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Harnessing Albumin as a Carrier for Cancer Therapies

Ella N. Hoogenboezem and **Craig L. Duvall**

Department of Biomedical Engineering, Vanderbilt University, Nashville, TN

Abstract

Serum albumin, a natural ligand carrier that is highly concentrated and long-circulating in the blood, has shown remarkable promise as a carrier for anti-cancer agents. Albumin is able to prolong the circulation half-life of otherwise rapidly cleared drugs and, importantly, promote their accumulation within tumors. The applications for using albumin as a cancer drug carrier are broad and include both traditional cancer chemotherapeutics and new classes of biologics. Strategies for leveraging albumin for drug delivery can be classified broadly into exogenous and *in situ* binding formulations that utilize covalent attachment, non-covalent association, or encapsulation in albumin-based nanoparticles. These methods have shown remarkable preclinical and clinical successes that are examined in this review.

Keywords

drug delivery; albumin; cancer; drug conjugate; prodrug; drug carrier

1. Introduction

Albumin, a long-circulating and highly-abundant protein in the blood, has unique promise as a carrier for cancer therapeutics based on several key characteristics: (1) it is a natural carrier of native ligands and other hydrophobic cargo (2) it is rescued from systemic clearance and degradation by natural mechanisms (3) it accumulates at sites of vascular leakiness and (4) it is more highly taken up and metabolized by rapidly growing, nutrient-starved cancer cells. Investigators have sought to leverage these characteristics for the delivery of several classes of approved and investigational anticancer agents, which will be reviewed herein.

1.1. Properties of Albumin

Albumin is the most abundant protein in human blood with a concentration of about 40 mg/mL and a molecular weight of ~67 kDa [1]. Notably, it exhibits an extraordinarily long half-life of 19 days [2] [3]. Albumin is synthesized in the liver with approximately 13–14g of albumin entering the circulation every day [2]. When albumin extravasates into tissue, it is returned to the vascular space via the lymphatic system through a natural recycling

Correspondence to: Craig L. Duvall.

mechanism. The same approximate mass of 13–14g of albumin entering the intravascular space is also catabolized from it every day. Importantly, albumin is known to be a carrier of a wide variety of both endogenous and exogenous compounds [4]. This facilitates the colloidal solubilization and transport of hydrophobic molecules such as long chain fatty acids as well as a variety of other ligands such as bilirubin, metal ions such as zinc and copper, and drugs such as warfarin and ibuprofen [5]. Figure 1 shows the crystal structure of albumin and the sites where these ligands can bind [6]. Of interest to the design of albumin-binding drugs is the distinct affinity and nature of each of these binding sites. For instance, Sudlow's site I is known to bind dicarboxylic acids and bulky heterocyclic molecules with a negative charge (e.g, warfarin) whereas Sudlow's site II is characterized by binding to aromatic carboxylic acids with a single negatively charged acid group separated by a hydrophobic center (e.g, diazepam, ibuprofen) [7].

Albumin naturally transcytoses across the vascular endothelium, a process which can ordinarily pose a significant barrier for drugs to reach cells inside the tissue [8]. This process is attributed to the receptor GP 60, also known as albondin. GP 60 is present in continuous vascular endothelium and alveolar epithelium. Albumin binds to GP 60, which induces clustering of albumin-GP 60 at the cell surface and association with Cav-1, the main protein critical to caveolae formation. Cav-1 induces the internalization of a vesicle composed of the surface membrane surrounding these clustered albumin-GP 60 receptors as well as compounds bound to albumin. Cav-1 is then transported to and fuses with basolateral membrane to complete transcytosis. [2] (Figure 2A) However, GP 60 only binds to native albumin. GP 18 and GP 30, by comparison, exhibit preferential binding to modified albumin to traffic it for lysosomal degradation and are widely distributed in the body [9]. These receptors may be part of a pathway to remove old, damaged, or potentially deleterious albumin marked by modifications such as oxidation, non-enzymatic glycation, and maleylation [10]. Albumin-gold conjugates and maleic anhydride treated albumin, for example, are modified forms of this protein subject to this pathway, necessitating careful design consideration for the modification of albumin for drug delivery [9].

The remarkable half-life of albumin makes it an attractive carrier for improving the pharmacokinetic properties of anticancer agents. This long half-life is attributed to the neonatal Fc receptor (FcRn), an intracellular receptor that is responsible for rescuing albumin from degradation. This receptor is widely distributed in the body and is known to extend the half-life of both serum albumin and immunoglobulin G. The FcRn functions by binding these proteins in the acidic endosome and diverting them from the highly-degradative lysosomal pathway. The FcRn and its bound ligand are exocytosed to the extracellular space where the ligand is released from the FcRn at physiological pH. Albumin can then re-enter the circulation through the lymphatics, prolonging its half-life. [2] This receptor interaction is pH dependent with strong affinity binding occurring at the low pH of the endosome (Figure 2B).

Additionally, albumin can avoid renal clearance by reabsorption through receptor-mediated endocytosis in the renal proximal tubule. The receptors megalin and cubilin are highly expressed in the proximal tubule brush border and have been demonstrated to be essential to this process. Cubilin is a 460 kDa glycoprotein which binds to transmembrane endocytic

receptor megalin [11]. Albumin binds to cubilin (K_d~0.6pM), and megalin subsequently facilitates the endocytosis and intracellular trafficking of the albumin-cubilin complex [12] [13]. It is thought that the FcRn may then redirect the endocytosed albumin complex to the interstitial space of the kidneys to ultimately return to the circulation via the lymphatics [14]. Using albumin as a carrier can leverage this natural pathway for extending drug circulation half-life. This approach circumvents the usage of synthetic systems which may involve complicated synthesis and confer some toxicity or immunogenicity. For instance, modifying drugs with poly(ethylene glycol) is a common method for improving hydrophilicity and circulation of a drug by making it larger than the renal size cutoff. However, humans can mount antibody responses to PEG, resulting in potentially dangerous allergic reactions and potentially limiting the utility of repeated administration of PEG-containing formulations [15]. The biocompatibility and physiological transport pathways used by albumin make it an exciting platform for anticancer drug development.

1.2. Cancer Implications of Albumin

1.2.1. Passive Albumin Tumor Accumulation—In addition to the more general advantages conferred by albumin as a carrier, it possesses unique characteristics that make it adept at targeting therapeutics to tumor sites. The internal vascular network of growing tumors often becomes insufficient for supplying the oxygen and nutrients necessary to support their aberrant, hyper-proliferative local environments. Consequent hypoxic conditions and cell death are associated with the release of angiogenic factors that cause rapid formation of new blood vessels at the tumor site. These new vessels are characterized by irregularities such as larger than normal fenestrations in the endothelium [16]. Tumors also have disruption of the lymphatic system, which in healthy tissues, continuously drains the extracellular fluid, facilitating reentry of macromolecules into the circulation. In tumors, there is either poor or heterogeneous lymphatic drainage due to the compression and eventual collapse of lymphatic vessels by rapidly growing cancer cells [16][17]. The tumor vasculature leakiness, paired with poorly formed lymphatic drainage, is thought to be responsible for the preferential tumor accumulation of nanostructures and macromolecules in the phenomenon known as the enhanced permeability and retention effect (EPR) (Figure 2C) [18].

Albumin is especially adept at accumulating in regions of proliferating tumor cells as a consequence of the EPR effect. This is because albumin is the most concentrated protein in the blood at the aforementioned concentration of 40 mg/mL compared to its interstitial concentration of about 14 mg/mL, driving its diffusional transport [19]. Additionally, the reliance of albumin on the lymphatic system to return to the circulation from the extracellular space makes it susceptible to accumulation in tumors with their poor lymphatic drainage [18] [20]. Indeed, the first observations of macromolecule accumulation in tumor interstitium were based on the preferential distribution and retention of radiolabeled albumin and other serum proteins [18]. Importantly, Evans blue dye, which naturally complexes with albumin, demonstrates prolonged retention in tumors compared to normal tissue, from which it is rapidly cleared [18]. Albumin accumulation has subsequently been observed in a variety of solid tumors animal models including sarcoma, ovarian carcinoma, and Novikof hepatoma [10].

The EPR effect has recently come under scrutiny based on the disparity observed between nanocarrier tumor biodistribution and therapeutic efficacy in preclinical tumor models versus human clinical trials. Tumor vasculature formation occurs at a faster rate in commonly used flank tumor mouse models compared to most human disease, and this physiologic difference may exaggerate the EPR effect in some animal models. Additionally, there is growing appreciation that the EPR effect may be more relevant for certain tumor or patient subsets among the wildly heterogeneous spectrum of human cancers. The development of companion diagnostic nanoparticles is a promising approach for predicting patient responses to nanomedicine, which may be key to best leveraging the EPR effect for clinical delivery [21]. Interestingly, in observations among a variety of tumor models including syngeneic breast tumors, mouse mammary intraepithelial neoplasia outgrowths, and epithelial-mesenchymal transition tumors, permeability to albumin is ~4 fold greater than 100 nm liposomes [22]. Further, the prolonged circulation time associated with albumin may further enhance tumor accumulation by EPR.

These findings suggest promise for the improved penetration of albumin therapeutics.

1.2.2. Albumin Tumor Internalization and Trafficking—In addition to its desirable passive tumor tissue accumulation, albumin also is preferentially internalized by tumor cells, which can be leveraged for intracellular delivery of therapeutic cargo. In 2013, Commisso et al observed a mechanism by which cancer cells can support their increased metabolic and growth needs by active uptake of extracellular proteins through macropinocytosis. They observed that cancer cells expressing oncogenic Ras, an inner plasma membrane protein whose aberrant activation is associated with virtually all aspects of the malignant cancer phenotype, more highly utilize extracellular proteins as a source of amino acids to drive cellular growth [23][24]. Indeed, pancreatic ductal adenocarcinoma cells have since been shown to grow indefinitely in media lacking essential amino acids in the presence of physiologic albumin [25]. These findings provide important insight into the ability of albumin to be internalized preferentially by cancer cells relative to normal cells. It has been additionally observed that hypoalbuminemia is a common characteristic of patients with advanced solid tumors [26]. Decreased serum albumin in these patients may be indicative of the increased catabolism of albumin due to the proliferating tumor processing albumin as an abnormal source of amino acids utilized to meet high metabolic demands [27].

Some cancer cells also preferentially use receptor-mediated albumin uptake pathways in addition to upregulated use of nonspecific macropinocytic mechanisms. This has become particularly evident based on the discovery that a correlation exists between expression of albumin-related receptors and relative efficacy of albumin-based therapies among different cancer types. Nab- paclitaxel (nab-P), also known as Abraxane, is an FDA approved albumin-bound paclitaxel particle. Recently, Chatterjee et al attempted to elucidate why certain pancreatic cancer patient populations responded better to treatment with nab-P than others [28]. It had previously been posited that SPARC (secreted protein acidic and rich in cysteine) was a critical albumin-binding protein that facilitates the efficacy of nab-P in metastatic pancreatic cancer. This idea was based on small scale retrospective studies (n=16) on nab-P treatment of head and neck cancer where the relationship was examined between SPARC tumor expression and patient outcomes [29].

This hypothesis was centered around the notion that the presence of SPARC in the tumor environment would concentrate nab-P and thus possibly enhance its therapeutic effect. However, further exploration in a phase III clinical trial on nab-P in metastatic pancreatic cancer patients demonstrated no association between SPARC level and treatment efficacy [30]. Indeed, additional preclinical studies in a mouse model of pancreatic cancer treated with nab-paclitaxel plus gemcitabine also demonstrated a lack of association with SPARC knockout and tumor progression [30].

Chatteij ee et al instead examined the role that caveolae, omega-shaped invaginations of the plasma membrane, play in albumin uptake in cancer cells [31]. Caveolae have previously been implicated in a wide variety of cellular processes including endocytosis, transcytosis, and signal transmission [32][33]. The primary protein necessary for caveolae formation, Cav-1, is upregulated in a wide variety of cancer types including pancreatic cancer, prostate cancer, and breast cancer, and Cav-1 upregulation is associated with cancer progression [28], [34]–[36]. It has been shown in Cav-1 deficient mice that caveolae are critical in albumin uptake and transport [37]. Interestingly, Chatterjee et al demonstrated that RNAi-mediated attenuation of Cav-1 in cancer cells reduces albumin and nab-paclitaxel uptake and causes resistance to nab-paclitaxel- induced apoptosis. This new development in the mechanistic understanding of albumin-bound chemotherapeutics may aid in better identification of patients who are likely to respond to albumin-based therapies based on prescreening of tumor biopsies for Cav-1 levels. By exploiting the natural affinity of albumin for tumor biodistribution and preferential tumor cell uptake, albumin provides great promise as a carrier for increasing the therapeutic efficacy of cancer drugs.

Upon elucidation of these albumin tumor uptake pathways, understanding and leveraging the intracellular trafficking of albumin-bound therapeutics has become of great interest for polyplexes, oligonucleotides, and small molecule drugs. In a study on the intracellular trafficking of cholesterol-PDMAEMA liposome complexes designed to bind to albumin, Szymanowski and colleagues showed that albumin-binding liposomes are not trafficked to the degradative lysosomal pathway in human epithelial-like cells [38]. Rather, they attribute uptake of the albumin-associated complex to the nonacidifying route of caveolar uptake. Complex uptake was thus decreased in the presence of caveolae inhibitors but remained unaffected by clathrin- mediated endocytosis and macropinocytosis inhibitors. They further demonstrated that lysotracker, a fluorescent dye that stains acidic organelles in live cells, did not colocalize with either the albumin or liposomes. On the contrary, a group investigating the intracellular fate of albumin-binding floxuridine homomeric oligonucleotides demonstrated that in cancerous HeLa cells, their albumin/oligonucleotide complex was trafficked to lysosomes using Lysotracker [39]. Indeed, they posit that these lipid-modified conjugates may therefore be trafficked through the degradative GP-18/GP-30 pathway. In terms of small molecule drugs, unmodified doxorubicin has been compared to albumin-conjugated doxorubicin, and in this study, confocal laser scanning images showed distinct differences in intracellular trafficking between the two formulations [40]. Namely, in LXF59 lung carcinoma cells, doxorubicin primarily localizes to the nucleus during the first few hours of incubation whereas doxorubicin conjugated to albumin mainly accumulates in the cytoplasm. This cytoplasmic albumin-conjugated doxorubicin was demonstrated to be localized to the Golgi apparatus and mitochondria. Notably, caveosomes are transported to

the Golgi and endoplasmic reticulum. The authors additionally did not observe a difference in lysosomal accumulation between free doxorubicin or albumin-bound doxorubicin using LysoTracker. Interestingly, the authors also concluded that the distribution of both acid-labile conjugates and stable amide conjugates is very similar, despite the lack of cytotoxicity associated with the stable conjugate compared to its labile counterpart. These findings taken together suggest that the role of albumin-binding in intracellular trafficking of therapeutic loads remains only partly elucidated and is likely dependent on both the type of formulation and therapeutic cargo. More investigation is needed into understanding how different types of albumin association impact the fates of the diverse spectrum of therapies that leverage albumin as a carrier.

2. General Albumin Binding Strategies

Multiple classes of albumin-based formulations have been tested for cancer-targeting therapies (Figure 3). Herein we will review two general categories- preformed albumin therapeutics and *in situ* binders. *In situ* binders can dock on to endogenous albumin after delivery into the body. Exogenous formulations, by comparison, rely on drug loading into or attachment to recombinantly produced albumin, bovine serum albumin, or human serum albumin isolated from donors prior to administration to patients. This discussion will focus specifically on surveying albumin-based cancer therapeutics, but other types of clinically-mature therapeutics will be highlighted in each class as applicable.

2.1. *In Situ* Binders

2.1.1 Covalent Conjugation—Covalent bonds between native albumin and therapeutics can be formed *in situ*. The primary method for *in situ* covalent attachment to albumin leverages the cysteine-34 amino acid of albumin. Importantly, albumin cysteine-34 represents the most abundant free thiol in the blood, and competitive side reactions with other free thiols are not a significant concern because cysteines are typically found in nonreactive disulfide bridges. Kratz and colleagues pioneered this method for binding endogenous albumin [41]. Their approach used a maleimide carboxylic hydrazone derivative of doxorubicin to form a covalent thioether bond *in situ* with the cysteine- 34 position of albumin. This approach is made possible by the unique properties of this amino acid residue, with approximately 70% of circulating albumin possessing the cysteine-34 amino acid in its accessible form. Indeed, the authors posit that the lack of a free thiol on the majority of other circulating serum proteins makes this a relatively specific reaction. The preferential reaction with albumin is further bolstered by the low cysteine 34 pKa of 7, making it the most reactive thiol group in human plasma. The incorporation of an acid-sensitive hydrazone linker was utilized by the authors to create triggered release of the doxorubicin cargo within the highly acidic environment of endosomes and lysosomes. A variety of groups have used the cysteine-34 approach to improve the pharmacokinetic properties of their cancer therapeutics ranging from small molecule drugs [42]–[46] to biologies [47]. Additionally, rather than employing the maleimide to sulfhydryl reaction, a disulfide bond with this free thiol can be formed to make a more readily reducible link between therapeutic cargo and native albumin. One such example involves the addition of a cysteine residue to a tumor penetrating peptide to form a disulfide bond with albumin *in situ* [48]. Finally, both

ruthenium-based anticancer complexes and copper pro-drugs have been synthesized to bind endogenously to the large hydrophobic cavity at the IIA subdomain of albumin, followed by subsequent exchange with the N-donor residues of Lys 199 and His 242 to form a stable albumin complex [49], [50].

Several strategies have been employed to facilitate the liberation of the therapeutic cargo from albumin after it reaches the site of interest. In addition to the acid-labile hydrazone and reducible disulfide linkages mentioned above, another example albumin-binding prodrug incorporated a caspase-cleavable peptide spacer [51] to promote drug release from albumin at sites of tumor radiotherapy. In this design, the DEVD peptide, a well-known substrate of caspase-3, was attached on one end to albumin cysteine-34 through a maleimide functional group and on the other end to doxorubicin by a self-immolative linker. Because the enzyme caspase-3 is upregulated during apoptotic cell death that occurs in an irradiated tumor, this design allows for targeted release of doxorubicin chemotherapy at the site of tumor radiotherapy. An alternative liberation approach from *in situ* covalently bound albumin involves cathepsin-cleavable linkers. Cathepsins B and D are lysosomal enzymes known to be overexpressed in a variety of malignant tumors [52]. In one example prodrug, Schmid and colleagues incorporated a pentapetide linker, Ala-Leu-Ala-Leu-Ala, which is a known cathepsin-cleavable sequence previously employed in targeted drug release [52]. These authors conjugated ε-maleimidocaproic acid to camptothecin (a small molecule inducer of apoptosis) and doxorubicin using this linker flanked by two arginines which were incorporated to promote water solubility. A final method for liberating cancer therapeutics covalently bound to endogenous albumin leverages matrix metalloproteinase (MMP)-cleavable linkers. For example, MMP-2 has been shown to be overexpressed in melanoma and plays a significant role in tumor proliferation, angiogenesis, and metastasis [53]. Mansour and colleagues introduced an octapeptide, Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln, that has been shown to be effectively cleaved by MMPs 2 and 9 when used to link doxorubicin and an albumin-binding maleimide group [53]. These covalent binding and targeted release strategies could be generalized for the delivery of a wide range of cargo including other small molecule drugs and biologies.

2.1.2. Native Ligand Conjugates—Another approach to promote *in situ* interaction with albumin to alter drug pharmacokinetics involves the conjugation of therapeutic agents to ligands that naturally bind albumin non- covalently in the body. For instance, fatty acids are naturally ferried by albumin in the bloodstream due to their insolubility in plasma. Indeed, albumin is known to have seven fatty binding sites distributed asymmetrically on the protein and to bind around ~0.1 mol of fatty acid per mol human serum albumin under normal physiological conditions [54]. A common approach to promoting *in situ* docking of therapeutics through the fatty acid binding pockets of albumin involves direct conjugation of fatty acids to therapeutic payloads. Notably, commercially available Semaglutide (Figure 4) uses an 18-carbon fatty diacid linked to a glucagon-like peptide-1 analog via a glutamyl ethylene glycol spacer for the treatment of diabetes albumin binding (K_d~0.38 nM and half-life of 46.1 hr in mini-pigs) [55]. This was a remarkable improvement compared to its precursor, Liraglutide, (16-carbon monoacid with γGlu spacer) which demonstrated an i.v. half-life of 8–10 hours. This drastic enhancement underscores the impact of both linker and

binding chemistry in the use of albumin as a drug carrier. Similar noteworthy approaches have used a diacyl lipid linked by polytheylene glycol to deliver antigen/adjuvant cargo [56], palmitoyl modifications to the 2' position of antisense oligonucleotides [57], DNA nanocages modified with branching 12-carbon chains [58], and fatty acid bound platinum prodrugs [59]. Interestingly, increasing the valency of lipid-modified drugs appears to result in superior albumin-binding and pharmacokinetics, but may hinder potency depending on modification site [60].

In the context of cancer treatment, Sarrett et al used lipid-mediated *in situ* albumin binding to deliver therapeutic short interfering RNA (siRNA) to solid breast tumors. Short interfering RNA is a powerful gene silencing platform that enables the selective degradation of a specific mRNA, which can be used to directly target expression and phenotypes associated with a wide spectrum of cancers. However, siRNA pharmacokinetics are typically poor because it is subject to rapid renal clearance and has no mechanism for targeting cancer cells. This approach involved using simple and specific "click" chemistry to conjugate modified siRNA to a PEGylated diacyl lipid, and resulted in a 5.7-fold increase in half-life with Kd~1.38 μM [61]. Similarly, cholesterol is another native ligand that is non-covalently bound to albumin in the body and has been used as a means to improve the pharmacokinetic properties of biologies [62], [63], [64]. In a study on a variety of lipophilic siRNA conjugates, it was demonstrated that uptake efficiency of these conjugates is dependent on the type of lipophilic moiety, length of linker between the lipophilic group and siRNA, and the cell type [65]. These naturally bound ligands allow for non-covalent association of cargo which may both improve pharmacokinetic properties but also allow for easier offloading into sites of interest due to the reversibility of the albumin interaction.

2.1.3. Small Molecule Binders—In contrast to leveraging native ligands, synthetically produced small molecules can also be used for binding to endogenous albumin. A notable example of these small molecule binders is the dye Evans Blue (EB). Remarkably, each albumin protein can bind up to 14 Evans Blue molecules [66]. Truncated versions of this molecule have been derived to retain its albumin-binding properties but allow for modifications such as conjugation to other drugs or imaging agents[67][68][69]. For instance, truncated EB conjugated to the diabetes drug Exendin-3 resulted in markedly improved half-life (5 to 32 hours) and yielded improvement in hypoglycemic effects [70] [71]. A recent and interesting use of this platform was the development of an albumin/ vaccine nanocomplex that conjugated molecular vaccines to EB derivatives for use in cancer immunity (K_d for mouse serum albumin =1.0 μM)[72].

Another synthetic small molecule binder was developed by Dumelin and colleagues through screening of aDNA-encoded chemical library comprised of 619 candidate molecules [73]. The resulting binders were characterized by a common butanoyl moiety and require hydrophobic groups in the *para* position of the phenyl ring for good retention. The most successful candidate was a 4-(p-iodophenyl)butyric acid derivative that demonstrated affinity for albumin with K_d=3.2μM. Conjugation with this low molecular weight albumin binding entity has prolonged half-life of antibody fragments (~30 mins to 1000 mins) [74] and reduced renal clearance of a chelator-folate conjugate (~70 %ID/g vs 28 %ID/g at 4 h after injection) [75]. Other groups have found that a wide variety of synthetic and naturally

occurring aromatic compounds can be used for non-covalent association with albumin, which may be attributed to their hydrophobic nature. For instance, the FDA-approved MRI agent gadofosveset trisodium utilizes a 4- diphenylccyclohexyl group to reversibly bind serum albumin, resulting in extension of elimination half-life from approximately 90 to 1000 minutes [76] [77]. Modifications with these simple albumin-binding moieties are a synthetically appealing approach for improving drug pharmacokinetic properties.

2.1.4. Albumin-Binding Domains and Nanobodies—Albumin-binding peptides have also been leveraged to non-covalently associate with albumin to improve the pharmacokinetics of cancer therapeutics. These albumin-binding domains (ABDs) are often genetically fused to recombinantly-produced therapeutic proteins. The heptapeptide WQRPSSW is an albumin-binding domain that was identified using phage display by Su and colleagues [78]. This group genetically fused the chimeric peptide BB28, a fusion of tumor homing bombesin and mitochondria disrupting peptide, to this albumin binding domain in order to extend its half-life from several minutes to 2 hours [78]. Li et al, by comparison, used a 46- amino acid albumin-binding domain derived from streptococcal protein G [79] [80].

Streptococcal protein G is a cell-surface exposed protein produced by gram-positive bacteria that is capable of binding to serum proteins, namely albumin [81]. This group sought to extend the half-life of human recombinant tumor necrosis factor-related apoptosis-inducing ligand (hTRAIL), a tumor specific inducer of apoptosis whose lack of efficacy in clinical trials has been attributed to its poor pharmacokinetic properties. Their study identified fusion of the N- terminus of this 46-amino acid ABD to hTRAIL as a promising technique for improving its antitumor efficacy. The resulting conjugate bound human serum albumin with high affinity (K_d=0.4nM) and extended the half-life of hTRAIL from 0.32h to about 14. lh. Other successful examples of this technique include ABD genetic fusion to human soluble complement receptor type 1 [82], insulin-like growth factor II [83], and respiratory syncytial virus subgroup protein [84].

Monoclonal antibodies are a common means for high affinity binding to biologic targets and have had marked success in clinical translation. However, intact IgG antibodies are large (~150 kDa), and thus are considered to have limited tumor penetration capability [85][86]. This challenge has prompted investigation into monoclonal antibody fragments to achieve high affinity target binding with a molecule that is smaller in size. However, these antibody fragments can have diminished target affinity and can be too small in size to avoid renal clearance. To overcome these obstacles, Tijink et al investigated the class known as nanobodies for binding to albumin [85]. Nanobodies are derived from the unique antibody format present in dromedaries that is comprised only of the heavy chain. These “heavy chain only” antibodies are approximately 15 kDa, but, upon the desired binding to albumin would be able to avoid renal clearance. Tijink and colleagues constructed a 50 kDa albumin-binding nanobody for the purpose of improving upon existing epidermal growth factor receptor (EGFR) antibody treatments. Their bivalent nanobody comprised two EGFR binding units and one albumin binding unit [85]. The group details that, by themselves, nanobodies are quickly excreted, making intermediate association with albumin an additionally appealing approach. The EGFR- EGFR-Alb nanobody showed faster and

deeper tumor penetration than the unmodified EGFR- EGFR binder in A431 xenograft-bearing mice with a tumor to blood ratio greater than 80 achieved after 6 hours.

Another interesting implementation of albumin-binding domains involves its fusion to an affibody molecule. Affibodies are a new class of small (~6.5 kDa) proteins with high target specificity derived from staphylococcal protein A [87]. Multiple groups have sought to fuse an albumin binding domain to an affibody targeting human epidermal growth factor receptors, which are overexpressed in a variety of cancers and are known to drive tumor cell proliferation [88] [89]. Interestingly, Orlova and colleagues incorporated a radiolabel into these affibody molecules and suggest the potential of labeling with therapeutic radionuclides for radioimmunotherapy of solid tumors [88]. The elimination half-life of the parent affibody after fusion to the ABD was increased 80-fold from 0.5 to 41h. Similarly, a 46-amino acid ABD derived from streptococcal protein G was used to create a bispecific single-chain diabody for the retargeting of cyototoxic T cells to carcinoembryonic antigen (CEA)-expressing tumor cells by genetic fusion. This diabody was directed against the CEA and T cell receptor complex protein CD, and the resulting complex resulted in a 6-fold circulation time extension with a K_d of ~4nM for human serum albumin [90].

2.2. Exogenous Formulations

2.2.1. Covalent Conjugation—Cancer drugs formulated with exogenous albumin prior to delivery into the body have also been pursued in several formats, including direct, covalent conjugation of drug to albumin. A common strategy involves conjugation of therapeutic cargo to a primary amine available within a free lysine residue on albumin. This strategy has been used for the conjugation of small molecule drugs such as methotrexate [91], curcumin [92], and doxorubicin [93]. These albumin-drug covalent conjugates were created through the formation of amide bonds and reductive amination. The benefits to this covalent method include avoiding use of albumin's free thiol which is not always available, and the presence of multiple lysine groups available during exogenous conjugation. However, Kuhlmann et al suggest that, although this method allows for the conjugation to multiple lysines, the absence of selectivity may compromise FcRn engagement and consequent albumin pharmacokinetics [94]. Additionally, it may be more difficult to control the number of modifications per albumin as well as site specificity. The aforementioned binding method for conjugating drugs to the cysteine-34 of albumin can also be used for covalent binding to exogenous human serum albumin. Indeed, this method was used for the conjugation of oligodeoxynucleotides to this position to allow for the subsequent annealing of various complementary strands such as aptamers [94]. However, these exogenous albumin binding methods require either isolation from donors or recombinant production, both of which are associated with their own challenges including possible transmission of disease and high cost.

2.2.2 Recombinant Albumin Fusion Proteins—Direct genetic fusion of therapeutic proteins to whole recombinant albumin is another alternative. For instance, this approach was used to link the N-terminus of proaerolysin, a potent toxin, to recombinant albumin [95]. This group has termed the resulting therapeutic a "pro-toxin". The goal is for these "pro-toxins" to only be cleaved by a defined protease that is present in the metastatic

prostate cancer tumor microenvironment. The authors specifically engineered a peptide linker into their recombinant protein that was specific for the protease prostate specific antigen. Another recombinant HSA fusion protein, Albuleukin, combines recombinant interleukin-2 (rIL-2) and human serum albumin. This strategy aimed to maintain the excellent pharmacokinetic properties of albumin while conferring the immunomodulatory and anti-tumor properties of rIL-2 [96]. Other examples of this strategy include an interferon-β [97], anticarcinoembryonic antigen single-chain antibody [98], and barbourin [99]. This method is elegant in the sense that it does not require any complicated conjugation chemistry, merely the production and purification of precisely-defined, recombinant protein.

2.2.3. Nanoparticle Formulations—One of the more widely explored methods that utilize albumin as a carrier for cancer therapeutics involves drug encapsulation into an exogenous albumin-based nanoparticle. The appeal of this method lies in the ability to leverage the native albumin mechanisms that facilitate its extraordinarily long half-life and cancer homing properties, while shielding therapeutic cargo until the particle is broken down at the therapeutic site of interest. The methods for synthesizing albumin nanoparticles can be generally categorized into the techniques of desolvation, emulsification, thermal gelation, nano spray drying, and self-assembly [100] (Table 1). The most notable of these albumin nanoparticles is the aforementioned FDA approved nab-paclitaxel or Abraxane. Nab-paclitaxel synthesis involves passing a lipophilic drug and human serum albumin in an aqueous solvent through a jet under high pressure to form nab-paclitaxel nanoparticles with a mean particle size of 130 nm [101][102], However, various albumin nanoparticle strategies have been employed for a wide variety of treatment agents and a myriad of chemical modifications. Herein, we will survey notable examples of the large pool of clinical and preclinical formulations that utilize the various aforementioned synthesis methods. Albumin nanoparticles can be decorated with a variety of targeting ligands to give additional specificity to cancer-associated receptors. For instance, Zhao et al sought to target drug resistant colon-cancer cells and tumor associated macrophages which both highly express mannose receptors and SPARC [111]. They used mannosylated bovine serum albumin which was synthesized using 4-isothiocyanatophenyla-a-mannopyranoside. The albumin was then denatured using urea/BH4, and the subsequent addition of hydrophobic drugs and changing of salt concentration resulted in self-assembly into drug-loaded nanoparticles. In an analogous approach, folate-decorated bovine serum albumin nanoparticles were developed for the targeted delivery of paclitaxel. This approach leverages the overexpression of folate receptor which is known to occur on a wide range of tumor cell types; importantly the folate receptor is continuously internalized as a mechanism for carrying folate (vitamin B9) into tumor cells and thus serves as a particular useful portal for cellular entry [104]. This receptor-targeted nanoparticle was formulated by a desolvation method where BSA was dissolved into water followed by the addition of paclitaxel in ethanol using a peristaltic pump to drive aggregation. Then, a gluataraldehyde solution was added to crosslink the amino groups of the nanoparticles. After formation of the nanoparticles, an N-hydroxysuccinimide ester of folate was conjugated to free amines of BSA under alkaline conditions [104]. The resultant particles were approximately 210 nm in diameter and achieved a reported drug loading efficiency of approximately 27%. In addition to decorating albumin-based nanoparticles with natural receptor ligands, similar nanoparticle formulations

have been targeted through functionalization with antibodies [113], [114]. One such example employed covalent coupling of DI17E6, a monoclonal antibody directed against αν integrins, which are cell membrane-spanning matrix adhesion domains that are highly expressed in a various cancer lines. Inhibitors of αvβ3 have been shown to inhibit growth and angiogenesis in melanoma. DI17E6-functionlized particles were formulated by ethanol desolvation of an aqueous solution of doxorubicin adsorbed to HSA followed by crosslinking with glutaraldehyde. Free amines on the surface of these particles were modified with thiolated antibody through a PEG maleimide-NHS ester. The resultant loaded drug particles showed very low dispersity at a size of approximately 380nm [115]. A wide variety of other targeting ligands have been used to modify the surface of albumin nanoparticles to further direct them towards cancer cells including aptamers against cancer cell membrane proteins [116], apolipoprotein E for facilitating transport across the blood-brain barrier [117], and lutenizing- hormone releasing hormone, whose receptor is overexpressed on a variety of cancer cells [118]. These examples underscore the versatility of albumin-based carriers for small molecule chemotherapeutics and also for modification to target specific cancer cell types.

Albumin nanoparticle composites have also shown efficacy in the encapsulation and delivery of anticancer agents. For example, silk fibroin-albumin blended nanoparticles have been developed with the goal of circumventing the leakage of encapsulated chemotherapeutics believed to occur due to the relatively hydrophilic nature of albumin [119]. It was hypothesized that because silk fibroin is a more hydrophobic protein, a composite nanoparticle would improve mechanical and encapsulation properties due to the electrostatic interactions between carboxyl groups of the silk fibroin and the amino groups of albumin. The authors applied this system to encapsulate the chemotherapeutic methotrexate into −100 nm particles; this was achieved by desolvation in acetone and glutaraldehyde crosslinking of a silk fibroin and albumin solution [119]. Additionally, albumin has been derivatized into a diblock format with traditionally-utilized, hydrolytically-degradable, and hydrophobic synthetic polymers such as polycaprolactone. In this setting, a micellar nanoparticle can be formed with the albumin serving as the hydrophilic block which is traditionally PEG. Jiang et al created two different polymers based on ring-opening polymerization: poly(oligo(ethylene glycol) methyl ether acrylate)-poly(ε-caprolactone) (POEGMA-PCL) and maleimide -functionalized polycaprolactone (MI-PCL). This maleimide PCL was then conjugated to the free cysteine on bovine serum albumin. The authors then synthesized nanoparticles with different albumin content through the co-assembly of POEGMA- PCL and BSA-PCL at different mixture ratios. They then loaded these particles with curcumin, and noted that for several cancer cell lines, cellular uptake of the nanoparticles positively correlated with the amount of albumin present on the nanoparticle surface [120]. Other interesting albumin-polymer investigations have shown that modifiying albumin with cationic polymers can be used to improve cell penetration in breast cancer [121] and using thermosensitive polymer-conjugated albumin can be used to thermally target cancer cells [122]. Albumin-modified nanoparticles may benefit from reduced degradation product toxicity, increased ease of injectability, and solubility in water. This overall body of literature suggests that in conjunction with the previously discussed tumor-tropic properties of albumin, it is a promising candidate for nanoparticle synthesis.

3. Survey of Results

Albumin has demonstrated benefits as a drug carrier in both preclinical models and clinical trials. In the following sections, we will highlight the ability of diverse strategies employing albumin to improve the pharmacokinetic properties of anticancer agents including biodistribution, circulation half-life, tumor targeting and penetration, and ultimately, tumor cytotoxicity and survival outcomes. Applications with *in vivo* results are preferentially highlighted in this section.

3.1. Chemotherapeutics

3.1.1. Nanoparticles—Albumin nanoparticles have been investigated in many cancer types, but are especially well- suited for the challenging cancers of the brain. The brain-blood barrier typically serves the important physiological purpose of protecting the brain from insults in the circulation. However, it also presents the greatest challenge for drug delivery to cancers of the brain. Despite the poor transport across the brain vascular endothelium, there is exchange of substances between the intravascular and tissue compartment of the brain through active nutrient transporters [123]. Lin and colleagues were motivated to leverage the increased demand for albumin from metabolically actively tumors to facilitate the intake of a drug-loaded albumin nanoparticle. Their self- assembled formulation included a cell penetrating peptide, low molecular weight protamine, on the outside of the particle to aid in tissue penetration and two hydrophobic chemotherapeutics, paclitaxel and fenretinide. Their nanoparticle demonstrated a tumor growth inhibition rate of 82% in a subcutaneous glioma mouse model compared to 40% for the free combination of drugs. Furthermore, in an orthoptopic glioma (Luc-U87) model, their particle administered with 2 mg/kg of each of their therapeutics demonstrated a greater survival time of 37 days relative to 24 days for negative control and 31 days for equivalent concentrations of free drug (Figure 5). Interestingly, protumor phenotype M2 macrophages were also suppressed by these nanoparticles.

For direct pulmonary treatment of lung cancer, Choi and colleagues synthesized inhalable albumin nanoparticles made of human serum albumin conjugated with doxorubicin and octyl aldehyde and absorbed with TRAIL [110]. Briefly, this involved thiolating doxorubicin and modifying HSA with a maleimide (sulfo-SMCC linker). These two components were then reacted, and the resulting conjugate was functionalized with octanal by reductive amination in the presence of sodium cyanoborohydride. The self-assembled particles were then loaded with TRAIL by sonication in an ice bath. These particles achieved anti-tumor efficacy in mice bearing lung H226 tumors, with lungs of mice treated with the TRAIL/Dox HSA nanoparticle (5μg TRAIL + 1μg Dox) showing fewer malignant surface lesions compared to mice treated with dose matched Dox or TRAIL HSA. Lung weight as a measurement of tumor expansion from groups treated with TRAIL particles or Dox particles was significantly greater than that of the combination TRAIL/Dox HSA-NP (678.2 ± 51.5, 598.9 ± 24.8, and 337.5 ± 7.5 mg respectively) (Figure 6). Additionally, histological samples of the lung specimens from H226- implanted mice demonstrated that TRAIL/Dox HSA-NP not only decreased lesion numbers and sizes, but exhibited significant induction of apoptosis whereas mice treated with TRAIL or Dox particles did not. With lung cancer presently

associated with a particularly low 5-year survival rate of less than 18%, high efficiency delivery of therapeutics directly to lung tumors using albumin presents a promising approach.

3.1.2. Covalent Conjugates—Chemotherapeutics directly bound to exogenous and endogenous albumin rather than encapsulated into nanoparticles have also demonstrated notable benefits. For the albumin-binding doxorubicin prodrug synthesized by Chung et al, the addition of an albumin-binding moiety resulted in the extension of half-life 38-fold from approximately 30 minutes to 19 hours [51]. Doxorubicin was linked via a self-immolative linker to a DEVD motif. This DEVD-S-DOX conjugate was linked to the albumin-binding maleimide functionalization, ε-maleimidocaproic acid (EMC) using an N-succinimide ester. The DEVD spacer is recognized and hydrolyzed by caspase-3, a protease upregulated in irradiated tumors. The administration of this conjugate labeled with Cy5 in lieu of doxorubicin resulted in significant accumulation in tumors with fluorescent intensity decreasing in other organs. Notably, *in vitro* there was a lack of toxicity associated with HSA-DEVD-S-DOX up to IOOμM until incubation with caspase 3, whereas, under these activated conditions, the prodrug created similar toxicity as free doxorubicin in SCC7 (IC_{50}=0.49 μM and IC_{50}=0.30 μM) and MDA-MB231 cells (IC_{50}= 4.17 μM and IC_{50}=2.04 μM respectively). Furthermore, insignificant accumulation of the compound within the cell nucleus was observed without the presence of caspase-3, further underscoring the ability of the system to avoid off target effects using environmentally targeted activation. When administered at 10 mg/kg with radiotherapy, the authors reported a 91% decrease in tumor volume compared to radiotherapy alone with no noticeable body weight changes or indications of systemic toxicity. According to the results, EMC-DEVD-S-DOX had negligible anti-cancer effect when used alone, underscoring the ability of this prodrug to overcome the nonspecific toxicity shown in traditional chemotherapy [51].

The same benefits conferred by binding to endogenous albumin can be achieved by the covalent conjugation of exogenous albumin to chemotherapeutics. Notably, methotrexate (MTX) conjugated at a 1:1 drug:HSA ratio through HSA lysine residues found significant success in preclinical animal models (clinical translatability reviewed in Section 3.4). Motivated by the short tumor exposure time of unmodified methotrexate, an albumin-MTX conjugate was developed to improve the MTX pharmacokinetic profile. These conjugates were evaluated in seven nude mouse human tumor xenograft models including bladder, breast, lung, osteosarcoma, soft tissue sarcoma, and prostate cancers [124], Notably, in soft tissue sarcoma SXF 1301, MTX- HSA treatment resulted in complete remission after a single injection at 12.5 mg/kg whereas an equivalent drug dose of free MTX resulted in short-lasting, partial tumor regression.

Additionally, in the prostate-cancer model PRXF PC3M, MTX-HSA demonstrated 92.8% growth inhibition of control. However, at a molar basis, the authors noted that MTX-HSA was more active than MTX but also more toxic. The authors cited the potential for this compound to overcome drug resistance due to transport deficiencies for native MTX, and suggested the improved pharmacokinetic properties, persistent high plasma levels, and demonstrated accumulation in solid tumors contributed to the potential efficacy of their compound.

3.2. Biologies

3.2.1 Oligonucleotides—Albumin-mediated delivery of therapeutic oligonucleotides has found success in multiple preclinical models of cancer. Conjugation of therapeutic short interfering siRNA via a PEGylated linker to an albumin-binding diacyl lipid (L_2) prolonged the half-life of siRNA by 6- fold. Notably, siRNA molecules can be degraded by nucleases and are rapidly cleared through the kidneys following intravenous delivery, and association with albumin appears to reduce both of these clearance mechanisms. In addition to systemic pharmacokinetic advantages, testing on *in vitro* MCF7 breast cancer spheroids showed that albumin-bound siRNA conjugates have increased model tumor tissue penetration and both higher and more homogenous tumor cell internalization than commercially available jetPEI nano-polyplexes (Figure 7). The siRNA-L_2 conjugates also localized to tumors significantly more than commercially available jetPEI in a patient derived xenograft model at 1 mg/kg (99% uptake vs. 60% uptake 30 minutes after i.v. injection), and tumor radiance as an indicator of Luciferase knockdown was significantly lower for this formulation than jetPEI in an orthotopic tumor of MDA-MB-231 cells. One striking finding from this study was a complete lack of toxicity at doses of 10mg/kg, which sharply contrasts with reported toxicity and immunogenicity of nanoparticle carriers [61]. Additionally, siRNA-L_2 demonstrated a tumor to liver ratio of 40:1 compared to 3:1 for jetPEI. This finding is particularly notable given that a main disadvantage of nanoparticles is their natural tendency to accumulate in the liver. These data suggest that albumin-based nucleotide delivery may be especially promising for nonhepatic, oncologic therapeutic applications relative to synthetic nanocarriers.

Short interfering RNA has also been delivered to tumor cells via albumin nanocarriers [125], [126]. Notable *in vivo* results were achieved using nanocomplexes were comprised of thiolated HSA that would interact with thiolated siRNA to self-crosslink (Figure 8). Tail vein injection of 50 mg of Cy5-labeled nanocomplex to SCC7 tumor-bearing mice resulted in 1.7 times the signal intensity in tumor tissue 12 hours post-injection than thiolated siRNA alone. Using RFP/B16F10 tumor-bearing mice, Son and colleagues were able to show their nanoparticle/siRNA formulation turning off the RFP signals at the tumor site (RFP signal diminished to 43% at 7 days compared to saline control and free thiolated siRNA). The authors also quantified gene expression by RT- PCR and showed that the amount of RFP mRNA after treatment with their nanoparticle was decreased to about 19% of the control while free precursor thiolated siRNA showed no significant reduction. Finally, they demonstrated anti-tumor therapeutic efficacy of their nanoparticle formulation containing siRNA against vascular endothelial growth factor, which has been implicated as a key angiogenic factor that drives tumor angiogenesis and growth. Their treatment (0.5 mg of siRNA/kg once per 3 days by i.v. injection) resulted in the reduction of tumor volumes by 80% compared to the control group at day 30 (no significant reduction from free thiolated siRNA siRNA).

Albumin-mediated delivery of antisense oligonucleotides (ASO) has also been investigated in the context of cancer treatment. For instance, Wartlick and colleagues first demonstrated uptake of ASO bearing albumin nanoparticles in the breast cancer cell lines MCF-7 and MDA-BM-435 administered at 1 mg/mL [103]. Subsequent efforts to mediate ASO delivery

with albumin have included the investigation of a folate receptor-targeted lipid-albumin nanoparticle for the delivery of Akt1, an ASO which has shown clinical efficacy in tumor inhibition but is limited by low membrane permeability and rapid clearance from the blood [127].

3.2.2. Immunomodulatory Drugs—Albumin mediated-delivery has also demonstrated success in the context of tumor immunotherapy. The albuleukin fusion protein of recombinant interleukin-2 and human serum albumin showed the ability of genetic fusion to albumin to confer its remarkable pharmacokinetic properties [96]. Indeed, pharmacokinetic studies of 500μg/kg of albuleukin versus the rIL-2 peptide alone in BALB/c mice demonstrated half-life extension from 19–57 minutes to 6 to 8 hours. Importantly, the immunomodulatory effects associated with the rIL-2 peptide alone were maintained in the fusion peptide. In Renca renal adenocaminoma tumors in mice, albuleukin suppressed tumor growth significantly compared to rIL-2 alone with an approximately 4-fold difference in tumor volume. Additionally, the fusion protein promoted infiltration of $CD4^+$ and $CD8^+$ T cells, which are associated with a beneficial anti-tumor immune response. Albuleukin additionally inhibited metastasis of hepatic tumors of B16F10 melanoma cells when administered to mice every other day at 1 mg/kg for three days with significant reduction in residual metastases compared to rIL-2 alone (~30 vs ~80 percent mice with residual metastases). Hepatic metastasis of melanoma continues to present a significant clinical challenge, underscoring the need for new treatment strategies such as albuleukin.

Despite the tremendous potential of vaccines in cancer immunotherapy, inefficient delivery of antigen and adjuvants to the lymph node, where the immune response of lymphocytes is coordinated, has limited their clinical success. At 67 kDa, albumin exceeds the cutoff for dissemination in to the blood from the interstitial space and is instead trafficked to the lymphatics [128]. Zhu et al sought to leverage this characteristic along with slow lymph flow to modulate lymphocytes within lymph nodes. Albumin can be efficiently endocytosed by antigen- presenting cells (APCs) via endocytosis for antigen processing and presentation making it an appealing platform for subunit vaccines. By conjugating molecular vaccines to Evans Blue, albumin-binding vaccines that self-assemble *in vivo* using endogenous albumin were found to be 100-fold more efficient at the co-delivery of CpG and antigens to lymph nodes. This albumin vaccine nanocomplex was additionally shown to elicit ~10 times more antigen-specific $CD8^+$ cytotoxic T lymphocytes with immune memory than the benchmark incomplete Freund's adjuvant. This formulation in conjunction with Abraxane or anti-PD 1 shows further potency and represents a robust platform for combination cancer immunotherapy [72].

3.3. Theranostics

The unique characteristics of albumin as a carrier lend themselves to utilization of this protein for theranostics, which can not only be used for diagnosis and imaging but also for treatment [129], [130], [131]. For instance, lymph node metastasis is associated with poor prognosis. Chen and colleagues demonstrated they could create nanoprobes for image-guided photoablation by adsorbing IR825, a near-infrared dye, to human serum albumin covalently linked with diethylenetriamine pentaacetic acid molecules chelating gadolinium.

(Figure 9). Specifically, they demonstrated inhibition of tumor metastasis after surgery by photothermally ablating sentinel lymph nodes (SNLs) with metastatic tumor cells. In an *in vitro* study, 4T1 murine breast cancer cells efficiently internalized HSA-Gd-IR825 nanoprobes with no difference in cell viability until photothermal ablation was induced. Mice inoculated with 4T1 murine breast tumor cells via footpad injection developed metastatic tumor cells in their sentinel lymph nodes after 12 days. When 0.03 μ mol of HSA-Gd-IR825 was administered intratumorally on the right foot pad, these sentinel lymph nodes were rapidly heated upon exposure to an 808-nm laser at a power density of 0.8 W/cm^2 for 10 min. For mice injected with the nanoprobe, temperatures of the SLNs reached ~55°C: high enough to ensure effective photothermal ablation of cancer cells, whereas saline injected mice under the same irradiation conditions showed little change. Excitingly, the researchers found that mice that underwent surgical dissection of their primary tumors as well as photothermal ablation of their SNLs showed greatly prolonged survival. Notably, 4 out of 6 mice remained alive after 70 days compared to mice with primary tumor isolation alone dying within 50 days. Such a strategy could be an alternative approach to assist surgery and reduce the risk of post-treatment metastasis via lymphatic systems [132].

Another exciting approach from Chen et al utilized a nanoscale delivery system based on albumin-coated MnO2, which can modulate the tumor microenvironment by mitigating hypoxia [133]. It is known that the rapid growth of cancer cells results in insufficient blood supply to tumors, and the resultant hypoxia may hinder treatments such as oxygen-dependent radiotherapy and photodynamic therapy. Manganese dioxide (MnO2) nanoparticles are known to have high reactivity toward H_2O_2 (solid tumors constitutively produce H_2O_2) to produce O_2. By leveraging MnO_2 decomposition in the reactive oxygen species rich environment of the tumor, the authors sought to overcome tumor-hypoxia associated resistance of photodynamic therapy. Importantly, the tumor growth in 4T1 tumor bearing nude mice after combination therapy using the HSA MnO_2 nanoparticles plus 660 nm light irradiation was significantly inhibited. The HSA MnO_2 system provided improved therapeutic efficacy compared to various control groups, including the combination therapy using HSA NPs plus light irradiation without the assistance of MnO_2 to relieve tumor hypoxia.

3.4. Clinical Results

The promising results from preclinical investigation of albumin-mediated cancer treatments have prompted several clinical trials. The first albumin-drug conjugate evaluated in phase I/II studies was the aforementioned methotrexate-albumin conjugate synthesized by the direct covalent conjugation to lysine residues of HSA. Seventeen patients were treated, and tumor responses were seen in three: partial and minor responses in patients with renal cell carcinoma and pleural cell carcinoma. A regimen with MTX-HSA injections of 50 mg/m^2 every 2 weeks was recommended for further investigation after this study [134]. However, a subsequent phase II study did not show any objective responses despite being well tolerated (n=17) [135]. A phase II study assessing administration in combination with cisplatin showed one complete response and one partial response in advanced bladder cancer (n=7) but no further clinical investigation was undertaken [136].

Abraxane (nab-paclitaxel) was the first albumin-based drug approved in oncology. Its development was motivated by the desire to avoid Cremophor-based toxicity in paclitaxel delivery [102]. It was first approved in 2005 for the treatment of metastatic breast cancer and subsequently approved in 2012 and 2013 for first-line treatment of non-small cell lung cancer and metastatic pancreatic cancer, respectively [137]. The design of both phase III studies combined Abraxane with the best chemotherapeutic agent of choice, and this combination was compared with the single standard agent alone (100mg/m^2 with carboplatin every 3 weeks for non-small cell lung cancer compared to Cremophor encapsulated paclitaxel plus carboplatin once every three weeks). For advanced pancreatic cancer, the study examined 125 mg/m^2 Abraxane combined with 1000 mg/m^2 gemcitabine compared to gemcitabine alone on a three-week schedule. Approximately 1 month and 2 month overall survival benefits were observed for the combination therapy in non-small cell lung cancer and pancreatic cancer respectively. The exceptional success of Abraxane has launched subsequent clinical investigation in to other nab- technology formulations in the cancer arena such as nab-docetaxel (Phase I/II completed 2011), nab-rapamycin (Phase I completed 2016), and nab-heat shock protein inhibitor [138] [139].

Phase 1 clinical studies of the (6-maleimidocaproyl) hydrazone derivative of doxorubicin (aldoxorubicin) showed a good safety profile at doses up to 260 mg/m^2 and induced tumor regression in breast cancer, small cell lung cancer, and sarcoma [140]. In 2015, aldoxorubicin was investigated in a phase 2b clinical trial for advanced soft-tissue sarcoma. A total of 123 patients were treated with 83 receiving aldoxorubicin at 350 mg/m^2 or doxorubicin at 75 mg/m^2 once every three weeks for up to 6 cycles. Median progression-free survival time was significantly improved (5.6 vs 2.7 months) with aldoxorubicin compared to doxorubicin as well as the rate of 6-month progression-free survival (46% and 23%) [141]. In 2017, a phase III clinical trial investigated aldoxorubicin at the same dose of 260 mg/m^2 against investigators choice of several standard chemotherapeutics in 433 patients with soft tissue sarcoma. Aldoxorubicin demonstrated a time to progression or death of 5.32 months vs 2.96 for investigator's choice therapeutics ($p=0.007$), suggesting it is a promising option for treating relapsed or refractory metastatic soft tissue sarcoma [142]. With the pioneering successes of these clinically examined formulations, the wide variety of preclinical albumin-based cancer therapeutics hold great promise for future translation.

3.5. Conclusions and Future Directions

Albumin-mediated delivery has demonstrated advantages for multiple categories of anti cancer agents. Its cancer-targeting properties, extraordinary circulation half-life, and natural ligand-binding capability make it a promising and already translatable medium for delivery of diverse therapeutic cargo. Excitingly, many high affinity albumin-binding platforms described in the synthesis portion of this review have yet to be examined in the context of cancer treatment. Moreover, as our understanding of molecular targets for treating the diverse spectrum of cancers is continually expanding, it is probable that new, tailored, and personalized medicine will be made possible through albumin-mediated delivery of biologies and immunomodulatory drugs.

Moving forward, it will be necessary to identify the cancer subsets that are most amenable to albumin-mediated treatment. Albumin is known to accumulate in tissues with vascular leakiness characteristic of tumors, but it has recently become more appreciated that vascular disruption and EPR-driven delivery is not consistent across tumor types or even within different regions of a single tumor. While the smaller size and higher diffusivity make albumin better suited to overcome this barrier than many synthetic nanoparticle carriers, partnering imaging reagents that can characterize tumor vasculature may confer key information for predicting which patients may benefit from albumin-based tumor therapies.

While tissue level pharmacokinetics and biodistribution into tumor tissue is often a primary focus, most molecularly-targeted small molecule and biologic therapies must also navigate the cell-level barriers of the cell and endosomal membranes. This is an especially important consideration for biologies that act in the cytoplasm as they are less able to diffuse through lipid bilayer membranes than small molecules. While it is clear that binding to albumin can extend circulation half-life through mechanisms such as the FcRn and megalin/cubilin reabsorption, what remains only partly elucidated is how albumin binding impacts cellular entry and subsequent intracellular trafficking of its bound cargo. For example, cancers with high expression of Cav-1 may be particularly well-suited to treatment with albumin-based formulations based on the aforementioned findings from nab-paclitaxel particles, suggesting that albumin can preferentially utilize caveolaer endocytosis mechanism for cellular entry. However, there is still little understanding for the mechanism of action for other albumin-based formulations. For example, the interaction between the type of therapeutic cargo (small molecule, biologic, etc) and the nature of the albumin association (nanoparticle, direct conjugation/fusion, non-covalent through a native or synthetic albumin-binding ligand) and the impact on intracellular therapeutic bioavailability is key for informing optimized therapeutic design but remains relatively unexplored. For biologic cargo, investigating how molecular design and the nature of the albumin association facilitates avoidance of endo/lysosomal entrapment is an area especially important future focus area.

Albumin is a remarkable carrier protein with the potential to overcome barriers to delivering a host of promising therapeutics in the cancer arena. Characterizing how different class of cargo and type of albumin engagement affect systemic and intracellular trafficking will be critical for optimizing future therapeutic candidates. Ongoing and future studies are expected to continue to define the design rules for successful clinical implementation of different classes of therapeutics and for treatment of various cancer types.

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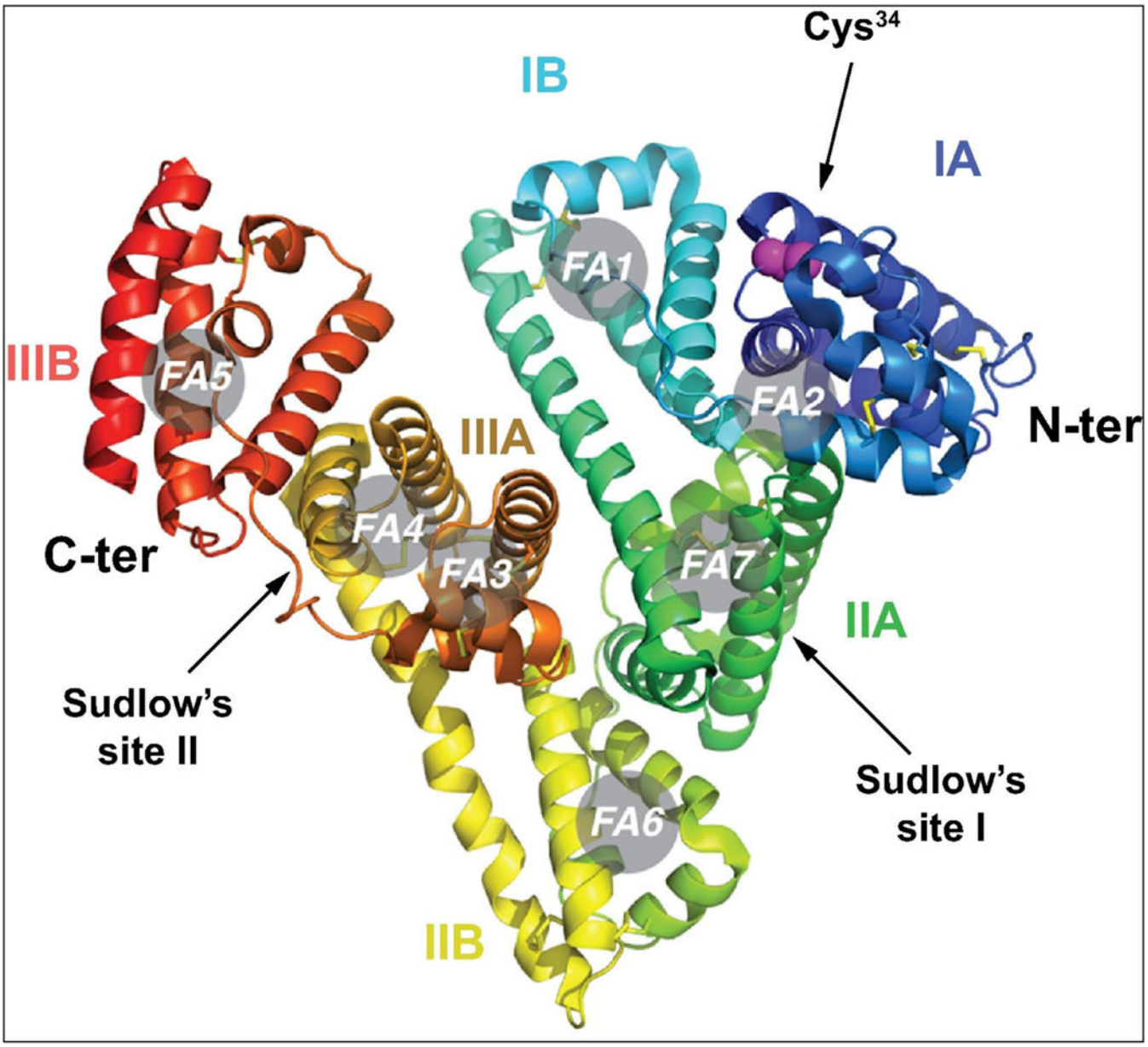


Figure 1:
Crystal structure of human serum albumin. Albumin contains three alpha helical domains each comprised of two subdomains. Its seven fatty acid binding sites are distributed asymmetrically across the protein. Additional sites of importance in binding include the free thiol located at the cysteine-34 amino acid residue and Sudlow's sites I and II, which bind a variety of nonspecific hydrophobic drugs. Figure reproduced from Arroyo et al [6] with permission of the Journal of Hepatology

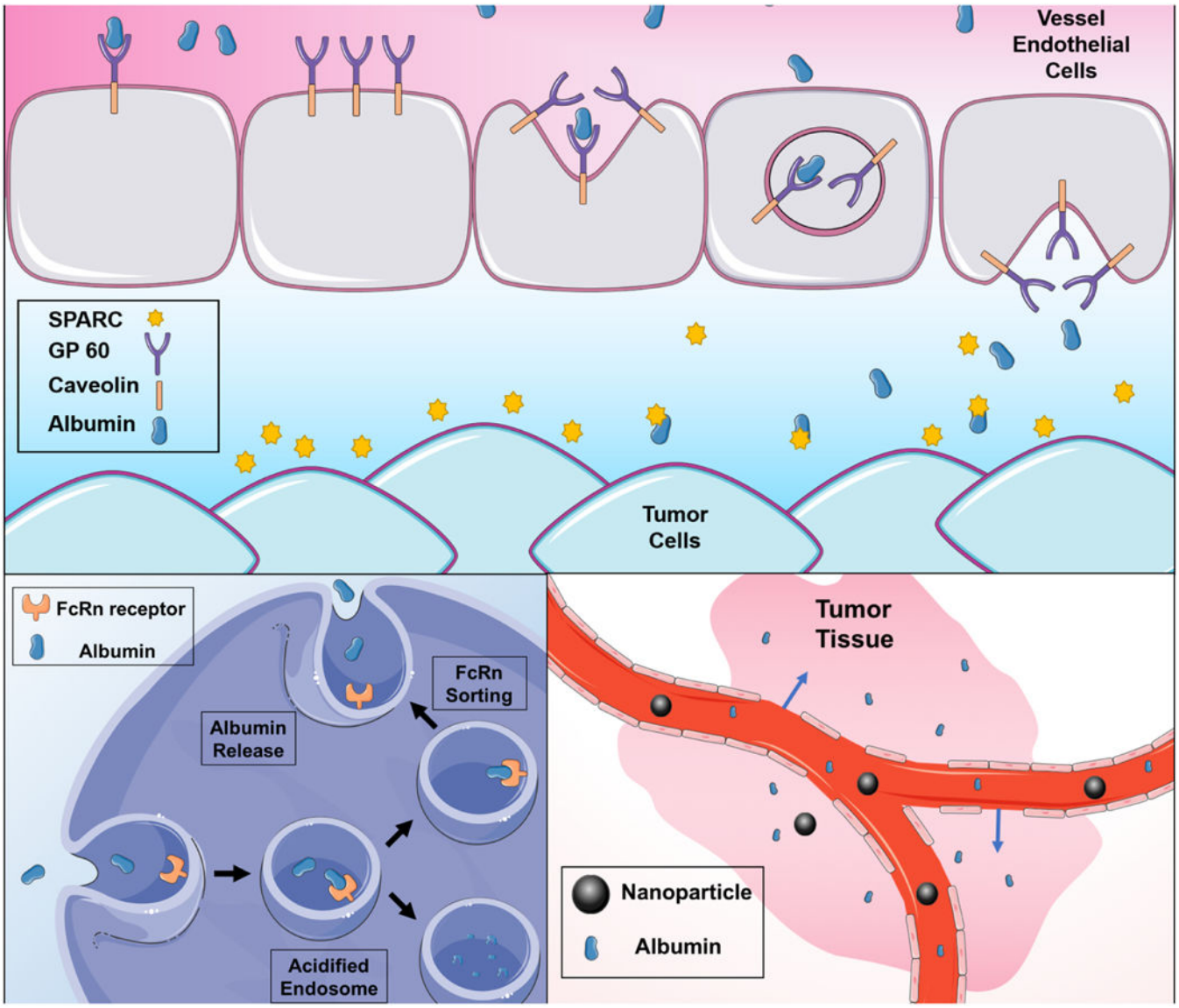


Figure 2:

Mechanisms of albumin transcytosis, recycling, and accumulation. A) Albumin binds to GP60 which is associated with Cav-1. Subsequent clustering results in caveolae formation and shuttling of receptor-bound albumin across the endothelium. Albumin is then hypothesized to bind SPARC and concentrate albumin-bound therapeutics in the tumor interstitium B) The neonatal Fc receptor binds albumin the acidic conditions of the endosome and rescues it from degradation in the lysosomal pathway. C) Vascular leakiness and lymphatic insufficiency results in the accumulation of macromolecules in tumors.

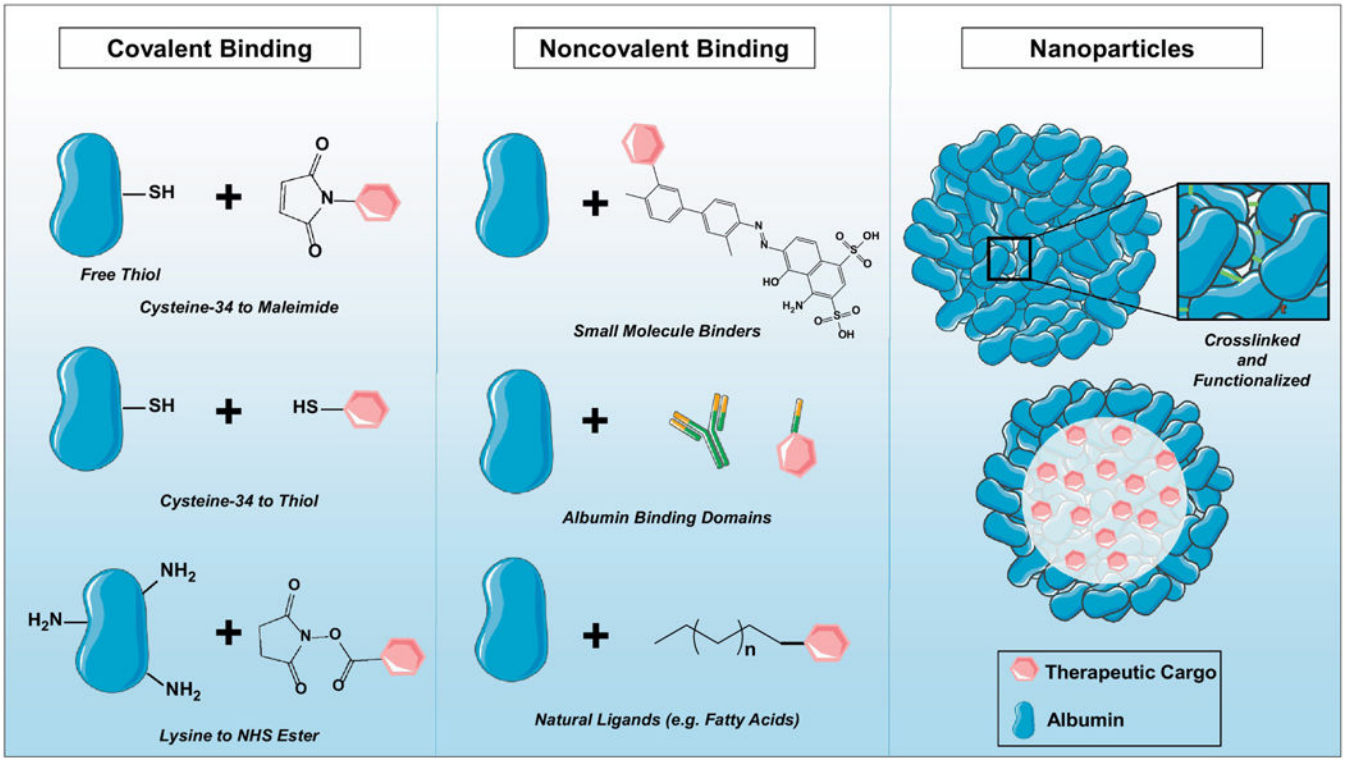


Figure 3:
Overview of albumin binding strategies.

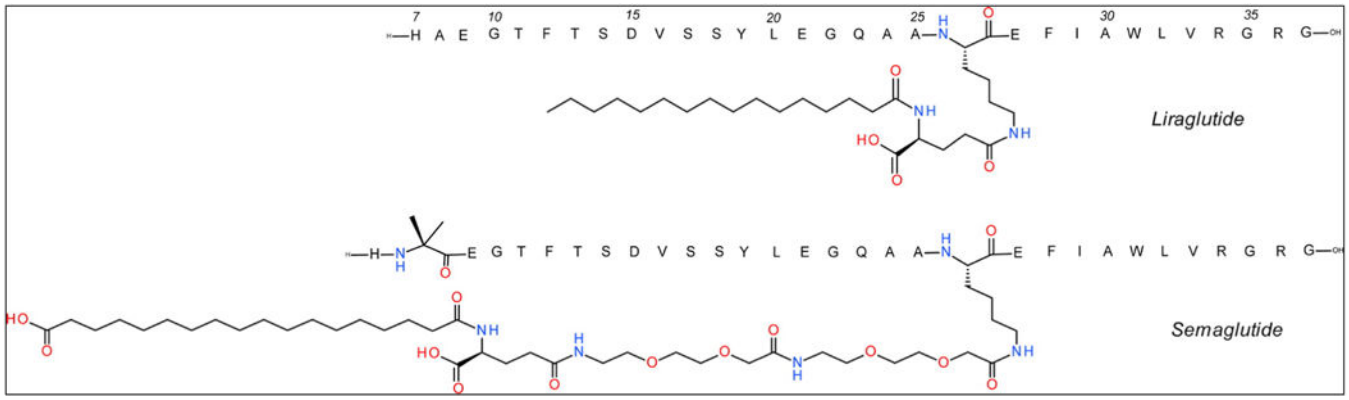


Figure 4:
Chemical structures of Liraglutide and Semaglutide. Figure reprinted from Lau et al [55] with permission of the Journal of Medicinal Chemistry

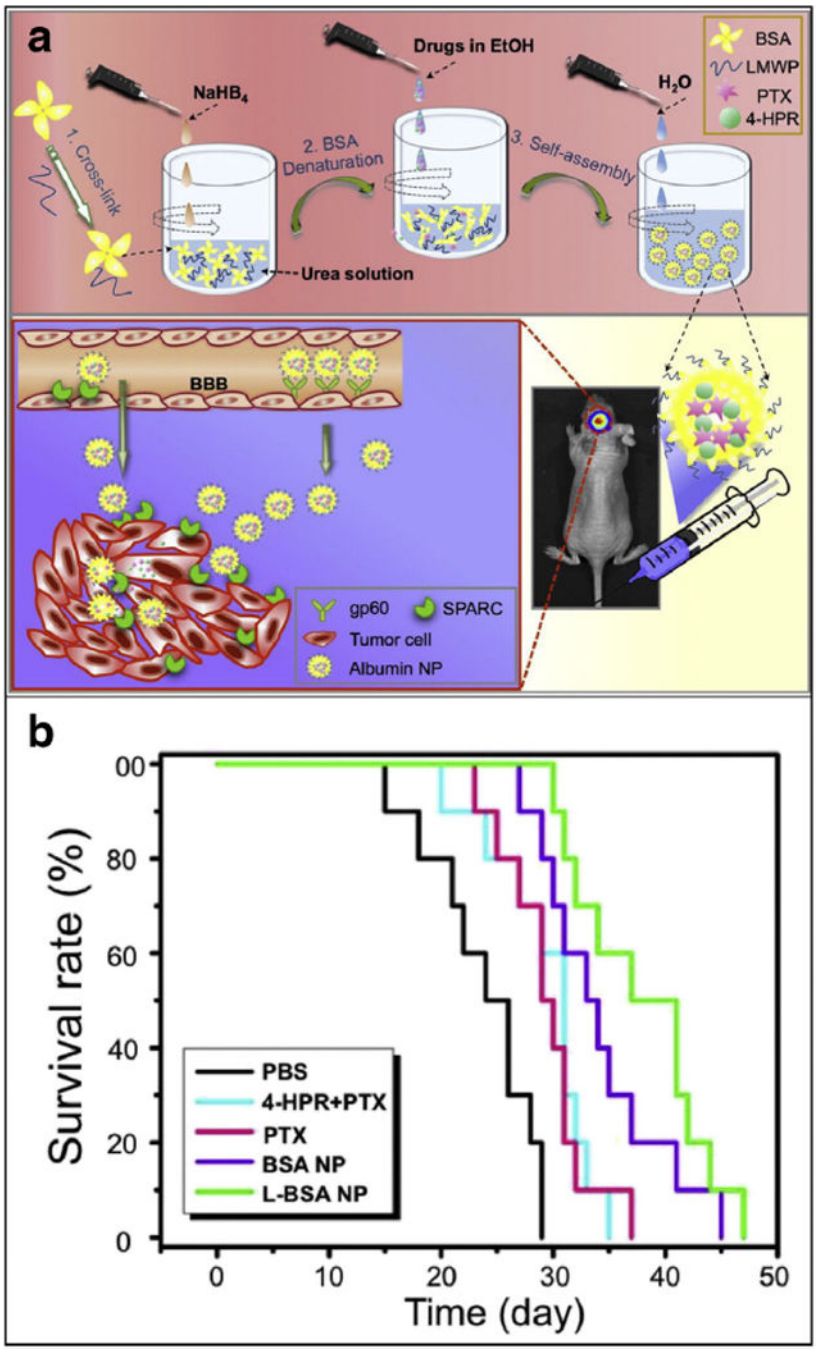


Figure 5:

A) Self-assembly of low molecular weight protamine-modified albumin nanoparticles. Upregulation of SPARC was observed in tumor blood vessels over normal capillaries of the blood brain barrier as well as colocalization with nanoparticle B) Survival curve of mice bearing orthotopic gliomas treated cell penetrating peptide modified nanoparticle (L-BSA-NP) encapsulating fenretinide+paclitaxxel, unmodified nanoparticle (BSA-NP) encapsulating fenretinide+paclitaxel, free fenretinide (4-HPR), free paclitaxel (PTX), free

drug combination (4- HPR+PTX), or phosphate buffered saline control (PBS). Figure reproduced from Lin et al [123] with permission of ACS Nano

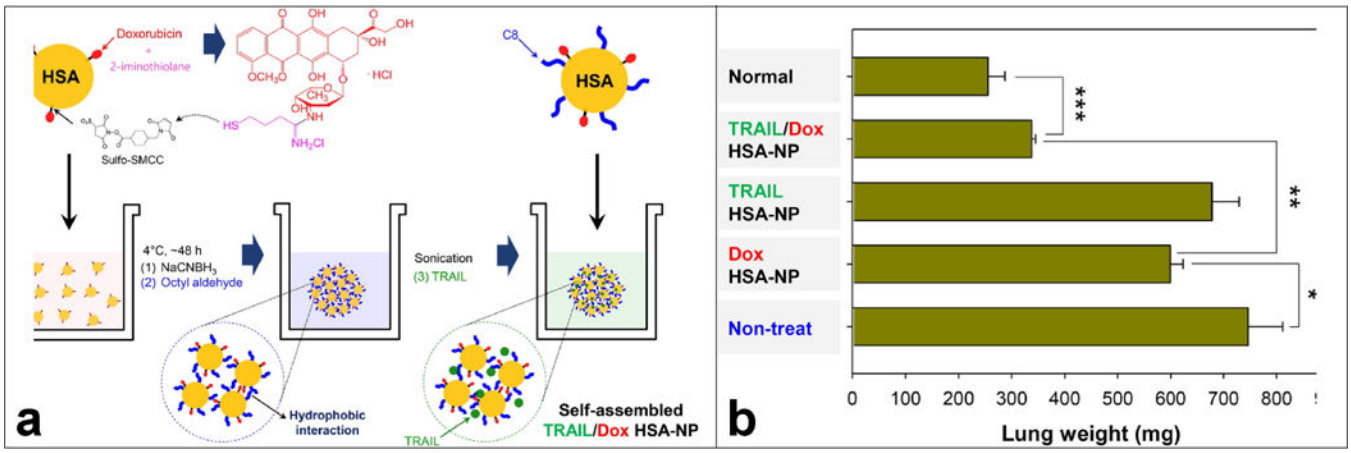


Figure 6:

A) Synthesis overview of TRAIL/Dox HSA-NP B) Lung weight as a measure of tumor burden in treated mice in normal, healthy mouse or tumor-bearing mice treated with human serum albumin nanoparticles delivering one or both or doxorubicin or trail Figure reproduced from Choi et al [110] with permission of the Journal of Controlled Release

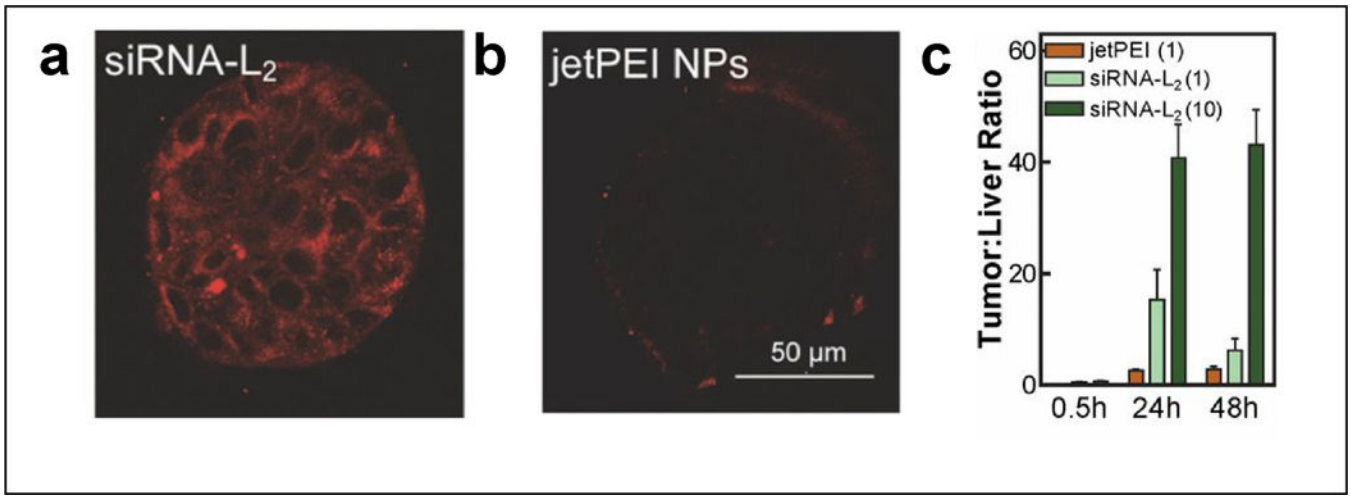


Figure 7:

Confocal images depicting superior tumor spheroid tumor spheroid penetration by (A) modified siRNA (siRNA-L2) compared to (B) commercially available jetPEI. C) Higher tumorliver ratio achieved by siRNA-L2 than jetPEI in orthotopic tumor mouse model Figure reproduced from Sarett et al [61] with permission of PNAS

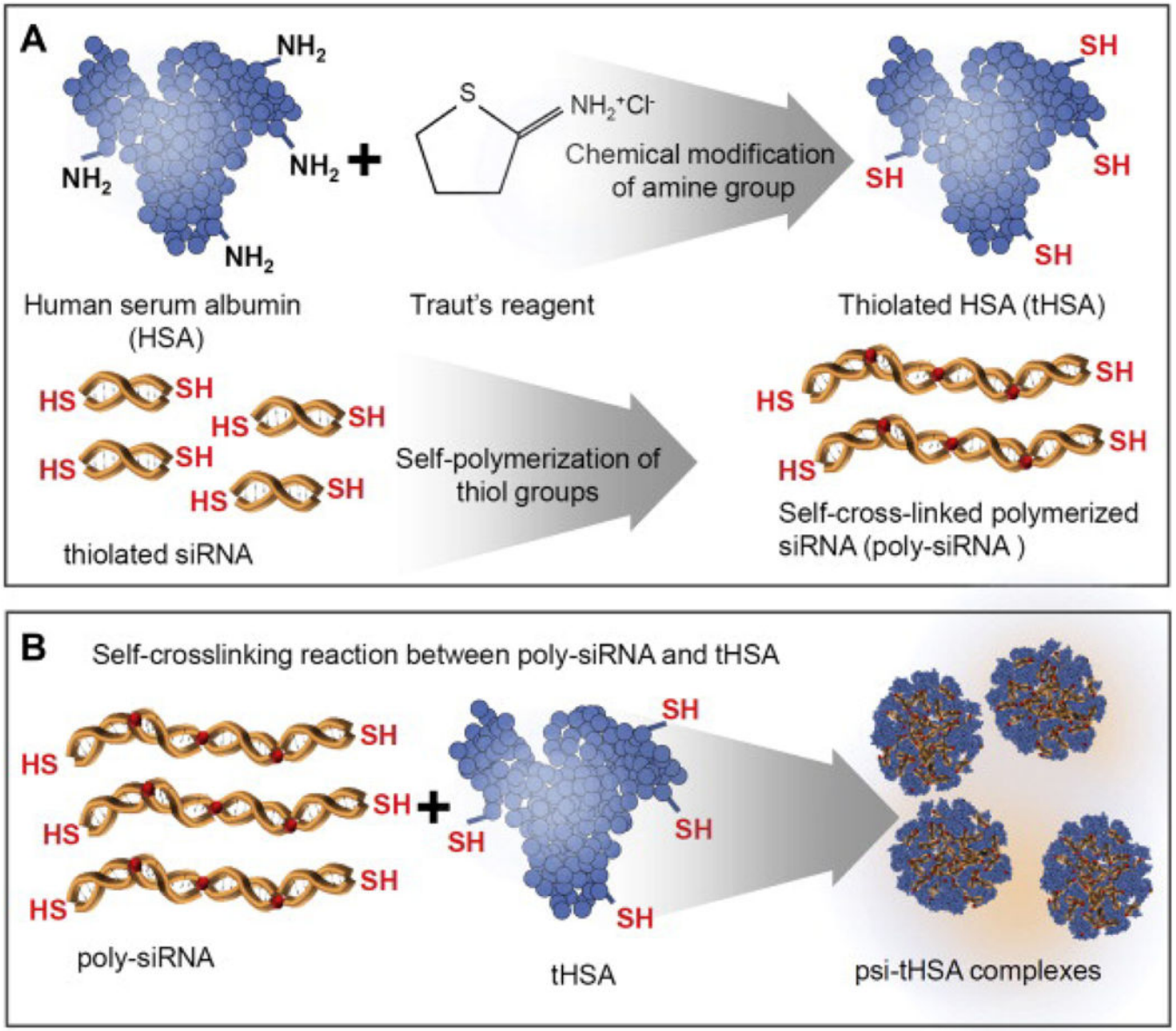


Figure 8:

A) Albumin lysine residues are chemically modified using Traut's reagent to create thiol groups. Thiolated siRNA is able to "self-polymerize" by interactions between terminal thiols. B) Thiolated albumin is complexed with thiolated siRNA through disulfide crosslinking Figure reproduced from Son et al [126] with permission of Biomaterials

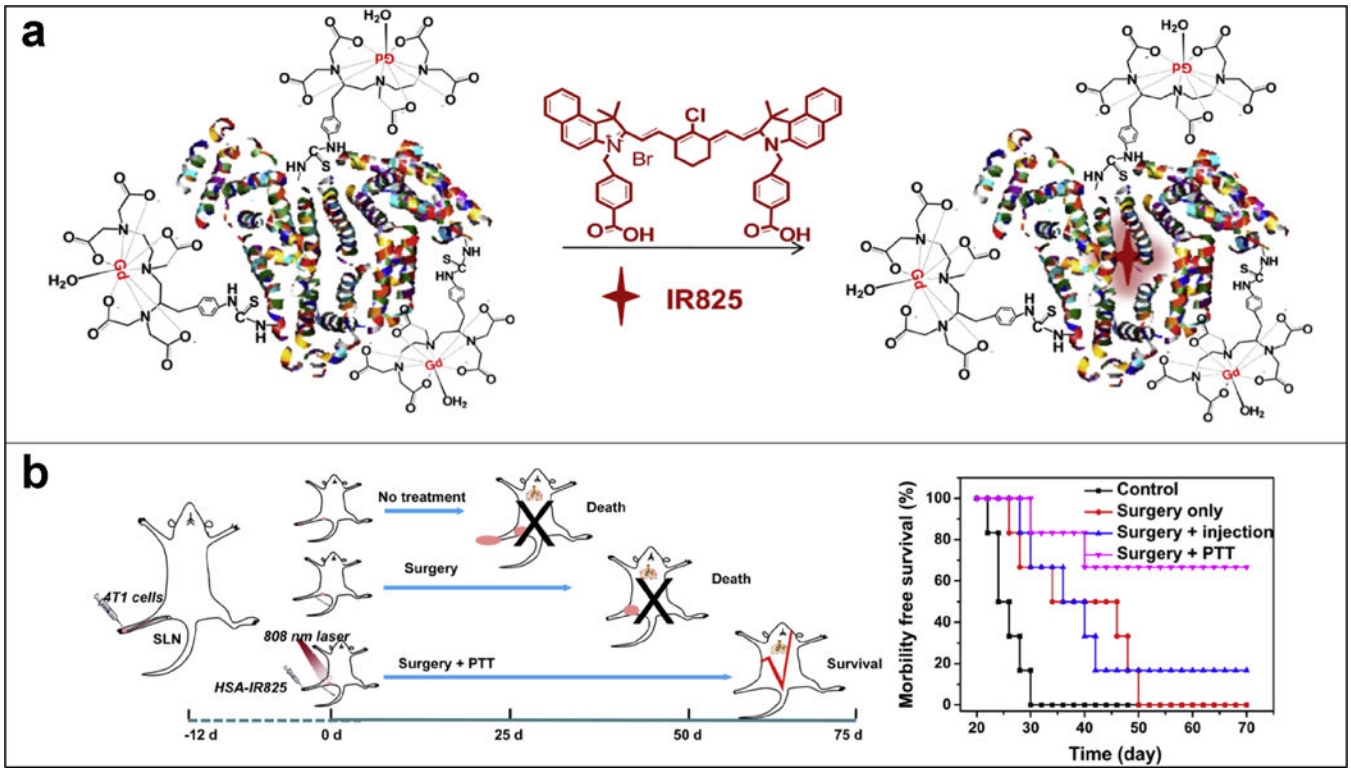


Figure 9:

A) Preparation of HSA-Gd-IR285 B) 4T1 cells were inoculated on hind paw of Balb/C mice. Cancer metastasis was noted by day 12, when HSA-Gd-1R825 was injected into the tumor. Photothermal ablation (PTT) of sentinel lymph nodes (SLN) was carried out. Albumin-mediated PTT of metastasized areas results in prolonged morbility free survival time. Figure reproduced from Chen et al [132] with permission of Biomaterials

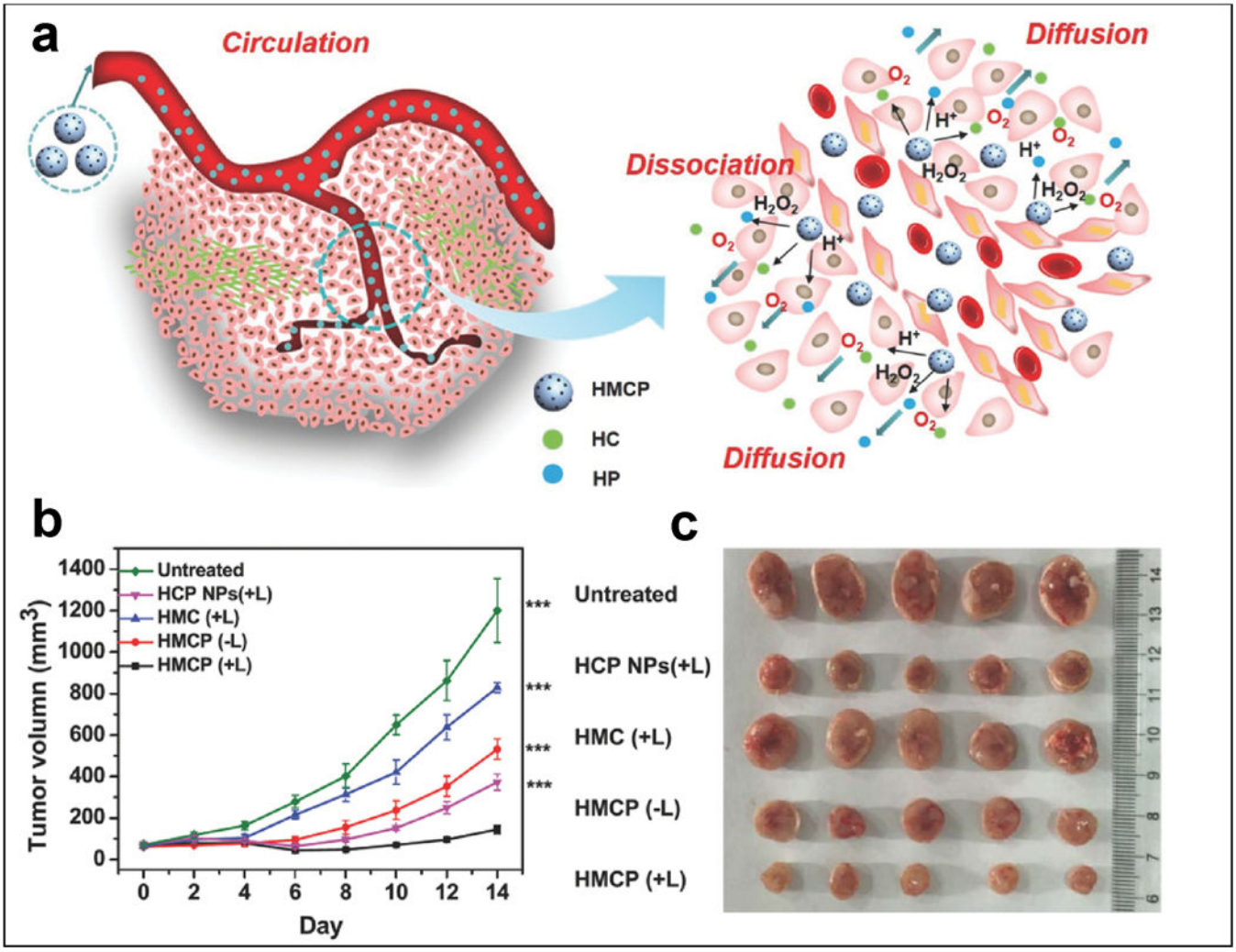
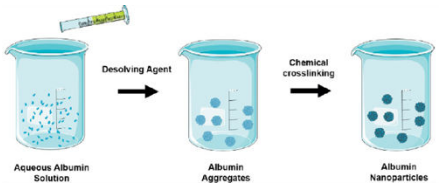
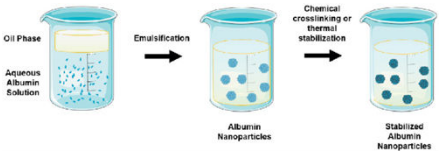
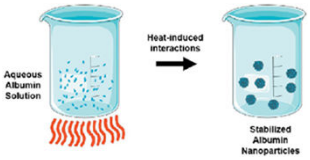
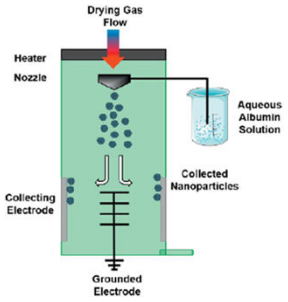
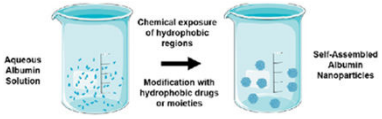


Figure 10:

Top: A) Overview of Mn02 albumin nanoparticle mechanism. B) Tumor growth curves of mice after various treatments (components indicated by designation below, irradiation indicated by L+). C) Photographs of tumors collected from different groups of mice 14 d after treatment. H=Human serum albumin, C=Chlorine e6, photosensitizer, M=Mn02, P=prodrug of cisplatin Figure reproduced from Chen et al [133] with permission of Advanced Materials

Table 1:

Overview of albumin nanoparticle synthesis strategies [100,112]

Fabrication Method	Description		Representative Examples
Desolvation (Coacervation)	Desolving agent such as ethanol or acetone is continuously added to an aqueous solution of albumin under continuous stirring. Unstable albumin aggregates are formed and are hardened by chemical crosslinking to prevent redissolving.		Human serum nanoparticles containing antisense oligonucleotides [103] Folate-decorated paclitaxel-loaded bovine serum albumin nanoparticles [104]
Emulsion	Dropwise addition of nonaqueous phase to aqueous albumin solution under continuous stirring. The resulting emulsion is then sonicated and a chemical crosslinking emulsion (e.g. glutaraldehyde) or high heat can be used to stabilize the resulting nanoparticles.		PEGylated albumin nanoparticles encapsulating azidothymidine [105] 10-hydroxycamptothecin-loaded bovine serum albumin nanoparticles [106]
Thermal Gelation	Heat-induced unfolding followed by protein-protein interactions such as hydrogen bonding, electrostatic, hydrophobic, and disulfide-sulfhydryl.		Bovine serum albumin-dextran-chitosan nanoparticles encapsulating doxorubicin [107] Albumin/lysozyme nanoparticles [108]
Nanospraying	Drying gas enters system through heater. Nozzle sprays fine droplets with narrow size distribution into chamber where they dry in to solid particles. These particles are then collected using electrostatic particle collector consisting of grounded star electrode and cylindrical collecting electrode.		Bovine serum albumin nanoparticles [109]
Self-Assembly	Increase hydrophobicity of albumin by breaking disulfide bonds, removing primary amines, or addition of lipophilic drugs.		Inhalable HSA nanoparticles with doxorubicin & TRAIL [110] Mannosylated albumin nanoparticles [111]