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Ferritin Drug Carrier (FDC) for tumor targeting therapy

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Abstract

Ferritin is an iron storage protein that plays a key role in iron homeostasis and anti-oxidation of cells. Due to its unique architecture of 24 self-assembling subunits and hollow cavity capable of encapsulating drugs, and an outer surface that can be modified genetically and chemically for additional functionality, ferritin has recently emerged as a promising drug delivery vehicle. Recent research demonstrated that unmodified human heavy chain ferritin binds to its receptor, transferrin receptor 1 (TfR1), in different types of tumor tissues, including lung and breast cancer, thus highlighting the potential use of ferritin for tumor-targeting applications. In this review, we consider the many favorable characteristics of ferritin drug carriers (FDCs) for tumor drug delivery. In particular, compared with antibody-drug conjugates (ADCs), ferritin exhibits superiority in a range of attributes, including drug loading ability, thermostability, and ease of production. Thus, the emergence of FDCs may be the next step in targeted cancer therapy.

Key words

ferritin drug carrier; (FDC); tumor targeting; TfR1; nanoparticles; nanocarrier; antibody-drug conjugates (ADC).

1. Introduction

Ferritin is a ubiquitously expressed iron storage protein, first identified by Laufberger in 1937 [1]. Classically, ferritin is considered a crucial protein with dual roles in iron storage and antioxidation, with its knockout in mice known to result in embryonic death [2, 3]. While ferritin has been well studied in relation to its physiological properties and mineral core formation, more recently it has been recognized for its potential as a drug delivery vehicle (Figure 1). Its self-assembly ability, symmetrical spherical architecture, and high thermal stability are important aspects driving interest in ferritin nanocarriers [4]. Furthermore, as an endogenous protein, ferritin also possesses excellent biocompatibility, biodegradability, and low toxicity, which are highly desirable features for nanocarriers in clinical use.

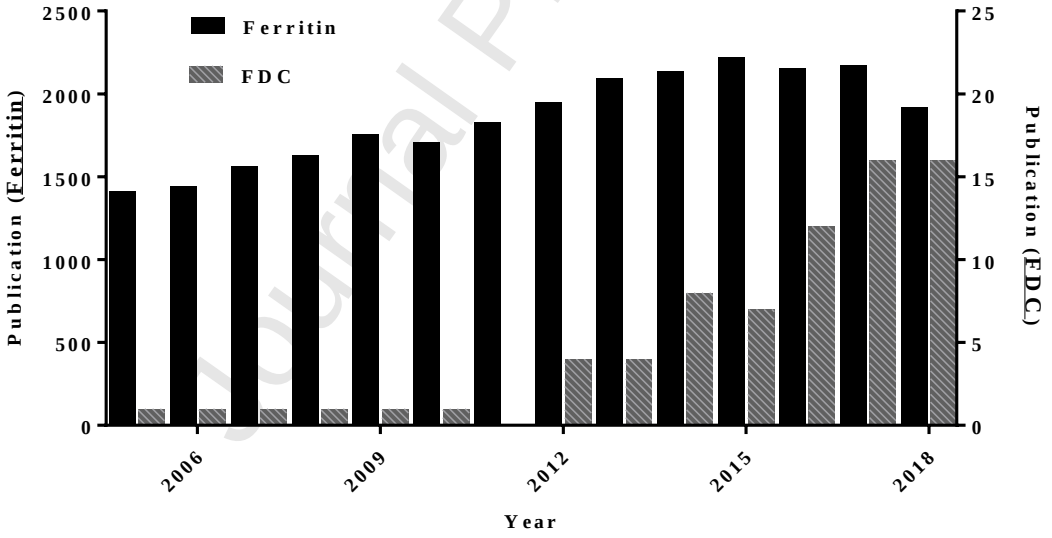

Figure 1. FDC-related publications by year (to 2018). Data based on Web of Science.

Due to its 8-nm diameter inner cavity, ferritin has the potential space to encapsulate many drug molecules, thus offering protection from degradation as well as limiting potential side effects to healthy cells. Furthermore, its outer 12-nm diameter is suitable for the enhanced permeability and retention (EPR) effect [5]. In addition, the sensitivity

of the stable cage-like structure of ferritin to pH facilitates the application of various drug-loading methods. For example, under extreme environments, such as strong acidic pH, the quaternary structure of ferritin disassembles but, interestingly, reassembles once pH returns to physiological conditions. Thus, by manipulating the disassembly and reassembly of ferritin, it is possible to encapsulate therapeutic drugs inside its structure. The first report of the use of ferritin for encapsulation and delivery of drug molecules was by Simek and Kilic in 2005, who encapsulated doxorubicin (Dox) into the protein cage structure [6]. In the following years, most research focused on how to provide this drug encapsulating protein with targeting abilities, with attempts at genetic and chemical modification achieving some success. Of particular interest, ferritin was identified as having the ability to naturally target tumor cells.

The direct interaction between ferritin and tumors has been studied since the 1960s, when several research groups reported that human ferritin could be selectively taken up by tumor cells [7-9]; however, the targeting mechanism was unknown. In 1988, Fargion *et al.* found that human ferritin was specifically bound to an unknown 100-kDa protein in several human cancer cell lines [10]. In 2010, Seaman's research group identified that human heavy chain ferritin (HFn) targets transferrin receptor 1 (TfR1) [11], a transmembrane glycoprotein previously identified as the receptor of iron binding blood plasma glycoprotein transferrin (Tf) [12]. In 2012, we found that HFn directly targets TfR1 in various tumor cells and tissues from nine types of cancer, including lung and breast cancer [13]. TfR1 is involved in regulating cell growth [14] and is overexpressed (100-fold) in proliferating cells that require more iron. Moreover, as TfR1 is extensively overexpressed in many malignant tumors, ferritin is able to target a broad spectrum of tumors. The biological identification of ferritin receptors, especially TfR1 as the receptor of human HFn, should encourage the development of ferritin as a drug carrier, especially given that unmodified ferritin nanocages also possess site-specific drug delivery ability [13, 15] (Figure 2).

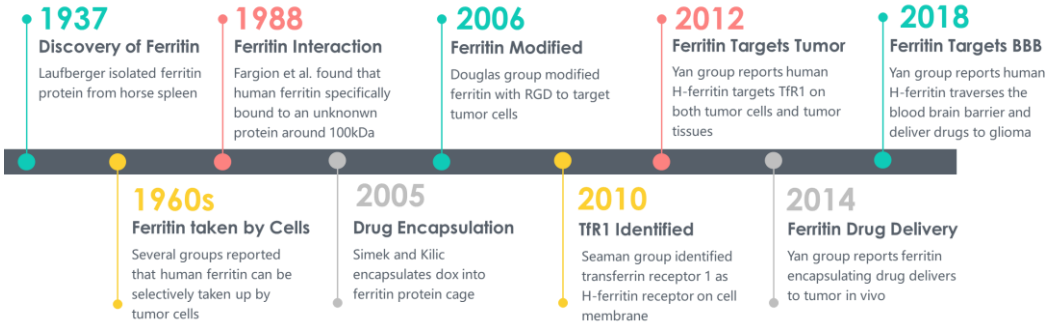

Figure 2. History of FDC research and discovery

Its intrinsic tumor-selective properties, in combination with high thermal stability and ease of production and modification, highlight the potential of ferritin-based drug delivery as a promising candidate in clinical use. In the current paper, we simplified ferritin-based drug delivery systems as ferritin drug carriers (FDCs) and summarized FDC tumor-targeting research to compare it with previously established tumor-targeting drug delivery systems, chiefly antibody-drug conjugates (ADCs). We briefly discuss the physiological properties of ferritin in the context of drug delivery, but to fully appreciate this unique protein from a biological standpoint, please read the reviews of Harrison and Theil [16-18]. This paper does not cover the various ferritin-based contrast agents and medical imaging, which should be a review topic on their own.

2. Ferritin

Ferritin was first isolated from the horse spleen by Laufberger in 1937 [1] and subsequently discovered in various organisms, including humans, other animals, plants, fungi, and bacteria [18]. Mammalian ferritin can be found both intracellularly and in circulation. Cytosolic ferritin plays an essential role in iron storage and detoxification. Furthermore, although the physiological function of secreted ferritin is yet to be fully elucidated, it is reported to be correlated with inflammation, angiogenesis, and tumors [19-21]. Thus, as ferritin exists physiologically inside the body, it is considered to be safe, stable, and biologically degradable.

Ferritin is a relatively large (450 kDa) spherical protein composed of 24 self-assembled subunits with an outer diameter of 12 nm and an internal cavity with a

diameter of 8 nm [17]. Subunit self-assembly occurs spontaneously in the physiological environment, without external force or additional modifications after protein expression [18], thus allowing the expression of functional ferritin in prokaryotic systems [22]. The large number of intra- and inter-subunit salt bridges and hydrogen bonds of ferritin provide excellent stability [17, 23]. Ferritin can withstand a wide range of pH and still maintain its hollow spherical structure, and its excellent physical and chemical properties set it apart from many other protein-based drug delivery vehicles. Unlike most other proteins, which are sensitive to temperature and pH, ferritin can withstand temperatures of up to 75 °C for 10 min. Furthermore, due to its high stability, ferritin nanocarriers are easy to produce. Protein purification typically requires complex procedures to isolate the target protein from those of the host cell. However, for ferritin nanocarriers, one-step heat treatment results in the denaturing of more than 80% of host cell proteins. Thus, ferritin nanocarrier purification is a simple and effective procedure, which should allow easy translation from bench to bedside. Ferritin architecture can be disassembled into its various subunits under extreme acidic or basic conditions but can be reassembled when pH returns to the physiological range [23-25], thus enabling convenient encapsulation of drug molecules inside the protein cage. Moreover, after drug loading, FDCs maintain excellent stability, which should facilitate their commercialization, transportation, and storage.

Eukaryotes typically have two ferritin genes encoding the immunoglobulin heavy (H) (21 kDa) and light (L) (19 kDa) chains, which co-assemble to form heteropolymers under physiological conditions. Depending on the tissue, the H-chain to L-chain ratio can differ drastically. For example, the H-chain is dominant in the heart, where it was first isolated, whereas the L-chain in dominant in the liver, where it was first isolated, thus was likewise named H-ferritin and L-ferritin (LFn) [26]. The main function of H-chain is the catalyzation of Fe(II) to its oxidized state Fe(III) [17], which is enabled by its ferroxidase center. However, the L-chain lacks this center and catalytic ability, which therefore assists iron nucleation [27]. As far as the encapsulation of drug molecules, the HFn and LFn is relatively similar. However, up to now, we have not yet identified a LFn receptor on human cancer cells, and whether or not LFn has the ability to site

specifically delivery therapeutic agents to tumor is questionable [28]. Thus, we favor HFn for drug delivery. Ferritin can accommodate up to 4500 Fe(III) atoms in the form of an iron mineral core [18, 29]. However, apoferritin, which does not have a mineral core, is formed after reassembly of ferritin following pH alternation, with the vacated cavity of apoferritin able to encapsulate therapeutic agents. There are three main differences between ferritin and FDC. First of all, ferritin is a natural protein in the body, while FDC is an engineered product by expression and purification. Secondly, natural ferritin usually consists of heavy and light chain in mammalian cell, while FDC contains purely of HFn. Third is that, natural ferritin normally has an iron oxide core formed by biomineralization of excess iron in the cell, while FDC are without iron ready for drug loading. (Figure 3).

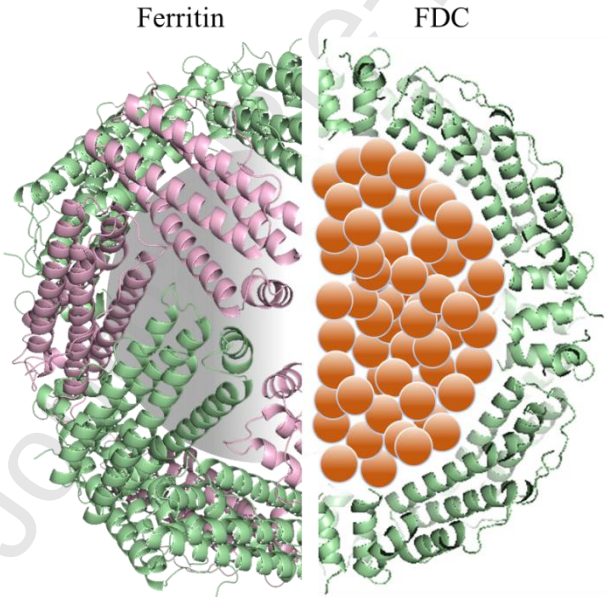

Figure 3. Ferritin and ferritin drug carrier (FDC). Green coils represent HFn subunit, pink coils represent LFn subunit, and orange circles represent payload.

3. Intrinsic tumor-targeting properties of ferritin

Commonly, tumor-targeting strategies of nanoparticles consist of passive and active targeting. Passive targeting typically relies on the enhanced permeability and retention effect (EPR). In this respect, FDC's uniform size of 12nm is ideal for taking advantage

of EPR effect caused by the disorders in blood and lymph vessel systems in tumor tissue. Thus far, clinically approved nanoparticle drug delivery system on the market, essentially liposomal doxorubicin (Doxil) and albumin-paclitaxel (Abraxane), relies on EPR effect alone. However, deeper analysis has shown that these delivery methods does not yield significant improvements in therapeutic index [30, 31].

Thus active targeting through either chemical modification or genetic modification is often introduced to nano-delivery system, which inevitably leads to complexity of the system, with a number of issues to be solved from both technological (i.e. production reproducibility and surface characterization) and biological points of view (i.e. interaction with bloodstream proteins, protein corona composition, safety, and immune reaction), and affects the overall the biocompatibility of the final structure [32, 33].

The key advantage for FDC as a tumor targeting nanocarrier is its innate specific affinity to TfR1 and the simplicity of the delivery system because of it. FDC require no additional modification to encapsulate drugs and target tumor specifically, up to 10 times higher than EPR effect alone can achieve [34].

While ferritin was known to be taken up by cells as early as the 1960s [8], it was not until 2010 that TfR1 was identified as the receptor of human H-ferritin and that after formation of the H-ferritin-TfR1 complex on the surface of the cell, it was internalized in the lysosome [15]. This finding was of great significance because TfR1, initially known as a receptor for the iron carrier Tf and transporter of iron within the cell, is highly expressed in a variety of malignancies and is efficiently internalized, thus making it an excellent target for tumor treatment and diagnosis [35]. In 2012, we demonstrated that clinical tumor tissue can be identified by HFn, thus indicating that HFn can specifically target tumors.

Interestingly, mouse and human HFn are able to interact with T-cell immunoglobulin and mucin domain-2 (TIM2) in mice [36]; however, no human ortholog for mouse TIM2 has yet been found. In regard to L-ferritin, scavenger receptor class A member 5 (Scara5) has been identified as its receptor in mouse kidney [37]; however, no studies have reported that human L-ferritin exhibits the same binding to

human Scara5. Thus, ferritin from different origins is not guaranteed to share the same receptor or binding activity. Furthermore, horse spleen ferritin (HosFn), which has been used in a number of ferritin drug delivery studies, is mostly composed of L subunits, unlike human HFn, and it likely binds to Scara5 in mice, but not mouse TfR1 [28].

Following the discovery of ferritin receptors, ferritin nanocarriers have gained increasing attention as candidates for tumor-targeted delivery platforms. Ferritin likely acts as an antibody and guides the payload *in vivo*. However, rather than using transferrin or antibodies to target TfR1, both of which are limited in their ability to load large amounts of drug molecules, ferritin nanocarriers provide a substantial cavity to encapsulate such drugs. Furthermore, HFn targeting of TfR1 allows for better biosafety, with recent study revealing that positive ferritin receptor cells incorporate ferritin nanocarriers in a threshold-dependent manner [38], indicating excellent tumor selective properties *in vivo*. Recently, Montemiglio *et al*. showed that HFn binds to a different TfR1 domain than that of transferrin [39], confirming that the binding of HFn to TfR1 does not disturb or compete with the binding of Tf [11, 13, 40] and minimally affects the physiological functions of TfR1.

We previously stated that HFn nanoprobes can be seen as a new ligand to successfully target TfR1 tumor tissues across a broad spectrum (Table 1) [13]. Consistent with our clinical tissue staining results, many studies have shown that HFn can deliver payloads specifically to cancer sites [40-42]..

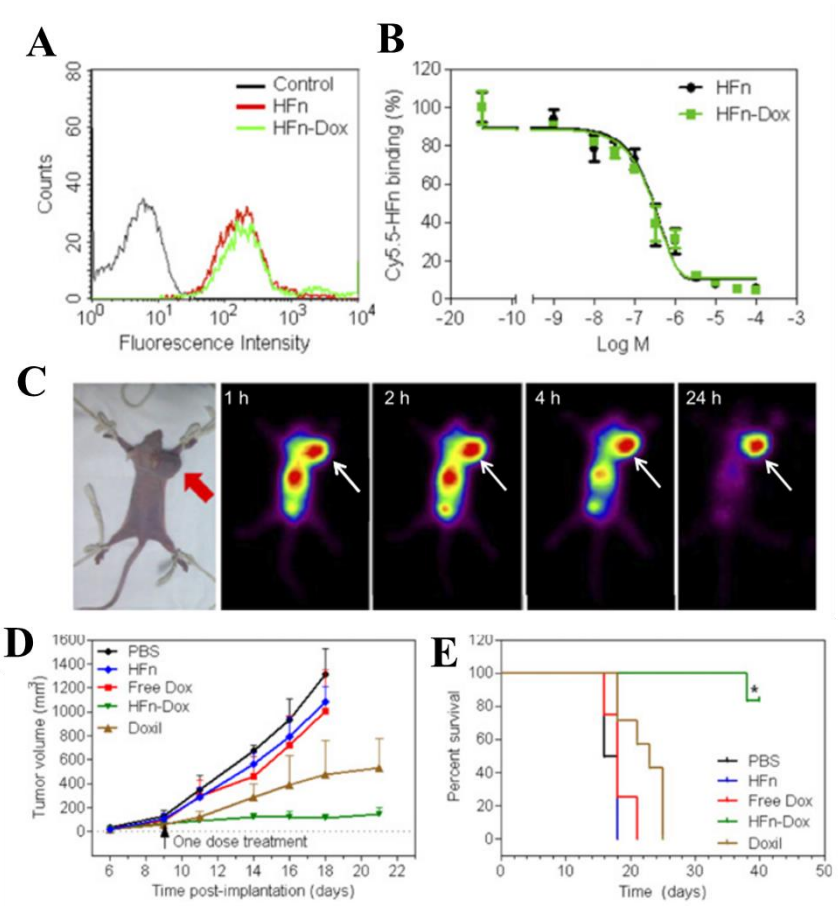

Figure 4. FDCs (HFn) specifically target tumor cells and achieve antitumor activity with lower toxicity. (A) Flow cytometry of HFn binding to HT-29 colon cancer cells. (B) Inhibition of binding of Cy5.5-labeled HFn to HT-29 by unlabeled HFn. (C) *In vivo* nuclear imaging of HT-29 tumors injected with 125I-labeled HFn. (D) Tumor growth curves for various mouse groups, with HFn-Dox group significantly lower than other groups. (E) Animal survival curves for various mouse groups, with HFn-Dox group exhibiting the highest survival percentage. Reproduced with permission from ref [40]

Table 1. Histological analysis of M-HFn nanoparticle staining of tumours in clinical tissue specimens, sensitivity represents the positive staining ratio of the tumor, specificity represents negative staining ratio of normal tissue.

Tumor type	Sensitivity	Specificity
Liver cancer	98%	86.67%

Lung cancer	96%	98.21%
Colorectal cancer	100%	97.56%
Cervical cancer	100%	100%
Prostate cancer	100%	87.5%
Ovarian cancer	98%	100%
Breast cancer	75%	75%
Thyroid cancer	100%	100%
Esophageal cancer	67%	100%
Stomach cancer	73.03%	84.21%

Note: Data obtained from [13].

The tumor-targeting ability of ferritin is efficient enough to target moiety in larger nanodrug carriers. Turino *et al.* covalently bound L-ferritin with PLGA-NPs to exploit the targeting capability of the LFn SCARA5 receptor, while also reducing its nonspecific drug release [43]. Their result was better than non- or albumin-decorated particles and could deliver paclitaxel and Gd MRI contrast agents at the same time. Fan *et al*. showed that ferritin could deliver nanozymes into cells, specifically into the lysosome, and boost reactive oxygen species generation [44].

Drug delivery to the brain is a complex process restricted by the special junctions of the blood-brain barrier (BBB). The BBB is a highly regulated barrier that protects the brain from outer toxins and regulates hemostasis, and thus is also highly impenetrable for drugs intended for delivery to the brain. Fan *et al.* found that HFn encapsulated Dox was able to cross the BBB and deliver drugs to the tumor site through HFn receptor (TfR1)-mediated endocytosis [45]. TfR1 is a promising receptor for crossing the BBB and is overexpressed in the BBB and in many types of brain tumor. Importantly, there are many TfR1-targeted nanodrugs currently under clinical trial for brain tumor therapy [46], further highlighting the central role of TfR1 in traversing the BBB and targeting brain tumors. In TfR1-targeting strategies, FDCs show better efficacy at crossing the BBB than transferrin-based nanocarriers as a high concentration

of endogenous transferrin may compete with nanocarrier binding, unlike ferritin, which it has a different binding site to TfR1. The relatively weaker binding affinity of FDC to TfR1 compared with TfR1-targeted antibodies allows FDCs to transfer into the endosomes of BBB endothelial cells and thus traverse the BBB. Furthermore, FDCs are preferentially incorporated by receptor-positive cells in a threshold-dependent manner, allowing for significant tumor cell uptake while also ensuring biosafety. Thus, taken together, FDCs are a promising strategy for TfR1-based BBB traversal and brain tumor therapy.

The crossing of the BBB by FDC does not appear to cause off-target affects. Fan *et al*. demonstrated that HFn can cross the BBB to specifically target glioma tumor cells via interaction with TfR1 (Figure 5) but is found in two different locations after the FDC enters the cell, namely the endosome of BBB endothelial cells and the lysosome of glioma cells. In a healthy brain, HFn does not accumulate as TfR1 is not excessively expressed. An accumulated 3 mg/kg-dose of HFn-Dox did not induce significant toxicity in the liver, kidney, or spleen, nor in healthy brain tissue.

The different localizations of HFn in endothelial and tumor cells are likely due to two important reasons: 1) The multiple binding sites of TfR1 on HFn, based on the symmetry structure of HFn; and, 2) When the binding ratio of the TfR1/ligand is greater than 2:1, the complex will be localized in the lysosome [47]. As the expression of TfR1 in tumor cells is dramatically higher than that in BBB endothelial cells, when HFn binds to endothelial cells, the relatively low level of TfR1 results in a TfR1/HFn binding ratio of less than 1:1, whereas in tumor cells, the high level of TfR1 results in a TfR1/HFn binding ratio of more than 2:1.

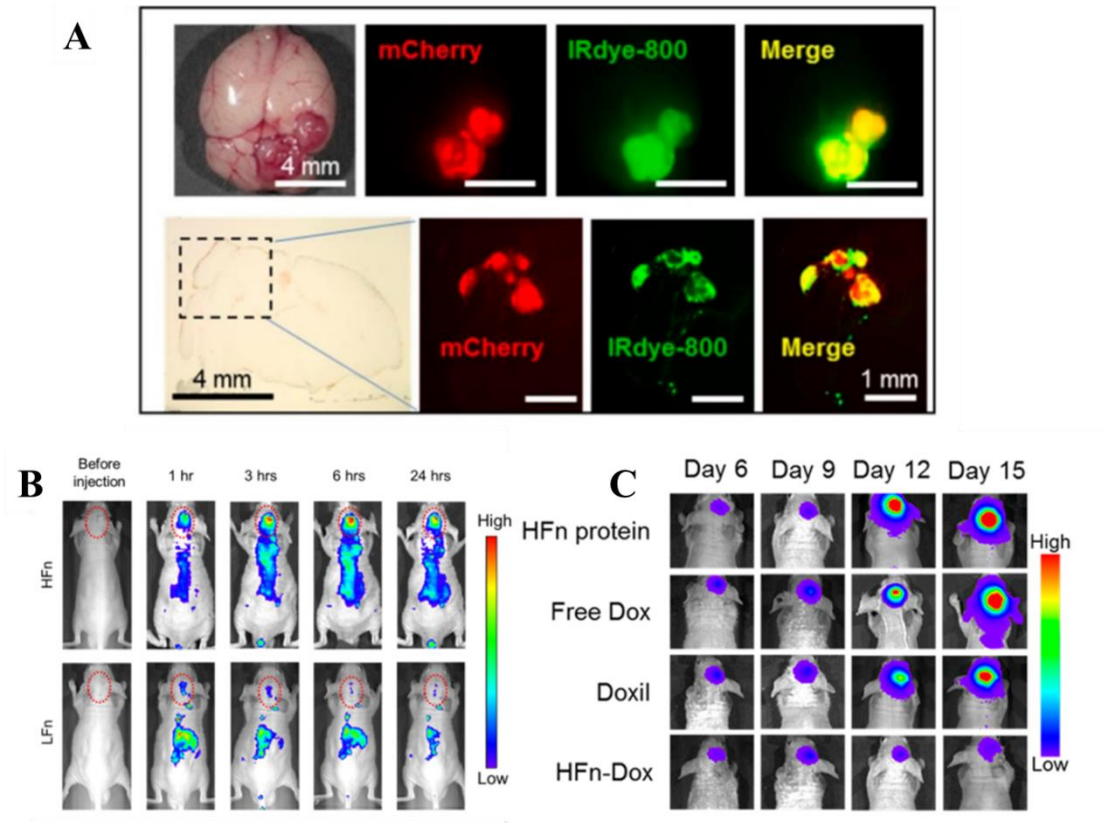

Figure 5. FDC successfully crosses the BBB and effectively targets glioma to improve anti-glioma tumor activity. (A) Irdye-800-labeled HFn overlapping with mCherry-labeled tumor upon intravenous injection of HFn. (B) *In vivo* imaging of Irdye-800-labeled HFn and LFn intravenously injected into healthy mice (C) *In vivo* BLI images of glioma-bearing mice intravenously injected with different formulations. Reproduced with permission from ref [34]

4. Ferritin modified with tumor-targeting moieties

While the intrinsic tumor-targeting capabilities of ferritin have enabled it to become a simple drug delivery vehicle, it is not limited to targeting its natural receptor TfR1 only but can be readily modified with additional targeting motifs for diverse applications (Table 2, Figure 6). Indeed, even before the cell membrane receptor of ferritin was identified, active tumor-targeting strategies for FDCs had already been investigated via bioengineering and chemical modification. Ferritin, which consists of 24 protein subunits encoded by genes, can be genetically engineered to be functionalized with

targeting motifs [48]. Furthermore, the lysine and cysteine residues exposed on the surface of ferritin nanocages can be exploited to conjugate with chemical groups via cross-linking with N-hydroxysuccinimide (NHS) ester or maleimide groups.

RGD modification is currently the most commonly used tumor-targeting modification for FDCs due to its small size and the simplicity of incorporating motifs on the external surface without disruption of the overall structure. Douglas *et al.* genetically modified human HFn with a RGD-4C(CDCRGDCFC) peptide, which increased specific targeting interaction with cancer cell C32 melanoma via binding to αvβ3 integrin molecules [49]. Following this, Chen *et al*. also confirmed that RGD-modified ferritin maintained its integrin selectivity with the loading of various cargo, including metal cations, Dox, and photosensitizers, while targeting glioblastoma [50-52]. To date, accumulating evidence has demonstrated the effectiveness of RGD modification [53-57].

The success of RGD modification suggests that the incorporation of peptides is a reasonable strategy for specific tumor targeting. Ceci *et al.* linked melanocyte-stimulating hormone to the exterior of ferritin by a linker peptide [58, 59]. Furthermore, the chimeric protein of epidermal growth factor and human HFn can specifically bind to and be taken up by breast cancer MCF-7 and MDA-MB-231 cells and accumulate in breast tumors in a mouse xenograft model [60]. In addition to traditional targeting moieties, more recently identified peptides have been genetically modified on the surface of HFn nanoprobes to target tumor cells [61, 62]. Jiang *et al*. identified peptide SP94, a novel peptide for hepatocellular carcinoma (HCC), and successfully displayed it on an FDC for effective delivery of an anti-HCC drug without damage to healthy tissue [63]. Innovative targeting methods, such as matrix metalloproteinase (MMP) modification, are also useful. Hwang *et al*. proposed that human apoferritin can be transformed into a modular construct for modification of different sets of components by expressing protein G genetically on ferritin, thus allowing for any antibody or Ni-NTA-functionalized nanoparticle to be bound to ferritin [64].

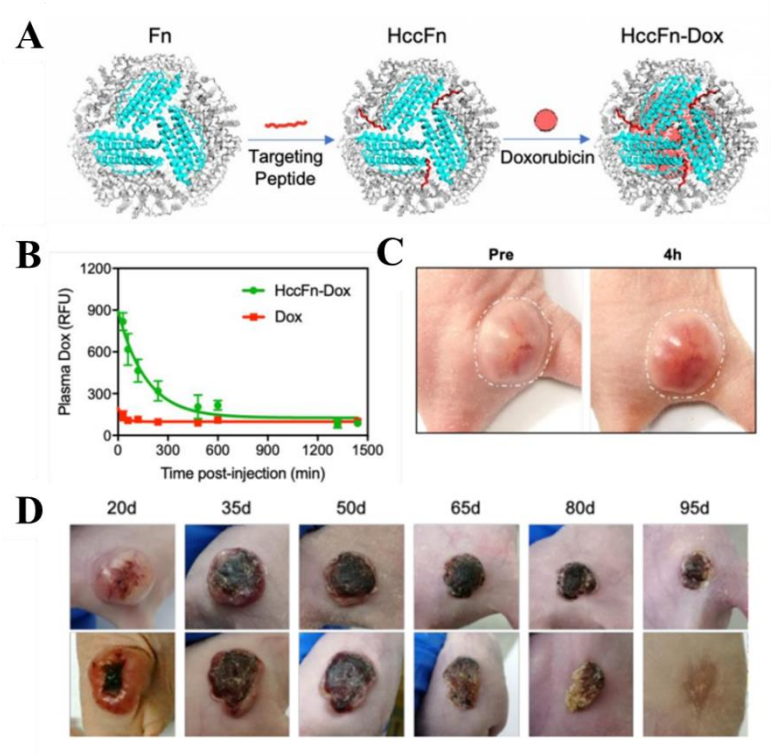

Figure 6. Construction of surface-modified FDC and its delivery and therapeutic efficacy. (A) Schematic of HccFn-Dox FDC construction. (B) Comparison of Dox concentration in plasma between FDC encapsulated and free Dox. (C) *Ex vivo* imaging of Dox accumulation in tumor. (D) HccFn-Dox nanocages effectively killed HCC tumors and exhibited less toxicity than Doxil. Reproduced with permission from [63]

Chemical modification can also be applied to alter the targeting ability of FDCs. For example, Ceci *et al.* directly conjugated monoclonal antibodies to the surface of ferritin to provide the FDC with melanoma targeting ability [65]. Biotinylation is a standard protein modification method that covalently attaches biotin to a biological molecule. Crich *et al.* used this method to conjugate a neural cell adhesion molecule (NCAM) targeting peptide to ferritin, thereby allowing ferritin to target tumor angiogenesis *in vivo* [66].

Normally, a drug delivery vehicle with fewer components provides more reliable and stable function. The intrinsic binding of ferritin to TfR1 enables ferritin to specifically target a wide range of tumors. The ability to modify makes FDC more versatile and capable of greater functions.

Table 2. Modifications of FDCs

FDC	Targeting moiety	Targeting receptor	Reference
PfFn	Trastuzumab	HER2	[67]
PfFn	SP94 peptide	GRP78	[63]
Human HFn	Anti-Claudin antibody	Claudin-4	[64]
Human HFn	Cetuximab, Trastuzumab	EGRF, HER2	[68]
Human HFn	EGF	EGFR	[60]
Human HFn	Vimentin recognizing peptide, EGFR recognizing peptides	Vimentin, EGFR	[69]
LFn	MMP-2 cleavage site, Thrombin receptor agonist peptide	MMP2, protease-activated receptor-1	[61]
eDPS	RGD	Integrin αvβ3	[57]
Fn	RGD	Integrin αvβ3	[51]
LFn	AP1, RGD	Interleukin-4 receptor, integrin αvβ3	[56]
Human HFn	α-melanocyte-stimulating hormone peptide	Melanocortin receptor	[59]
Horse Spleen Fn	C3d peptide	NCAM	[66]
Human HFn	Anti CSPG4 antibody	Melanoma-specific antigen CSPG4	[65]
Horse Spleen Fn	Anti-PSMA antibody	PSMA	[70]

Abbreviations: PfFn: *Pyrococcus furiosus* ferritin; eDPS: *Escherichia coli* DNA-binding protein; HER2: human epidermal growth factor receptor 2; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; MMP2: matrix metalloproteinase-2; RGD: arginylglycylaspartic acid; AP-1: IL-4 receptor-targeting peptide; C3d peptide: NCAM-binding peptide; PSMA: prostate-specific membrane antigen.

5. Ferritin drug-loading strategies

The cage structure of ferritin enables it to encapsulate a relatively large quantity of drugs, which is crucial for a successful drug delivery carrier. The amount of drug that can be stably loaded can affect the efficacy of the carrier. Many drug-loading methods have been developed in an attempt to identify the optimal strategy for FDC application (Table 3). There are three main methods used to encapsulate payloads into the ferritin

protein cage, i.e., passive loading, protein structure disruption through denaturing buffer, and pH-mediated disassembly and reassembly, which exhibit varying degrees of success depending on the type of drug.

The ferritin protein shell features eight hydrophilic and six hydrophobic channels. d. Drug encapsulating strategies relying on passive loading have been attempted in various studies [71, 72]. For example, Cu^{2+} has been used to load doxorubicin into the cavity of ferritin protein, whereby doxorubicin incubates with Cu^{2+} to form a complex, which can then pass through the hydrophilic channel [50]. These channels are sufficiently flexible to allow entry of molecules larger than the channel, with a maximum dimension of 13 Å. However, it becomes less efficient when loading drugs with higher molecular weight. The hydrophobic channels have also been used for the loading of drugs such as Gefitnib, an EGFR tyrosine kinase inhibitor [72]. Recently, a high hydrostatic pressure encapsulation method was developed to load doxorubicin, which maximized the drug-loading potential of ferritin channels [73].

The encapsulation of large drug molecules into ferritin can be promoted by denaturing protein using detergents. For example, a previous study used 8 M urea to widen the channels and allow additional drugs to pass through, with a gradient then used to remove urea, leaving the drugs encapsulated in the protein cage [40]. This strategy has also been incorporated for the encapsulation of Dox, carbachol, and atropine [74].

Furthermore, drug loading can also be achieved by pH-mediated disassembly and reassembly of ferritin [75, 76]. The ferritin protein can be disassembled by altering the buffer pH to extremely acidic or basic (e.g., for wild type HFn, the pH required for encapsulation is below 2 or higher than 11, for HFn variant, the pH required is below 4), with the drug then mixed with the disassembled protein. Restoration of normal pH allows ferritin nanocage reassembly, with a substantial amount of drug molecules entrapped inside. This is the most commonly used strategy in FDC research, in which the ferritin nanocage is thoroughly disassembled into subunits, allowing for sufficient exposure and blending of drug and protein. However, the use of extreme pH permanently damages ferritin, compromising drug encapsulation ability and stability,

which is a complication in the translation of such methods into clinical practice [77].

Table 3. FDCs and their encapsulated drugs.

Ferritin carrier	Encapsulated drug	Encapsulating method	Loading efficiency (molecule/per nanocage or weight percentage)	Reference
Horse spleen Fn	Doxorubicin	pH	28	[78]
Human HFn	Doxorubicin	Urea	33	[40]
Human HFn	Doxorubicin	Urea	32.5	[74]
Human HFn	Doxorubicin	Urea	29	[79]
Human HFn	Doxorubicin	Urea	36	[45]
PfFn	Doxorubicin	Urea	400	[63]
Human HFn	Doxorubicin	Pre-complexation with Cu(II)	73.41 wt%	[50]
Human HFn	Doxorubicin	pH	90	[76, 80]
Human HFn	Doxorubicin	pH	86	[41]
Human HFn	Doxorubicin	Chemical conjugation	88	[81]
LFn	Doxorubicin	pH	—	[82]
Horse spleen Fn	Methylene blue	pH	1	[83, 84]
Horse spleen Fn	Daunomycin	Loading with poly-L-aspartic acid	0.2	[85]
Human HFn	ZnF16Pc	Incubation in PBS.	60 wt%	[51, 55, 86, 87]
Human HFn	IR820	pH	17.32 wt%	[88]
Human HFn	Sinoporphyrin sodium	pH	66.67 wt%	[54]
Horse spleen Fn	Cisplatin	pH	30	[89]
Horse spleen Fn	Cisplatin	pH	20-55	[75]
Pig pancreas Fn	Cisplatin	pH	11.26	[90]
Human HFn	Cisplatin	pH	50	[65]
Horse spleen Fn	Carboplatin	pH	15	[91]

Human HFn	Curcumin	pH	90	[42]
Human HFn	Curcumin	pH	14.7	[92]
Human HFn	Curcumin	pH	14	[93]
Horse spleen Fn	Curcumin	pH	9.6	[94-96]
Human HFn	Atropine	Urea	46.7	[74]
Human HFn	β-carotene	pH	12.4	[97]
Horse spleen Fn	^{235}U	Direct incubation	800	[98]
Pig spleen Fn	5-fluorouracil	Direct incubation	45.5	[99]
Human HFn	siRNA	pH method	0.2	[100]
Human HFn	Gefitinib	Direct incubation	10	[72]
Human HFn	Doxorubicin Curcumin Quercetin	Direct incubation	121 26 440	[101]
Human HFn	mitoxantrone	pH	47	[102]
Horse Spleen Fn	DBN, EBN	pH	1.04%, 13.34%	[103]

Abbreviations: DBN: 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide; EBN: chemotherapeutic epirubicin

6. FDCs and their anti-tumor effects

Although the mechanism for drug release from ferritin in the cytosol or nucleus has not yet been fully elucidated, there is strong evidence that payload delivery by ferritin is an effective method that results in reduced nonspecific cytotoxicity and higher drug efficacy. The delivery of drugs using ferritin carriers can also lessen the side effects of various drugs, such as seizure risk of cisplatin or cardiotoxicity of doxorubicin [104]. Furthermore, as a water-soluble protein, ferritin can be readily dissolved, which is essential for delivery of hydrophobic drugs [105]. Our group previously showed that HFn can be used to target tumor cells that overexpress TfR1 due to the high metabolism and demand for iron in tumor cells, and thus ferritin is an ideal protein for tumor drug delivery. Moreover, after interaction with TfR1, HFn can be efficiently delivered to the lysosome via TfR1-mediated endocytosis [40]. Current evidence suggests that during

acidification in the lysosome, drugs contained in ferritin are gradually released. Animal studies have also shown significant tumor reduction while reducing measurable side-effects [34, 40, 50, 63, 106]. These positive results reflect the ability of FDCs to accumulate and release at the specific site required (Figure 4).

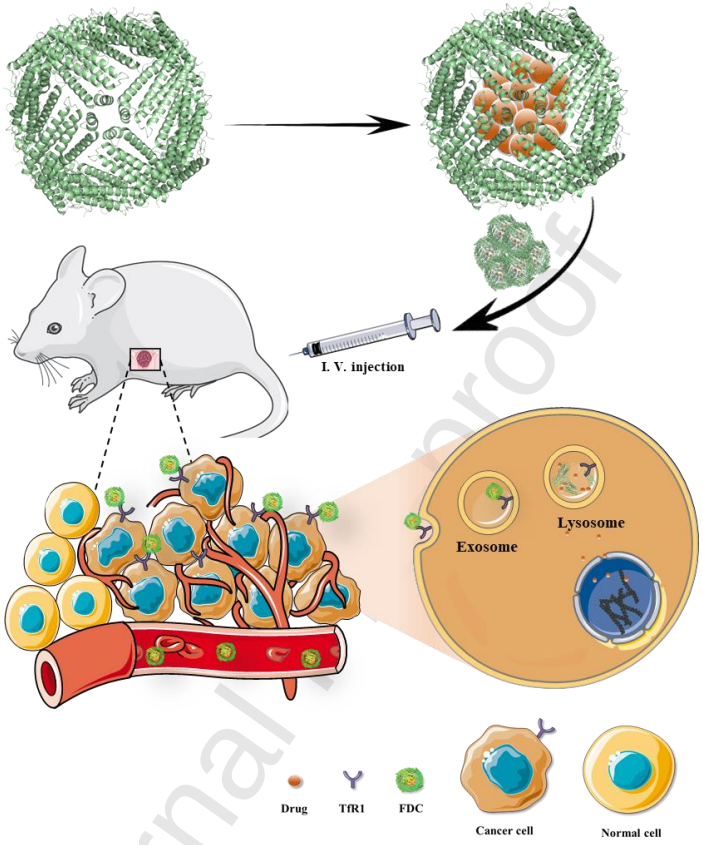

Figure 7. Process of drug loading and delivery of FDCs.

When ferritin is first introduced into circulation, it undergoes a biodistribution step before reaching the target site. With the help of a ferritin cage, the blood elimination half-life and area under the concentration-time FDC are significantly longer than free drug: e.g., 256 ± 19.0 min and 1192 ± 99.38% ID·mL^{-1}·min^{-1} vs. 21 ± 6.4 min and 5.7 ± 0.33% ID·mL^{-1}·min^{-1} in mice [40]. The passive targeting of FDC mainly depends on the enhanced permeability and retention effects caused by blood and lymph vessel disorders in tumor tissues [107]. The EPR effects of nanoparticles rely on their size distribution [108], which is often poorly controlled in chemically synthesized nanoparticles [109, 110]. This lack of control over nanoparticle size is a common issue and makes it difficult to achieve predictable clinical results. On the other hand, ferritin

shows excellent size distribution, with different bioengineered ferritin typically ranging from 12 to 20 nm, and minimal change in size observed when loaded with drugs [40].

A variety of drugs have been tested using ferritin for drug delivery. Drugs based on metals, such as cisplatin and carboplatin, can be easily encapsulated in the ferritin shell. Cisplatin is a platinum-based antineoplastic medication that kills cancer cells by binding to DNA and inhibiting its replication. However, heart disease and kidney disorder are among its side effects, and thus its clinical application has been largely restricted by high toxicity and tumor resistance [111]. The encapsulation of cisplatin and platinum-based carboplatin was first reported by Yang *et al*. [71]. However, ferritin-entrapped cisplatin can induce apoptosis in cancer cells. Falvo *et al*. used ferritin to load cisplatin to improve the therapeutic index of anti-blastic therapy in melanoma [65, 88].

Compared to metal-containing drugs, non-metal-containing drug loading is more complex due to poorer interaction with ferritin. Dox was the first chemotherapeutic drug to be encapsulated in ferritin by Simek and Kilic in 2005 [6]. Dox is a widely used drug for a large spectrum of cancers, but is associated with major toxicities at high doses. However, encapsulation of Dox in the ferritin nanocage can negate these unwanted effects. For example, Dox pre-complexed with Cu(II) loaded inside the ferritin nanocage was evaluated in U87MG glioblastoma tumor models with significant tumor suppressing results, although with the added toxicity of Cu(II). We also previously showed that, without any targeting ligand functionalization, ferritin nanocarriers could target tumors *in vivo*, exhibiting more than 10-fold higher drug concentration while significantly reducing healthy organ drug exposure [40]. High quality encapsulation and delivery of Dox have been achieved by a number of studies [62].

In addition to Dox, a wide variety of drugs have been loaded in ferritin. For example, daunomycin, which is structurally similar to Dox, has been successfully added to horse spleen through poly-L-aspartic acid-assisted loading, though it did not show antitumor activity [85]. Curcumin, which is relatively therapeutically ineffective in its free form due to its chemical instability, water insolubility, and absence of potent and selective targets, has been effectively loaded into HFn, truncated HFn, and HosFn [42,

93, 96], with HosFn-curcumin demonstrating significant antitumor activity. These studies indicate that organic compound anti-cancer drugs with poor bioavailability, may be enhanced by ferritin-cage encapsulation.

Ferritin can also deliver a variety of payloads, including radioisotopes. For example, in 1992, Hainfeld *et al.* developed a ferritin nanocage with about 800 ^{235}U atoms per nanoparticle [98]. Gene delivery and therapy have also become useful ways in which to correct genetic issues, such as cancer, at their source. Research has shown that ferritin can encapsulate, protect, and deliver nucleic acid [100]. Even with RNase present, ferritin encapsulated group shows no sign of siRNA reduction, thus demonstrating the protective ability of the ferritin nanocage of its payload.

The above studies demonstrate that ferritin is capable of encapsulating a wide range of drugs with beneficial effects on pharmacological properties. Drugs encapsulated within ferritin are shielded, which reduces their toxicity to healthy cells significantly, while maintaining their effects on cancer cells. The maximum tolerated dose of ferritin-encapsulated drugs is reported to be four times higher than that for free drugs [40] and is comparable to that of Doxil and albumin delivery [112, 113]. Current evidence suggests that during acidification in the lysosome, drugs contained in ferritin are gradually released.

7. Ferritin drug carriers vs antibody drug conjugates

A variety of unique characteristics make ferritin a promising drug delivery candidate, including its remarkable thermal stability, pH stability, uniform size, biocompatibility, biodegradability, low cost, large-scale production, hollow cavity, natural targeting ability, and ease of conjugation by chemical procedures and genetic means. Despite this, research on FDCs remains in its infancy. Thus, it is important to compare ferritin with well-established platforms. Li *et al*. previously compared various aspects of ferritin and exosomes [114], and here we shall discuss how ferritin measures against ADCs, which have similar traits in terms of size, targeting ability, and composition.

The concept of targeted drug delivery can be traced to Paul Ehrlich, who described a “magic bullet” that could selectively deliver a toxophore to a tumor [115]. This idea

led to the development of ADCs as well as other targeted drug delivery systems. ADCs take advantage of the specificity of monoclonal antibodies to deliver cytotoxic drugs to selective sites. Through a long process of research and optimization, Mylotarg was the first FDA-approved ADC drug on the market. However, not long after approval, Mylotarg administration resulted in multiple clinical failures due to increased fatalities caused by toxicities related to hydrazine linker instability [116]. Today, only a handful of ADC drugs have been approved by the FDA, and their long-term performance is yet to be evaluated.

FDCs exhibit similar targeting abilities as ADCs, especially since the discovery of TfR1-specific binding. Even though the binding affinity is ten times weaker than that of some TfR1 antibodies, it has not hindered its ability to deliver drugs to the intended tumor. It is even arguable that high affinity may be impractical in the development of targeted drug delivery. Stronger affinity over a certain binding strength hinders the ability of the antibody to cross barriers into the tumor. It is, however, a huge advantage to have flexibility in the development of different antibodies for different tumors, a quality that FDC can accomplish by adding targeting peptides or antibodies.

The drug loading process of ADCs requires chemical conjugation of the payload by linkers, which limits the amount of drug that can be linked to the antibody without affecting the targeting ability of the ADC. In contrast, FDCs are loaded with drugs by disassembly and reassembly or passively through ion channels and are only limited by the space of the ferritin protein nanocage and the method of encapsulation optimization. One ferritin nanocage can encapsulate up to hundreds of small molecule drugs, whereas an antibody can only link to 2–6 drugs at a time. In the initial failures of ADCs, the antibodies were conjugated with clinically approved drugs with well-established mechanisms. For example, due to low doxorubicin potency, doxorubicin-conjugated (BR96-DOX) ADC failed to achieve preclinical activity because of the necessary high drug to antibody ratio, which led to significant toxicity from the nonspecific cleavage of the linker before the drug was delivered to the targeted site [117]. However, next-generation ADCs in current clinical development are using much more potent payloads with lower drug to antibody ratios, which should exhibit cytotoxic potency at a

picomolar range to kill tumor cells intracellularly. Clinical studies with radiolabeled antibodies have demonstrated that only 0.01% of the injected antibody is localized to a tumor after 24 h [118]. Thus, a carrier that can deliver a higher ratio of drug is important in clinical use.

The stability of ADCs is greatly influenced by the linker and is crucial for successful delivery. Linker stability enables the conjugate to circulate in the bloodstream for an extended period of time [119]. However, if the linker is too stable, it is unable to release the drug once inside the cell. In contrast, FDCs rely on the protein shell to keep the payload from prematurely releasing as a free drug, which has been shown to be relatively effective in leaking assays [40]. A protective shell also extends the shelf life of the drug, which is an advantage not shared by ADCs.

Ferritin nanocages can be produced with *Escherichia coli* and can self-assemble without any further modifications. This has improved the ability to undertake ferritin research and should be translatable to larger scale operations. Antibodies, on the other hand, require eukaryotic cells or mouse ascites for production [120], which are relatively expensive. In contrast, recombinantly produced mammalian ferritin is relatively cheap, with established protocols to separate ferritin from other proteins achieved simply through heating to ~75 °C and removing the denatured proteins, with a ferritin supernatant remaining. With further purification through chromatographic separation, the final material exhibits very high purity.

FDC and ADC are very similar in that they are both protein-based drug delivery system with specific affinity to tumor markers, and thus have similar traits in terms of size, targeting ability, and composition. Over the course of research on ADC, a number of limitations have been exposed in which shows considerable strength in. An antibody is typically conjugated with 2-6 drug molecules, resulting in low potency drugs which is hard to achieve clinical benefits; ferritin on the other hand encapsulates up to 400 drug molecules, significantly increasing the ratio of drug to carrier. The conjugation of drug molecule onto the antibody affects the pharmacokinetics parameters of antibody in vivo; this may be due to the hydrophobicity of the drug molecule commonly used for ADC. However, Since FDC encapsulates drugs within the cavity of ferritin protein shell,

it is expected that FDC should not have similar problems as ADC.

ADC also faces challenges of toxicity caused by nonspecific targeting of antibody to normal cells. In comparison, FDC shows a TfR1 expression threshold dependent incorporation into cancer cells, which means the expression of TfR1 on a normal cell does not facilitate endocytosis of FDC, only high expression in tumor cells cause significant incorporation of FDC into the cells. There are multiple binding sites on HFn to TfR1 based on the symmetry structure of HFn, a single binding does not trigger the internalization of HFn, but binding to multiple receptors does allowing for a threshold dependent incorporation into the cell [121]. Meanwhile, monovalent binding to TfR1 facilitates transcellular transport [47].

With that being said, FDC has yet to be in a clinical trial, so it is still early to say for sure that FDC will perform better than ADC. This is in fact why we want to write this review, is to get more people to be interested and involved in developing FDC so that we can find an ideal drug delivery vehicle for cancer treatment. We believe that FDC is a promising candidate to be the next generation of tumor targeted drug delivery, and in many situations may be even better than ADC, these details can be found in the comparison section for ADC and FDC.

Thus, FDCs have many qualities that are on par with or superior to those of ADCs. Despite this, research and optimization of FDCs are lacking compared to that for ADCs (Table 4). Overall, FDCs are strong alternatives to ADCs, and comparisons with other drug delivery systems, such as exosomes, has also shown the advantages of using FDCs as a drug delivery system. Further clinical studies should be conducted to determine under what situations these two platforms are suitable.

Table 4. FDC vs ADC comparison

	FDC	ADC
Targeting tumor	TfR1receptor overexpression in various tumors	Specific tumor antigen
Loading method	Disassembled/ reassembled	Chemically conjugated

Drug/Carrier ratio	400 Dox per 1 HFn	1:2-6
Stability	Stable at 70 ℃	Unstable at 40 ℃
Cellular uptake	Threshold dependent endocytosis	Receptor mediated endocytosis
Production	Easy to produce	Relatively expensive

8. Future Perspectives

Inspired by Ehrlich's "magic bullet" [115], various promising drug delivery systems that selectively target the site of disease have been developed in the last few decades. Among them, nanocarriers capable of loading drugs, improving circulation, and reducing toxicity have already been used clinically [122, 123]. In particular, several nanotherapeutic platforms, including liposomes, albumin nanoparticles, and polymeric micelles, have been approved for clinical cancer treatment due to their passive tumor-targeting properties (e.g., enhanced penetration and retention, EPR effects) [124]. However, recent systematic analysis of clinical trials for these EPR effect-based nano-formulations has demonstrated that only a limited amount of drug is delivered to the tumor site, and efficacy in patients is not significant compared with conventional chemotherapy [30, 31]. On the other hand, nanoparticles developed to target specific tumors and modified with targeting ligands can result in an excess of ligands or overcrowding, causing nonspecific binding and aggregation [32, 33, 125]. Thus, nanocarriers with reliable targeted delivery to the diseased site are in urgent demand for effective tumor therapy.

Ideally, the characteristics of a tumor-targeting drug delivery system should include a) tumor-targeting ability, b) safety (i.e., nontoxic, biocompatible, biodegradable), and c) excellent pharmacological properties (i.e., easy drug loading, controlled drug release, and physiochemically stable both *in vivo* and *in vitro*) [126, 127]. Although diverse chemical synthetic nanocarriers have been designed to fit these criteria, none has achieved translation from bench to bedside due to their toxicity, poor biocompatibility,

weak homogeneity, and complex production procedures [128]. Considering this, many researchers have turned to existing natural materials, particularly endogenous self-assembling proteins, as new candidates for drug delivery [129].

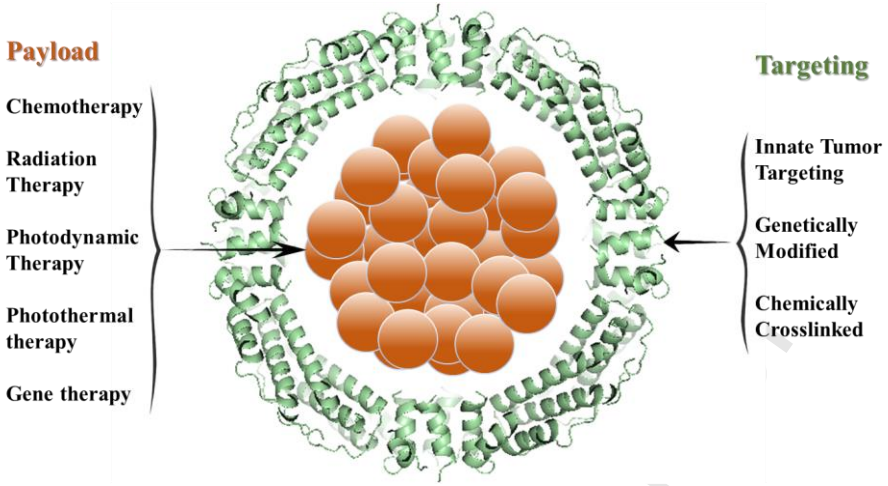

Figure 8. FDC platform carrying different types of drugs into the cavity and displaying a variety of moieties to target tumors.

With its unique natural protein properties, such as low toxicity, no immune response, easy biomimetic synthesis, and simple modification, ferritin has great potential for the delivery of drugs or imaging probes (Figure 5). However, several issues currently limit the clinical translation of ferritin and require further study. Firstly, its drug loading efficacy remains relatively low, which can be affected by a number of factors such as buffer pH and loading parameters. At present, most drugs are encapsulated in ferritin through charge interaction; however, this requires further investigation and could be important to its release mechanism. Another limiting factor is its targeting. When using its natural TfR1 binding, different tumor cells have various expression, thus some will have better efficacy than others. On the other hand, additional targeting modification increases production difficulties and new issues such as biosafety.

Furthermore, in terms of clinical use, the fact that ferritin is a protein limits its delivery method; for example, it cannot be delivered orally but must be injected into the system. In addition, its storage state in solution is not as stable as in the solid state. However, protein lyophilization may cause ferritin to lose its activity.

Although these limiting factors are all very important questions that need to be addressed, they are common in the development of drug delivery systems and do not overshadow the advantages of FDCs. We hope this review will encourage further research and development of FDCs, particularly in regard to their high potential for therapeutic use.

FDCs have seen great improvements and new opportunities in the last decade. The ability of ferritin to incorporate iron in its cavity has allowed researchers to use the empty space inside (with an inner diameter of 8 nm) for substantial drug encapsulation. However, optimization of drug loading is needed to further improve its therapeutic effects. Using ferritin from *Pyrococcus furiosus*, Jiang *et al*. were able to achieve drug loading of more than 400 Dox molecules per ferritin, much higher than previously reported [63]. This suggests that the capacity for optimization of human ferritin-based nanocarriers still has enormous potential for improvement.

How to maximize ferritin's intrinsic ability to target specific sites *in vivo* is arguably the most crucial aspect of ferritin drug delivery. For instance, the ability of FDCs to cross the BBB is difficult to achieve for most nanocarriers. To find a reliable delivery method to the central nervous system has been a difficult task throughout drug delivery history. With better understanding of the TfR1-mediated delivery mechanism, it is likely that FDCs will see greater use in brain-related drug delivery. Using the targeting ability of ferritin to direct nanocarriers to diseased sites also allows ferritin to act as an antibody to be labeled onto and guide the nanocarrier *in vivo*. Using ferritin's natural targeting ability and ease of labeling are intriguing approaches for ferritin delivery of nanomaterials.

In addition, over the past few years, considerable effort has been expended to lengthen the half-life of HFn *in vivo*. HFn-based constructs involve the genetic modification of the N-terminus of each HFn subunit to fuse with a PAS sequence or tumor-selective sequence responsive to tumor proteases (MMPs) [80]. Further modifications can be made to add a glutamate residue in the PAS sequence to eliminate the negative charge residue. Other methods to lengthen the half-life include using biosilica to envelope the ferritin cage [130]. In addition, Dox release can be controlled

according to the extracellular matrix. Wang *et al.* demonstrated that by fusing with albumin binding domain, HFn can dramatically improve its pharmacokinetics, thus increasing its plasma half-life to approximately 17.2 h [131]. Although these attempts have extended the circulation half-life of FDCs, how these modifications affect the affinity of FDCs to the cell is not yet fully understood.

Nature creates both simplistic and fascinating designs. In the case of ferritin, we, among many others, have sought to harness its storage and targeting potentials to deliver life-saving medications to the site of need. Whether or not it can be translated to clinical practice remains a challenge. However, promising results have shown FDCs to be an excellent drug delivery vehicle in many aspects. Thus, we feel optimistic in our continued endeavor to develop FDCs into clinically reliable drug carriers.

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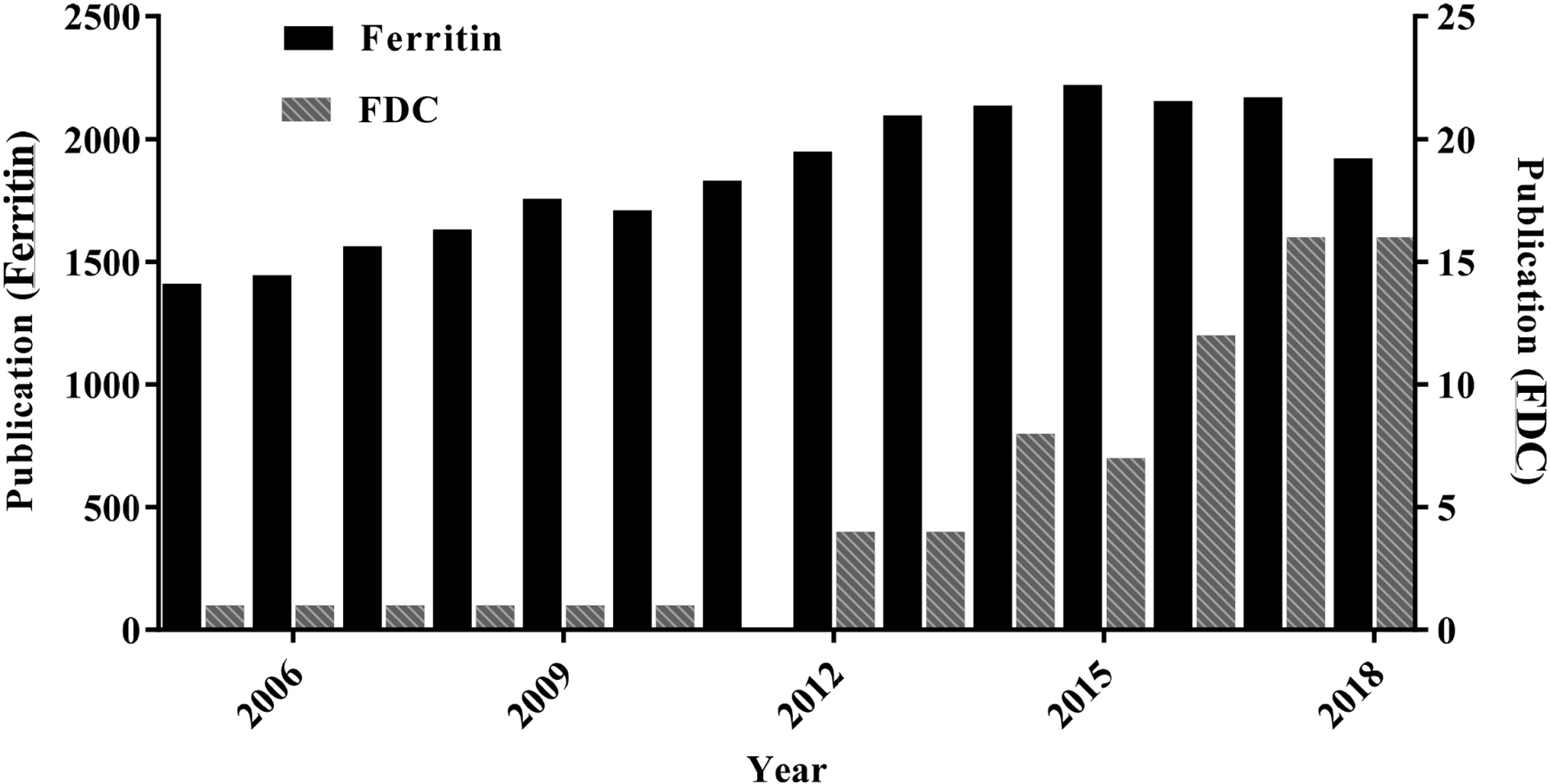
Ferritin
FDC
Publication (Ferritin)
Publication (FDC)
2500
2000
1500
1000
500
0
25
20
15
10
5
0
2006
2009
2012
2015
2018
Year

Figure 1

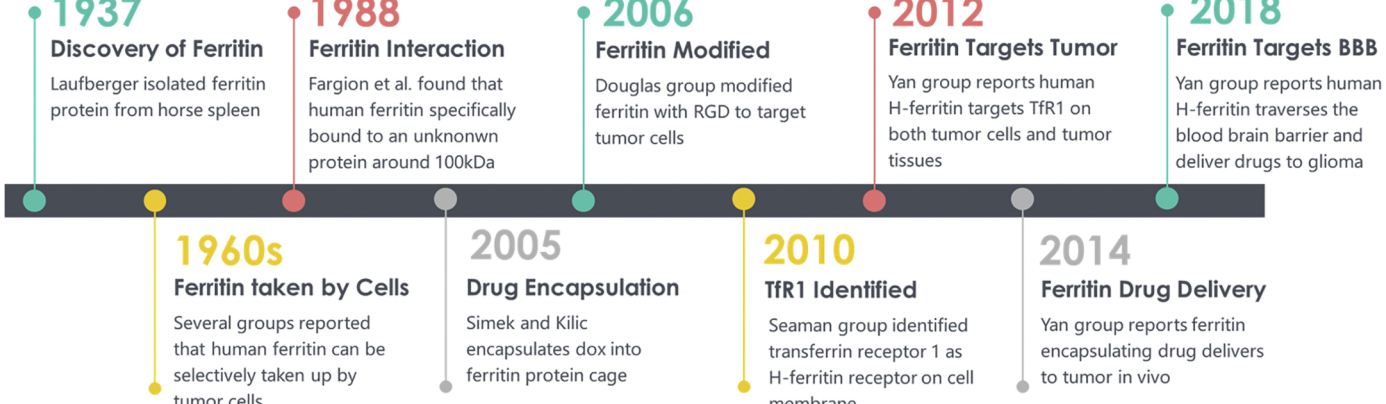
1937
Discovery of Ferritin
Laufberger isolated ferritin protein from horse spleen
1960s
Ferritin taken by Cells
Several groups reported that human ferritin can be selectively taken up by tumor cells
1988
Ferritin Interaction
Fargion et al. found that human ferritin specifically bound to an unknonwn protein around 100kDa
2005
Drug Encapsulation
Simek and Kilic encapsulates dox into ferritin protein cage
2006
Ferritin Modified
Douglas group modified ferritin with RGD to target tumor cells
2010
TfR1 Identified
Seaman group identified transferrin receptor 1 as H-ferritin receptor on cell membrane
2012
Ferritin Targets Tumor
Yan group reports human H-ferritin targets TfR1 on both tumor cells and tumor tissues
2014
Ferritin Drug Delivery
Yan group reports ferritin encapsulating drug delivers to tumor in vivo
2018
Ferritin Targets BBB
Yan group reports human H-ferritin traverses the blood brain barrier and deliver drugs to glioma

Figure 2

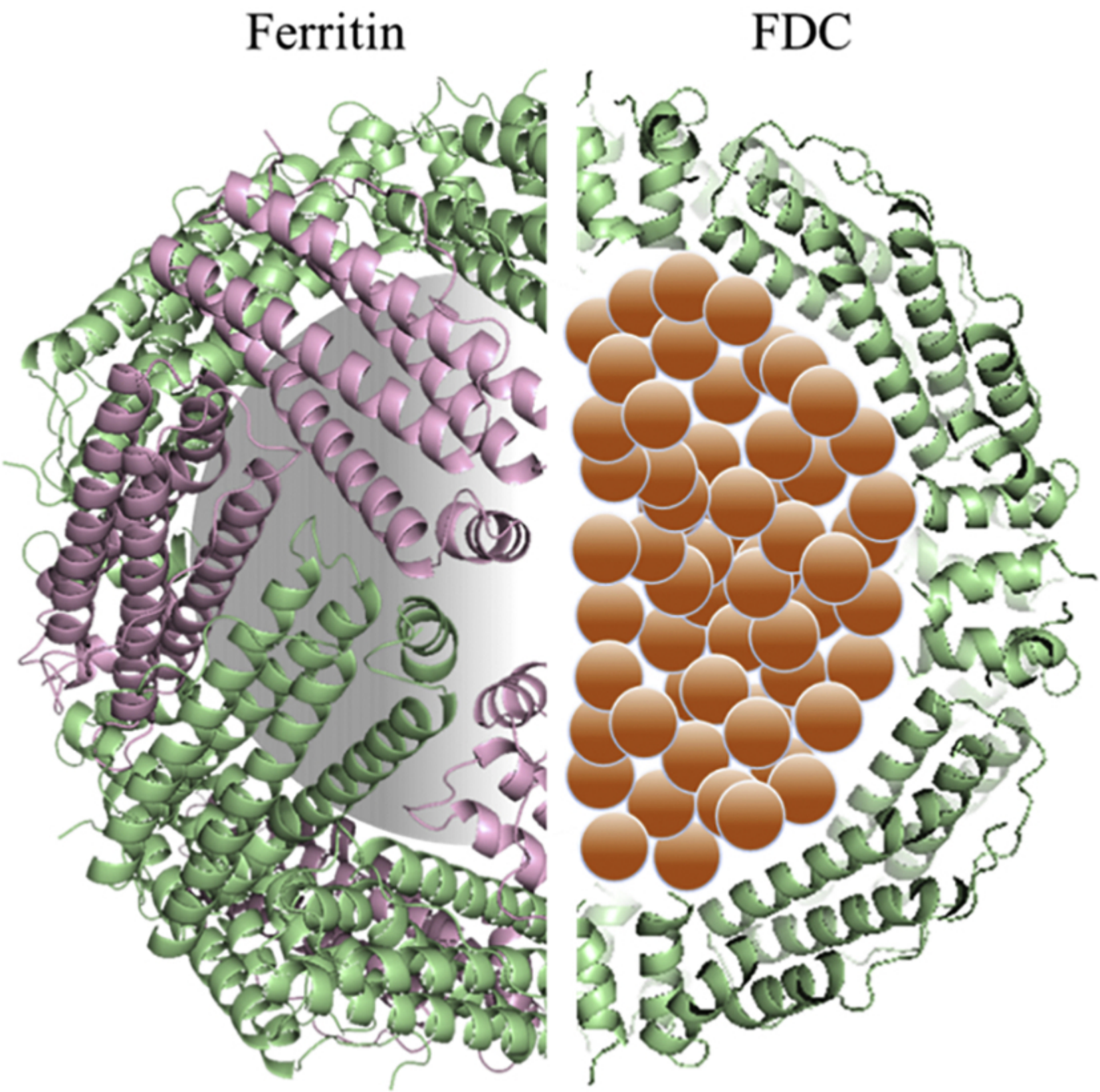

Figure 3

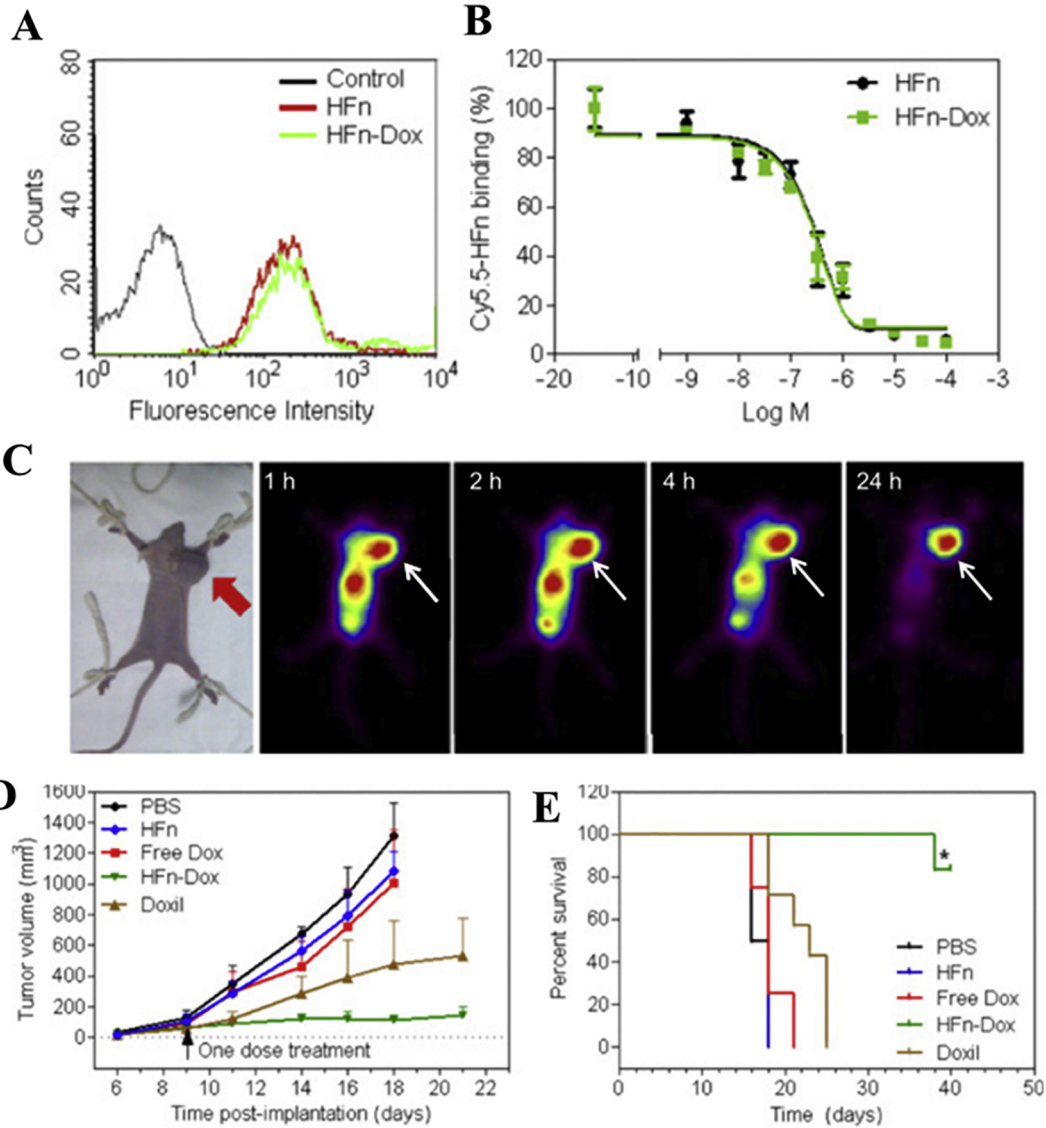
A
Control
HFn
HFn-Dox
Counts
Fluorescence Intensity
B
HFn
HFn-Dox
Cy5.5-HFn binding (%)
Log M
C
1 h
2 h
4 h
24 h
D
PBS
HFn
Free Dox
HFn-Dox
Doxil
Tumor volume (mm3)
One dose treatment
Time post-implantation (days)
E
Percent survival
PBS
HFn
Free Dox
HFn-Dox
Doxil
Time (days)

Figure 4

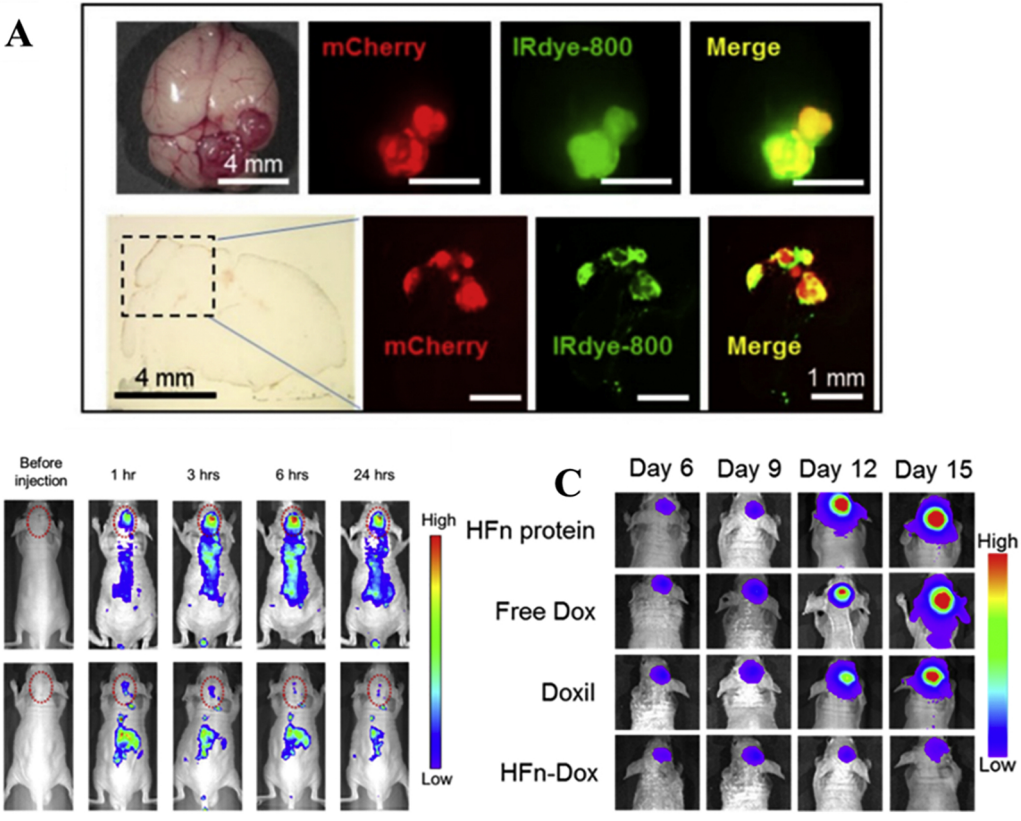
A
mCherry
IRdye-800
Merge
4 mm
4 mm
mCherry
IRdye-800
Merge
1 mm
B
Before injection
1 hr
3 hrs
6 hrs
24 hrs
HFn
LFn
High
Low
C
Day 6
Day 9
Day 12
Day 15
HFn protein
Free Dox
Doxil
HFn-Dox
High
Low

Figure 5

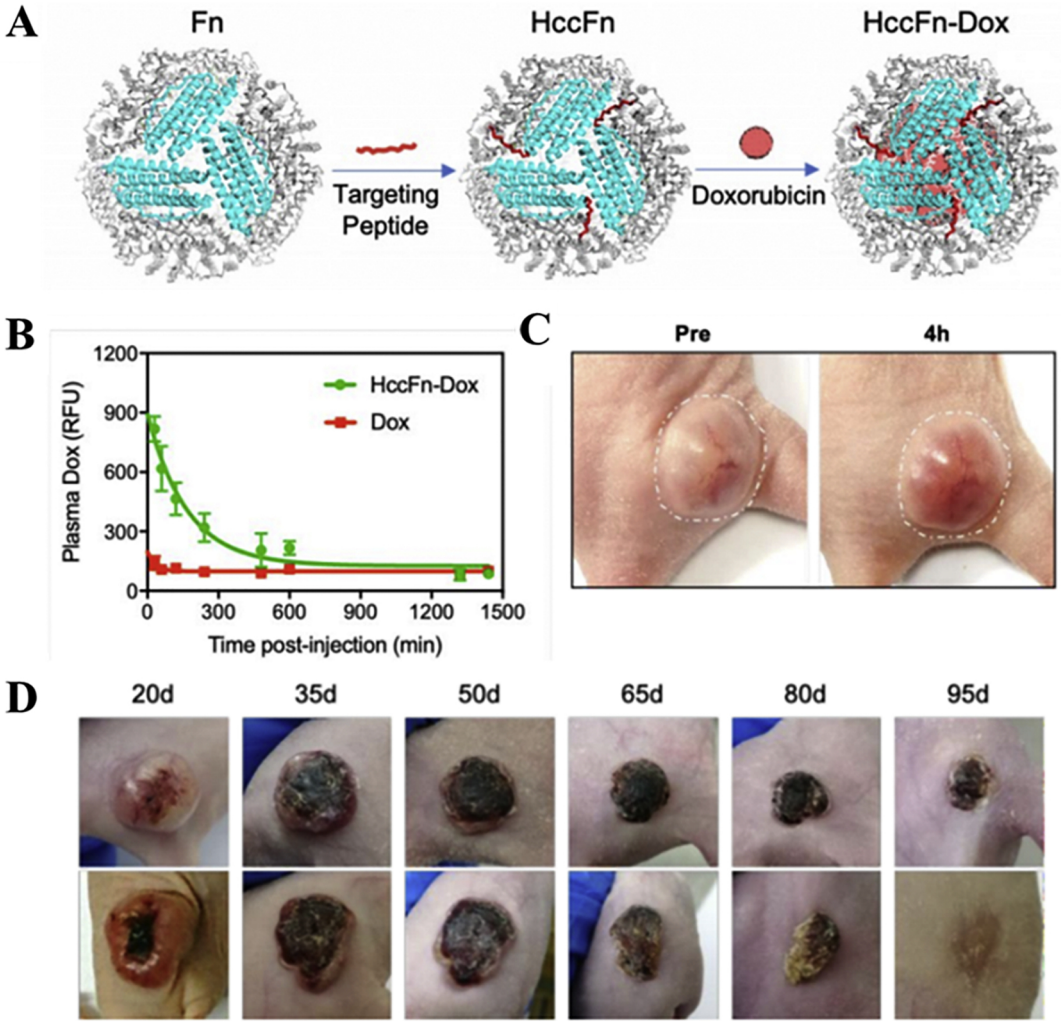
A
Fn
HccFn
HccFn-Dox
Targeting Peptide
Doxorubicin
B
Plasma Dox (RFU)
1200
900
600
300
0
HccFn-Dox
Dox
0
300
600
900
1200
1500
Time post-injection (min)
C
Pre
4h
D
20d
35d
50d
65d
80d
95d

Figure 6

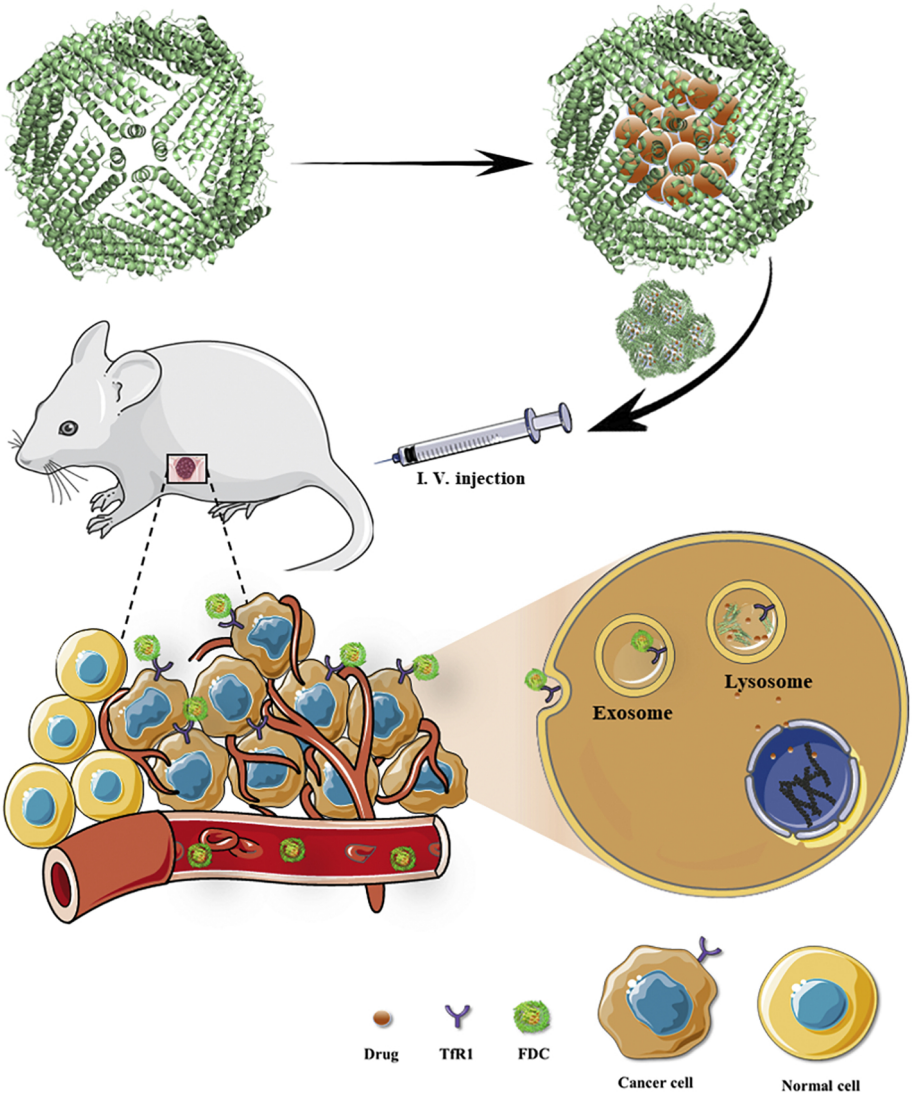
I. V. injection
Exosome
Lysosome
Drug
TfR1
FDC
Cancer cell
Normal cell

Figure 7

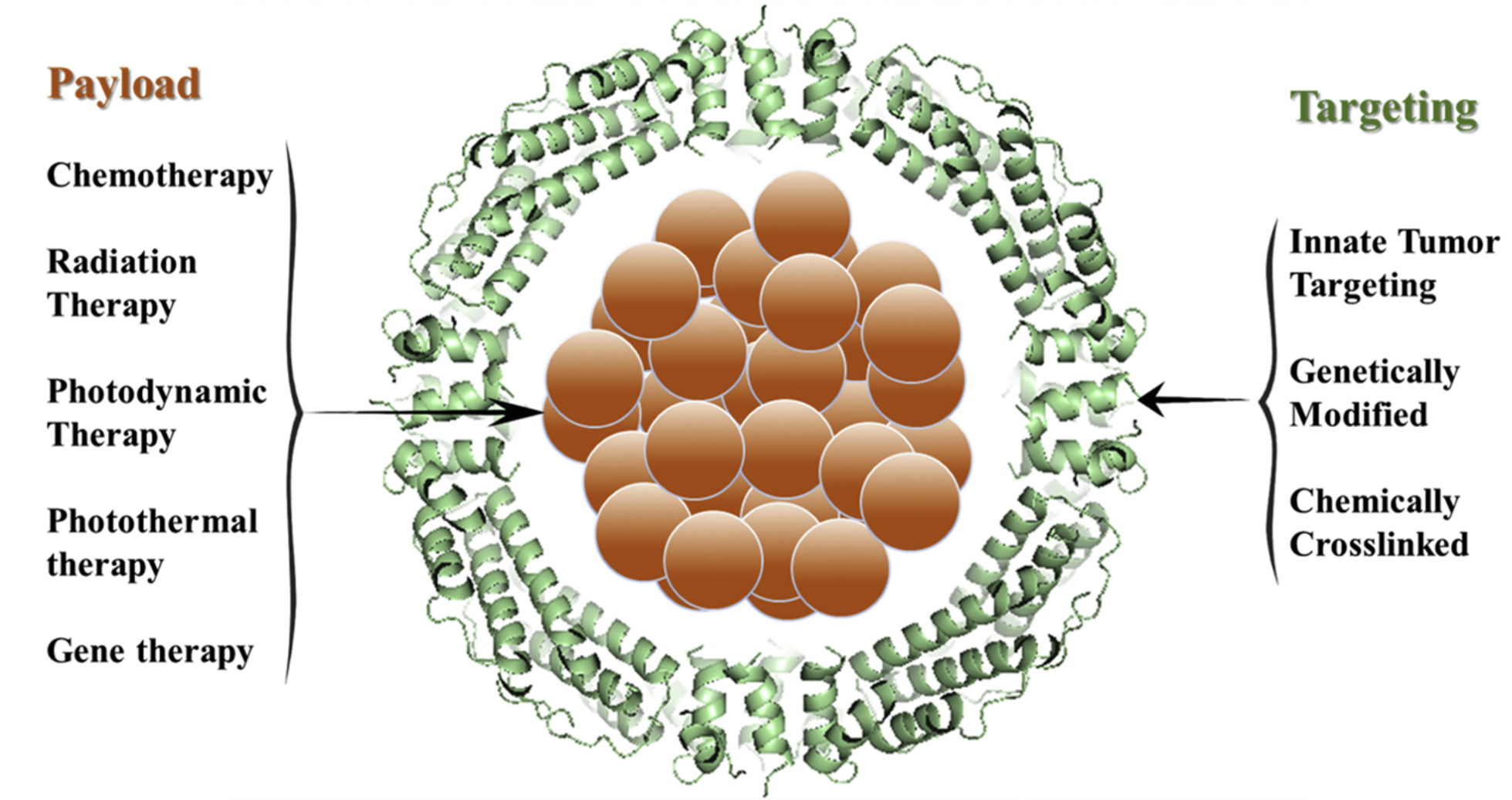
Payload
Chemotherapy
Radiation Therapy
Photodynamic Therapy
Photothermal therapy
Gene therapy
Targeting
Innate Tumor Targeting
Genetically Modified
Chemically Crosslinked

Figure 8