo* in a mouse model.[57] The evaluation of the *in vivo* behavior of the two NP system demonstrated that 1 min after injection, only 30% of the non-PEGylated albumin NPs, used as control, were found in the blood, whereas at the same time point, ∼60% of the PEGylated NPs were still circulating in the blood. Compared to unmodified nanoparticles, the PEGylation led to four times larger plasma half-life. It is important that the PEG chains cover the whole surface of the nanoparticle (high density), and it is preferable to have long chains. The PEG chains can be found in two main conformations, brush and mushrooms. Kouchakzadeh et al.[58] used the phase separation method to produce BSA nanoparticles. After that, the obtained nanoparticles were PEGylated, using the available amino groups on the surface for covalent binding, and the formulation was optimized, modulating different process parameters. Optimum conditions were found to be as follows: 32.5 g/L PEG concentration, 10 min incubation time, incubation temperature of 27 °C, and pH of 7 for 5 mg of BSA in 1 mL of PBS. The obtained nanoparticles had a hydrodynamic size of 217 nm and ζ potential of −14 mV, highlighting the fact that the addition of PEG reduced the negative surface charge compared to the nonfunctionalized nanoparticles. The release of 5-Fluoracil from loaded-PEGylated nanoparticles was initially slower than bare 5-FU-loaded-nanoparticles and became almost identical after 3 h, probably because of the steric presence of a layer of PEG surrounding the nanoparticles and opposing resistance to the delivery of the drug. Furthermore, the addition of PEG layers affected the flexibility of the nanoparticles, and it decreased the amount of attraction between the nanoparticles by augmenting their steric distance.[59] Even though the use of PEG has various advantages, it still has some drawbacks that limit its applications. The toxicity of PEG is low, but sometimes it can be toxic with a frequency inversely proportional to the molecular weight of the layer. PEG can be degraded by light, heat, or shear stress, leading to fragmentation of the layer. Alternatives to this polymer have been investigated, and they are mainly constituted by PEG hybrids, chitosan, dextran, polyvinylpyrrolidone (PVP), poly(vinyl alcohol) (PVA), and poly(acrylic acid) (PAA). Du and Wang developed BSA/poly(acrylic acid) (BSA/PAA) nanoscaled particles via noncovalent bonds induced self-assembly method in acid pH, and they optimized the formulation by tuning the process variables, such as pH, BSA/PAA weight ratio, and PAA molecular weight.[60] PAA increased particles' hydrodynamic diameters, and their distribution became narrower. The appropriate BSA/PAA weight ratio was around 3/8, and the suitable self-assembly pH for the BSA/PAA stable nanoparticle was around 2.4−3.1. BSA chains are supposed to be partly trapped in the nanoparticle core after interaction with PAA due to the formation of H-bonding at pH lower than 2.4 and electrostatic attraction at higher pH values while the rest of the BSA chains should form the shell of the nanoparticles. However, to the best of our knowledge, there are no report *in vivo* evaluation of the formulation.

4. PREPARATION METHODS OF ALBUMIN NANOPARTICLES

There are different fabrication methods to produce albumin-based nanoparticles. They are classified in chemical-based methods, which use chemical additives, such as ethanol, cottonseed oil, or β-mercaptoethanol, to induce nanoparticle formation, and physical-based methods, which take advantage of physical factors, such as heat or pressure, in order to generate nanoparticles.[61] Among the chemical-based techniques, desolvation, emulsification, and self-assembly are the most commonly used methods. Nanospray drying, thermal gelation, and NAB-technology, which will be described later, belong to the physical-based techniques. Reproducibility is a key feature that has to be achieved, and every production technique should aim to produce nanoparticles characterized by predictable and reproducible properties (Table 1).

4.1. Desolvation (Coacervation). Desolvation is one of the most used procedures to prepare albumin nanoparticles. It involves the addition of a desolvating agent such as ethanol or acetone in a continuous and dropwise manner to an aqueous

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solution of albumin under stirring until turbidity of the solution is reached.[62] The desolvating agents work by changing the tertiary structure of albumin gradually, leading to phase separation and aggregation of the protein. In fact, the homogeneous solution separates into two phases, one of which constituted mainly of solvent and the other of solute, albumin, that forms submicronic aggregates. Most of the time, the obtained formulation is not stabilized enough, and a cross-linker, such as glutaraldehyde, is used to further preserve and stabilize the morphology of the resulted nanoparticles. The properties of the resulting formulation depend on the conditions of the process, such as pH, protein concentration, cross-linker concentration, desolvating agent level, ionic strength, and stirring speed.[63] The preparation process is shown in Figure 3A.

4.2. Emulsification. The emulsification process (Figure 3B) involves the addition of a nonaqueous solution (oil phase) into an albumin solution (water phase), under stirring, generating a crude emulsion.[64] The emulsion can be made uniform by homogenization using a high-pressure homogenizer. After that, there are two possible methods to stabilize the nanoparticles, thermal heating (temp >120 °C)[65] or chemical treatment using a cross-linker, such as glutaraldehyde.

4.3. Self-Assembly Method. Self-assembly relies on the formation of albumin nanoparticles due to the increase in the hydrophobicity of the protein by breaking of disulfide bonds caused by the use of β-mercaptoethanol or reduction of primary amine groups on the surface of the protein caused by the addition of a lipophilic compound.[66] The result is the self-assembly of albumin and the formation of nanoparticles in an aqueous environment. The self-assembly procedure is illustrated in Figure 3C.

4.4. Thermal Gelation. Thermal gelation, as can be seen from Figure 3D, is characterized by heat-induced protein conformational change and unfolding, followed by protein−protein interactions, such as the formation of hydrogen bonds, electrostatic, hydrophobic interactions, and disulfide-sulfhydryl interchange reactions.[67] The properties of the obtained formulation depend on conditions of the process, such as pH, protein, concentration, and ionic strength.

4.5. Nanospray Drying. Nanospray drying (Figure 3E) is a versatile technique,[68] commonly used to produce a dry powder from a liquid phase. One of the main advantages of this method is that particles are dried and produced in a continuous and single-step process.[69] It is characterized by the spray generation of droplets from a liquid solution. The process includes different steps, such as atomization of feed into a spray, spray-air contact, drying of spray, and separation of dried product from the drying air.[70] A liquid feedstock is atomized into a spray of droplets and brought into contact with a drying gas, at a sufficient temperature to obtain moisture evaporation. The contact takes place in a drying chamber, where an aqueous solution of albumin is held. As the moisture evaporates, the solid dried particles are formed and collected using an electrostatic particle collector. Optimization of the nanospray drying parameters allows regulating the properties of the nanoparticles, making them suitable for specific applications.[71]

4.6. Microfluidic Mixing. Even though less investigated, another technique used to produce albumin nanoparticles is the microfluidic technology.[72] It provides an effective alternative for the fabrication of lipid,[73] polymeric,[74] and serum albumin nanoparticles. This technique is a controllable

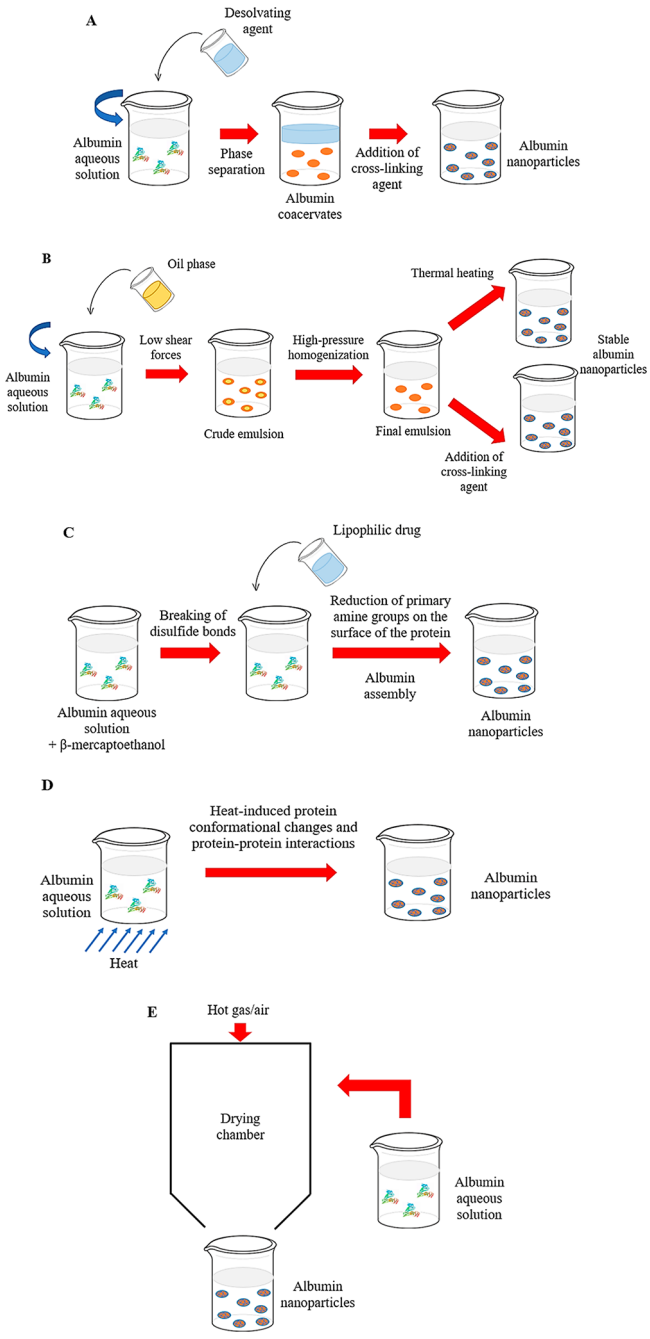

Figure 3. Main methods for the preparation of albumin nanoparticles: (A) desolvation, (B) emulsification, (C) self-assembly, (D) thermal gelation, (E) nanospray drying.

preparation process, which results in particles with tunable size and narrow size distribution. Furthermore, it provides a unique opportunity for automatized large-scale, pharmaceutical production. In the literature, there are few studies on the production of albumin nanoparticles underflow conditions. Successful results have been obtained in a recent study conducted in 2020,[75] which was focused on the preparation of core−shell type, drug-loaded albumin-based nanoparticles. The stabilizer poly(allylamine hydrochloride) (PAH) was added to channel 1 (v1) in the first syringe pump, while the solution containing the carrier and the drug (BSA/KYNA) was filled into the channel 2 (v2) in the second syringe pump. After passing through the syringe pumps, the two solutions were

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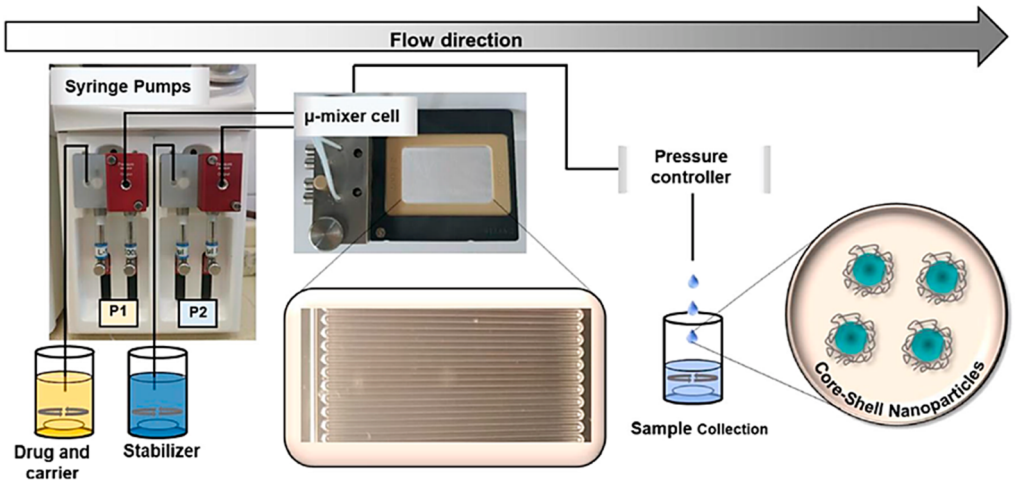

Figure 4. Flow system used to prepare BSA core−shell nanoparticles with the pumps and the μ-mixer cell. Adapted from ref 75 with permission.

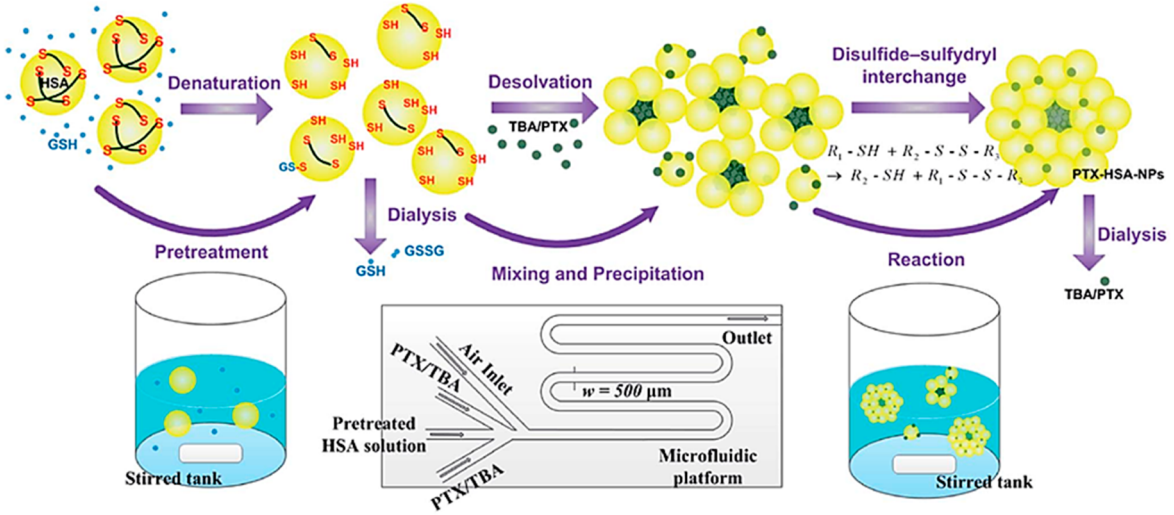

Figure 5. Scheme and procedure for the preparation of PTX-HSA nanoparticles. Adapted from ref 76 with permission.

mixed in the μ-mixer cell, with a volume of 250 μL, and a pressure controller apparatus. After that, the sample was collected in defined time intervals. The schematic representation of the preparation of the core−shell NPs by this microfluidic device is presented in Figure 4.

A paclitaxel-loaded disulfide-cross-linked (biocompatible alternative to cross-linking with glutaraldehyde) HSA nanoparticles were produced in a microfluidic platform.[76] The entire process includes four steps, as shown in Figure 5. In the first step, the pretreatment step, HSA was incubated with deionized water with GSH to reduce the 17 disulfide bonds to free sulfhydryl groups. In the second step, the mixing and coprecipitation, the HSA/water solution was mixed with paclitaxel/tertiary butyl alcohol (TBA, an organic solvent used as antisolvent to water) in a microchannel reactor: therefore, there were three liquid inlets and an additional air inlet to form a segmented gas−liquid flow. In the mixing solution, both paclitaxel and albumin were subjected to a huge decrease of solubility, and they precipitated out together, forming PTX-HSA nanoparticles. The third step was the reaction step, where the suspension was incubated at 37 °C to form disulfide bonds. In the final step, dialysis, the suspension was dialyzed against deionized water at 4 °C to remove TBA. Two fundamental factors to obtain stable nanoparticles in the microfluidic technique are choosing an adequate antisolvent in the precipitation step and modulating the reaction time in the reaction step.

5. DRUG INCORPORATION IN ALBUMIN NANOCARRIERS

Albumin can incorporate drugs in two main ways:[81] either by covalent binding or noncovalent interaction. The reversible noncovalent binding of drugs to albumin is mostly based on electrostatic/hydrophobic interactions. This mode of interaction is usually preferred because it allows faster availability and release of the drug where and when needed. However, several studies have also provided evidence for the potential of albumin in covalent drug interactions. Of note is a study from the University of Torino,[82] which demonstrated the efficiency of an albumin-based vehicle containing covalently attached paclitaxel, making the formulation more stable. In this study, the 2-hydroxyl group of paclitaxel was esterified with succinic anhydride and derivatized to give the *N*-hydroxy-3-sulfosuccinimide active ester, which then reacted with the free amine group of lysine in albumin structure. Because of the strong covalent binding, after bolus intravenous administration,

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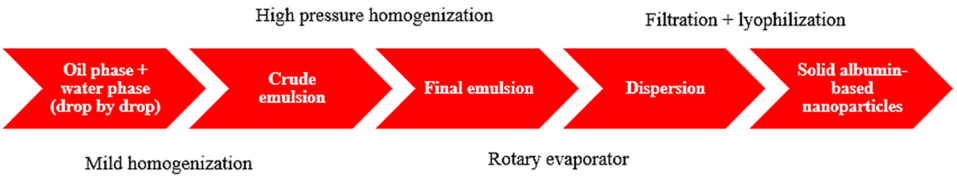

Figure 6. NAB-Technology process.

the conjugate was distributed and slowly eliminated, compared to the free drug that was cleared much faster ($t_{1/2}$ β = 8.39 h for the conjugates, compared to 1.3 h for the free drug). Paclitaxel plasma concentration in mice at 24 h was around 1 μM for albumin-paclitaxel conjugate and 0.6 μM for the free drug. The enhanced pharmacokinetic profile of the conjugate was also shown by the increased AUC (256.9 ± 18.5 μMh for the conjugate, compared to 68.7 ± 4.5 μMh for the free drug) and MRT (4.85 ± 0.5 h for the conjugate, compared to 1.13 ± 0.12 h for the free drug). In another study, the use of an acid-sensitive hydrazine linker, which can be cleaved upon delivery at acidic pH of extracellular space or intracellular organelles at tumor site, has been pursued.[83] The use of ligands that noncovalently bind to albumin represents an effective approach to prolong the elimination half-life of small biotherapeutics with improved pharmacokinetics by sterically shielding from proteolytic degradation and protecting from rapid renal filtration.[84] For example, conjugation of exendin-4 with ABD35 (a protein derived from a naturally occurring 46-residue three-helix bundle albumin binding domain of nanomolar affinity for HSA[85]) showed a 32-fold increase in its plasma elimination half-life in rats. IL-1rα (interleukin-1 receptor antagonist) plasma elimination half-life of 2 min achieved 4.3 h after conjugation with an albumin-binding molecule. IFN-α2 (interferon alpha-2) has a half-life of 1.2 h when administered intravenously, whereas when carried by albumin, it has a blood residence time of 22.6 h. Furthermore, a reversible, noncovalent interaction with albumin allows the faster dissociation of the biotherapeutics, promoting its interaction with the target site and its faster penetration and diffusion into regions that would not allow the entrance to larger molecules.

6. NANOPARTICLE ALBUMIN-BOUND (NAB)-TECHNOLOGY

NAB-Technology is one of the most used and popular techniques involving albumin. It is a modified version of the emulsion method described before. NAB-Technology is a nanotechnology-based drug delivery system that takes advantage of the intrinsic properties of albumin to obtain a selective and efficient delivery of hydrophobic drugs without using toxic solvents.[77] This breakthrough drug delivery platform allows us to overcome the limitations imposed by the hydrophobic nature of many chemotherapeutic drugs, such as paclitaxel or docetaxel. It was developed to solve the problems that the conventional formulations of these drugs had raised. Taxol (cremophor-ethanol-based paclitaxel[78]) and Taxotere (polysorbate 80-ethanol based docetaxel) have shown acute toxicity, including neuropathy, and hypersensitivity reactions, which are partly due to due to the use of cremophor and ethanol for the former formulation, and polysorbate 80 and ethanol for the latter.[79] It would then be necessary to administer premedication to prevent the deleterious side effects, and as a consequence, the maximum tolerated dose of the drug is low. Another reason why nanoparticle formulations are superior to the alternatives is that solubilizing agents such as cremophor and ethanol can leach plasticizers from PVC bags, which are the commonly used infusion systems. The NAB-technology process is based on an emulsion-based method described before. As shown in the process in the scheme in Figure 6, an oil phase (containing the drug) is added dropwise to an aqueous phase (containing HSA/BSA, presaturated with 1% chloroform), and the mixture is subjected to mild homogenization at low rpm to form a crude emulsion. The final emulsion is obtained by using a high-pressure homogenization, and after transferring the mixture in a rotary evaporator, the solvent is removed, and the nanosuspension is produced. It is translucent, and the drug-loaded albumin nanoparticles have a diameter of hundreds of nm (generally <200 nm). A 0.22 μm filter is used to control the size of the nanoparticles and to sterilize the formulation, filtering out the impurity and bacteria. After that, the nanoparticles are lyophilized (without adding any cryoprotectant) to obtain solid powders. The original dispersion can be recreated by the addition of water or saline to the solid nanoparticles.

6.1. Nab-Paclitaxel (Abraxane). Paclitaxel is an antimitotic drug that belongs to the family of Taxanes, which are microtubule stabilizers. They bind to the β-tubulin chain and enhance polymerization, inhibiting mitosis, motility, and intracellular dynamics, resulting in cell death (apoptosis). Paclitaxel summary formula is $C_{47}H_{51}NO_{14}$, and its molecular weight is 853.91 g/mol.[86] It is hydrophobic, and its melting temperature is 216−217 °C. Nab-paclitaxel (Abraxane for injectable suspension, ABI-007 manufactured by Abraxis Bioscience) is a formulation constituted of paclitaxel loaded albumin-based nanoparticles obtained through NAB technology.[80] This technology was FDA approved in 2005, and it has been commercialized to increase the efficiency and targeting, while reducing the side effects due to the solubilizing agents in the conventional formulation of paclitaxel, previously discussed. It is currently used for the treatment of metastatic breast cancer, nonsmall cell lung cancer, metastatic adenocarcinoma of the pancreas (in this case, a combination of Nab-paclitaxel with gemcitabine, an antimetabolite, shows optimal results), bladder cancer, and gastric cancer (in Japan). The formulation is composed of three-dimensional nanoparticles (size of approximately 130 nm) of paclitaxel encapsulated, in an amorphous state, in HSA, through noncovalent hydrophobic interactions. It is free of any toxic solvents/surfactants, and its ζ potential is −31 mV, which shows stability in the aqueous phase.

6.1.1. Clinical and Preclinical Advantages of Abraxane over Taxol. Abraxane (or ABI-007) can be injected at concentrations of 2−10 mg/mL, compared to the 0.3−1.2 mg/mL of Taxol. For this reason, the volume and infusion time of Taxol is higher (3−24 h compared to 30 min for Abraxane), making its administration time-consuming. The

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Table 2. Blood Pharmacokinetics Variable of Two Paclitaxel Formulations: Abraxane and Taxol (Animal Studies)[a,b]

	Abraxane		Taxol		
variable	5 mg/kg(radioactivity)	5 mg/kg(HPLC)	5 mg/kg(radioactivity)	5 mg/kg(HPLC)	10 mg/kg(radioactivity)
$T1/2$ (h)	19.01	11.42	20.78	7.24	7.58
T_{max} (h)	0.033	0.033	0.033	0.033	0.033
C_{max} (μg/mL)	4.2	4.0	13.5	11.8	32.3
C_0 (μg/mL)	8.1	7.3	20.5	17.7	40.2
AUC_{last} (μg h/mL)	6.14	3.78	10.44	5.60	36.43
AUC_∞ (μg h/mL)	9.86	4.59	15.69	5.85	41.13
$AUC_{\infty\%extrap}$ (%)	37.80	17.6	33.4	4.3	11.4
V_z (L/kg)	14.18	18.33	9.36	8.75	2.66
CL (L/h kg)	0.517	1.112	0.312	0.837	0.243

[a] $T_{1/2}$, elimination half-life; T_{max}, time to the maximum concentration; C_{max}, maximum concentration; C_0, concentration at time zero; AUC_{last}, area under the curve to the last time point; AUC_∞, area under the curve to infinity; $AUC_{\infty\%extrap}$, percentage of AUC_∞ extrapolated after the last quantified concentration; V_z, the volume of distribution; CL, total body clearance. [b] The methods used for blood paclitaxel evaluation are radioactivity and HPLC. From ref 77 with permission.

Table 3. Blood Pharmacokinetics Variables of Abraxane and Taxol (Human Studies) from Ref 77[a]

	Abraxane 260mg/m^2, 30 min(n = 14)			Taxol 175mg/m^2, 3 h(n = 12)		
variable	mean	% CV	range	mean	% CV	range
$T1/2$ (h)	21.6	17.2	16.5−29.6	20.5	14.6	17.5−26.3
T_{max} (h)	0.36	45.3	0.0−0.5	2.65	27.6	1.0−3.5
C_{max} (ng/mL)	22968.6	112.5	4060−86700	3453.3	57.2	1540−9380
C_{maxda} (ng/mL)	88.69	114.2	15.64−346.8	20.14	55.8	8.8−52.4
V_{ss} (L/m^2)	230.7	54.3	53.2−492.9	156.3	43.2	99.7−346.0
λ_z (h^{-1})	0.033	16.9	0.023−0.042	0.034	13.0	0.026−0.040
$AUC_{\infty\%extrap}$ (%)	2.8	41.3	1.0−5.0	2.8	52.6	1.4−6.8
AUC_∞ (ng h/mL)	14788.6	45.3	5981.7−28680.2	12602.7	21.0	60871.1−17081.2
$AUC_{\infty da}$ (ng h/mL)	56.84	46.3	23.04−114.7	71.90	21.1	34.78−98.00
V_z (L/m^2)	663.8	48.1	296.3−1347.3	433.4	31.1	309.7−809.7
CL (L/h m^2)	21.13	43.8	8.72−43.41	14.76	31.8	10.20−28.75

[a] $T_{1/2}$, elimination half-life; T_{max}, time to the maximum concentration; C_{max}, maximum concentration; C_{maxda}, dose-adjusted C_{max}; V_{ss}, the volume of distribution at steady state; λ_z, rate constant of the terminal phase; $AUC_{\infty\%extrap}$, percentage of AUC_∞ extrapolated after the last quantified concentration; AUC_∞, area under the curve to infinity; $AUC_{\infty da}$, dose-adjusted AUC_∞; V_z, the volume of distribution; CL, total body clearance.

maximum tolerated dose for Taxol is 175 mg/m^2, whereas for Abraxane, it is 300 mg/m^2. For an every-3-weeks regimen, the dose is 80 mg/m^2 for Taxol versus 150 mg/m^2 for Abraxane in a weekly regimen. Preclinical and clinical studies were conducted to highlight the differences in the pharmacokinetics between the two formulations.[77] Radioactive Abraxane and Taxol were prepared and given both to Harlan Sprague−Dawley male rats and 27 patients with advanced solid tumors (260 mg/m^2 of Abraxane, 30 min and 175 mg/m^2 of Taxol , 3 h) with cycles repeated every 3 weeks. For the animals, blood samples were collected from the tail vein of each rat at specific time points, and the radioactivity and tissue distribution were determined after having homogenized large tissues and organs in distilled water. For the patients, blood samples were taken at serial time points. The results obtained from the animal studies were the following. The total body clearance (CL) and the volume of distribution (V_z) were higher in Abraxane compared with Taxol. Both the formulations showed a gradual buildup of circulating paclitaxel metabolites, expressing a similar rate of metabolite formation. The distribution of paclitaxel in tissues was similar for both formulations, and the tissues containing the greatest drug concentration were the liver, carcass, and gastrointestinal tract. The excretion of paclitaxel was mainly through the faecal route and was similar for both formulations. After 48 h, the excretion was almost complete (Table 2). In animals, the LD_{50} and maximum tolerated dose for Abraxane and Taxol were 47 and 30 mg/kg/d and 30 and 13.4 mg/kg/d, respectively.

In the human studies, of the 27 patients enrolled in the trial, 26 had complete data for pharmacokinetic analysis. Fourteen of them were administered Abraxane at a dose of 260 mg/m^2 for 30 min, and the remaining 12 received Taxol at a dose of 175 mg/m^2 for a 3 h infusion preceded by premedication. As expected, C_{max} is obtained at the end of the administration for both formulations. T_{max} was less for Abraxane than for Taxol, highlighting the shorter infusion time of the former. The CL and V_z of paclitaxel were higher in Abraxane than in Taxol (Table 3).

Phase I trials reported no infusion-related acute hypersensitivity reactions, mild and not cumulative hematological toxicity.[87] Also, phase II studies showed no severe hypersensitivity reactions despite the lack of premedication, and the toxicities noted were typical of paclitaxel and included grade 4 neutropenia, grade 3 sensory neuropathy, and grade 4 febrile neutropenia.[88] In a phase III study comparing Abraxane (260 mg/m^2) and Taxol (175 mg/m^2 every 3 weeks) in 454 patients with metastatic breast cancer, the patients' response was higher with Abraxane, and the incidence of grade 4 neutropenia was lower with Abraxane than with Taxol (9% vs 22%).[89] The results obtained by phase III studies confirm the outcomes of phase I/II that claim the use of Abraxane improves both the toxicity and the efficacy of the drug. Despite an increasing dose

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of paclitaxel, the incidence of grade 3 and 4 neutropenia was significantly lower with Abraxane compared to Taxol. Grade 3 sensory neuropathy was higher with Abraxane but easily manageable. Abraxane shows distinct linear pharmacokinetics, characterized by rapid tissue distribution, increased distribution volume, and a higher rate of clearance.[90] It resulted in being more effective than Taxol, with an IC_{50} (related to a hepatocellular carcinoma cell line) being 15-fold lower than that of paclitaxel alone.[91] The noncovalent bond between albumin and paclitaxel and the presence of the drug in a noncrystallized amorphous state allows access of the drug to the target site.[92] Preclinical results showed that, following equal doses of paclitaxel injected intravenously (20 mg/kg), transcytosis of Abraxane across endothelial cell monolayers was increased compared with Taxol, and the albumin-based formulation achieved 33% higher ($P < 0.0001$) intratumoral paclitaxel concentration in mice bearing human breast tumor xenografts (MX-1 tumor-bearing nude mice). Paclitaxel biodistribution was monitored by radioactivity. In live human umbilical vascular endothelial cells (HUVEC), binding and transport across the endothelial layer were higher (respectively, 9.9- and 4.2-fold) with Abraxane, but this difference is inhibited by methyl β-cyclodextrin, an inhibitor of the endothelial gp60 receptor and caveolar-mediated transport.[93] A key factor is that the tumor itself requires albumin; the protein is highly accumulated in tumors and inflamed tissues, because it plays a vital role in their proliferation and sustainment, functioning as a nutrient source. On the other hand, Taxol showed nonlinear pharmacokinetics, slower tissue distribution, reduced activity and efficacy as well as increased toxicity. The presence of paclitaxel in plasma is of the greatest importance for the antitumor activity of the drug. The CrEL micelles hinder the release of the compound and the slower elimination from the blood determines protracted systemic drug exposure and a higher risk of neutropenia. Overall, the preclinical and clinical studies conducted on paclitaxel demonstrated that the properties of the drug are widely influenced by the composition of the pharmaceutical preparation.[94] To sum up the evidence, the potential advantages of Abraxane over Taxol in preclinical animals are higher tolerated dose and consequent greater efficacy, longer drug residence in the tumor, reduced infusion time, reduced risk of hypersensitivity and no required premedication, and more rapid paclitaxel tissue distribution, based on pharmacokinetic data. In human, advantages of Abraxane over Taxol include the avoidance of solvents/solubilizing agents in its formulation, an acceptable toxicity profile and most importantly, limited taxane cross-resistance, as demonstrated in a study conducted on women with metastatic breast cancer, between 2006 and 2010.[95] Even though Abraxane has been formulated to overcome the limitations imposed by the cremophor vehicle used in solvent-based paclitaxel and has attracted much attention, it certainly still has some drawbacks, mainly due to the drugs that it carries. Examples are some reported cases (much less recurrent than in Taxol) of neutropenia, anemia, dyspnoea, flushing, hypotension, chest pain, arrhythmia, cardiovascular side effects, arthralgia, myalgia and gastrointestinal side effects. However, it is important to highlight the fact that these side effects are mainly drug-related, and they can potentially be overcome through further optimization of the nanoformulation.

7. OTHER ALBUMIN-BASED FORMULATIONS OF PACLITAXEL

Apart from the already FDA-approved Nab-Paclitaxel, paclitaxel has been tested and investigated in different albumin-based formulations. In particular, paclitaxel has also been delivered to the target sites in combination with other chemotherapeutic agents to obtain an enhanced synergistic antitumor effect, overcoming the chemoresistance of the tumor. For example, paclitaxel-loaded albumin nanoparticles were incorporated in PEGylated hybrid liposomes encapsulating curcumin, and the two agents were released sequentially. The dual-drug-loaded nanocarriers resulted in a superior cytotoxic activity compared to that of the paclitaxel-loaded albumin nanoparticles alone. Another example of codelivery is the combination of pirarubicin (a DNA intercalation agent and inhibitor of DNA topoisomerase II) and paclitaxel in HSA nanoparticles to improve the antitumor efficacy and reduce toxicity in breast cancer.[96] This combination not only improved the efficacy by overcoming tumor resistance, but it also exhibited synergistic cytotoxicity (the drug ratios of 2:1, 1:1, and 1:2 all showed synergistic effects), higher drug accumulation at tumor sites, and decreased distribution in off-target organs and tissues, resulting in higher efficacy and reduced toxicities (10% higher apoptosis compared to the curcumin-loaded albumin nanoparticles and 25% higher apoptosis compared to paclitaxel-loaded albumin nanoparticles). The delivery of the two drugs, as already known, would cause various deleterious side effects, such as cardiotoxicity, neurotoxicity, and leukopenia. For these reasons, HSA nanoparticles allow the simultaneous delivery of multiple drugs with low toxicity and immunogenicity. The *in vivo* antitumor effect was evaluated in a xenograft 4T1 murine breast cancer model. The combination administration of the two compounds showed a better inhibitory effect against tumor growth than the single-drug treatment. A study conducted at the University of California[97] allowed the development of the formulation of Nab-paclitaxel modified with the two tumor-homing peptides, CREKA (cysteine-arginine-glutamic acid-lysine-alanine) and LyP-1 (Cys-Gly-Asn-Lys-Arg-Thr-Arg-Gly-Cys). This new formulation showed greater tumor inhibition compared to the untargeted Abraxane on mice bearing MDA-MD-435 human cancer xenografts. CREKA is a pentapeptide that binds to clotted plasma proteins, and it targets tumors because the interstitial tissue of tumors and the vessel wall contain clotted plasma proteins. LyP-1 is a cyclic nine-amino acid peptide that targets p32, a protein overexpressed in tumors cells and lymphatics. Peptide-Abraxane conjugates were produced by binding peptides to Abraxane through their cysteine sulfhydryl group using a sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate) cross-linker. Abraxane was suspended in nitrogen-purged sterile PBS (5 mg/mL), and 0.3 mg of sulfo-SMCC was added. After 30 min, the excess cross-linker was removed by filtration (Nap-10 column), and a peptide labeled with FAM was dissolved in sterile nitrogen-purged water and added to the Abraxane-sulfo-SMCC conjugate for 1 h. Finally, the excess peptide was removed by repeated washing using an ultracentrifuge. The two peptide-coated Abraxane formulations were tested for *in vivo* homing to MDA-MB-435 xenograft tumors grown in nude mice. The coating of the surface of Abraxane with tumor-homing peptides further increased the accumulation of albumin-bound paclitaxel in the target sites.

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Quantification of fluorescence intensities showed that LyP-1 Abraxane delivered 4-folds more fluorescence into the tumors than unmodified Abraxane (P = 0.007), whereas CREKA-Abraxane was not significantly different from the unmodified Abraxane. The LyP-1 particles were not detectable in blood vessels but accumulated outside the blood vessels in islands of cells positive for p32, the known receptor for this peptide. Future studies will investigate the possible synergistic effect of LyP-1-Abraxane and CREKA-Abraxane since the two peptides deliver the formulation to nonoverlapping sites in tumor tissue, and combining them should result in a broader distribution of the drug than either one alone. Another example of decorated-Nab-paclitaxel is an antibody-nanoparticle conjugate constituted of trastuzumab (Herceptin) and paclitaxel-loaded albumin-based nanoparticle for the targeting of human epidermal growth factor receptor 2 (HER2) positive gastric cancer.[98] Trastuzumab is a monoclonal antibody that targets HER2 (expressed by 20% of the gastric tumors). The conjugates were produced by "one-step" synthesis using EDC/NHS, attached by covalent bonding, they had a spherical shape, and their size was 139.18 ± 32.06 nm. Compared with untargeted Abraxane, Trastuzumab/Nab-paclitaxel generated higher tumor apoptosis, evaluated both *in vitro* and *in vivo*: free paclitaxel, nab-paclitaxel, and trastuzumab/nab-paclitaxel were tested in HER2-positive GC NCI-N87 cells at 48 h. The IC_{50} was found to be 0.24 ± 0.08, 0.13 ± 0.03 and 0.048 ± 0.01 μg/mL of paclitaxel, nab-paclitaxel and trastuzumab/nab-paclitaxel, respectively, with excellent dose−effect relationship. In the same way, the apoptosis rate was higher in the trastuzumab/nab-paclitaxel group (51.30 ± 2.28%) than in the paclitaxel group (43.34 ± 1.08%) and nab-paclitaxel group (46.64 ± 1.47%) in NCI-N87 cells. In 2018, Ge et al. followed the example of Nab-paclitaxel and optimized the formulation by using human albumin fragments nanoparticles as a paclitaxel carrier to improve the anticancer efficacy.[99] Human albumin was broken into fragments via degradation and then cross-linked by genipin (at various concentrations) to generate HSAF nanoparticles, achieving reduced toxicity, due to the use of the naturally biocompatible cross-linker, compared to the widely used glutaraldehyde. In particular, HSA was dissolved in deionized water before 70% formic acid was added. CNBr was added to degrade the HSA into fragments, which were then separated and purified. The obtained fragments were used to prepare the PTX-loaded-HSAF NPs, which were first cross-linked using a disulfide bond and then using genipin as a second cross-linking agent (1 h). After the excess of cross-linkers was removed, the HSAF NPs were collected by lyophilization. The nanoparticles obtained with albumin fragments had a smaller size compared to the ones obtained with albumin, and the paclitaxel loading efficiency was higher (7−8.3% in HSAF nanoparticles compared to 5.1−6.5% in HSA nanoparticles). Furthermore, paclitaxel was released more slowly from HSAF nanoparticles; within 48 h, less than 50% of paclitaxel was released from HSA nanoparticles, but only less than 25% from HSAF nanoparticles, showing that HSAF nanoparticles could provide better ability to deliver the drug in the specific organ, reducing the off-target delivery. The activity of PTX-loaded HSAF was evaluated *in vivo* in nude mice. After 28 days of treatment, the results demonstrated that the tumors were significantly (P < 0.01) inhibited after being treated with HSA NPs and HSAF NPs compared to free PTX. The tumor inhibitory rate in mice treated with HSA NPs and HSAF NPs were 63.3 and 71.4, respectively. The dose of HSAF used in the NPs was lower than that of HSA. However, the effect was better, and the tumor volume was the smallest. Chen et al.[100] synthesized cationic BSA nanoparticles assembled with hyaluronan (HA) via simple electrostatic interactions in various size distributions and structures and used them to encapsulate paclitaxel for the treatment of metastatic lung melanoma. They tested hyaluronans with different molecular weights and found out that the 49 kDa HA achieved the best results, in terms of size, stability, and drug loading. The obtained spherical nanoparticles (HNPs) showed an average particle diameter of 331.4 ± 5.29 nm (PDI 0.051 ± 0.021) and ζ potential of −36.0 ± 2.0 mV, and the encapsulation efficiency of paclitaxel was found to be 74.5% ± 3.0% (wt %). The formulation exhibited higher cytotoxicity, showing IC_{50} values of 12.96 ± 1.34 μg/mL in comparison to the values of 19.04 ± 4.12 μg/mL and 28.34 ± 5.28 μg/mL of Taxol and PTX-NPs, respectively, against B16F10 cells. Furthermore, they showed enhanced cell uptake, most likely due to the presence of HA on the surface of the nanoparticles, which could selectively target B16F10 cells via CD44 receptor-mediated endocytosis. The *in vivo* distribution of HNPs in mice was investigated using a near-infrared fluorescent dye (DiD), and the HNPs showed significantly higher lung accumulation at 1, 2, and 4 h postinjection than that of DiD-loaded NPs and DiD solution. Gharbavi et al.[101] designed a versatile and smart microemulsion system for paclitaxel delivery, using folate conjugated- BSA NPs for its development. Microemulsions are typically made by four components: the surfactant, cosurfactant, oil, and water phase. One of the drawbacks of the use of microemulsions is the difficulty in surface functionalization. Hybridization of MEs with functionalized BSA NPs has been proposed as a novel strategy to overcome this limitation, leading to efficient modified MEs. Therefore, in this study, FA-conjugated BSA-PEG NPs were used as a fraction of the MEs' aqueous part, providing stability through the negative charge and possibility of active targeting, since the FA receptor is generally overexpressed in breast cancer cells. The cytotoxicity of the formulation was evaluated on MCF7 cell line at 48 h. The results showed that the PTX ME FA-BSA-PEG NPs exhibited an IC_{50} of 6.04 nM on the cell line, compared to the IC_{50}s of 12.04 nM and 10.80 nM of PTX ME and PTX ME BSA NPs, respectively.

8. CROSS-LINKING OF ALBUMIN-BASED NANOPARTICLES

Typically, the albumin-based nanoparticles are prepared by coacervation, emulsification, or thermal gelation methods, which are followed by a cross-linking step with an aldehyde-group containing reagent, such as glutaraldehyde, to increase the stability and limit the degradation rate and hydration potential of the resulting nanoparticles. Abraxane is a non-cross-linked albumin-bound paclitaxel formulation that has been approved for the treatment of various cancers. However, a systematic comparison between cross-linked and non-cross-linked albumin-bound paclitaxel nanoparticles was essential to figure out which kind of nanoparticles is more advantageous. To address this issue, Li et al.[102] compared the *in vitro* stability, pharmacokinetics, biodistribution, and efficacy of two formulations of non-cross-linked and cross-linked paclitaxel-loaded albumin-based nanoparticles. The cross-linking had no effect on the size distribution. Both formulations had a mean particle diameter of about 130 nm with a narrow size distribution indicated by the polydispersity index (PDI) < 0.1.

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In vitro disintegration experiments showed that the cross-linked particles, as compared to non-cross-linked nanoparticles, were stable, and no significant disintegration and changes in particle size were observed. The plasma paclitaxel profiles after intravenous injection of both albumin-bound paclitaxel formulations exhibited biexponential kinetics and were significantly different. At 5 min postinjection, the maximal plasma paclitaxel concentrations of the cross-linked formulation were considerably greater than those of non-cross-linked formulation (10.38 ± 2.05 vs 4.32 ± 0.31 μg/mL). Slower clearance of paclitaxel in the cross-linked nanoparticles, as compared to the non-cross-linked nanoparticles, resulted in an increase in the area of paclitaxel plasma concentration−time profile (5.06 ± 0.80 vs 4.08 ± 0.55, μg h/mL) and a longer terminal half-life of the drug (5.94 ± 3.34 h vs 13.24 ± 4.04 h). Overall, the amount of drug located in the tumor was found to be higher for non-cross-linked nanoparticles compared to the cross-linked ones, whereas the amount of drug found in the other off-target organs was lower. Therefore, the targeting efficiency of the nanoparticles into the tumor following treatment with a non-cross-linked particle was greater than that of the cross-linked formulation, resulting in a considerably improved antineoplastic activity. This could be attributed to the higher targeting efficiency of non-cross-linked nanoparticles. The non-cross-linked albumin nanoparticles showed to rely mainly on the gp60/SPARC pathway for internalization, whereas the cross-linked ones depended on the passive targeting (EPR effect). The non-cross-linked formulation was concluded to be more advantageous for the delivery of paclitaxel. Furthermore, after the cross-linking, the albumin could be denatured, and the toxicity of glutaraldehyde and its possible reaction with encapsulated drug make it an undesirable cross-linker, which could also affect the bioactivity of the drug. The researchers Niknejad and Mahmoudzadeh[103] suggested that there are other ways for cross-linking the nanoparticles. If the purpose is to create stable, cross-linked nanoparticles, a possible alternative to glutaraldehyde could be ultraviolet irradiation (physical cross-linking) or glucose (chemical cross-linking). This study investigated the efficiency of these methods and their comparison with glutaraldehyde in a docetaxel-loaded albumin-based formulation obtained by a desolvation technique. The UV used alone showed more toxicity than the cross-linking with glutaraldehyde (cell viability of 16.48% ± 3.87, compared to 40.08% ± 6.6) because of the toxic free radicals generated by the UV irradiation to the nuclei of aromatic residues, such as those in tyrosine and phenylalanine, and also glucose was not better than glutaraldehyde (cell viability of 14.29% ± 3.60, compared to 40.08% ± 6.6). However, when a combination of UV and glucose was used, the cell viability rose to 76.59% ± 7.67 ($P < 0.01$). the free radicals generated by the UV formed complexes with the glucose molecules, giving rise to reactive linear glucose molecules. Therefore, once the toxic free radicals are produced by UV irradiation, they immediately form cross-linked complexes with the free glucose molecules, thus reducing the toxicity of the system. In 2019, Amighi et al.[104] investigated the effects of different "natural" cross-linking agents such as tannic acid, ascorbic acid, citric acid, sorbito, and glucose and optimized the particle production in order to obtain the smallest particle size and the most stable particle during storage. The different cross-linkers had no significant impact on efficiency, but they affected the size, the stability and the PDI of nanoparticles. By using ethanol instead of acetone as desolvating agent, it was possible to achieve smaller nanoparticles. The nanoparticle size obtained were 368 ± 16.1 nm (without cross-linker), 76 ± 1.2 nm (with glutaraldehyde), 125.1 ± 3.5 nm (for glucose), 150.3 ± 2.5 nm (with sorbitol), 905.8 ± 8.1 nm (for ascorbic acid), 1201 ± 58.4 nm (for citric acid), and 240.4 ± 3.2 nm (for tannic acid). As it is possible to observe, the smallest size was related to the glutaraldehyde, whereas the most significant sizes belonged to the citric acid and tannic acid. This trend is explained by the ζ potential results, which showed that particles produced using glutaraldehyde, glucose and sorbitol were more stable (ζ potential values of −18.19 ± 0.47 mV, −23.29 ± 2.27 mV, and −19.1 ± 0.47 mV, respectively) than the ones produced with other cross-linkers, such as ascorbic acid, citric acid and tannic acid (ζ potential values of 2.54 ± 0.17 mV, 8.13 ± 0.93 mV and −9.29 ± 0.67 mV, respectively), leading to a bigger size and PDI. Furthermore, the cross-linker addition changed the surface charge of nanoparticles, modulating the electrostatic potential of the nanoparticle. The cross-linking of protein occurred by nucleophilic attack of the amino groups of lysine and arginine residues in albumin to two carbonyl groups of the cross-linker, forming Schiff bases in solution, which showed to be stable at basic pH but unstable in acidic conditions. After having observed the effects of the different cross-linkers, the researchers concluded that glucose-cross-linked nanoparticle had the same physical characteristics as nanoparticles prepared with glutaraldehyde and that could be considered as a nontoxic alternative for glutaraldehyde. Weber et al.[105] used a desolvation process of HSA for the preparation of nanoparticles and then stabilized them with varying amounts of glutaraldehyde. Modulating the percentage of cross-linker, the particle size and the number of the available free amine groups on the surface of the nanoparticles were determined. The results pointed out that the cross-linker did not influence the particle size, but the number of free amine groups on the surface was affected. The objective of the research was to develop the albumin-based nanoparticle via the desolvation method and investigate the possibilities of surface modification in order to obtain covalent drug attachments. Different amount of aldehyde concentrations in the range between 0 and 200% of the theoretic amount, needed to cross-link all of the 59 amino groups in the HSA molecule, were added to the formulation. The lowest required glutaraldehyde concentration for the generation of stable nanoparticles was 40%. If the purpose was to prepare a drug delivery system characterized by the covalent binding of the drug to the carrier, the number of free amino groups on the particle surface would be very important. The results showed that this number decreased significantly with increasing dosage of glutaraldehyde, affecting the capability of the drug to create covalent attachments. A possible solution to overcome this phenomenon and preserve some amino groups for further modifications or binding has been found in a study conducted by Bae et al.[106] The surface of HSA nanoparticles loaded with doxorubicin prepared via desolvation method was modified with tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and transferrin. TRAIL is a homotrimeric type 2 transmembrane protein−ligand on the TNF receptor superfamily, which binds to two death receptors overexpressed in cancer cells. Furthermore, it is a safe anticancer agent that has little or no toxicity to normal cells. Transferrin, on the other hand, induces receptor-mediated endocytosis into tumor cells. To reversibly protect some amine-groups during the cross-linking step,

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albumin was preconjugated with dimethyl maleic anhydride, allowing the conjugations of TRAIL or transferrin to the preserved surface amines of HSA-nanoparticle. An alternative was provided by a group of Italian researchers,[107] who developed HSA nanoparticles as nose-to-brain carriers and achieved cross-linking through electrostatic interactions by using natural substances, such as chitosan (CS). CS-HSA nanoparticles were obtained by the electrostatic interaction between negatively charged HSA nanoparticles and positively charged low molecular weight CS. The positive surface charge of CS-HSA nanoparticles was required for the stability of the formulation, providing an electrostatic repulsion and preventing the aggregation, and for the interaction with negatively charged cell membranes. A further solution was provided by Wang et al., who developed a novel self-cross-link strategy to fabricate the HSA-nanoparticle by a reduction and desolvation method without using any toxic chemicals.[108] They worked on the formation of intramolecular disulfide bonds between the albumin sulfhydryl groups, which could not only stabilize the nanoparticles against dilution, but also make the particles have the redox responsiveness in the reducing environment. The obtained self-cross-linked nanoparticles were stable in the blood circulation system and could be degraded by glutathione (which broke up the intramolecular disulfide bonds) to release the payloads in the cytoplasm of cells. Therefore, the developed nanoparticle was still dissolvable in the physiologically relevant reducing environment and released albumin monomers. Another study that used the desolvation technique to produce cross-linked salicylic acid-loaded BSA nanoparticles took place in Brazil in 2016.[109] Salicylic acid and salicylates come from plants and are characterized by anti-inflammatory, antibacterial, and antifungal properties. Furthermore, they have been used for the prevention of cardiovascular disease and cancer. They have often been incorporated into specific drug delivery systems in order to improve their therapeutic index. Sustained and slow releasing of salicylic acid is more appropriate for lowering the risk of cancer and cardiovascular diseases, whereas rapid release is required for antimicrobial and anti-inflammatory effects, such as the treatment of infections. In the study, Bronze-Uhle et al. synthesized BSA nanoparticles by a desolvation process, using salicylic acid as the active agent. The experiment demonstrated that the process of protein nanoparticle formation depended on the amount of added desolvating agent, pH of the starting solution and amount of cross-linking agent (glutaraldehyde, in that case). In this process, the glutaraldehyde added was part of the protein surface nanoparticles through covalent bonds, and the nanoparticles resulted in being nontoxic. However, the free glutaraldehyde in the solution might exhibit toxicity. Therefore, the unreacted glutaraldehyde remaining in the solution after the cross-linking process was removed by centrifugation and purification of the nanoparticles obtained. As already seen in the previous studies, this research reported that pH change and glutaraldehyde addition modified the surface charge of nanoparticles. In fact, Langer et al.[110] demonstrated the effect of cross-linking on the isoelectric point of HSA nanoparticles, depending on the amounts of cross-linking agent used. The nanoparticles were cross-linked with 0.235, 0.588, or 1.175 μL of an 8% aqueous solution of glutaraldehyde per mg of albumin, which corresponds, respectively, to 40, 100, and 200% of the calculated amount required for the quantitative cross-linking of the 59 amino groups of lysine in the HSA matrix. No influence was seen on the particle size, whereas the increase in the glutaraldehyde concentration resulted in the decrease of the isoelectric point of the nanoparticles. Altering the charge on the nanoparticle surface led to the modification of the electrostatic repulsion between molecules and of the hydrophobic interactions, changing the interaction and/or binding of drugs as well as the electrostatic potential and colloidal stability of the formulation. As pointed out from the previous examples, chemical cross-linking allows for the production of a very stable final product, even within the circulatory system.[111] However, as highlighted above by Cui et al., sometimes non-cross-linked formulations exhibit better efficacy than cross-linked ones. Usually, formulations obtained by high-pressure homogenization are not cross-linked, and the comparison between the cross-linked nanoparticle and non-cross-linked nanoparticle produced by Nab-Technology showed that the former relied on the EPR effect and were less efficient, whereas the latter took advantage of albumin-mediated gp60/SPARC endocytosis pathways to enter the tumor and had a higher internalization and cytotoxic activity. They disintegrated faster inside the blood vessels, releasing the drug that soon bound to endogenous albumin and was transported inside the tumor through endocytosis. Therefore, fast disintegration and instability is not always an undesirable characteristic of a drug delivery system. If the formulation relies on internalization via gp60/SPARC pathways, which is more efficient than passive targeting,[112] it is useful to immediately have a free drug that is able to form complexes with endogenous albumin and reach the target site. On the other hand, if the formulation depends on the EPR effect, it is preferable to have more stable nanoparticles and, therefore, longer circulation residence time can be achieved with the nanoparticle formulation. Conversely, formulations generated by desolvation methods are almost always followed by a cross-linking step that stabilizes the nanoparticles. This type of technique is simpler than Nab-Technology and the elimination of the chlorinated organic solvents makes the formulation safer for intravenous injection.

9. ALBUMIN-BASED FORMULATIONS FOR THE DELIVERY OF OTHER THERAPEUTIC AGENTS

9.1. Docetaxel. Docetaxel is an FDA-approved antimitotic chemotherapeutic agent derived from taxoid, which promotes microtubule stabilization leading to cell cycle arrest and death.[113] It can be used as a monotherapy or in combination with other anticancer drugs to treat nonsmall cell lung cancer, gastric, breast, pancreatic, and ovarian cancers.[114] Similar to paclitaxel, its hydrophobicity and low biocompatibility have limited its therapeutic applications. As already described, it was originally formulated with Polysorbate 80 and ethanol (50:50), but these organic solvents were demonstrated to cause hypersensitivity reactions and harmful side effects, such as muscle pain, joint pain, diarrhea, constipation, vomiting, nausea, injection site reactions (pain, redness, or swelling), temporary hair loss and fingernail or toenail changes. Qu et al.[115] demonstrated the efficacy of docetaxel-loaded albumin-based nanoparticles and investigated the requirements for the optimization of the formulation. The independent variables chosen as parameters influencing the resulting formulation were the drug-to-HSA (w/w) ratio, the duration of HSA incubation, and the choice of the stabilizer. The particle diameter increased with the incubation time, with the drug-to-HSA ratio and with the use of sodium gluconate over sodium tartrate as a stabilizer. Modulating these parameters, Gao et al.

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tried to reach the optimized formulation. The final size of the docetaxel-nanoparticles remained below 200 nm, the cellular uptake resulted to be higher compared to the free drug, and the maximum tolerated dose was almost 75 mg/kg for the docetaxel-loaded nanoparticles, compared to the 30 mg/kg for the free docetaxel, in mice. Docetaxel-loaded albumin nanoparticles were also used successfully by Ertugen et al.,[116] labeled with ^{131}I, in both nuclear imaging and radiotherapy of prostate cancer. In 2015, Tang et al.[117] developed docetaxel-loaded HSA nanoparticles by a novel, simple self-assembly method. The efficacy was evaluated both *in vitro* and *in vivo*. A549 xenograft mice were intravenously injected with the formulation. At 7.5 mg/kg, DTX-NPs exhibited an extremely significant tumor inhibition rate (62.64%), compared to the 52.32% of free DTX. A further improvement of docetaxel-loaded nanoparticles is to functionalize the surface of the albumin-based nanoparticles with ligands able to recognize specific receptors on the surface of the tumor. In the case of docetaxel, Cheng et al.[118] formulated biotin-decorated docetaxel-loaded BSA nanoparticles to specifically target the corresponding receptors of carcinoma. The nanoparticles were prepared via desolvation technique and cross-linked with glutaraldehyde at the end. For the surface decoration, *N*-hydroxysuccinimmide (NHS) ester of biotin was developed and, after being dissolved in carbonate/bicarbonate buffer, was added to the suspension of nanoparticles. The carboxylic groups of NHS-biotin created covalent bonding with amino groups of the BSA-nanoparticle. The results demonstrated that the cellular uptake of the biotin-decorated nanoparticles was much higher than the nondecorated nanoparticles. Moreover, the cytotoxicity of the biotin-decorated nanoparticles in MCF-7 cells, SGC7901 cells, LS-174T cells, and A549 cells resulted in being higher than the one of the free drug with IC_{50} of 0.26 ± 0.07 μM and 0.43 ± 0.04 μM in MCF-7 cells ($P < 0.05$), 0.23 ± 0.06 μM and 0.46 ± 0.04 μM in SGC7901 cells ($P < 0.01$), 0.21 ± 0.04 μM and 0.34 ± 0.04 μM in LS-174T cells ($P < 0.05$), 0.27 ± 0.03 μM and 0.42 ± 0.04 μM in A549 cells ($P < 0.01$), for DTX-BIO-BSA-NPs compared to free docetaxel, respectively).

9.2. Doxorubicin. Doxorubicin[119] is an anthracycline antibiotic with antineoplastic activity, widely used against breast cancer, but also for soft-tissue sarcomas, esophageal carcinomas, and osteosarcoma. There are two main modes of action of doxorubicin in cancer cells. First, it intercalates into DNA, disrupting the topoisomerase-II-mediated DNA repair, and second, it generates free radicals causing damage to cellular membranes.[120] However, its application is limited by some serious side effects, such as dose-dependent toxicity[121] (cardiotoxicity), low specificity, and drug resistance. The FDA-approved doxorubicin nanoformulation is a PEGylated liposomal platform[122] which reduces the side effects of doxorubicin and prolongs its blood residence time. In 2016, Li et al.[123] developed albumin-based nanoparticles for improving the therapeutic and safety profiles of chemotherapeutic agents. They cross-linked BSA using a Schiff base-containing vanillin into nanoparticles and loaded doxorubicin inside the NPs by incubation, obtaining DOX-BSA-V-NPs. The images obtained by confocal laser scanning microscope (TCS-SP5) demonstrated that the uptake of DOX-BSA-V-NPs by BGC-823 cells resulted in being higher than that of free DOX at 37 °C for 4 h. The IC_{50} value of DOX-BSA-V-NPs was 3.693 ± 0.525 μg/mL, compared to 4.007 ± 0.378 μg/mL of free DOX in BGC-823 cells. To evaluate the antitumor effect of the NPs, *in vivo* studies on Heps tumor-bearing mice were performed. The results demonstrated that the NPs were more effective than the free DOX to increase the lifespan of the tumor-bearing mice (+39.29% for DOX-BSA-NPs and +57.14% for DOX-BSA-V-NPs) and the group treated with the DOX-BSA-V-NPs had the longest mean survival time (24.83 ± 6.43 days, compared to 19.0 ± 4.34 days for DOX-BSA-NPs and 15.33 ± 1.75 days for free DOX). Furthermore, the results showed that the tumor volume of the mice treated with DOX-BSA-V-NPs was smaller than the mice treated with free DOX (0.760 ± 0.4666 cm^3 compared to 1.42 ± 1.19 cm^3, respectively), whereas no significant difference was found in this study between the tumor volume of the two nanoparticles. However, the tumor tissues treated with DOX-BSA-V-NPs had the largest necrotic area. A recent study conducted in 2020 developed HSA-DMDOX (a pH-sensitive prodrug of DOX) nanoparticles via self-assembly driven by hydrophobic interactions.[124] At pH 6.5, the average IC_{50} values of HSA-DOX and HSA-DMDOX on 4T1 cells were 1.15 and 0.53 μg/mL, respectively. The flow cytometry, used to investigate the cellular uptake, revealed that HSA-DOX and HSA-DMDOX had much higher intensity in fluorescence than that for the DOX group in 4T1 cells. In particular, the two formulations showed slightly higher uptake at pH 6.5 than 7.4 by the same cell line, providing evidence of the efficacy in the acid environment.

9.3. Other Therapeutic Agents. Many other albumin-based nanoparticles have been formulated because of the high versatility of this carrier and its binding to a number of different drugs (Table 4). Cabazitaxel (Cbz) is an active chemotherapy drug for taxane-resistant metastatic castration-resistant prostate cancer (mCRPC).[125] It is a microtubule-stabilizing agent which promotes the polymerization of β-tubulin, leading to cell death. It has shown better antiproliferative activity than docetaxel against chemotherapy-resistant tumor cell lines and better antitumor activity *in vivo*. To overcome the limitations imposed by the hydrophobicity of the drug and the use of toxic organic solvents (such as polysorbate 80) in the clinical product Jevtana, albumin-based nanoparticles have been proposed. Cabazitaxel-loaded HSA-based nanoparticles[126] were produced by a salting-out method and then cross-linked. The nanoparticles demonstrated high efficacy and great advantages compared to the solvent-based formulation, reducing the toxic manifestation of the drug.[127] The therapeutic efficacy of Cbz-NPs and Cbz-Tween in a murine prostate cancer xenograft model bearing human prostate cancer PC3 cells was evaluated. Both formulations suppressed the growth of the tumors, and both of the treatments caused a reduction in body weight after therapy. The mice treated with Cbz-Tween showed slightly greater reduction (around 1 g) in body weight, compared to those treated with Cbz-NPs, but the difference was not statistically significant.

Another compound that has been entrapped in albumin nanoparticles is all-trans-retinoic acid (ATRA),[128] a derivative of retinoic acid, known as vitamin A acid, in a study conducted by Li et al.[129] ATRA is a potent agent involved in various signaling pathways related to stem cell maintenance. It activates retinoic acid receptors, regulates the transcription of genes, and induces differentiation of stem cells. However, ATRA is characterized by low hydrophilicity and instability. Nanoparticle encapsulation can preserve its stability and prevent its degradation. Evidence exists that cancer stem

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Table 4. Albumin-Based Formulations beyond Abraxane

compound	formulation	binding strategy	indication	studies performed	ref
paclitaxel	(1) PEGylated PTX-HSA nanoparticle, (2) PTX/HSA nanoparticle encapsulated in PEGylated liposomes containing curcumin, (3) coloaded PTX-Pirarubicin HSA nanoparticle, (4) CREKA and LyP-1-decorated PTX/HSA nanoparticle, (5) trastuzumab-decorated PTX/HSA nanoparticle, (6) PTX/HSAF (albumin fragments) nanoparticle, PTX/HSAF (albumin fragments) nanoparticle, (7) PTX-loaded cationic BSA nanoparticles assembled with hyaluronan (HA), (8) PTX-loaded microemulsion-folate-conjugated BSA-PEG NPs	(1) physical encapsulation, (2) physical encapsulation, (3) physical encapsulation, (4) physical encapsulation and chemical conjugation with peptide on the surface of NPs, (5) physical encapsulation with chemical conjugation with antibody, (6) physical encapsulation, (7) physical encapsulation, (8) physical encapsulation and microemulsion	metastatic breast cancer, non-small lung cancer, pancreatic cancer, gastric cancer	(1) *in vitro* and *in vivo*, (2) *in vitro*, (3) *in vitro* and *in vivo*, (4) *in vitro* and *in vivo*, (5) *in vitro* and *in vivo*, (6) *in vitro* and *in vivo*, (7) *in vitro* and *in vivo*, (8) *in vitro*	(1) 57, (2) 168, (3) 96, (4) 97, (5) 98, (6) 99, (7) 100, (8) 101
docetaxel	(1) DTX-HSA nanoparticle, (2) biotin-decorated DTX-BSA	(1) physical encapsulation, (2) physical encapsulation with chemical conjugation with biotin on the surface	nonsmall cell lung cancer, gastric, breast, pancreatic, and ovarian cancers	(1) *in vitro* and *in vivo*, (2) *in vitro* and *in vivo*	(1) , 117, 113, 114, and 115, (2) 118
doxorubicin	(1) self-assembled microbubble and albumin-doxorubicin nanoparticles PEI-coated DOX-HSA nanoparticle, (2) DOX-HSA NPs cross-linked by a Schiff base-containing vanillin, (3) HSA-DMDOX (pH-sensitive prodrug of DOX) NPs by self-assembly	(1) physical encapsulation, (2) physical encapsulation, (3) physical encapsulation	breast cancer, soft-tissue sarcomas, esophageal carcinomas, and osteosarcoma	(1) *in vitro* and *in vivo*, (2) *in vitro* and *in vivo*, (3) *in vitro*	(1) 169, (2) 123, (3) 124
cabazitaxel	Jevtana	physical encapsulation	metastatic castration-resistant prostate cancer	*in vitro* and *in vivo*	125, 127, 126
all-*trans*-retinoic acid	ATRA-encapsulated in HSA nanoparticles/codelivery of ATRA and PTX in HSA nanoparticles	physical encapsulation	targeting of cancer stem cells	*in vitro* and *in vivo*	128−131
methotrexate	(1) MTX-HSA, (2) LHRH-decorated MTX-HSA, (3) biotin-decorated MTX-HSA, (4) trastuzumab-decorated MTX-HSA	(1) covalent conjugates, (2) covalent conjugates, (3) covalent conjugates, (4) covalent conjugates	breast cancer, lung cancer, lymphoma, leukemia, inflammatory conditions, autoimmune disorders	(1) *in vitro* and *in vivo*, (2) *in vitro*, (3) *in vitro* and *in vivo*, (4) *in vitro*	(1) 135, 136, and 137, (2) 138, (3) 139, (4) 140
dexamethasone	E-selectin-binding peptide (Esbp)-modified BSA NPs	physical encapsulation	acute lung injury	*in vitro* and *in vivo*	141
lapatinib	lapatinib-HSA NPs	physical encapsulation	triple-negative breast cancer	*in vitro* and *in vivo*	142
atorvastatin	atorvastatin calcium-loaded HSA nanoparticle	physical encapsulation	decrease cholesterol level and prevent cardiovascular diseases	*in vitro*	144
5-FU	5-FU-rHSA-PEG-NPs	covalent conjugates	colorectal cancer	*in vitro*	145, 146
palladium complex with a dioxime ligand derived from isophthalaldehyde	palladium complex-loaded BSA NPs	physical encapsulation	myelogenous leukemia, pulmonary adenocarcinoma	*in vitro*	147−149
sulfasalazine	SSZ-loaded BSA nanoparticles	physical encapsulation	advanced gastric cancer	*in vitro*	150, 151
Kolliphor HS15 and THP	THP-HSA complexes	physical encapsulation	antimetastasis activity	*in vitro* and *in vivo*	152
proaerolysin	HSA/PA	genetic fusion	castration-resistant prostate cancer	*in vivo*	153
fusion inhibitor C34	PC-1505	covalent conjugates	HIV-1	*in vitro*	154, 155

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Table 4. continued

compound	formulation	binding strategy	indication	studies performed	ref
lysostaphin		protein fusion	bacterial infections	*in vitro*	156
Myr FARKALRQ	RH01 (SAFETY technology)	physical encapsulation	bacterial infections	*in vitro*	157
molecular vaccines and Evans blue	albumin/AlbiVax	self-assembly	cancer immunotherapy	*in vivo*	81, 158, 159
piceatannol	PIC-HSA nanoparticle	physical encapsulation	colorectal cancer	*in vitro* and *in vivo*	160, 161
rutin	rutin-loaded BSA nanoparticles	physical encapsulation	antioxidant activity (ROS scavenging)	*in vitro*	162
curcumin	Curc-Alb conjugates	chemical conjugates	breast, lung, hematological, gastric, colorectal, pancreatic, and hepatic cancers	*in vitro* and *in vivo*	163, 164
nutraceuticals (β-carotene)	thermally treated chondroitin sulfate-loaded BSA NPs	physical encapsulation	food technology		165
gambogic acid	gambogic acid-loaded HSA nanoparticles	physical encapsulation	lung cancer	*in vitro* and *in vivo*	166
	BPE-modified BSA vehicles		glioma	*in vitro* and *in vivo*	167

cells (CSCs) play a vital role in tumor progression, sustainment, and chemoresistance. Despite the numerous breakthrough techniques able to fight the tumors by reducing their bulk, there are few developments in the strategies for eliminating CSCs. The surface of cationic albumin nanoparticles has been functionalized with hyaluronic acid (HA), a natural polysaccharide used as a targeting ligand due to its high affinity for the CD44 receptor, overexpressed on the surface of the cancer stem cells in many cancer types. The nanoparticles showed high cytotoxicity and specific target delivery of antitumor drugs to the cancer stem cells.[130] Furthermore, codelivery of ATRA and paclitaxel using albumin-bound nanoparticles exhibited a significantly improved antimetastatic effect on breast cancer, both *in vitro* and *in vivo*.[131] In particular, the *in vivo* antitumor and antimetastasis effects of drug-loaded nanoparticles were evaluated using a 4T1 breast tumor mice model. The drug-loaded HSA nanoparticles were injected intravenously every other day 11 times, and the average tumor size and body weight were monitored. Codelivery in PTX/ATRA-NPs presented higher antitumor effects to all other treatments. Codelivery of PTX and ATRA showed more pronounced synergistic effects than the simple mixture of the two drugs. In all treatments, animal body weights were slightly altered. Treatment with PTX-NPs almost did not decrease the number of tumor nodules, providing evidence of a limited antimetastasis effect. Treatment with the ATRA-loaded nanoparticles reduced the number of metastatic tumor nodules on the lung surface, and the codelivery PTX/ATRA-NPs resulted in fewer metastatic tumor nodules, indicating the synergistic effect of PTX and ATRA in the antimetastasis effect. Analysis with H&E staining showed that almost no tumor was observed inside the lung following treatment with the codelivery nanoparticles, representing that the codelivery of PTX and ATRA significantly inhibited cancer metastasis *in vivo*.

Methotrexate (MTX) is a chemotherapy drug and immune system suppressant that belongs to the family of antimetabolites, and it is used to treat various types of cancer (e.g., breast cancer, lung cancer, lymphoma, and leukemia), inflammatory conditions, and autoimmune disorders.[132] It is a folate antagonist, and it has been the first drug to demonstrate efficacy against metastatic choriocarcinoma in women.[133] Side effects of methotrexate high doses may be deleterious. Bone marrow, gastrointestinal mucosa, and hair are very vulnerable to the activity of this drug because they are characterized by a high rate of cellular turnover.[134] Methotrexate can cause acute renal failure, chronic neurotoxicity, thrombocytopenia, cutaneous side effects, gastrointestinal disorders, and pneumonitis. For this reason, to overcome the limitations and increase the specificity to the target tissues, this potent drug has been bound to albumin in an albumin-coupled methotrexate (MTX-HSA) formulation, used both as an anticancer platform and for the treatment of rheumatoid arthritis.[135] Methotrexate has been bounded covalently (via amide bonding) to the plasma protein albumin in a 1:1 molar ratio,[136] and the efficacy of the complexes generated has been evaluated in xenografts, showing high cell internalization and no immunogenicity or toxicity.[137] The efficacy of MTX-HSA conjugate as an antitumor agent in comparison to conventional MTX has been evaluated in 6 different human tumor xenografts, including bladder, breast, lung, prostate, soft tissue sarcoma, and osteosarcoma. Three of the tumors responded to MTX-HSA and MTX with tumor growth inhibition of >50% of control. MTX-HSA conjugate

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was significantly more active ($P < 0.01$) than free MTX, and it was capable of producing complete remission in the fast-growing SXF 1301 soft tissue sarcoma model. Moreover, MTX-HSA induced dose-dependent antitumor activity, *in vivo*. In another study,[138] methotrexate-HSA conjugates were functionalized with the luteinizing hormone-releasing hormone (LHRH) as an active targeting ligand to specifically deliver the drug to the cancer cells (the LHRH receptors are overexpressed by many cancer cells). LHRH-targeted nanoparticles showed higher cytotoxicity, internalization, and antitumor activity than nontargeted nanoparticles on the LHRH receptor-positive T47D cells. The average IC_{50} values for free MTX, MTX-HSA nanoparticles, low, medium, and high LHRH-targeted MTX-HSA nanoparticles on the T47D cells (that are LHRH receptor-positive) were 78.23 ± 3.12, 49.2 ± 2.12, 19.3 ± 1.98, 9.12 ± 1.36, and 5.82 ± 1.08 nM, respectively. Other examples of the increased selectivity and efficacy allowed by active targeting in MTX-HSA nanoparticles is a biotin-decorated MTX-HSA formulation investigated by Taheri et al.,[139] evaluated both *in vitro* and *in vivo*. Conjugation of biotin on the surface of MTX-HSA NPs significantly enhanced the inhibition of tumor growth by MTX-HSA NPs on the 4T1 tumor model. At the end of the study of antitumor effect (day 21), the mean tumor volume in the groups treated with biotin 9.41-MTX-HSA NPs, biotin 7.02-MTX-HSA NPs, and biotin 3.66-MTX-HSA NPs was 8.5-, 4.9-, and 3.7-fold lower than that in the group treated with nontargeted MTX-HSA NPs (at doses equivalent to 12.5 mg/kg of free MTX). The average tumor volume in the groups treated with biotin 9.41-MTX-HSA NPs, biotin 7.02-MTX-HSA NPs, and biotin 3.66-MTX-HSA NPs was only 17.7, 28. 6%, and 34.4% of the initial tumor volume, whereas the average tumor volume in mice treated with nontargeted MTX-HSA NPs grew rapidly and reached 250.78% ± 29.01% of the initial tumor volume. Another example is trastuzumab-decorated MTX-HSA nanoparticles for the targeted delivery of HER2 positive tumor cells.[140] The cytotoxicity of MTX, MTX-HSA NPs, TMAB-MTX-HSA NPs, and free TMAB were investigated *in vitro* on SKOV3, T47D, and HeLa cells, for 24 h. The IC_{50} values of free MTX were 36.03 ± 4.13, 77.89 ± 7.09, and 102.10 ± 4.56 nM on SKOV3, T47D, and HeLa cells, respectively. The IC_{50} values of nondecorated MTX-HSA were 20.61 ± 1.56, 54.02 ± 2.34, and 82.91 ± 4.67 nM in the three cell lines, respectively, indicating higher cytotoxicity for the nanoparticle-loaded MTX. Medium TMAB-MTX-HSA NPs exhibited very low IC_{50} values of 3.53 ± 4.41 and 11.51 ± 5.45 for SKOV3 cells and T47D cells, respectively.

Liu et al.[141] produced E-selectin-binding peptide (Esbp)-modified BSA NPs using the desolvation method to reduce the toxicity of dexamethasone, a synthetic glucocorticoid often used in the treatment of acute lung injury (ALI). The formulation showed an average particle size of 266.7 ± 2.7 nm (PDI 0.165 ± 0.02) and a ζ potential of −33.64 ± 1.23 mV. It exhibited long-term sustainable drug release (51.2% released in 12 h, compared to the 88.2% in 12 h of the free drug) and an encapsulation efficiency of 84.3 ± 2.3%. Hemolysis and MTT tests demonstrated the good blood biocompatibility of the formulation at concentrations lower than 0.5 mg/mL and its safety for intravenous injection. The *in vivo* distribution studies in ALI mice showed that the formulation could last longer in the blood circulation, leading to accumulation in the lungs.

Lapatinib is an inhibitor of the intracellular tyrosine kinase domains of the epidermal growth factor receptor, and it has been encapsulated in albumin nanoparticles to enhance the therapeutic effect in fighting triple-negative breast cancer (TNBC) metastasis to the brain. Wan et al. developed lapatinib-loaded HSA nanoparticles for treating TNBC brain metastasis.[142] The antitumor efficacy of the system was investigated in 4T1 brain metastatic mouse models. Lapatinib-loaded HSA nanoparticles at a medium dose (10 mg/kg) or higher (30 mg/kg) not only suppressed tumor growth tremendously but efficiently inhibited the further transfer of the established metastasis to other brain regions, demonstrating superior therapeutic effects on brain metastasis. Furthermore, the median survival time of metastatic mice treated with lapatinib-loaded HSA nanoparticles at medium and high doses (29.6 days and 36.4 days, respectively) was significantly longer than those of mice treated with physiological saline (19.1 days, $P < 0.05$).

Statins, the 3-hydroxy-3-methylglutaryl (HMG)−CoA reductase inhibitors, are lipid-lowering medications that decrease the cholesterol level and prevent cardiovascular diseases.[143] When used in combination with other anticancer drugs like doxorubicin, they can help fight certain types of cancer. The low solubility and availability of atorvastatin calcium can be overcome by the use of albumin-based nanoparticles and noncovalent bonding between the cargo and the carrier. The BSA nanoparticles were produced via the desolvation method and cross-linked at the end. The main parameters that influenced the size of the nanoparticles were as follows: the pH of the medium, the glutaraldehyde concentration, and the rate of addition of ethanol. After the optimization of the previous factors (pH: 4.0−9.0, 8% glutaraldehyde, 0.8 mL/min ethanol), small and spherical atorvastatin calcium-loaded albumin-based nanoparticles were obtained and tested successfully. The anticancer activities of the ATV-BSA NPs against MiaPaCa-2 cell lines have been evaluated where dose−response effect was observed. Free ATV showed toxicity as well, but to a much lower extent than ATV-BSA NPs. The enhanced activity for nanoparticle formulation was attributed to the improved cellular uptake of ATV-BSA.[144]

5-Fluorouracil (5-FU) is an analogue of the pyrimidine, and it is a chemotherapeutic anticancer agent that belongs to the family of the antimetabolites.[145] It exerts its anticancer effects through inhibition of thymidylate synthase, followed by incorporation of its metabolites into RNA and DNA, and it was widely used, in particular, for colorectal cancer. It has been encapsulated in albumin-based nanoparticles thanks to the hydrophobic interactions with the protein, and it has been examined in preclinical studies *in vitro*. Sharma et al.[146] conducted an experiment where 5-FU was conjugated to poly(ethylene glycol) anchored recombinant human serum albumin nanoparticles (5-FU-rHSA-PEG-NPs). The obtained 5-FU-rHSA-PEG-NPs displayed an IC_{50} of 3.7 μM, significantly lower ($P < 0.05$) than 6.8 and 11.2 μM of 5-FU-rHSA-NPs and 5-FU solution, respectively, in the colorectal cancer cell line HT-29.

Many palladium compounds with oxime ligands have been synthesized over the years, and their anticancer activity has been evaluated. In previous studies, palladium complexes with various ligands including amines, oximes, and phosphorus ylides were demonstrated to be effective against different cancer cell lines.[147,148] For this reason, Karami et al.[149] developed BSA nanoparticles to control the release of a new palladium complex with a dioxime ligand derived from isophthalaldehyde, in order to achieve the desired therapeutic

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effects, protect the drug from degradation, and improve its characteristics. The obtained palladium complex-loaded nanoparticles exhibited a mean size of around 400 nm, and the entrapment efficiency ranged between 96.63 and 97.78%. The results of an MTT assay showed that the loaded BSA nanoparticles exhibited cytotoxicity on A459 and K562 cell lines, with IC_{50} values at 48 h of 26.4 ± 1.6 μg/mL and 128.7 ± 4.8 μg/mL, respectively.

Sulfasalazine (SSZ) is a serine-threonine-specific protein kinase activation inhibitor that inhibits x_c^- cysteine/glutamate antiporter reducing cellular glutathione level. In a phase 1 clinical study, a solid oral form of SSX in combination with cisplatin was administered to patients with advanced gastric cancer.[150] The results of the trial showed that there was a reduction in the CD44v-positive cancer cell population, but the objective of the study was not achieved since the blood concentration of SSZ did not reach the tumor cell killing level with the current oral SSZ dose. In order to overcome this limitation, Olaitan and Chaw[151] developed an SSZ-containing BSA nanoparticle formulation using a desolvation technique. The obtained nanoparticles were spherical in shape, and the particle sizes ranged between 200 and 400 nm, with a low PDI. The maximum drug encapsulation efficiency was 28%. SSZ has poor aqueous solubility, which is predicted to be 0.0024 mg/mL. When encapsulated in BSA nanoparticles, the drug solubility increased to 1 mg/mL. Therefore, the nanoparticles can be administered as a water-soluble injection with an enhancement in drug solubility.

Zhou et al.[152] combined HSA, Kolliphor HS 15 (HS15), and pirarubicin (4′-*O*-tetrahydropyranyl-adriamycin, THP), an anthracycline antibiotic that acts as a topoisomerase II inhibitor and used in various types of cancer. A thin-film hydration method was used to obtain albumin-bound complexes of HSA-THP via purely physical forces, and the absence of chemical reactions allowed for the preservation of HSA bioactivity. *In vitro*, the formulation showed higher cytotoxicity and cellular uptake than free pirarubicin in 4T1 breast tumor cells and B16F10 melanoma cells. Uptake efficiency was much higher for HSA-THP than THP, which was probably due to high SPARC expression in the cell lines investigated. In 4T1 cells, the HSA-THP complex showed an average IC_{50} of 18.02 μg/mL, compared to 31.61 μg/mL for THP, providing evidence that the formulation was more cytotoxic than free THP. *In vivo*, BALB/c mice bearing 4T1 tumors were treated with THP or HSA-THP to evaluate the antitumor efficacy. HSA-THP showed a tumor inhibition rate of 75.88 ± 10.65%. Median survival time was 38, 41, and 44days for mice treated with saline, THP, and HSA-THP, respectively. Among the mice treated with the nanoparticles, 25% exhibited tumor elimination and lived ≥60 days without any abnormality. Other than inhibiting proliferation, the formulation seemed to inhibit also tumor angiogenesis. Moreover, from an *in vivo* imaging system, it was proven that HSA-THP also suppressed tumor metastasis.

The N-terminus of proaerolysin (PA), a potent toxin, was linked to recombinant albumin via a peptide linker specific for the protease-specific antigen (PSA) in order to aid the targeted delivery of the toxin. This formulation has been studied preclinically, *in vivo*.[153] The recombinant HSA/PA showed selective activation in the presence of PSA expressing PCa cell lines with toxicity at low nanomolar concentrations. The PA toxin is extremely toxic when intravenously injected in mice, displaying an LD_{100} of 100 ng. The vital role of the albumin delivery system was to restrict the potent killing ability of PA to sites of PCa so that it could be delivered as a system therapeutic. Furthermore, the binding with HSA allowed to reduce the extreme toxicity observed when PA alone was injected directly in mice.

Early inhibitors of human immunodeficiency virus, type 1 (HIV-1), have been widely investigated and studied in the past few years.[154] The vulnerability to degradation by peptidases, the lack of bioavailability, the rapid renal clearance, and the poor distribution make the therapeutic peptides limited in their application. T-20, sold under the name of fuzeon, or enfuvirtide, is the first of a novel class of antiretrovirals called fusion inhibitors approved by the FDA in 2003 for clinical use.[155] It is a 36 amino-acid synthetic peptide that targets gp41, a glycoprotein subunit that remains noncovalently bound to gp120 and facilitates the second step through which HIV enters T-cells, inhibiting the entry of HIV into uninfected cells. C34, a potent fusion inhibitor of HIV-1 derived from the HR2 region of gp41, was engineered into a 1:1 human serum albumin conjugate through stable covalent attachment of a maleimido-C34 analogue onto cysteine 34 of albumin. The obtained formulation, PC-1505, required less frequent dosing and less peptide than T-20 while being equipotent to the C34 peptide and to T-20 *in vitro*. Despite the belief that cross-linking of peptide inhibitors to larger proteins would likely reduce their antiviral activity, the conjugation with albumin resulted in superior pharmacokinetics profiles of C34 peptides. The conjugation reaction could be accomplished either *in vivo*, by administering the compound into the human body which is followed by conjugation with endogenous albumin, or *in vitro*, by reacting the maleimido-peptide with albumin before administering the formulation to the patient.

Another application of albumin-based formulations is the field of bacterial infections. *Staphylococcus aureus* is a multidrug-resistant bacterium that can cause life-threatening diseases. Antibacterial lysins are promising proteins that can fight both antibiotic-susceptible and antibiotic-resistant bacteria.[156] One of their limitations is that they have a short half-life, being eliminated rapidly from the systemic circulation. PEGylation increases their half-life but makes them inactive. Genetic fusion to a small albumin-binding peptide or domain is an alternative to genetic fusion to albumin. Lysostaphin, a potent antistaphylococcal lysin, was fused with an albumin-binding domain from streptococcal protein G. The residence time in the systemic circulation of the protein was exponentially increased compared to lysostaphin, and the pharmacokinetics was improved. Moreover, a family of new antibacterial inhibitors, cation peptide FARKGALRQ (non-apeptides), showed efficacy against *S. aureus*, but they are very vulnerable to degradation. In order to develop a novel drug delivery system for cationic peptides, FARKALRQ was derivatized with myristic acid as an albumin-binding moiety to yield the N-myristoylated nanopeptide RH01 (Myr FARKALRQ).[157] It was demonstrated that, in a 6:1 ratio, RH01 was bound slowly and inefficiently to native human serum albumin, which contains two molecules of fatty acid bound to the five fatty acid binding sites, but effectively to a commercially available fatty acid-free albumin. The technology, also known as SAFETY, showed higher efficacy of RH01 bound to essentially fatty acid-free albumin (HSAff) than to native human serum albumin against *S. aureus*. The most likely reason why RH01 formulated with HSAff was more effective at

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killing *S. aureus* is a protective effect of HSAff toward enzymatic degradation of the RH01.

Albumin can also show its potential in the formulation of vaccines. An efficient vaccine (comprised of an antigen and an adjuvant) must allow the antigen to effectively reach the lymph nodes and activate both the innate and adaptive immune system. Despite the remarkable advances in cancer immunotherapy, the ineffective delivery of antigens and adjuvants to the desired site has limited the success of vaccines and has led to a constant search for next-generation technologies. A linear dependence of lymphatic uptake and molecular weight, up to a plateau of around 45 kDa in size, has been shown. Above which proteins show nearly 100% trafficking to lymph nodes.[158] Albumin, characterized by a molecular weight of around 67 kDa, potentially prevents the dissemination of the antigen into the blood and instead allows the efficient trafficking to the lymphatics, leading to enhanced accumulation in the lymph nodes and to increased immunogenicity, compared with the equivalent soluble antigen. Furthermore, albumin can be efficiently endocytosed by antigen-presenting cells (APCs), which can facilitate antigen processing and presentation. Following this path, Zhu et al.[159] hypothesized that nanovaccines assembled *in vivo* from exogenous molecular vaccines and endogenous nanocarriers (such as albumin) could enhance vaccine bioavailability in lymph nodes and potentiate immune responses. They conjugated molecular vaccines with Evans blue (EB) into albumin-binding vaccines (AlbiVax), developing clinically promising albumin/AlbiVax nanocomplexes that self-assembled *in vivo* from AlbiVax and endogenous albumin. PET pharmaco-imaging, super-resolution microscopies, and flow cytometry showed that this formulation allowed almost 100-fold more efficient codelivery of CpG and antigens to lymph nodes. Moreover, albumin/AlbiVax demonstrated to elicit around 10 times more frequent peripheral antigen-specific $CD8^+$ cytotoxic T lymphocytes with immune memory than benchmark incomplete Freund's adjuvant (IFA)-emulsifying vaccines. In conclusion, these albumin-based nanocomplexes have proven to be a robust platform for combination cancer immunotherapy.[81]

Albumin has also been used as a drug delivery carrier for effective and safe novel anticancer molecules derived from natural products.[160] In the past few years, the potential of natural compounds such as flavonoids, polyphenols, carotenoids, and anthocyanins has been widely studied. Piceatannol (PIC) is a natural polyphenol,[161] which has anticancer and anti-inflammatory properties, and it works by downregulating the pro-inflammatory cytokines and transcription factors, such as the nuclear factor-$\kappa\beta$ and the hypoxia-inducible factor 1-α, that lead to proliferation and development of the tumor. Because of low solubility and stability, PIC has been encapsulated in BSA nanoparticles generated by a desolvation method and then cross-linked with glutaraldehyde. The cytotoxicity of PIC-BSA NPs and free PIC in colorectal cancer cell lines, CaCo-2 and HT29, was investigated. The use of the nanoparticle formulation significantly increased cell cytotoxicity, compared to free PIC. Time- and dose-dependent responses were observed in both preparations. The experimental animal protocols were performed in C57 BL/6 mice bearing colon cancer. In the groups treated with free PIC and PIC-BSA NPs, the disease severity showed its highest level on day 7. PIC-BSA NPs exhibited a significant effect on the reduction of the disease severity, scored as DIA ($P < 0.05$). The inhibition of the colitis by PIC-BSA NPs was not only apparent during the treatment but also evident after cessation of the administration. This provides evidence that PIC in nanoformulation significantly reverses colitis.

Pedrozo et al.[162] developed rutin-loaded BSA nanoparticles by nanospray drying. Rutin is a flavonoid compound derived from different vegetables and fruits. It exhibits several biological properties, including antiviral, vasoprotective, anti-inflammatory, and anticarcinogenic activities. Among these features, its antioxidant activity is the most well studied. The obtained spherical nanoparticles presented a mean diameter of 316 ± 210 nm (PDI 0.27 ± 0.11) and a ζ potential of -32.1 ± 2.6 mV. The encapsulation efficiency was $32 \pm 9\%$. Rutin-loaded BSA nanoparticles were evaluated for their ability to scavenge ABTS+ through an ABTX radical cation decolorization assay, to assess whether nanoencapsulation influenced the antioxidant activity of rutin. The formulation showed a concentration-dependent scavenging activity. The IC_{50} values for free rutin were about 2-fold lower than those of rutin-loaded BSA nanoparticles.

Another natural product that has been demonstrated to possess several pharmacological properties and an anticancer effect against various types of cancer is curcumin, as already mentioned above. It was encapsulated in HSA nanoparticles[163] and covalently conjugated with BSA,[164] and the water solubility of the drug has been increased by conjugation of curcumin to albumin. The activity of the curcumin-BSA conjugates has been investigated preclinically *in vitro* and *in vivo*. The efficacy of the system was tested on DLA (Dalton lymphoma ascites) murine lymphoid cancer cell lines, and dose-dependently, the growth of DLA cell was inhibited. In particular, the IC_{50} value was found to be 0.0093 μg/mL for DLA cells. Cell toxicity significantly increased when cultures were incubated for 48 h as compared to 24 h. However, at 72 h, no further increase was found. The studies showed that the Curc-Alb conjugates acted on cancer cell lines by inducing apoptosis and causing the arrest of the cell cycle. Administration of up to 11.4 mg of conjugated curcumin per kg body weight to healthy animals was nontoxic, in terms of lethality and weight loss. In addition to being safe, Curc-Alb conjugates also demonstrated their ability to reduce cancer growth in the DLA mice model. 0.57 mg/kg body weight concentration of the conjugates significantly reduced the tumor volume, packed cell volume, and tumor viable cell count. Moreover, the system caused the prolongation of the life span of animals. Mean survival time showed a 2-fold increase in the Curc-Alb conjugate-treated group.

Papagiannopoulos and Vlassi[165] evaluated the electrostatic complexation of chondroitin sulfate (CS) with BSA in acidic conditions (pH 4.2), by citric acid, in order to form well-defined biocompatible NPs. The integrity of the formulation was strongly pH-dependent since, at a neutral pH, both the protein and the polysaccharide were negatively charged. When the NPs were thermally treated, the denaturation of BSA caused irreversible changes that made the NPs resistant to pH-induced disintegration, leading to a salt content- and pH-responsive formulation. In fact, the temperature-induced conformational change of BSA led to aggregation in a solution of free BSA to stabilize the CS/BSA NPs against basic pH. The results showed that the thermally treated CS/BSA NPs were very stable upon pH increase. The encapsulation efficiency of β-carotene in these NPs was around 46% and independent of the CS content.

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Gambogic acid (GA) has been proven to be an efficient chemotherapeutic agent for lung cancer treatment. However, it exhibits poor water solubility and low chemical stability. Zhang et al.[166] synthesized an HSA-based delivery system for GA, which helped to improve the solubility of the drug, its stability, and enhance its antitumor efficacy. The GA-HSA nanoparticles were produced using nab-technology and exhibited a particle size of 135.2 ± 35.03 nm, a ζ potential of −21.81 ± 1.24 mV. The in vitro drug release studies suggested that GA-HSA NPs showed a better sustained release than GA-Arg solution, providing 57.4% and 68.9% releases in pH 7.4 PBS medium containing 30 and 40% ethanol, respectively, compared to the 89.9% and 92.7% releases obtained from the GA-Arg solution. Furthermore, in A549-bearing mice, HSA NPs improved the therapeutic efficacy of GA (the TGI of the GA-HSA NPs group was approximately 1.25-fold higher than that of the GA-Arg solution group) and were less toxic (the % survival of the GA-Arg solution group was 33% at day 19, which was significantly lower than the 83.3% in the group treated with GA-HSA NPs).

In search of effective brain penetration enhancers (BPEs), Liang et al.[167] screened the active components of aromatic resuscitation drugs used in traditional Chinese medicine. A novel glioma-targeting system based on enhancer-modified albumin NPs was consequently developed to safely and effectively deliver drugs to the glioma regions of the brain. Borneol, muscone, and menthol were conjugated with BSA for the preparation of BPE-modified BSA vehicles. The formulation facilitated the entry to the brain through both transcellular and paracellular pathways, and endothelial cells fenestrae in the pineal gland. After penetration of the blood−brain barrier, the presence of BSA allowed the accumulation in the glioma region. In particular, the formulation induced the downregulation of tight junction TJs-associated proteins, leading to the penetration of the blood−brain barrier. In conclusion, the study demonstrated that the obtained nanoparticles have the potential to deliver drugs to the brain without obvious tissue damage because of their good biocompatibility.

10. ALBUMIN-BASED DRUG FORMULATIONS CURRENTLY IN CLINICAL TRIALS

There are several albumin-based anticancer formulations that are currently undergoing clinical trials and are being tested on various types of cancer. The albumin-based anticancer therapeutics can be classified as micro or nano albumin-based particles, albumin-drugs covalent conjugates, and genetic fusions. The most popular formulation is, as already pointed out, ABI-007 (Nab-paclitaxel, Abraxane), an albumin-based nanoparticle-containing paclitaxel, which was the first albumin-based drug delivery system approved by the FDA[170] for the treatment of metastatic breast cancer. Abraxane was approved, respectively, in 2012 and 2013 for the treatment of nonsmall cell lung cancer and pancreatic cancer. For these indications, in phase III clinical trials, Abraxane was combined with the most effective anticancer agent of choice and compared with the single standard drug alone. For nonsmall cell lung cancer, Abraxane at 100 mg/m^2 once in a week was combined with carboplatin (at a target AUC 6 mg/mL/min) once every 3 weeks and compared to 200 mg/m^2 Cremophor EL paclitaxel plus carboplatin at a target AUC 6 mg/mL/min once every 3 weeks. For advanced pancreatic cancer, 125 mg/m^2 Abraxane was combined with 1000 mg/m^2 gemcitabine and compared to gemcitabine alone on a three-week dosing regimen. Overall survival benefits of about one month for nonsmall cell lung cancer and two months for pancreatic cancer were observed for the combination therapies.[171,172] The exceptional success of Abraxane has led to subsequent clinical investigations for other nab-technology formulations in the cancer arena.

ABI-008 (Nab-docetaxel) is an albumin-based formulation in the form of nanoparticles containing docetaxel. ABI-008 phase I/II trial for metastatic breast cancer and hormone-refractory prostate cancer was completed in 2008 and 2011, respectively (Clinicaltrials.gov, 2019), but further clinical studies are not reported.

Aldoxorubicin (INNO-206) is a 6-maleimidocaproyl hydrazone prodrug formulation of doxorubicin currently under investigation for the treatment of soft tissue sarcomas. Upon i.v. administration, aldoxorubicin rapidly binds to the circulating albumin covalently and is transported to the acid-rich environment of the tumor, where it cleaves and accumulates. Early studies have demonstrated a promising reduction in the cardiotoxicity of aldoxorubicin compared with equivalent doses of doxorubicin.[173] A phase I clinical study of aldoxorubicin at a dose level of 20 to 340 mg/m^2 doxorubicin equivalents in participants with localized and metastatic anthracycline sensitive solid tumors demonstrated a good safety profile at 260 mg/m^2 and was able to induce tumor regression in breast cancer, small cell lung cancer, and sarcoma.[174] In 2015, a multinational Phase II/b trial compared the efficacy and safety of aldoxorubicin with doxorubicin for advanced soft-tissue sarcoma. In this study, 123 participants were randomized to receive aldoxorubicin (350 mg/m^2 equivalent to doxorubicin 260 mg/m^2) or doxorubicin (75 mg/m^2) in a 2:1 ratio (83:40 patients) once every 3 weeks for up to six cycles. Median progression-free survival was significantly improved (5.6 vs 2.7 months, P = 0.02) with aldoxorubicin compared with doxorubicin, as well as the rate of 6-month progression-free survival (46% and 23%, P = 0.02). The median overall survival was 15.8 months with aldoxorubicin and 14.3 months (P = 0.21) with doxorubicin.[175] In 2017, in a pivotal international, multicenter, phase III trial evaluating progression-free survival as the primary end point, 433 patients with relapsed or refractory soft tissue sarcoma were randomly assigned to receive IV aldoxorubicin 350 mg/m^2 (260 mg/m^2 doxorubicin equivalent) every 3 weeks or investigator's choice of several standard treatments including dacarbazine, doxorubicin, pazopanib, ifosfamide, and gemcitabine/docetaxel. Aldoxorubicin demonstrated a time to progression or death of 5.32 months vs 2.96 for investigator's choice therapeutics (P = 0.007), suggesting that it is a promising option for treating relapsed or refractory metastatic soft tissue sarcoma. Aldoxorubicin (up to 40 cycles) had minimal or no cardiotoxicity compared to doxorubicin.[176]

Methotrexate is an antimetabolite that has been conjugated covalently with HSA (HSA-MTX) and evaluated in phase I/II trials for renal cell carcinoma. In the phase I study of the MTX-HSA conjugate, 17 cancer patients who were no longer amenable to standard treatment were treated on a weekly basis up to 8 injections, and dose-limiting toxicity (DLT) and maximum tolerated dose (MTD) were evaluated. A regimen with MTX- HSA injections of 50 mg/m^2 every 2 weeks was recommended to complete clinical phase I study.[177] Subsequent phase IIa study with 17 renal cell carcinomas was conducted but did not show any significant responses, although MTX-HSA was generally tolerated.[178] A phase II

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study of MTX-HSA in combination with cisplatin as the first-line treatment in patients with advanced or metastatic transitional cell carcinoma showed an overall response of 29% in 7 evaluable patients, but no further clinical investigation was undertaken.[179]

ABI-009 (Nab-rapamycin or Nab-sirolimus) is an albumin-bound nanoparticle formulation containing rapamycin, an immunosuppressor drug inhibitor of mTOR. Phase I/II trials are currently in progress for severe pulmonary arterial hypertension, advanced sarcoma, advanced malignant PEComa, low or intermediate grade neuroendocrine tumors of the lung or gastroenteropancreatic system, metastatic colorectal cancer, high-grade glioma and newly diagnosed glioblastoma, soft tissue sarcomas, and recurrent or refractory solid tumors. It is estimated that these trials will be completed by the end of 2020 or in 2021, and the results of the studies have not yet been reported in the literature (Clinicaltrials.gov, 2020).

Thiocolchicine dimer, an inhibitor of both microtubule and topoisomerase I (TOP1) with antineoplastic and vascular disrupting activities, has been encapsulated in albumin nanoparticles (Nab-5404, ABI-011). Nab-5404 has shown potent antiangiogenic and antimicrotubule activity *in vitro* and antitumor activity *in vivo*, leading to cytotoxic efficacy against solid tumors and lymphomas, and clinical study of nab-5404 is, therefore, recommended.[180] A Phase I trial of ABI-011 in patients with advanced solid tumors or lymphomas has been completed. However, the results of this study have not yet been published. (Clinicaltrials.gov).

A cytotoxic benzoquinoid ansamycin antibiotic, 17-allylamino-17-demethoxygeldanamycin (17AAG), possesses anticancer properties due to its ability to inhibit the HSP-90.[181] Albumin-bound 17AAG nanoparticles (Nab-17AAG, ABI-010) were successfully produced using Nab-Technology. A phase I trial (NCT00820768) was planned with this therapy in combination with Nab-PTX for advanced nonhematologic malignancies, but the study was withdrawn prior to enrolment for an unknown reason (Clinicaltrials.gov).

Another example is the recombinant interleukin-2 (IL-2), an important therapeutic protein for the therapy of melanoma and renal cell carcinoma.[182] The fusion protein obtained by IL-2 genetically fused with HSA (Albuleukin, rIL-2-HSA) showed an extended half-life in plasma compared to IL-2 alone, an increased targeting capability, and improved efficacy.[183] Albuleukin was introduced in clinical practice to assess its therapeutic benefits in a variety of cancers.[184] Two phase I clinical trials of Albuleukin in two series of different doses in patients with solid tumors were conducted to determine the safety, tolerability, and pharmacokinetics of subcutaneously administered Albuleukin.

CYC-1134-PC, a recombinant HSA-exendin-4 conjugated protein, is a drug delivery system for the treatment of type 2 diabetes that has passed phase II trials. Exendin-4 is an incretin mimetic peptide, composed of 39 amino acids, and it is a peptide agonist of glucagon-like peptide receptor that promotes insulin secretion.[185] In CJC-1134-PC, Exendin-4 has been covalently bound to recombinant HSA via a chemical linker. The formulation has the ability to mimic a full spectrum of glucagon-like peptide (GLP)-1R-dependent actions, including activation of central nervous system circuits regulating gastric emptying, food intake, and body weight. Despite the larger size, CJC-1134-PC had the same capacity as Exendin-4 in its ability to stimulate cAMP production, in contrast to albiglutide, which is an approved glucagon-like peptide-1 (GLP-1) mimetic, generated by genetic fusion of a dipeptidyl peptidase-IV-resistant GLP-1 dimer to HSA. The difference in potency is due to the different methods of preparation of the formulations. Albiglutide is a fusion protein, whereas CJC-1134-PC is an albumin-drug conjugate, where the Exendin-4 moiety inside the formulation is less constrained and more mobile than the GLP-1 epitope in albiglutide.[186] A phase II clinical trial was performed to evaluate the efficacy and safety of twice-a-week CJC-1134-PC in patients with type 2 diabetes who were on metformin monotherapy. The principal investigator has been restricted from discussion, or published trial results after the trial are completed (Clinicaltrials.gov).

Neutropenia, a major complication of chemotherapy, is a potentially serious and life-threatening event that consists of a decrease in the number of neutrophils.[187] Reduction in doses, delays in treatment administration, and the use of granulocyte colony-stimulating factors (G-CSF) are recommended to prevent the loss of neutrophils, but the use of G-CSF is limited by their short half-life. Balugrastim is a long-acting recombinant G-CSF obtained via genetic fusion between recombinant HSA and G-CSF.[188] Albumin binding increases the molecular weight and imparts high plasma stability, leading to a half-life of about 19 days. The phase II trials of balugrastim both in neutropenia and pediatric patients with solid tumors were terminated by the sponsor or withdrawn (Clinicaltrials.gov, 2015).

Hemophilia B, also known as factor IX (FIX) deficiency, is characterized by a deficiency in factor IX clotting activity that results in prolonged oozing after injuries, surgeries, and recurrent bleeding before wound healing.[189] Frequent intravenous injections of FIX are required for the treatment of the disease since factor IX has a short half-life. Recombinant fusion protein linking recombinant coagulation factor IX with recombinant HSA (rIX-FP) is generated as a single protein with a cleavable linker between FIX and albumin. A global phase III study evaluated the pharmacokinetics, efficacy, and safety of rIX-FP in 27 male children with severe and moderately severe hemophilia B. Respectively, the mean terminal half-life and clearance of rIX-FP was 4.3-fold longer and 6.4-fold slower than FIX treatment. Overall, 97.2% of bleeding episodes were successfully treated with one or two injections of rIX-FP. The results indicated that rIX-FP is safe and effective for preventing and treating bleeding episodes in children with hemophilia B with weekly prophylaxis (Clinicaltrials.gov, 2019).[190]

Hemophilia A, also called factor VIII (FVIII) deficiency or classic hemophilia, is a genetic disorder caused by the lack of factor VIII, a clotting protein. Administration of recombinant factor VIIa (rFVIIa),[191] which has passed phase I trials (Clinicaltrials.gov), is the treatment of choice for controlling bleeding in people affected by hemophilia A, but its applications are limited by the short half-life. In order to overcome this drawback, recombinant fusion protein linking coagulation factor VIIa with albumin (rVIIa-FP) has been developed to extend the circulating half-life of the formulation and improve its efficacy. Compared with rFVIIa, rVIIa-FP showed enhanced *in vivo* recovery, reduced clearance resulting in an approximately 3- to 4-fold increase in half-life and extended activity, making the formulation a potential solution to be administered less frequently than rFVIIa.[192]

Chronic hepatitis C is an infection of the liver caused by the hepatitis C virus (HCV), which prevents the liver from functioning normally. If not treated, this condition can be fatal,

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Table 5. HSA-Based Formulations Currently in Clinical Trials

formulation	compound(s)	binding strategy	indication	clinical status	ref
Abraxane (ABI-007)	paclitaxel	physical encapsulation	nonsmall cell lung cancer, pancreatic cancer	FDA-approved (phase IV)	170−172
ABI-008 (Nab-docetaxel)	docetaxel	physical encapsulation	metastatic breast cancer, hormone-refractory prostate cancer	phase I/II	Clinicaltrials.gov, 2019
INNO-206	aldoxorubicin	covalent conjugate	breast cancer, small cell lung cancer, and soft tissue sarcomas	phase III	173−176
MTX-HSA	methotrexate	covalent conjugate	renal cell carcinoma	phase II	177−179
ABI-009 (Nab-rapamycin or Nab-sirolimus)	rapamycin	physical encapsulation	severe pulmonary arterial hypertension, advanced sarcoma, advanced malignant PEComa, low or intermediate grade neuroendocrine tumors of the lung or gastroenteropancreatic system, metastatic colorectal cancer, high-grade glioma and newly diagnosed glioblastoma, soft tissue sarcomas, and recurrent or refractory solid tumors	phase I/II	Clinicaltrials.gov, 2020
Nab-5404 (ABI-011)	thiocolchicine dimer	physical encapsulation	solid tumors and lymphomas	phase I	180
Nab-17AAG (ABI-010)	17-allylamino-17-demethoxygeldanamycin	physical encapsulation	advanced nonhematologic malignancies	withdrawn prior to phase I	181
Albuleukin (rIL-2-HSA)	recombinant interleukin-2 (IL-2)	fusion protein	melanoma and renal cell carcinoma	phase I	182−184
CYC-1134-PC	exendin-4	covalent conjugate	type 2 diabetes	phase II	185, 186
balugrastim	granulocyte colony-stimulating factors (G-CSF)	fusion protein	neutropenia, solid tumors	phase II, withdrawn	187, 188
rIX-FP	factor IX	fusion protein	hemophilia B	phase III	189, 190
rVIIa-FP	factor VIII	fusion protein	hemophilia A	phase I	191, 192
albIFN	recombinant interferon (IFN)-α-2b	fusion protein	chronic hepatitis C	phase II	193, 194
Albu-BChE	mutated human butyryl-cholinesterase (BChE)	fusion protein	cocaine addition	phase II	195−198, Clinicaltrials.gov 2015
ALX-0061	IL-6R-targeting nanobody	fusion protein	rheumatoid arthritis	phase II	199, Clinicaltrials.gov 2019

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leading to liver damage or liver cancer. Albumin-Albinterferon alpha 2b (albIFN)[193] is a fusion protein of recombinant human albumin/recombinant interferon (IFN)-alpha-2b, with about 200-h half-life used for the treatment of hepatitis C. The safety/efficacy of albIFN was evaluated in a phase II clinical trial in 391 treatment-naive patients with chronic hepatitis C virus (HCV). The sustained virologic response rate (SVR) was not significantly different between the albIFN and peg-interferon; however, rapid VR was significantly lower with albIFN. No serious/severe respiratory events were noticed with albIFN. Fewer absolute neutrophil count and hemoglobin reductions were associated with albIFN[194] (Clinicaltrials.gov, 2016).

Another application of an albumin-based formulation in clinical trials is the treatment of cocaine intoxication and addiction. The human butyrylcholinesterase (BChE), known as cocaine hydrolase-1, is the principal endogenous enzyme responsible for converting cocaine to biologically inactive metabolites through cocaine hydrolysis.[195] TV 1380, also known as Albu-BChE, is a recombinant protein containing HSA fused at its amino terminus to the carboxyl terminus of a mutated form of human plasma BChE. The fusion protein containing four amino acid mutations is able to metabolize cocaine more than 3 orders of magnitude compared to the wild-type enzyme. In a phase I study,[196] healthy male and female subjects received up to a total of four weekly IM injections of TV 1380 at 50, 150, and 300 mg. At doses of up to 300 mg, Albu-BChE showed good safety and tolerability with a dose-proportional increase in systemic availability. Median t_{max} and $t_{1/2}$ ranged between 12 and 36 h and postdose and 50−77 h, which were independent of the multiple dosing. In agreement with preclinical results, the ex vivo cocaine catalytic activity demonstrated a high correlation with plasma concentrations in subjects following treatment. Anti-TV 1380 antibodies were detected in about 12% of subjects receiving repeated doses, but no changes in either AChE or BChE activities were reported. In a follow-on study in recreational nondependent cocaine users, subjects were given IM a single 50, 100, and 300 mg dose of Albu-BChE and challenged with an intravenous dose of cocaine (40 mg) administered at baseline and at 1, 4, and 7 days after the Albu-BChE dosing.[197] Administration of Albu-BChE resulted in a significant dose-dependent reduction in cocaine C_{max}, AUC, and $t_{1/2}$. Maximal effects were achieved at 24 h after Albu-BChE dose and sustained up to 168 h. One day following the 300 mg dose of Albu-BChE, the C_{max} of a 40 mg dose of cocaine was reduced from 260 to 36 ng/mL, and the $t_{1/2}$ from 1.76 to 0.14 h, respectively. One week later, Albu-BChE was still able to significantly reduce both the C_{max} and $t_{1/2}$ of the challenge dose of cocaine compared to the vehicle; 257 to 96 ng/mL and 1.71 to 0.5 h, respectively. These observations are compatible with the Albu-BChE plasma profiles exhibiting a mean C_{max}, T_{max}, and $t_{1/2}$ of about 4396 ng/mL, 18 h, and 68 h, respectively. Based on these exclusive effects of Albu-BChE on the disposition of cocaine, the sponsor has completed a multisite, phase II stud,y assessing the efficacy and safety of AlbuBChE on facilitation of abstinence in treatment-seeking, cocaine-dependent individuals (Clinicaltrials.gov, 2015) comparing weekly IM administration of either 150 mg or 300 mg of TV 1380 to placebo. The primary end point was the percentage of participants who were abstinent from cocaine during the last 3 weeks of the 12-week treatment period, based on the report of no cocaine uses each day and one negative urine sample for cocaine metabolite each week. Statistically significant differences were not observed between different treatment groups in terms of abstinence from cocaine during the last 3 weeks of the treatment period. However, 6% of subjects both in the 150 mg and 300 mg TV-1380-treated groups and no participants in the placebo group were considered abstinent. Statistical analysis of the percentage of urine samples testing negative for cocaine metabolites during the last 8 weeks of a 12-week treatment period considered as the secondary end point revealed an increase in negative urine dose-dependently; (4.7%, 8.1%, and 14.6% of urines were negative in the placebo, 150 mg and 300 mg TV-1380 groups, respectively, P = 0.0056 for 300 mg vs placebo). There were no notable differences in adverse events seen between placebo and TV-1380 groups. The clinical and safety profiles following the weekly administration of TV-1380 to cocaine-dependent individuals appear positive enough to develop improved cocaine catabolic enzyme products with greater catalytic efficiency.[198]

Apart from albumin-coupled methotrexate (MTX-HSA) formulations, used both as an anticancer platform and for the treatment of rheumatoid arthritis, other albumin-based formulations have been investigated for the treatment of rheumatoid arthritis, an autoimmune disease that can cause joint pain and damage throughout the body. IL-6 is a cytokine that plays a vital role in the pathogenesis of rheumatoid arthritis. ALX-0061 is composed of an affinity-matured IL-6R-targeting nanobody domain fused to an albumin-binding domain, generating a two-domain structure,[199] with a high affinity and potency for IL-6 receptor (IL-6R). An apparent plasma half-life of 6.6 days and significantly increased drug exposure was observed after a single intravenous administration of 10 mg/kg ALX-0061. A 200-fold increase of target affinity was obtained through affinity maturation of the parental domain, which translated to a concentration-dependent and complete neutralization of IL-6R *in vitro*. In cynomolgus monkeys, ALX-0061 led to a dose-dependent and complete inhibition of hIL-6-induced inflammatory parameters, including plasma levels of C-reactive protein (CRP), fibrinogen, and platelets. ALX-0061 demonstrated a marked decrease in serum CRP levels in rhesus monkeys collagen-induced arthritis model.[199] An open-label extension phase II study assessing the long-term efficacy and safety of ALX-0061 in subjects with rheumatoid arthritis is underway (Clinicaltrials.gov, 2019) (Table 5).

11. PROTEIN CORONA

Apart from being used as the main component in the synthesis of nanoparticles, functioning as a matrix, albumin can also form a corona on the surface of the nanoparticles, changing the delivery and the targeting properties of other drug delivery systems. Nanoparticles present in any biological environment are subjected to the absorption of extracellular proteins on their surface, generating a "protein corona". This shell, or halo, will inevitably mask the original nanoparticle and becomes what the biological environment really sees. This dynamic phenomenon is characterized by an initial absorption of the most abundant proteins (soft corona) and a subsequent interaction with proteins that bind the particle with more affinity (hard corona).[200] Preformed albumin corona regulates the interaction between nanoparticle and cellular receptors, and it has shown to reduce nonspecific association and the clearance rate, increasing the circulation properties of the carriers.[201] Drug release from albumin-coated nanoparticles is

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slower, and the *in vitro* metabolism of the nanoparticle is reduced due to the physicochemical barrier effect of the preformed HSA corona. One of the major limitations of nanotherapeutic delivery is its incapability to reach therapeutic levels of drugs at the target sites due to the nonspecific uptake of nanoparticles in healthy organs.[202] When they are injected into the bloodstream, nanoparticles are exposed to various physiological fluids, mostly blood, and are sequestered by the mononuclear phagocyte system (MPS), a system of phagocytic cells, including bone marrow progenitors, blood monocytes, and tissue macrophages. This family of cells plays two main roles: phagocytosis of foreign material and presentation of antigens to T cells by antigen-presenting cells.[203] The process begins with the opsonization, which is the absorption of proteins on the nanoparticles' surface, altering the diverse physicochemical properties of nanoparticles such as size, surface charge, composition, and functionality. The proteins interact with specific receptors on the surface of the immune cells and are internalized and eliminated.[204] For this reason, decreased opsonization is essential in avoiding the clearance of nanoparticles by phagocytic cells and allowing them to reach the tumor sites. As already said before, antifouling functionalization with PEG,[205] with zwitterionic polymers,[206] or with peptides such as CD47[207] has demonstrated to reduce protein corona and increase circulation times. Nonetheless, it is now agreed that controlling the protein corona, rather than trying to achieve its total elimination, would be a better choice for nanomedicine.[208] Therefore, an *in vitro* preforming albumin corona on nanoparticles is highly recommended to inhibit opsonin proteins (IgG and complement) adsorption and prolong the blood circulation time, acting as a protective coating.[209] However, these interactions could lead to changes in the protein's secondary structure, giving rise to the denaturation of the protein. In particular, larger nanoparticles induce a bigger conformational change of the protein adsorbed, causing it to stretch more to cover the entire particles. Even a small modification in protein structure may provoke an enormous impact on the pharmacological activities, interaction with other proteins, and biological responses.[210] Factors to consider are concentration, the composition of the adsorbed proteins, and types of interaction with the nanoparticle surface. The uncontrollable plasma protein adsorption is one of the main causes of the rapid clearance and toxicity of nanoparticles. That is the reason why researchers thought to use one optimal protein to interact with nanoparticles and form a pure protein corona in advance.[211] After the formation of the albumin corona, the nanoparticle size became larger, the negative ζ potential higher, and the biological properties were improved. Most of the delivery systems including metallic,[212] magnetic, silica, polymeric, lipid nanoparticles, carbon nanotubes, and quantum dots can interact with albumin in their pristine form. The best techniques to provide an efficient albumin coating are the emulsion-solvent evaporation and microemulsion. To summarize, albumin coatings on nanoparticle surfaces may have the following advantages: increased stability, presence of a loadable coating, increased biocompatibility, prolonged release of the payload, protection of the payload, increased circulation time, decreased aggregation and enhanced SPARC and gp60 targeting. These features provide evidence that preformed albumin corona on the surface of nanoparticles could be an effective and versatile strategy for optimizing the nanoparticles-based drug delivery systems and develop long-acting formulations.[213] However, there is still lack of ability to predict how proteins will interact and absorb on a specific nanoparticle surface and whether these proteins will experience conformational changes. Furthermore, the formation of the "corona" is a much broader concept and it does not involve only proteins, but also other components of the environment, such as lipids. For these reasons, the mechanisms of formation of albumin halo on nanoparticles need to be further investigated and understood.

Since human blood can contain biomarkers for various diseases, plasma proteins are often used as potential prognostic or diagnostic biomarkers to discriminate between sick and healthy patients. Accordingly, the composition of the protein corona is strongly affected by the disease. Besides, the plasma proteasome is not always consistent even in healthy individuals, and it depends on lifestyle, health conditions, and genetic background.[214] This gave rise to the concept of "personalized protein corona", which refers to the formation of different protein coronas on the same nanomaterial incubated with plasma proteins of patients with different pathological conditions.[215] Hajipour et al.[216] employed two types of nanoparticles, hydrophobic sulfonated polystyrene NPs and hydrophilic amorphous silica NPs, and incubated them with various plasma samples. The results showed that the proteins constituting the coronas associated with various diseases were completely different, in terms of composition and amount. In agreement with their results, Colapicchioni et al.[214] analyzed the protein coronas formed on clinically approved liposomes (AmBisome) in breast, pancreatic and gastric cancer patients. The results of their study showed that the protein coronas surrounding the liposomes in pancreatic cancer were less negatively charged than the ones in gastric and breast cancer. This provides evidence of the changes in the concentration of Igs, correlated with the presence of autoantibodies produced by the immune system. In another study, Hajipour et al.[217] investigated the therapeutic/toxic impact of graphene oxide (GO) sheets after incubation with the plasma of patients with seven different diseases (blood cancer, rheumatism, diabetes, hypercholesterolemia, fauvism, and major and minor thalassemia) on breast cancer cell lines, MCF-7 and MDA-MB-231. They investigated cellular toxicity, apoptosis/necrosis, production of reactive oxygen species, cellular uptake, lipid peroxidation, and cellular inflammation. As expected, it was demonstrated that in different pathological conditions, the coated NPs showed significantly different effects. In addition to the concentration and composition of the protein corona, diseases can induce modifications in the conformation of proteins, altering their interactions with the nanoparticles and, therefore, of the protein coronas. Therefore, the patients' clinical history, condition, and lifestyle must always be taken into account in order to develop *ad hoc*-designed nanomaterials. In conclusion, the precise characterization of disease-related personalized protein coronas can help to predict the biological outcomes and responses of nanodelivery systems, leading to the development of disease-specific custom NPs and to the acceleration of clinical translation. To the best of our knowledge, studies on this effect have not been conducted specifically on albumin nanoparticles and deserve more attention.

12. FUTURE PERSPECTIVES

To date, the great potential of albumin has been widely demonstrated by various preclinical and clinical studies. While gp60 and SPARC are recognized to be responsible for the

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active internalization of albumin in tumors and inflamed tissues, more studies on the involvement of gp30 and gp18 and their interactions with albumin nano vectors should be carried out. Furthermore, it is important to clarify how the binding of ligands to albumin affects their activity. The methods of preparation of albumin and their mechanisms of loading should be studied further. In this context, the development of safe cross-linkers for the stabilization of albumin NPs deserves more attention. It is known that tumors are able to trap plasma proteins and use their degradation products for proliferation and development.[218] It is believed that the albumin nanoparticles take advantage of the active targeting as well as the EPR effect to enter and accumulate in the solid tumors and the inflamed joints. The enhanced accumulation of albumin nanoparticles in solid tumors cannot be solely explained by the presence of a leaky vasculature and impaired lymphatic drainage but also by the "desire" for albumin, an essential protein for the sustainment and growth of the tumors. The involvement of this mechanism needs more evidence in patients. As in cancer patients, people affected by rheumatoid arthritis often develop hypoalbuminemia,[219] caused by the high albumin consumption in the sites of inflammation. All the pathological conditions that are linked to albumin accumulation in the affected tissue, such as cancer, infections, and immune disorders, are characterized by cachexia, which is a multifactorial syndrome that causes extreme weight loss and muscle wasting. For these reasons, it would be interesting to investigate whether the accumulation of injected albumin-based formulations, regardless of the drugs they carry, could mitigate or exacerbate tumor development. Furthermore, studies conducted on mice bearing syngeneic ovarian and mammary carcinomas suggested that Nab-paclitaxel acts as a radiosensitizer, improving the effect of radiotherapy if used in combination with radiation (in particular, if administered before radiation).[220] In contrast to the strong enhancement of tumor radio-response, there was a complete lack of modification of healthy tissue radio-response. Therefore, Nab-paclitaxel showed antitumor effects when administered as a single agent, and it enhanced the antitumor activity of radiotherapy in a supra-additive manner. Its efficacy in increasing the therapeutic gain of radiotherapy is an interesting feature that could be investigated further in the future. Next developments in the area of drug delivery with the aid of albumin can focus on combination therapy and chemical modifications of albumin toward the accommodation of more diverse therapeutic entities as well as imaging agents. In this context, Lian et al.[221] developed multifunctional nanoparticles incorporating IR780 (a near-infrared dye) and docetaxel in human serum albumin nanoparticles. This formulation showed high targeting and theranostic potential for the treatment of castration-resistant prostate cancer. In 2019, Li et al.[222] developed stimuli-responsive albumin nanoplatforms for combinational theranostic and cancer multimode therapy. Multimode therapy relies on chemotherapy, radiotherapy, hyperthermia, and other monotherapy and cooperates with imaging technology to achieve both the diagnosis and the treatment. In 2020, Zhang et al. realized a human serum albumin-based dual-agent delivery system for combination therapy for the inhibition of cancer cells and neovascularization in the tumoral microenvironment.[223] The payloads were 2-acetylpyridine.4,4-dimethyl-3-thiosemicarbazone-copper(II) [Cu(Ap44 mT)]Cl and paclitaxel, for improving both the therapeutic activity and the targeting ability *in vivo*.

13. CONCLUSIONS

The field of nanomedicine is becoming more and more appealing as it provides efficient and smart solutions for the delivery of therapeutics in the treatment of cancer, inflammatory diseases, and other conditions. Over the past few years, the great potential of albumin as a drug delivery system attracted the attention of many researchers due to its biocompatibility, biodegradability, nonimmunogenicity, and nontoxicity. It is not a foreign body; it is not rejected by the immune system since it is the most abundant protein in the plasma, and that makes it even more appealing. Its high affinity for hydrophobic drugs, the possibility for surface modification, and the high loading capability allow us to overcome the great barriers imposed by the nature of many compounds available in the market nowadays. It is a versatile drug carrier, which could be used not only for the transport of therapeutics but also for imaging applications and gene therapy. Moreover, its binding affinity for specific receptors on the surface of endothelial cells and other cells in diseased organs permits the active targeting and the specific recognition of the albumin-based formulation by the target site. This is the most important and unique feature of albumin, which makes it different and unique compared to the other nanocarriers. This feature, perhaps, has provided the inspiration for the use of albumin as a preformed corona over several other nanodelivery systems. With several albumin-based formulations already in clinical trials and the already approved Abraxane formulation, which has shown outstanding results in cancer patients, albumin nanobased formulations provide a safe and potentially effective strategy for the formulation of many existing and emerging drugs with enhanced therapeutic index.

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Notes

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