Review

Yuanjun Zhu, Yuefeng Zhu, Tianmiao Cao, Xiaoyu Liu, Xiaoyan Liu, Yi Yan, Yujie Shi and Jian-Cheng Wang*

Ferritin-based nanomedicine for disease treatment

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Abstract: Ferritin is an endogenous protein which is self-assembled by 24 subunits into a highly uniform nanocage structure. Due to the drug-encapsulating ability in the hollow inner cavity and abundant modification sites on the outer surface, ferritin nanocage has been demonstrated great potential to become a multi-functional nanomedicine platform. Its good biocompatibility, low toxicity and immunogenicity, intrinsic tumor-targeting ability, high stability, low cost and massive production, together make ferritin nanocage stand out from other nanocarriers. In this review, we summarized ferritin-based nanomedicine in field of disease diagnosis, treatment and prevention. The different types of drugs to be loaded in ferritin, as well as drug-loading methods were classified. The strategies for site-specific and non-specific functional modification of ferritin were investigated, then the application of ferritin for disease imaging, drug delivery and vaccine development were discussed. Finally, the challenges restricting the clinical translation of ferritin-based nanomedicines were analyzed.

Keywords: drug loading; ferritin-based nanomedicine; functional modification; tumor therapy; vaccine.

Introduction

Ferritin is a natural protein first isolated by Laufberger from horse spleen in 1937. Since then, the ferritin protein superfamily is discovered from other animals, humans, plants, fungi, and bacteria [1]. Ferritin is composed of 24 subunits, which is self-assembled into a regular protein nanocage (Figure 1A). Human ferritin is made of two types of subunits, heavy (H) ferritin chain (21 kDa) (Figure 1B) and light (L) ferritin chain (19 kDa) [2]. H- and L-ferritin subunits could assemble, either separately or H–L combined, into a 24-mer spherical nanocage, including an outside protein shell and an inside hollow cavity with an outer and inner diameter of 12 and 8 nm (Figure 1C), respectively. The inner cavity can store up to 4500 Fe(III) atoms, which makes ferritin a vital iron storage protein and maintenance of cellular and systemic iron homeostasis, thus playing important role in both physiological function and pathological process, such as iron deficiency anemia, hemochromatosis, cell antioxidant and related inflammation, vascular proliferation and tumor [3].

Advantages of ferritin nanocage in drug delivery

Ferritin nanocage has shown bright application future in biomedical field, such as drug delivery and antigen displaying of vaccines, due to its special characters and advantages. Firstly, its hollow cavity can encapsulate various drugs inside, and the reversible self-assembly property makes it feasible to load drugs in the process of disassembly/reassembly, while the inter-subunit channels also enable drugs to enter the cavity. Secondly, ferritin shell can be easily engineered by

Yuanjun Zhu, Yuefeng Zhu and Tianmiao Cao contributed equally to this work.

***Corresponding author: Jian-Cheng Wang**, Beijing Key Laboratory of Molecular Pharmaceutics and New Drug Delivery Systems, State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China; and Laboratory of Innovative Formulations and Pharmaceutical Excipients, Ningbo Institute of Marine Medicine, Peking University, Ningbo 315832, Zhejiang Province, China, E-mail: wang-jc@bjmu.edu.cn. https://orcid.org/0000-0002-1651-8977

Yuanjun Zhu and Xiaoyan Liu, Beijing Key Laboratory of Molecular Pharmaceutics and New Drug Delivery Systems, State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing, China; and Department of Molecular and Cellular Pharmacology, School of Pharmaceutical Sciences, Peking University, Beijing, China

Yuefeng Zhu, Tianmiao Cao, Xiaoyu Liu, Yi Yan and Yujie Shi, Beijing Key Laboratory of Molecular Pharmaceutics and New Drug Delivery Systems, State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing, China

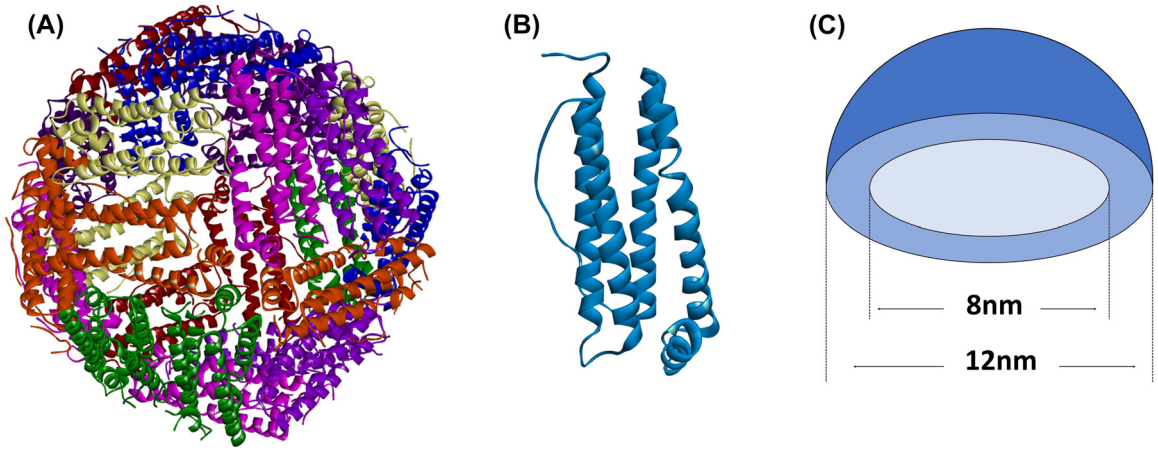

Figure 1: The structure of ferritin nanocage. (A) Structure of human H-ferritin (PDB ID: 6GSR). The 24 subunits were showed in cartoon representations and eight different colors. (B) The ferritin subunit monomer structure, with five α helixes. (C) The cage-like diagram of ferritin, with inner cavity and outer surface of diameter 8 nm and 12 nm, respectively. H-ferritin, heavy chain ferritin.

genetic or chemical modification to display vaccine antigens, increase targeted delivery or achieve other functions. Thirdly, as natural protein in human, ferritin is excellent in safety which shows low toxicity and immunogenicity, high biocompatibility and biodegradability. Fourthly, with the small and uniform particle size, 12 nm, ferritin can take advantage of enhanced permeability and retention effect (EPR), increasing the accumulation of drugs in tumors and enhancing the anti-tumor efficacy. Fifthly, ferritin shows high specific binding to transferrin receptor 1 (TfR1) that was overexpressed in cancer cells and brain blood barrier (BBB), suggesting an excellent intrinsic targeting ability for tumors and a great potential to cross BBB for brain drug delivery. Sixthly, ferritin can be kept at up to 75 °C for more than 10 min and maintain the original hollow structure in a wide pH range, which is beneficial for the preparation and storage of ferritin-based pharmaceuticals. Finally, ferritin can be easily produced in *Escherichia coli* at large scale and purified, with high yield and low cost [4–6]. In general, with these properties and advantages, ferritin nanocage shows great potential to be a drug carrier in nanomedicine.

Actually, besides ferritin, other natural proteins, such as albumin and hemoglobin, gelatin [7], casein [8], transferrin [9] and collagen, were used as drug carrier in nanomedicine. For example, albumin is the most abundant protein in serum, and has high biocompatibility, biodegradability, non-immunogenicity and safety, showing great advantages as a nanodrug delivery carrier especially for hydrophobic drugs [10]. Using albumin to deliver Paclitaxel (Abraxane®) has been approved by FDA to treat cancer. Albumin-based nanoparticles are constructed by desolvation, emulsification, and thermal gelation, etc. and most of small molecular chemical drugs need to be chemical cross-linked with albumin. However, ferritin can self-assemble into a nanocage with a hollow cavity, and drugs can be encapsulated in ferritin inner cavity in non-chemical linked manner. Albumin-based nanoparticles usually show particle size in the range of 100–300 nm [11], larger than ferritin-based nanoparticles of 12–50 nm [12]. Hemoglobin has similar advantages with albumin, and has also been used to construct hemoglobin-based nanoparticles for delivering drugs to treat cancers [13, 14]. In brief, ferritin-based nanoparticle is different from these proteins mentioned above derived nanoparticles, since ferritin shows several advantages, including self-assembly into nanocage, small size, easy modification, intrinsic tumor-targeting and BBB-crossing ability.

Loading methods for ferritin-based nanomedicine

Ferritin nanocage has excellent drug loading capacity, making it a good delivery vehicle in nanomedicine. As shown in Table 1, different types of cargo, including hydrophilic molecules, hydrophobic molecules and biomacromolecules, can be loaded into the ferritin nanocage. The cargo loading methods mainly include reversible disassembly/reassembly-mediated loading, channels-dependent loading and some other methods.

Reversible disassembly/reassembly-mediated drug loading

The inner cavity of ferritin has been explored as a promising cargo container. Ferritin has a natural property of

Table 1: Drugs and the related loading methods in ferritin.

Drugs	Category	Loading method
Saponin	Mixture	pH-induced reversible disassembly/reassembly [15]
Cisplatin	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [16–18] Channel-dependent loading [16]
Paclitaxel	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [19–21]
Quercetin	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [22]
Curcumin	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [22, 23]
Epirubicin	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [24]
Mitoxantrone	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [25]
Kartogenin	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [26]
$ZnF_{16}Pc$	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [27] Channel-dependent loading [28]
Idarubicin	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [29]
Vandetanib	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [30, 31] Channel-dependent loading [30]
Lenvatinib	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [30] Channel-dependent loading [30]
Camptothecin	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [32]
Yttrium phosphate	Hydrophobic molecules	Channel-dependent loading [33]
β-Lapachone	Hydrophobic molecules	Channel-dependent loading [34]
Gefitinib	Hydrophobic molecules	Channel-dependent loading [35]
FITC-AD	Hydrophobic molecules	Binding the outer shell [36]
Erastin	Hydrophobic molecules	Emulsion [37]
Rapamycin	Hydrophobic molecules	Emulsion [37]
Pseudolaric acid B	Hydrophobic molecules	Emulsion [38]
Lapatinib	Hydrophobic molecules	Emulsion [38]
Navitoclax	Hydrophobic molecules	Channel-dependent loading [39]
Hydroxychloroquine	Hydrophobic molecules	pH-induced reversible disassembly/reassembly [40]
Cyclosporine A	Hydrophobic molecules	Solvent-induced disassembly/reassembly [41]
Atropine	Hydrophilic molecules	pH-induced reversible disassembly/reassembly [42]
Doxorubicin	Hydrophilic molecules	Reversible disassembly/reassembly (pH-induced [43–46] Urea-induced [47, 48]) Channel-dependent loading [49, 50]
Streptomycin	Hydrophilic molecules	pH-induced reversible disassembly/reassembly [51]
Carbachol	Hydrophilic molecules	pH-induced reversible disassembly/reassembly [42]
ATP	Hydrophilic molecules	Channel-dependent loading [52]
Epigallocatechin gallate	Hydrophilic molecules	Channel-dependent loading [53, 54]
Chlorogenic acid	Hydrophilic molecules	Channel-dependent loading [53]
Anthocyanin	Hydrophilic molecules	Channel-dependent loading [53]
siRNA	Nucleic acid	pH-induced reversible disassembly/reassembly [55–57] Binding the outer shell [58]
dsRNA	Nucleic acid	pH-induced reversible disassembly/reassembly [59]
+36GFP	Protein	Salt-induced reversible disassembly/reassembly [60]
Ferredoxin	Protein	Salt-induced reversible disassembly/reassembly [61]
Artificial transfer hydrogenase	Protein	pH-induced reversible disassembly/reassembly [62]
GFP	Protein	Genetic fusion [63]
SIRPα	Protein	Genetic fusion [64]
TRAP	Peptide	Genetic fusion [65]
PD-L1 binding peptide	Peptide	Genetic fusion [66]

ZnF16Pc, zinc hexadecafluorophthalocyanine; FITC-AD, fluorescein isothiocyanate-conjugated adamantane; ATP, Adenosine triphosphate; siRNA, small interfering RNA; dsRNA, double-stranded RNA; GFP, green fluorescent protein; +36GFP, positively supercharged green fluorescent protein; SIRPα, Signal regulatory protein α; TRAP, thrombin receptor agonist peptide; PD-L1, programmed death-ligand 1.

pH-responsive disassembly/reassembly [67], which was first used to entrap small molecules (the pH indicator, neutral red and phenolphthalein; the iron chelator, bipy; the redox active molecules, MV and FMN) within the interior of horse spleen ferritin by Webb et al. [68]. The procedures were as follows: ferritin disassociated into subunits by adjusting the buffer pH to acidic 2.0; after adding small molecules to be wrapped, the pH was then adjusted to neutral 7.0, resulting in the reassembly of

the nanocage. Small molecules were encapsulated in the inner cavity during this disassembly/reassembly process (Figure 2A). Doxorubicin (Dox) was the first anti-tumor drug loaded in ferritin nanocage using this method, which was reported by Simsek and Kilic at 2005 who directly and step wisely changed the pH of the solution from 2.0 to 7.4 and encapsulated five Dox molecules per ferritin nanocage [43]. Afterwards, they continued to optimize this drug loading methods and improve the loading efficiency that up to 28 Dox molecules were entrapped into a single apoferritin nanocage at 2012 [44]. Besides, Yang et al. [16] managed to encapsulate platinum anticancer drugs into apoferritin after pH-mediating disassembly/reassembly that cisplatin or carboplatin were loaded in apoferritin. Other small molecules drugs, including paclitaxel [19–21], streptomycin [51], quercetin [22], curcumin [22, 23], saponin [15], epirubicin [24], carbachol [42], atropine [42], mitoxantrone [25], kartogenin [26], $ZnF_{16}Pc$ [27], idarubicin [29], vandetanib [30], lenvatinib [30], vandetanib [31] and camptothecin [32] were also encapsulated in ferritin nanocage by this loading method.

In addition to small molecules, biomacromolecules such as siRNA and protein can also be loaded through pH-induced reversible disassembly/reassembly. Li et al. [55] firstly reported that siRNA could be encapsulated into apoferritin by pH-induced procedure. However, the encapsulation efficiency of nucleic acids was low for lack of electrostatic binding between the negatively charged ferritin inner surface and siRNA [70]. To improve nucleic acids loading efficacy, Huang et al. [56] introduced Ca^{2+} into the process of pH-induced reversible disassembly/reassembly. Ca^{2+} formed complexes with phosphate groups of nucleic acids through coordination, which provided positive charge to bind ferritin inner cavity through electrostatic interaction. The encapsulation efficiency could be enhanced 1.65-fold compared to that without incorporated Ca^{2+}. Yuan et al. [57] provided another strategy to increase the encapsulation efficiency of siRNA. Six positively charged arginine mutation were introduced into the inner cavity of ferritin, endowing it with strong binding ability to negatively charged nucleic acid yet it did not impede the stabilization of ferritin nanocage. Hestericová et al. [62] reported that horse spleen ferritin can encapsulate an 66 KDa artificial transfer hydrogenase (ATHase) through pH-dependent disassembly/reassembly method. The encapsulation not only preserved but also further increased ATHase enzyme activity, because ferritin provided additional coordination domain for cyclic imine reduction.

However, the pH-induced ferritin disassembly/reassembly process was not fully reversible that the ferritin cage structure was not completely recovered but showed some defects such as two holes [69]. The irreversible damages to ferritins would cause leakage of loaded drugs and limit the *in vivo* stability, affecting the delivery efficiency [47]. A new strategy of protein mutation has been used to improve pH-induced disassembly/reassembly method for working at milder condition. Choi et al. [71] substituted ferritin C-terminal 22 residues (161–182) by GALA peptides with ability of reversibly transforming the structure from a random coil to an α-helix when the pH is reduced from 7.0 to 5.0. The obtained GALA-ferritin could dissociate at pH 6.0 and reassemble by neutralization to biological pH, yet it wasn't tested for drug loading. Similarly but differently, Chen et al. [45] deleted human ferritin C-terminal 23 residues (including DE turn and E helix involved in fourfold axis interaction) and engineered a deletion mutant rHuHF-ΔDE, which could disassemble/reassemble at the pH from 4.0 to 7.5 and encapsulate 14 curcumin molecules. Yet the mutation left six holes on ferritin surface with 1.8 nm diameter which might result in leakage of molecules encapsulated inside. Wang et al. [72] deleted two residues (D45–D46) or three residues (R44–D45–D46) of the AB loop to obtain two mutants, rHuHFΔ2 and rHuHFΔ3, respectively. rHuHFΔ2 disassembled at pH 3.0 and rHuHFΔ3T disassembled at pH 4.0, while both mutants could reassemble at biological pH to load Dox or curcumin. Gu et al. [73] constructed a ferritin mutant His_6-HuHF by substituting six residues (^{159}GAPESG^{164}) of DE loop with six ionizable histidine, which could be protonated under neutral conditions, affecting 24-mer assembly through steric effects. His_6-HuHF formed natural form of tetramers at pH 7.5 and could be self-assembled into 24-meric nanocage at pH 7.5 with the presence of transition metal ions Ni^{+} or pH 10.0, respectively. Both Dox and curcumin could be efficiently encapsulated into His_6-HuHF cage during this process.

Besides pH-induce loading method, the urea-induced disassembly/reassembly (Figure 2A) was also reported by Liang et al. [47]. Briefly, ferritin disassembled in 8 M urea and then reassembled into nanocage during gradient dialysis to remove urea. Dox was added during the dialysis process to be encapsulated in ferritin (33 Dox molecules per nanocage), while the CD spectra showed no significant difference of ferritin nanocage structure between before and after Dox loading, suggesting that the process was not harmful [47]. Jiang et al. [48] engineered a targeting peptide-modified *Pyrococcus furiosus* ferritin, which encapsulated up to 400 Dox molecules in a single ferritin using urea-induced disassembly/reassembly method, remarkably increasing the loading efficiency. Comparing the pH- and urea-induced

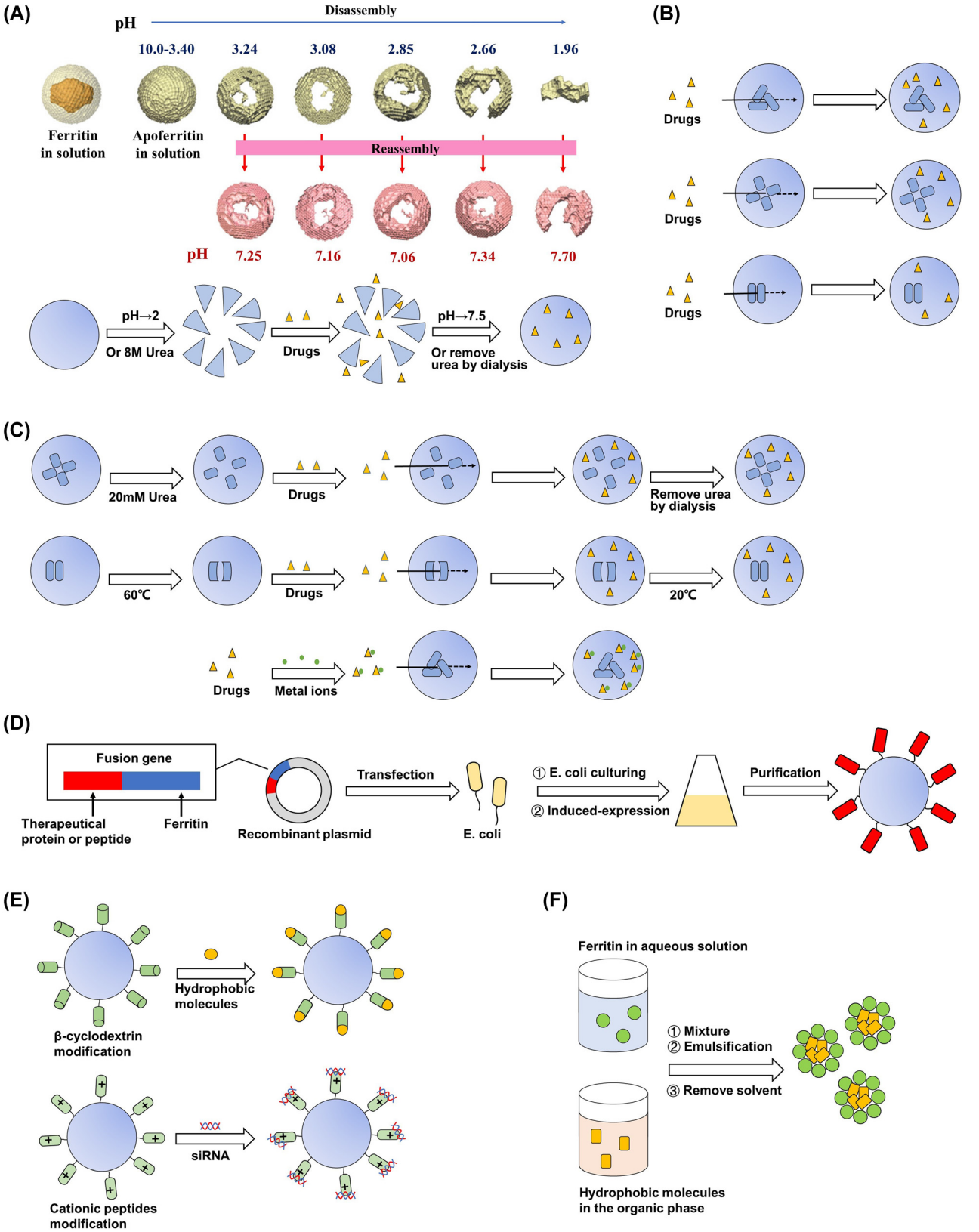

Figure 2: Drug loading methods of ferritin-based nanomedicine. (A) The pH-dependent disassembly/reassembly process of ferritin and the illustration of drug loading by pH/urea-induced disassembly/reassembly (image source: Kim et al. [69] with permission). (B) Channel-dependent drug loading through the three-fold, four-fold or two-fold axis. (C) Enhanced channel-dependent drug loading, assisted by 20 mM urea, temperature of 60 °C or metal ions coordination. (D) Loading protein/peptide by genetic fusion. (E) Loading drugs by binding the outer shell. (F) Loading drugs by emulsification. *E. coli*, *Escherichia coli*.

disassembly/reassembly drug loading method, urea was not as harsh as the extremely low pH, but caused enormous waste of drugs during dialysis process to remove urea.

In recent years, a new salt-induced ferritin disassembly/reassembly method has been developed. Ferritin from the archaea *Thermotoga maritima* (TmFtn) displays distinct assembly properties from mammalian ferritin. TmFtn recombinantly expressed in *E. coli* could reversibly dissociate from 24-mer to dimers at low ionic strengths, and reassemble to 24-mer at the presence of 50 mM $MgCl_2$ [74]. Chakraborti et al. [60] employed TmFtn to encapsulate positively supercharged green fluorescent protein (+36GFP) that four +36GFP molecules were loaded into one TmFtn nanocage. Bacterioferritin (Bfr), which contained a heme prosthetic group at the interface of each subunit dimer, showing similar salt-induced disassembly/reassembly behavior [75, 61]. Bradley et al. [61] also utilized this loading strategy to encapsulated ferredoxin into Bfr nanocage. Although salt-induced drug loading method was gentler, it has not been widely used since these archaeobacterial ferritins may cause stronger immune reaction compared with human ferritin.

Channel-dependent drug loading

Composed of 24 subunits, ferritin nanocage has eight hydrophilic threefold channels and six hydrophobic fourfold channels, both ranging from 0.3 to 0.5 nm [76]. These channels could serve as loading path for small molecules to enter the cavity by diffusion (Figure 2B). In addition, twofold axis was also found to be a potential pathway for drug entry [49].

The threefold channels are the native pathways for Fe^{2+} to traverse into the cavity in biological condition [77]. Other metal ions can also traverse freely into the inner cavity through the threefold channels and accumulate in it since there are adequate metal binding sites in the inner surface [78]. Yang et al. [16] efficiently loaded cisplatin ($PtCl_2(NH_3)_2$) into the ferritin nanocage, that K_2PtCl_4 passed threefold channels and bound apoferritin inner surface by diffusion at pH 8.5. Then NH_4^+–NH_3 solution was added to react with $[PtCl_4]^{2-}$, generating $PtCl_2(NH_3)_2$ *in situ*. Drugs that contain metal atoms, such as radioyttrium (90Y) [33] and Zinc hexadecafluorophthalocyanine ($ZnF_{16}Pc$) [28], were also reported to be encapsulated in ferritin by simply diffusion. Moreover, some non-metal small molecules can also be loaded into the interior of ferritins by diffusion through other channels. The fourfold hydrophobic channels permit hydrophobic molecules smaller than 0.3 nm to pass into ferritin. Hydrophobic gefitinib was encapsulated in ferritin by incubation at 4 °C with a quite high encapsulation efficiency of 55%. Other hydrophobic drugs such as β-lapachone [34], vandetanib [30] and lenvatinib [30] were also reported to be loaded in ferritin by diffusion. In addition, another potential pathway at two-fold axis identified by Jiang et al. [49] was used for drug loading. By mutating several residues at the twofold axis to shut down or enlarge the presumed pathway, the encapsulation of DOX was inhibited or increased, respectively.

Since the size limitation of these channels and the hydrophilic channels' preference for metal ions, the types of drugs that can be loaded by passive diffusion are limited [79]. Several methods, either assisted by urea, metal ions or temperature, were developed to broaden the application and enhance the loading efficiency of channel-dependent loading (Figure 2C). Scientists have managed to enlarge the size of channels to permit molecules pass through them. Inspired by the high concentration of urea (8 M) used for protein denaturation, Yang et al. [53] applied low concentration urea (20 mmol/L) to uncoiling α-helix structure around the fourfold channels, which increased the channels width and improved encapsulation efficiency of epigallocatechin gallate, chlorogenic acid and anthocyanin. In follow-up studies, they also found that guanidine hydrochloride (2 mmol/L) had similar effects in epigallocatechin gallate encapsulation [54]. Metal-assisted passive loading method was also proven useful for non-metal molecules' encapsulation. Dox was pre-complexed with a transition metal Cu(II) to generate Cu(II)–Dox complex, which traversed ferritin threefold hydrophilic channels by passive diffusing and was encapsulated with a quite high loading rate of 73%, while pre-incubation of ferritin with Cu(II) alone significantly reduced Cu(II)–Dox loading rate to 8.28%, suggesting the encapsulating efficiency of Cu(II)–Dox was highly depend on metal-binding sites on the inner surface which could be competitively occupied by Cu(II) [50]. Two other molecules, navitoclax [39] and ATP [52], were also reported to be encapsulated by metal ion's assisting. In addition, the drug entry tunnel at the two-fold axis could be expanded when the incubation temperature was raised to 60 °C, which significantly increased Dox loading efficacy [49].

Since channel-mediated drug loading works under normal condition, the drugs avoid from being harmed by pH- or urea-induced ferritin disassembly/reassembly. Kubalova et al. [30] compared diffusion with pH-induced loading method in encapsulating tyrosine kinase inhibitors (TKIs) vandetanib and lenvatinib. Both methods showed similar loading efficiency, however TKIs loaded by diffusion exerted markedly higher anticancer activity than loaded by

pH-induced disassembly/reassembly, indicating that the harsh acidic condition during pH-induced encapsulation was detrimental to the TKIs' structure and activity.

Other loading methods

Therapeutical or diagnostic proteins and peptides can be genetically fused to ferritin subunits as fusion proteins (Figure 2D) that antibodies [64, 66], antigens [80, 81], receptor agonist peptides [65] and fluorescent peptides [63] could be displayed on the ferritin nanocage. For example, Lee et al. [64] fused ferritin C-terminus with a SIRPα variant, a ligand of CD47, which was displayed on ferritin surface and bound tumor CD47 to block tumor from immune escape. Li et al. [63] presented GFP on ferritin outer surface by fusing GFP to the N-terminal of ferritin, obtaining a ferritin-based nanoprobe for fluorescent imaging.

Besides being encapsulated in the inner cavity, drugs can bind to ferritin shell for delivery (Figure 2E). Kwon et al. [36] precisely conjugated 24 β-Cyclodextrins (β-CD) to the specific sites on the outer surface of one modified ferritin through Michael-type addition followed by click reaction. Hydrophobic molecules such as FITC-AD could be loaded in the cavity of β-CD by hydrophobic interaction. Lee et al. [58] tandemly fused ferritin C-terminus with two peptides, enzymatically cleaved peptide (ECP) and cationic protamine, which provided enzymatic responsive cleavage and positive charges for siRNA binding, respectively. The siRNA-absorbed ferritin was endocytosed by cell and then released free siRNA under the reductive condition of cytosol through the ECP cleavage by lysosomal cathepsin B.

The emulsion-based method has also been used to prepare ferritin-based nanomedicine (Figure 2F). Fujii et al. [82] found that ferritin, as an emulsifier, could be applied to form stable 'Pickering-type' emulsions, with five kinds of oil phase, in the absence of any surfactant molecules. Li et al. [37] constructed a carrier-free nanodrug called nanoparticle ferritin-bound erastin and rapamycin (NFER) by the emulsification technique that erastin and rapamycin dissolved in dichloromethane and ferritin dissolved in PBS were mixed together and homogenized by ultrasonic to form a homogeneous emulsion, yielding nanoparticles of about 78 nm size after solvent removal by dialysis and freeze-drying. The drug-loading ratios (weight of the drug/weight of the nanoparticle) for erastin and rapamycin was 0.35 and 5.6%, respectively. Following similar procedure, Wu et al. [38] used ferritin to co-load lapatinib and pseudolaric acid B by emulsification.

Functional modifications of ferritin-based nanoparticle drug delivery system

As a protein-based nano-therapeutic platform, the surface of ferritin nanocages have multiple sites to be easily functionalized by chemical or biological modifications (Table 2). These modifications could provide ferritin with additional one or multiple capabilities, including improving the pharmacokinetics, enhancing the targeting ability, acquiring responsive release of loaded cargos, or displaying therapeutic functional modules.

Site nonspecific modification—chemical crosslinking

Covalent conjugation of functional modules through chemical reactions is one of the classic methods for functional modification of ferritin nanocage. Amino groups of lysine residues and sulfhydryl groups of cysteine residues groups on ferritin surface are often used as sites for reaction with functionalized fragments or chemical cross-linkers. Since there are lots of lysine and cysteine on ferritin surface, this kind of modification is often site nonspecific.

EDC/NHS catalyzed esterification of amino groups is one of the most widely used chemical modification methods to conjugate motifs with carboxyl to ferritin nanocage. Luo et al. [83] used EDC/NHS to react with hyaluronan (HA), a ligand of CD44, to generate active ester which subsequently covalently cross-linked with amino groups on ferritin surface, producing an HA-ferritin for lung cancer-targeted drug delivery. Other peptides, such as KGDS peptide, could also be conjugated to ferritin by the same method, preparing an activated platelets-targeted nanoparticle for the MRI diagnosis of thrombus [84].

Glutaraldehyde linker has been used to modify ferritin with motifs containing amino groups. For example, the reactive amino groups of ferritin and bovine serum albumin (BSA) both reacted with glutaraldehyde aldehyde groups to get ferritin and BSA covalent crosslinked by an acid-cleavable Schiff base bonds [85]. The product ferritin@BSA complexes showed significantly improved half-life, and could acid-responsively dissociate in lysosome.

Maleimides (MAL) can react with sulfhydryl groups to form thioether bonds, which has been broadly used to modify proteins. Vannucci et al. [86] incubated ferritin with methoxypolyethylene glycol maleimide to prepare PEGylated ferritin nanocage, which showed more persistent

Table 2: Methods and applications of ferritin's functional modification.

Functional modules	Modification site of ferritin	Method	Benefits
Hyaluronan (HA) [83]	Amino groups	Chemical crosslinking (catalyzed by EDC/NHS)	Binding to CD44 for targeting lung tumor
KGDS peptide [84]	Amino groups	Chemical crosslinking (catalyzed by EDC/NHS)	Binding to activated platelets integrin GPIIb–IIIa receptor for thrombus targeting
BSA [85]	Amino groups	Chemical crosslinking (catalyzed by glutaraldehyde linker)	Extended half-life; acid-responsively dissociation in lysosome
PEG [86]	Sulfhydryl groups	Chemical crosslinking (reacted with methoxypolyethylene glycol maleimide)	Extended half-life
Polyvalent mannoses or galactoses [87]	Sulfhydryl groups	Chemical crosslinking (crosslinked by thiol-maleimide)	Enhanced tumor targeting
Monoclonal antibody Ep1 [88]	Sulfhydryl groups	Chemically crosslinking (crosslinked by NHS-PEG-MAL)	Melanoma targeting
Fab fragment of anti-FAP antibody [39]	Sulfhydryl groups	Chemically crosslinking (crosslinked by NHS-PEG-MAL)	Cancer-associated fibroblasts (CAFs) targeting
α-Melanocyte-stimulating hormone peptide [86]	N-terminus	Genetic fusion	Melanoma targeting.
RGD4C [28, 89] or RGDK [90] peptide	N- or C-terminus	Genetic fusion	Binding to integrin GPIIb–IIIa receptor on tumor vasculature for tumor targeting
Clot targeting peptide CNAGESSKNC [91]	N-terminus	Genetic fusion	Binding of fibrin-fibronectin complexes for thrombus targeting
PAS polypeptide [90, 92]	N- or C-terminus	Genetic fusion	Extended half-life
Albumin binding domain [93]	N-terminus	Genetic fusion	Extended half-life
Cationic peptide composed of eight lysine residues [94]	N-terminus	Genetic fusion	Lysosomal escape
Ovalbumin-derived peptides [80]	C-terminus or Asp146 residue	Genetic fusion	Antigen displayed for DCs activation
The ectodomain of influenza hemagglutinin (HA) [81]	Asp5 residue	Genetic fusion	Ferritin-based influenza vaccine
preS1 domain of the large HBV surface protein [95]	SpyTag fused to ferritin N-terminus, preS1 fused with catcher	SpyTag/Catcher	Ferritin-based HBV vaccine
SARS-CoV-2 spike protein [96]	Spike protein fused to cather, SpyTag fused to ferritin N-terminus	SpyTag/Catcher	Ferritin-based SARS-CoV-2 vaccine
SARS-CoV-2 spike protein RBD [97–99]	RBD fused with SpyTag, catcher fused to ferritin N-terminus	SpyTag/Catcher	Ferritin-based SARS-CoV-2 vaccine
Scarf1 [94]	SpyTag fused to ferritin N-terminus, Scarf1 fused with catcher	SpyTag/Catcher	Binding to C1q on dying cells surface for targeting acute myocardial infarction injury
DBCO-functionalized anti-ICAM-1 antibody [100]	4-AzF inserted in ferritin sequence	Genetic code expansion-based click reaction	Pulmonary vascular targeting
CY3 or/and CY5 [101]	4-AzF inserted in ferritin sequence	Genetic code expansion-based click reaction	Fluorescent imaging

EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; NHS, N-hydroxysuccinimide; BSA, bovine serum albumin; PEG, polyethylene glycol; FAP, fibroblast activation protein; CAFs, cancer-associated fibroblasts; MAL, maleimide; Fab, fragment antigen-binding region of antibody; RGD4C, Cys–Asp–Cys–Arg–Gly–Asp–Cys–Phe–Cys peptide; RGDK, Arg–Gly–Asp–Lys peptide; PAS peptides, polypeptides rich in proline (P), serine (S), and alanine (A) residues; DCs, dendritic cells; HBV, *hepatitis B virus*; SARS-CoV-2, *severe acute respiratory syndrome coronavirus 2*; RBD, receptor-binding domain; Scarf1, scavenger receptor class F member 1; DBCO, azadibenzocyclooctyne-amine; CY5, sulfo-cyanine5; CY3, sulfo-cyanine; ICAM-1, intercellular adhesion molecule-1; 4-AzF, 4-p-azido-L-phenylalanine.

circulation. Kang et al. [87] displayed polyvalent mannoses or galactoses on ferritin using thiol-maleimide linkers, enabling the reconstructed nanocage to recognize and bind to DC-SIGN or ASGP-R lectins on the surface of DCEK or HepG2 cells. NHS-PEG-MAL crosslinker was used to conjugate the ferritin with monoclonal antibody to endow the

construct targeting ability to specific tissues or cells, while the PEG groups improved the conjugates' water solubility and half-life in animals [39, 88].

Since multiple sites-based chemical crosslinking usually results in poor formulation uniformity, a compromise to improve specificity is engineering mutants containing only one reactive group. Since each ferritin subunit outer surface contains three cysteines, Kwon et al. [36] mutated two cysteines to serine and left only one cysteine. The cysteine was then chemically conjugated with β-CD through thiol-maleimide Michael-type addition followed by click reaction, resulting in another particular site-specific modification.

Site-specific modification-genetic fusion

In order to obtain homogeneous functionalized nanoparticles and make the conformation of functional modules more controllable, site-specific modification on ferritin is needed. Engineered ferritin for nano-therapeutics is usually produced using *E. coli*. The functional peptides or proteins for ferritin modification could be linked to ferritin subunits as fusion proteins, which is easy to implement by molecular cloning. This modification is either N- or C-terminal fusion, or both, which is site-specific and the product is highly homogeneous.

Even with high intrinsic targeting ability for TfR1-overexpressing cancer, additional targeting ability of ferritin nanocages for some kinds of cancer could be achieved by genetic fusion of targeting peptide or protein. Uchida et al. [89] fused RGD4C peptide, ligand of $\alpha_v\beta_3$ integrins upregulated on tumor vasculature, to the N-terminal of human H-ferritin subunit, incorporating it on the exterior surface of ferritin, which resulted in enhanced melanoma targeting ability. α-melanocyte-stimulating hormone peptide was also fused to the N-terminal of ferritin subunit and displayed at the outer surface, which significantly enhanced ferritin's ability to target melanoma [86]. Lee et al. [102] fused SIRPα to the C-terminal of ferritin obtaining additional $CD47^+$ tumor cells targeting ability.

The half-life of ferritin nanocage could be extend through genetic fusion. PAS polypeptide, rich in proline (P), alanine (A) and serine (S) residues, was designed to imitate the mechanism of PEG to extend the half-life [103]. PASylated-ferritin generated by fusion protein significantly extended the half-life of ferritin from 1 to 15.6 h and showed unique homogeneity, and could be degraded by enzymes *in vivo* thereby preventing severe toxicity and immunogenicity [92]. Moreover, compared with PEGylation, PASylation avoided the complicated process of *in vitro* synthesis and purification. Wang et al. [93] fused ferritin subunit N-terminus with albumin binding domain (ABD), which could hijack albumin to achieve longer circulation of ABD-ferritin, while the half-life of Dox encapsulated in ABD-ferritin was extended more than 10 times compared with free Dox and Dox loaded in native ferritin.

Genetic fusion was also widely used for ferritin-based vaccine and immunotherapy. More importantly, in order to elicit and maximize the host's immune response, the fusion site for antigenic epitope is critical and needs to be rationally designed, which may influence the conformation of modified motifs. The genetic fusion of antigens at proper site on ferritin surface may produce native virus-like vaccine which induced much stronger immune protection. To mimic the natural trimer form of influenza virus hemagglutinin (HA), Kanekiyo et al. [81] constructed a ferritin-based influenza vaccine by fusing the ectodomain of HA to *Helicobacter pylori* ferritin at Asp5, with a short Ser–Gly–Gly linker between HA and ferritin. They found that the distance among Asp5 of the three ferritin subunits at the threefold axis was quite close to the distance among the native HA subunits trimer in influenza virus. TEM imaging verified the trimer HA spike structure on surface of HA-ferritin which showed stronger immunogenicity than HA Spike protein in monomer and even trivalent inactivated influenza vaccine in animals.

Site-specific modification-bioconjugation strategies

Several bioconjugation strategies have been applied to protein modification, such as Tag/Catcher technique and genetic code expansion. Compared to site non-specific chemical modification, these bioconjugation strategies could introduce genetically encoding linkers at specific sites and conjugate with functional modules efficiently.

SpyTag/Catcher technique is a typical Tag/Catcher strategy for irreversible conjugation between proteins in a click-link manner [104]. A domain of FbaB protein is split into two parts including a SpyTag (13 residues) and SpyCatcher (135 residues) which would react with each other spontaneously to form a structural stable SpyTag/Catcher complex. Wang et al. [105] constructed a SpyTag/Catcher- and ferritin-based vaccine platform to display single or multiple antigens. SpyCatcher was genetically fused to the N-terminus of ferritin, and SpyTag was genetically fused to the C-terminus of HPV16 oncogene E7 peptide antigen or MC38 tumor derived mutant neoantigens. When the two recombinant fusion proteins were mixed, SpyTag/Catcher pair mediated conjugation will produce ferritin nanoparticles displaying antigens.

Similarly, vaccines against hepatitis B virus [95] and SARS-CoV-2 [96–99] have also been developed. Besides, targeting peptides have been modified on the ferritin nanocage through SpyTag/Catcher, for example, Cheng et al. [94] displayed a C1q ligand, Scarf1, on ferritin nanocage to endow it targeting ability for dying cells at acute myocardial infarction site.

Genetic code expansion is a rapidly developing technique to genetically incorporate unnatural amino acids (uAAs) into proteins, which have been widely used in construction of antibody-drug conjugates (ADCs) [106]. Functionalized side chains, including reactive groups like azides, alkynes, alkenes and tetrazines, could be site-specifically conjugated on uAAs, which was introduced genetically by an orthogonal amber suppressor tRNA/aminoacyl-tRNA synthetase pair [107, 108]. Then, functional modules could be site-specifically linked to reactive groups via the click reaction. Khoshnejad et al. [100] incorporated an amber stop codon (TAG) at fifth residue position of ferritin yielding a 4-azidophenylalanine (4-AzF). Pulmonary vascular targeting antibody was conjugated with Dibenzylcyclooctyne (DBCO) and then reacted with 4-AzF via click reaction between azide and alkyne group, generating a pulmonary vascular targeting-ferritin nanocages. Using similar procedure, Wang et al. [101] Linked DBCO-conjugated multiple fluorophores or Dox to the ferritin shell.

Application of ferritin-based nanomedicine

Tumor visualization

Benefited from its intrinsic affinity for TfR1 or additional acquired targeting ability by surficial modification, ferritin can be targeted accumulated at certain disease sites. Once carrying agents that can produce color, fluorescence or radiation, ferritin may serve as a promising nanoprobe candidate for disease visualization (Figure 3).

Ferritin can catalyze color reactions for disease visualizing. Fan et al. [113] constructed magnetoferritin nanoparticles (M-HFn) by diffusion of Fe^{2+} into ferritin inner cavity, and Fe^{2+} was then oxidated by H_2O_2, generating an iron oxide (Fe_3O_4) core in ferritin. The M-HFn could catalyze the oxidation of peroxidase substrates 3,3,5,5-tetramethylbenzidine (TMB) or di-azo-aminobenzene (DAB) to produce a color reaction, for quickly distinguishing and visualizing tumor cells from normal tissues in tissue section analysis. M-HFn was able to distinguish nine types of cancerous cells from normal cells with a sensitivity of 98% and specificity of 95%, which made it an efficient and low cost nanoprobe candidate for tumor diagnosis. They further reported a hepatocellular carcinoma (HCC) cell-specific peptide SP94 modified ferritin with cobalt compound (Co_3O_4) loaded (Figure 3A) [109]. Co_3O_4-ferritin possessed higher peroxidase-like activity than formal Fe_3O_4 core to catalyze DAB oxidation at tumor site, showing strong blue color to visualize clinical HCC tumor tissues, which exhibited comparable sensitivity and specificity to the clinically used HCC-specific marker alpha-fetoprotein (AFP).

Fluorescent peptide or protein could also be modified on ferritin to achieve disease imaging. Fibroblast activation protein-α (FAP-α), which shows proteolytic activity, is over expressed by cancer-associated fibroblasts (CAFs) and pericytes in epithelial tumors. Nie group [110] constructed a hybrid ferritin-based FAP-α-responsive fluorescence probe for CAFs positive carcinoma by genetically fusing ferritin with a fluorescence (FAM)-tagged peptide that can be specifically cleaved by FAP-α, which was hybridized with FAM's black hole quencher (BHQ) linked ferritin (Figure 3B). This probe was silent and showed little fluorescence until activated by FAP-α cleavage.

Ferritin can realize magnetic resonance imaging (MRI) by encapsulating contrast agents in the cavity. Aime's team conjugated the Gd-HPDO3A-loaded ferritin with neural cell adhesion molecule (NCAM) that could target tumor vessels, constructing a nanoprobe for tumor vessel imaging by MRI [114]. Since melanogenesis is generally up-regulated in malignant melanoma, Szabo et al. [111] loaded apoferritin with Mn(III), which could be quickly reduced to Mn(II)-apoferritin by the oxidation of L-DOPA to melanin, working as an MRI sensor of melanin formation to detect melanoma cells (Figure 3C).

Moreover, ferritin could load multiple agents in cavity and modify multiple modules on outer shell, which combines many imaging techniques to become a multimodal imaging probe, or co-delivery of diagnostic and therapeutic modules to become a multifunctional and integrated nanomedicine. Zhao et al. [113] conjugated ^{125}I radionuclide to the outer shell of the M-HFn mentioned above to combine single photon emission tomography (SPECT) and MRI. This study broke through apparent sensitivity limitations between MRI and SPECT, enabling single-dose MRI/nuclear tumor-targeted imaging. Chen group modified ferritin surface with RGD4C peptide and Cy5, and loaded $^{64}CuCl_2$ inside [115]. These modules gave ferritin three additional functions, integrin $\alpha_v\beta_3$ targeting, near-infrared fluorescence (NIRF) imaging and positron emission tomography (PET), respectively.

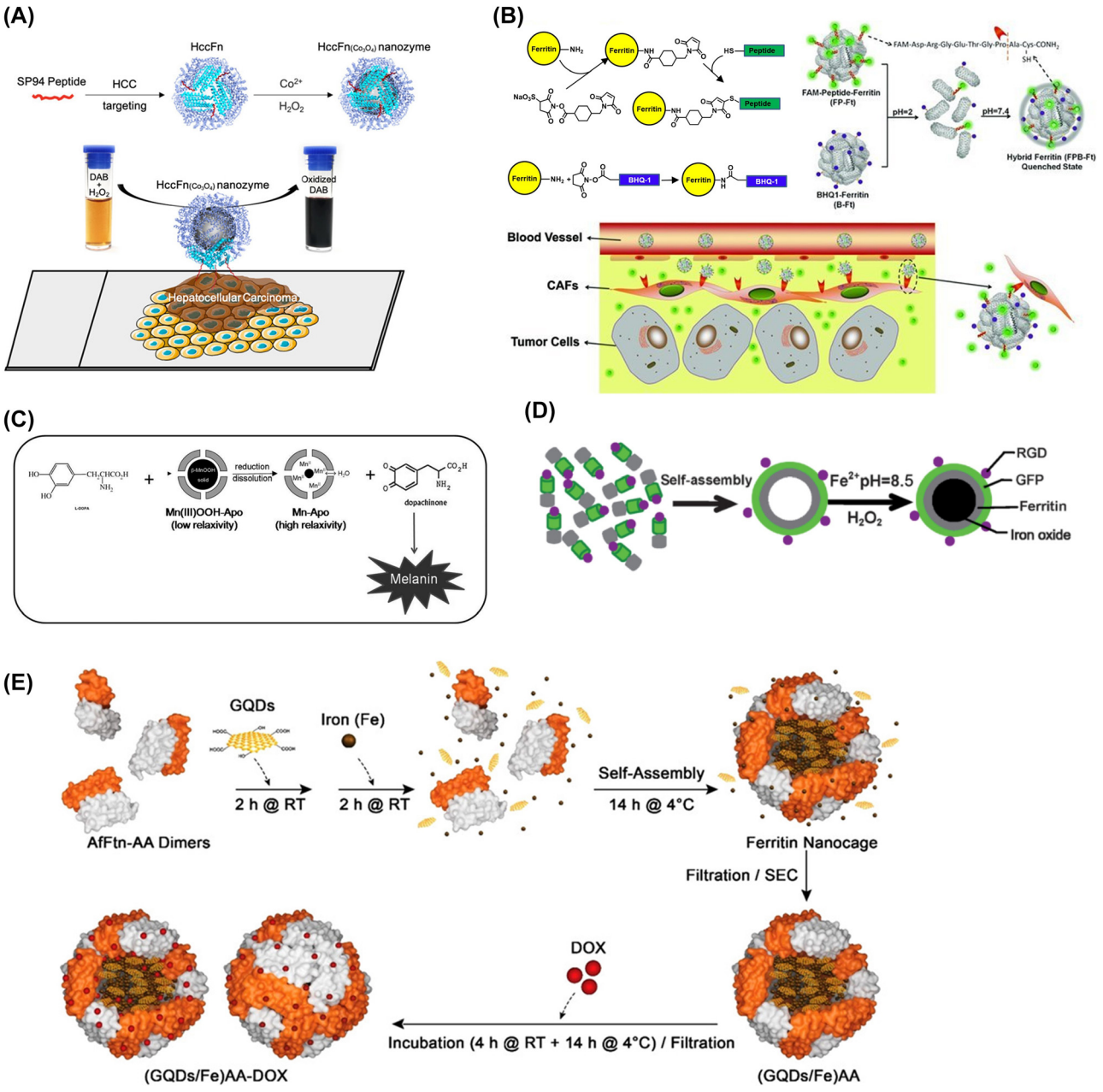

Figure 3: Ferritin-based nanoprobe for tumor visualization. (A) Tumor diagnosis by catalyzing color reaction (image source: Jiang et al. [109] with permission). (B) Tumor imaging by modifying fluorescent peptide or protein (image source: Ji et al. [110] with permission). (C) Tumor imaging by encapsulating contrast agents for magnetic resonance imaging (image source: Szabo et al. [111] with permission). (D) Multimodal imaging probe by combining different imaging agents (image source: Li et al. [63] with permission). (E) Combination of tumor visualization and treatment (image source: Nasrollahi et al. [112] with permission). RT, room temperature; HCC, hepatocellular carcinoma; HccFn, HCC-targeted ferritin; DAB, diaminobenzidine; CAFs, cancer-associated fibroblasts; FAM, carboxyfluorescein; BHQ, black hole quencher; Apo, apoferritin; RGD, Arg-Gly-Asp peptide; GFP, green fluorescent protein; GQDs, graphene quantum dots; DOX, doxorubicin; AA, an engineered ferritin nanocage derived from the archaeon *Archaeoglobus fulgidus*.

Inner Fe_3O_4-loaded and outer GFP-modified ferritin, could be used for fluorescence and MR imaging of tumor cells simultaneously (Figure 3D) [63]. Similarly, inner Dox-encapsulated and outer radiosensitizer bismuth sulfide nanocrystals-modified ferritin inhibited the tumor growth and imaged tumor in mice concurrently [116]. Dox and graphene quantum dots (GQDs), a fluorescent bioimaging agent, were co-loaded in ferritin, showed significant inhibitory effect on MDA-MB-231 breast cancer cells and a pH-responsive fluorescence (Figure 3E) [112].

Tumor chemotherapy

Small molecule anti-tumor chemotherapeutic drugs, including the "old" cytotoxic drugs [117] and targeted therapeutic drugs [118], have played a central role in tumor treatment for decades. However, the application of some tumor chemotherapy drugs is limited by their water solubility, pharmacokinetic properties, non-tumor tissue distribution, and toxic side effects. Ferritin, with advantages including excellent histocompatibility, low immunogenicity, size uniformity, intrinsic tumor targeting, rich functional modification sites and BBB-crossing ability, has been widely studied for delivery of small molecule anti-tumor drugs (Table 3). It can improve the solubility and druggability of insoluble drugs, such as paclitaxel [19, 119, 120], cisplatin [16, 88, 17], curcumin [22, 23], gefitinib [35], vandetanib [30] and lenvatinib [30].

Ferritin has been extensively researched for tumor-targeted delivery of small molecule drugs. Ferritin has high intrinsic affinity for TfR1 over expressed tumors, including liver, lung and breast cancer, etc. [113]. Liang et al. [47] encapsulated Dox in ferritin, which exhibited more than tenfold higher tumoral Dox concentration than free Dox treatment in mice, and even showed longer survival and lower toxicity than clinically approved liposomal Dox (DOXIL)-treated mice. Pandolfi et al. [121] loaded curcumin in human H-ferritin nanocage, which showed improved targeting ability to triple negative breast cancer cells and better therapeutic efficacy. Besides solid tumor, non-solid tumor also got targeted delivery using native ferritin as drug carrier. Wang et al. [123] discovered that different types of Leukemia cells highly expressed TfR1 so that they loaded trivalent arsenic (AsIII) in ferritin, which efficiently delivered AsIII into leukemia cells, enhanced the leukemia cells' uptake of AsIII, extended its half-life and significantly reduced the off-target cytotoxicity of AsIII. In addition to its intrinsic TfR1-binding mediated targeting, ferritin can be modified with other targeting motifs to gain additional targeting ability for some tumor cells or other diseases without TfR1 overexpressing. Targeting proteins or peptides such as RGD peptide [28], melanoma-targeting peptide [86], and pulmonary vascular targeting antibody [100] could be modified on ferritin surface to improve the targeted delivery of chemotherapeutic drugs.

Ferritin could display enzymes to dissolve dense extracellular matrix (ECM) for enhancing penetration of encapsulated chemotherapy drugs. Tumor microenvironment (TME) consists of ECM and various stromal cells [130]. Tumor ECM is mainly composed of collagen, fibrin, laminin, proteoglycans, and glycoproteins, etc. [131], limiting the drug penetration to deeper tumor sites [132, 133]. Yao et al. [125] cross-linked collagenase (Col) with glycerol dimethacrylate to form collagenase nano-capsules (Col-nc) which was then modified on Dox-loaded ferritin generating Col-nc/HFn(Dox) complex. Col-nc/HFn (Dox) released Col through the acid-responsive glycerol dimethacrylate (degradable below pH 6.5) in TME to dissolve the ECM collagen, promoting the penetration of Dox deep into the tumor interior. Seo et al. [126] also fused ferritin with fibrin-targeting peptide and fibrin-dissolving tissue plasminogen activator. This ferritin encapsulated Dox, and could dissolve the fibrin in TME and enhanced the penetration of Dox-loaded ferritin, thereby enhancing the anti-tumor efficacy significantly.

Ferritin also shows bright prospect for delivering tumor chemotherapeutic drugs to brain. BBB is one of the most restrictive barriers that over 98% of small molecules and almost all large molecules fail to pass the BBB [134]. Due to the high expression of TfR1 in the BBB endothelial cells [135], ferritin could encapsulate drugs and cross BBB through TfR1-mediated transcytosis, thus delivering drugs to the central nervous system. Chen et al. [127] found that Dox delivered by H-ferritin could accumulate specifically in brain tumor tissues, showing significantly longer survival period compared to free Dox-treated mice. More importantly, Fan et al. [128] found that TfR1 was high expressed in glioma cells. After traversing BBB, Dox-loaded ferritin accumulated specifically in glioma tumors, with little distribution in the healthy brain tissue, showing possibilities of ferritin as an ideal nanocarrier for glioma therapy. Besides, ferritin was also proven effective to encapsulate other drugs including paclitaxel [19, 20, 124], camptothecin [32], epirubicin [32] and vincristine sulfate [129] to treat brain glioma.

Tumor immunotherapy

As a drug carrier and displaying platform for functional module, combining the inherent tumor-targeting ability and easy surface modification, ferritin has been demonstrated great potential to be a promising tumor immunotherapy platform (Figure 4). Ferritin-based drug delivery could enhance tumor immunotherapy in many manners, such as surficial displaying proteins or peptides to reduce tumor immune escape, delivering tumor antigens to dendritic cells (DCs) for increasing antigen presentation, and carrying drugs to cross BBB for brain tumor immunotherapy.

Proteins or peptides blocking tumor immune escape can be displayed on the surface of ferritin to achieve stronger anti-tumor immunotherapy. CD47 is a "don't eat me" signal molecule expressed on cell surface [139, 140]. Macrophages

Table 3: Ferritin-based tumor chemotherapy.

Anti-cancer drugs	Ferritin type	Cancer types	Benefits
Doxorubicin	Human H-ferritin	Colorectal cancer, breast cancer and melanoma [47]	Tumor targeted delivery
Curcumin	Human H-ferritin	Triple negative breast cancer [121]	Tumor targeted delivery
Curcumin	Hybrid horse spleen ferritin (85% L chains and 15% H chains)	Breast cancer [122]	Tumor targeted delivery
Epirubicin	Mucin 1 aptamer-modified horse spleen apoferritin	Colorectal cancer [24]	Tumor targeted delivery;
Doxorubicin	Sirpα variant-displayed human H-ferritin	Colorectal cancer and melanoma [64]	Tumor targeted delivery; improve tumor immunotherapy
As_2O_3	Human H-ferritin	Leukemia [123]	Enhanced leukemia cells targeted delivery; extended As_2O_3 half-life
Paclitaxel	Human H-ferritin	Breast cancer [19]	Improved solubility; tumor targeted delivery
Paclitaxel	Human H-ferritin modified with tumor-penetrating peptide	Lung cancer [124]	Tumor targeted delivery; better penetrability; improved solubility
Cisplatin	Monoclonal antibody conjugated human H-ferritin	Melanoma [88]	Improved solubility; enhanced melanoma targeting
Gefitinib	Human H-ferritin	SKBR3 breast cancer cells [35]	Tumor targeted delivery; improved solubility
Doxorubicin	Human H-ferritin conjugated with collagenase	Breast cancer [125]	Enhanced tumor penetration; tumor targeted delivery;
Doxorubicin	Human H-ferritin modified with fibrin clot-targeting peptides and microplasmin	Melanoma [126]	Dissolve clots in the tumor microenvironment and improve drug distribution
Doxorubicin	Horse spleen apoferritin	Glioma [127]	Crossing BBB
Doxorubicin	Human H-ferritin	Glioma [128]	Crossing BBB
Paclitaxel	Human H-ferritin	Glioma [20]	Crossing BBB
Vincristine sulfate	Horse spleen apoferritin modified with GKRK peptide	Glioma [129]	Crossing BBB and enhanced glioma targeting
Doxorubicin	Human H-ferritin modified with PAS peptides	PaCa44 human pancreatic tumor cells	Extended half-life [92]
Doxorubicin	Human H-ferritin modified with albumin binding domain	A549 human lung cancer cells	Extended half-life [93]
Camptothecin and epirubicin	Human H-ferritin fusing with hydrophobic peptides at C-terminus	Glioma, metastatic liver cancer, and chemo-resistant breast tumors [32]	Tumor targeted delivery; loading hydrophobic and hydrophilic drugs simultaneously for synergistical antitumor effect
Rapamycin and erastin	Horse spleen apoferritin	Breast cancer [37]	Tumor targeted delivery; combining autophagy and ferroptosis for anticancer therapy
Pseudolaric acid B and lapatinib	Human H-ferritin	Triple-negative breast cancer [38]	Tumor targeted delivery; synergistic effects of blocking EGFR and inhibiting cancer stem cell
Quercetin and curcumin	Horse spleen apoferritin	Breast cancer [22]	Tumor targeted delivery; improved solubility; synergistic effect on cancer cell apoptosis
Vandetanib and lenvatinib	Horse spleen apoferritin modified with folic acid	Breast cancer [30]	Tumor targeted delivery; improved solubility

H-ferritin, heavy chain ferritin; BBB, blood–brain barrier; Sirpα, signal regulatory protein α; EGFR, epidermal growth factor receptor; PAS peptides, polypeptides rich in proline (P), serine (S), and alanine (A) residues.

and dendritic cells recognizes tumor CD47 through its surficial signal regulatory protein-α (SIRPα) [141], thus inhibiting the phagocytosis of tumor cell [142]. Blocking CD47 and SIRPα interaction could abolish tumor immune escape [139]. Lee et al. [102] genetically fused H-ferritin C-terminus with SIRPα variants which forms a tetramer bundle on ferritin surface, demonstrating much stronger affinity to CD47 than SIRPα monomer. SIRPα-ferritin effectively competitively inhibited the binding of CD47 to SIRPα, promoted the phagocytosis of tumor cells and induced stronger immune response, and simultaneously released Dox loaded inside to kill tumor cells. Programmed cell death ligand 1 (PD-L1) is another immunosuppressive molecule highly expressed on tumor cells, which binds to programmed cell death protein 1 (PD-1) on the T cells, leading to inhibition of T cell response to tumor cells [143]. Block the binding of PD-1 to PD-L1 could

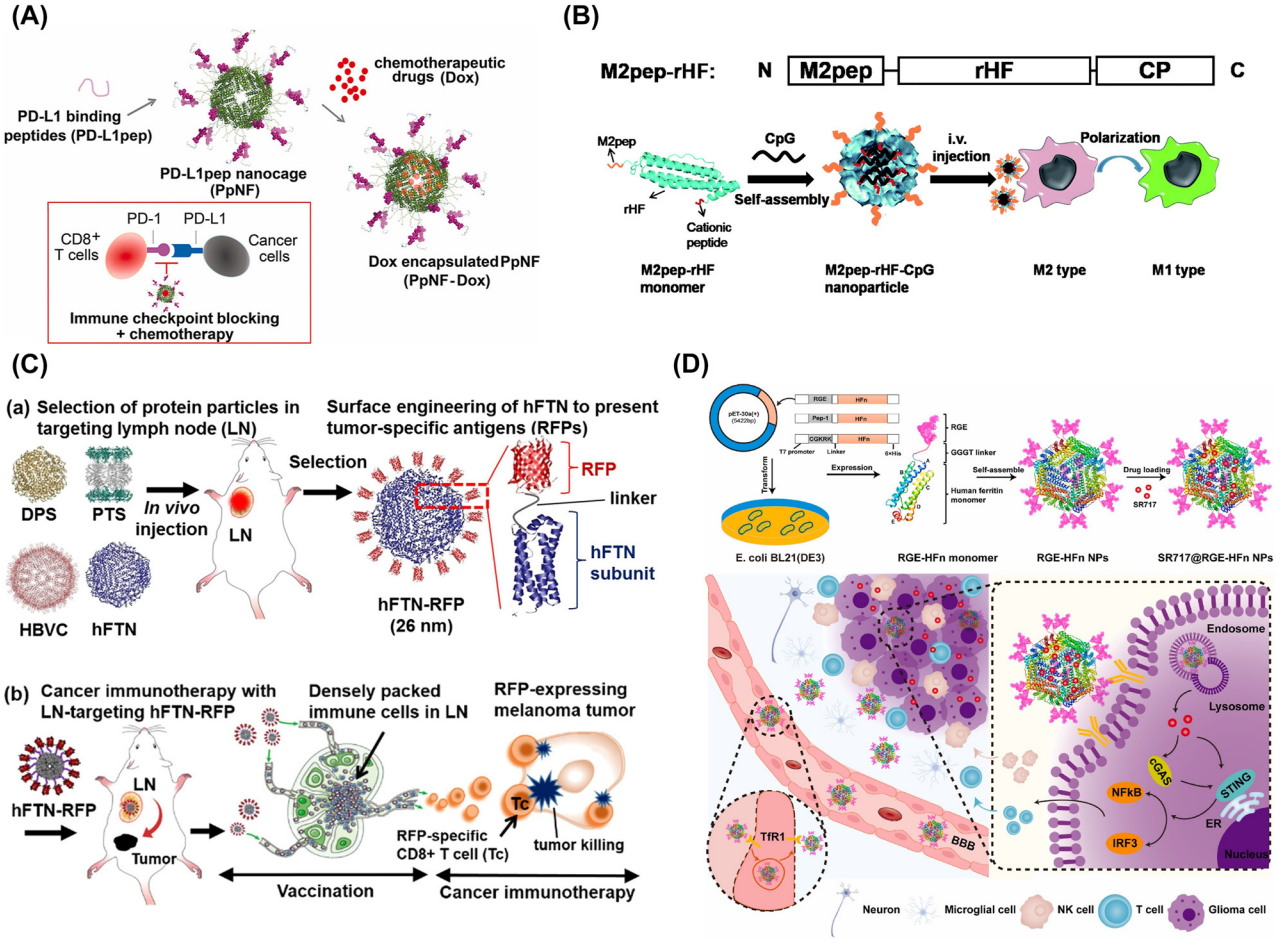

Figure 4: Ferritin-based nanomedicine for tumor immunotherapy. (A) Displaying proteins or peptides that block tumor immune escape (image source: Jeon et al. [66] with permission). (B) Reprograming tumor-associated macrophages (image source: Shan et al. [136] with permission). (C) Delivering tumor antigens to lymph nodes (image source: Lee et al. [137] with permission). (D) Delivering STING-activator to brain for glioma immunotherapy (image source: Wang et al. [138] with permission). M2pep, M2-macrophage targeting peptide; rHF, human ferritin heavy chain; DPS, *Escherichia coli* DNA binding protein; PTS, *Thermoplasma acidophilum* proteasome; HBVC, hepatitis B virus capsid; hFTN, human ferritin heavy chain; TfR1, transferrin receptor 1; STING, stimulator of interferon genes; IRF3, interferon regulatory factor 3; ER, endoplasmic reticulum; NF-κB, nuclear factor kappa-B; BBB, blood–brain barrier; HFn, heavy-chain ferritin.

enhance the T-cell mediated antitumor activity [144]. Jeon et al. [66] fused ferritin N-terminus with PD-L1 binding peptides which could bind PD-L1 on tumor cells and block the immunosuppressive effect of PD-1/PD-L1 pathway, while Dox encapsulated in ferritin was released to further kill tumors (Figure 4A).

Moreover, ferritin can also be used to targeted delivery drugs that could reprogram tumor-associated macrophages (TAMs). Shan et al. [136] encapsulated CpG oligodeoxynucleotides (CpG ODNs) on the surface of a murine M2 macrophage targeting peptide (M2pep)-modified ferritin, which could be targeted delivered to pro-tumor M2 type TAMs and repolarize M2-type TAMs to anti-tumor M1-type, inhibiting tumor growth in 4T1 tumor-bearing mice (Figure 4B). These immune checkpoint-blocking and TAM-repolarizing ferritin nanocages may have promise as an immunotherapeutic nanomedicine for tumors.

Ferritin has been applied to enhance tumor vaccines effect and show potential as an ideal carrier for lymph nodes (LNs) targeting delivery. Tumor antigen recognition and presentation by DCs is very often obstructed by the difficulty of tumor antigens reaching DCs [145]. Since LNs contain a high population of immature DCs which could be activated to mature DCs under the stimulation of tumor antigens [146, 147], nanoparticles targeting LNs are investigated to enhance tumor immunity [148, 149]. The transport of nanoparticles to the LNs is dependent on their size, and nanoparticles of 10–80 nm can migrate to the LNs through the lymphatic system [150, 151]. Lee et al. [137] found that human H-ferritin presenting a model antigen

red fluorescent protein (RFP) fulfilled the prerequisites for *in vivo* LN targeting (10–80 nm) (Figure 4C). RFP-ferritin rapidly accumulated in LNs for a long time after subcutaneous injection in mouse footpads and given sufficient exposure time of RFP antigen to LN immune cells, inducing strong RFP-specific cytotoxic $CD8^+$ T cell responses for RFP-expressing tumor cells. Native human ferritin nanocage shows a size of 12 nm, while surface modification increases ferritin size a little yet still small than 80 nm, matching very well with the size required for LN-targeted delivery.

Since most drugs and the carriers cannot cross BBB, ferritin-based drug delivery can overcome this problem and is beneficial for brain tumor immunotherapy, The cyclic guanosine monophosphate–adenosine monophosphate synthase (cGAS)-stimulator of interferon gene (STING) signaling pathway is important in tumor immunity, and activation of STING in immune cells can activate the immune cells themselves and the surrounding immune cells [152]. Wang et al. [138] fused a glioma-targeting peptide (RGERPPR) on ferritin to deliver an STING-activator SR717 for glioma immunotherapy (Figure 4D). After crossing BBB by TfR1-mediated transcytosis, SR717-loaded ferritin accumulated more in brain glioma facilitated by RGERPPR mediated deep penetration and glioma binding. SR717 was then released to activate the STING pathway, recruiting $CD8^+$ T cells, natural killer cells (NKs) and DCs to tumor sites, ultimately leading to significant inhibition of tumor growth.

Tumor photodynamic and photothermal therapy

Photodynamic therapy (PDT) is an emerging tumor treatment method [153]. When the tumor site is irradiated with light of a specific wavelength, photosensitizers are excited and transfers energy to O_2 to form cytotoxic reactive oxygen species (ROS) which can kill neighboring tumor cells, destroy tumor vasculature and activate the immune system [154, 155]. To avoid light damage to normal tissues, patients undergoing PDT usually need to avoid sunlight or even indoor light exposure for up to 1–2 months [156]. Tumor photothermal therapy (PTT) refers to the destruction of cancerous tissues/cells by absorbing the energy of light produced by photothermal agents (PTA), and converting it into heat energy under the irradiation of near-infrared light [157]. PTT for tumor is also limited by tumor nonspecific distribution of PTA, causing significant side effects [158]. Therefore, there is an urgent need of tumor-targeted delivery agents for tumor PDT and PTT. Ferritin can serve as a natural good carrier for photosensitizers and photothermal agents delivery due to its intrinsic targeting ability for tumors.

Ferritin-based PDT and PTT showed specifically distribution of photosensitizers and photothermal agents at tumors sites, thus leading to less side effects. Zhen et al. [28] encapsulated a potent photosensitizer, Zinc hexadecafluorophthalocyanine (ZnF16Pc), in RGD4C-modified ferritin to achieve tumor site-specific PDT. ZnF16Pc-loaded ferritin showed a high tumor accumulation rate (tumor/normal tissue ratio of 26.82), a good tumor inhibition rate (83.64%), and little toxicity to the skin and other major organs. ZnF16Pc was also reported being encapsulated in a folic acid-modified ferritin and targeted delivery to 4T1 tumors-bearing mice, which suppressed tumor growth and metastasis [159]. Other photosensitizers such as methylene blue (MB) [160], rose bengal (RB) [161] and manganese phthalocyanine (MnPc) [162] also achieved tumor-targeted delivery and tissue-specific PDT when they were loaded in ferritin. Huang et al. [163] encapsulated a photothermal agent, indocyanine dye (IR820), in ferritin, which exhibited enhanced tumor-specific accumulation, much stronger anti-tumor PTT effects and lower side effects than free IR820 in nude mice bearing 4T1 tumors. Wang et al. [164] loaded ferritin within copper sulfide (CuS), which showed higher tumor accumulation in mice compared to free CuS.

Ferritin was also used for combined therapy to achieve better anti-tumor effects, through co-delivering PDT, PTT agents, and/or other drugs. For example, ferritin-based delivery strategy has been applied to alleviating hypoxia limitation of TME in order to improve the therapeutic effects of photosensitizers. Zhu et al. [165] co-encapsulated MnO_2 and a photosensitizer chlorin e6 (Ce6) in ferritin. After being excited by light, Ce6 transfers energy to O_2, generating ROS with cytotoxic effects [154], which is severely limited by TME hypoxia [166]. MnO_2, could catalyze high concentrations of H_2O_2 in TME to O_2, alleviating the resistance of the tumor hypoxic environment to PDT. This MnO_2–Ce6-ferritin exerted more potent anti-tumor efficacy and negligible damage to normal tissues compared to free Ce6. Guo et al. [167] encapsulated both photothermal agent IR780 and anti-cancer agent resveratrol (RSV) in ferritin, which inhibited ovarian cancer in mice, prolonged the survival and showed lower side effects, indicating a strong synergistic effect of photothermal and chemotherapy. Huang et al. [168] loaded photosensitizer sinoporphyrin sodium (DVDMS) in ferritin, which not only generated ROS for PDT but also produced local hyperthermia for PTT, resulting single-wavelength NIR-triggered synergistic PDT and PTT.

Diagnosis and treatment for other diseases

Ferritin-based nanoprobe was also used to detect or visualize other disease. He et al. [169] constructed an osteoarthritis (OA) nanoprobe by genetically modifying ferritin shell with NIR dye-labeled matrix metalloproteinases-13 (MMP-13) responsive peptide which was cleaved by MMP-13 overexpressed in OA and then induced fluorescence. Human ferritin subunit was fused with a mercury (Hg^{2+}) binding peptide (MBP) which was then labeled with green fluorescent dye fluorescein isothiocyanate (FITC) [170]. The fluorescence of FITC-labeled HuHF-MBP was quenched by fluorescence resonance energy transfer (FRET) which was induced by graphene oxide (GO) added in solution. However, addition of Hg^{2+} to the above solution recovered strong FITC fluorescence through their specific and high binding affinity for Hg^{2+}, showing potential to be a heavy metal ion sensor. FITC-labelled thyroglobulin antibodies (FITC-TgAb) were conjugated to ferritin, which showed good selectivity and specificity to rapidly capture and detect thyroglobulin by fluorescence quenching [171]. FITC-loaded ferritin was used to visualize and validate whether ferritin could pass through BBB to deliver drugs for central nervous system [172, 173], and also to identify and characterize a ferritin receptor on rat lipocytes [174]. Moreover, a Cy5-labeled bone-targeting peptide was genetically fused to ferritin N-terminus which was used for imaging bone osteoblast [175]. Cy5.5-labeled ferritin was also used for fluorescence imaging of vascular macrophages which could accumulate in human atherosclerotic plaques [176].

Ferritin was used to delivery drugs for other diseases treatment (Table 4). OA is the most common form of arthritis, and articular cartilage damage is its typical feature. Liu Gang group [26] loaded Kartogenin (KGN) in ferritin to enhance cartilage regeneration in OA. The ferritin surface was bifunctionally modified with RGD peptide for binding integrin GPIIb–IIIa of bone marrow mesenchymal stem cells (BMSCs) and WYRGRL peptide for targeting cartilage matrix component of collagen II protein, respectively. This KGN-loaded ferritin leveraged its small size to penetrate the proteoglycan network of cartilage, and the targeting peptide-modification endowed the nanoparticle with prolonged retention in cartilage matrix to prevent rapid release and clearance of KGN. Meanwhile, they also loaded metformin in WYRGRL-modified ferritin and injected articular, which remarkably prolonged the retention time of metformin for 3 weeks and significantly reduced OA inflammation [169]. Moreover, ferritin was used as dual-functional platform for both OA imaging and precision therapy. Chen et al. [40] mounted a fluorescent probe on a WYRGRL-modified ferritin surface for examining the degree of OA severity. The fluorescent probe could be controlled released by matrix metalloproteinases-13 (MMP-13) overexpressed in OA microenvironment for imaging, while anti-inflammatory drugs hydroxychloroquine (HCQ) encapsuled in inner could be sustained released under acidic pH conditions at the OA joint for treatment.

Ferritin is also a promising drug delivery platform for anti-inflammation, anti-thrombosis, central nervous system disorders and even reproductive system diseases. Shuvaev et al. [177] modified ferritin with plasmalemmal vesicle-associated protein (Plvap), an endothelial glycoprotein involved in control of vascular permeability. Superoxide dismutase (SOD) was encapsuled into the Plvap-modified ferritin which was internalized through caveolin-dependent pathway. Then it dissociated from caveolin-containing vesicles and transported to intracellular endosomes where the SOD played an anti-inflammatory role. Two different peptides, thrombin receptor agonist peptide (TRAP) and γ-carboxyglutamic acid of protein C (PC-Gla), were fused to ferritin N- and C-terminus respectively in order to achieve sepsis treatment [65].

Saponin-loaded ferritin was administered orally in mice to attenuate the murine pneumococcal pneumonia caused by *Streptococcus pneumoniae* infection [15]. Microplasmin presented at ferritin surface was used to dissolve clots in arterial and venous thromboses models [91], and also in the tumor microenvironment [126]. Cyclosporine A-loaded ferritin crossed BBB efficiently and showed a significant therapeutic effect on cerebral ischemia/reperfusion injury in mice [41]. Bisdemethoxycurcumin-loaded ferritin penetrated BBB model and showed potential to regulate inflammation in Alzheimer's disease [178]. Ferritin carrying ATP inside could cross blood-testis barrier (BTB) and accumulate in the sperm heads, improving sperm motility and facilitating ATP-dependent asthenozoospermia treatment [52].

Ferritin-based vaccine for infectious diseases

Subunit vaccines, composed of antigenic proteins or peptides, were safer compared with attenuated vaccines and inactivated vaccines [179]. However, when proteins or peptides antigens were solely used, the immune response may be weaker compared with native pathogens. A good platform to display antigens similar with native molecular structures is needed to improve its effect. Ferritins showed advantages as subunit vaccines displaying platform which could display antigens on its surface in the form of monomer, dimer or trimer (Figure 5), largely depending on the structure of antigens and the fusion site on ferritin. Ferritin-based

Table 4: Ferritin-based therapy for other diseases.

Ferritin-based drug delivery	Disease	Benefits
KGN encapsulated in RGD- and cartilage targeting peptide-modified ferritin	OA [26]	Enhanced KGN solution; dual targeting of bone marrow mesenchymal stem cells and cartilage matrix
HCQ encapsulated in cartilage targeting peptide-modified ferritin	OA [40]	Cartilage-targeting delivery; prolonged drug retention and sustained drug release
Metformin encapsulated in cartilage-targeting peptide-modified ferritin	OA [169]	Cartilage-targeting delivery; prolonged drug retention and sustained drug release
SOD encapsulated in Plvap/PV1-modified ferritin	LPS-induced inflammation [177]	Precise caveolae-dependent delivery and intracellular transportation to endosomes
Saponins encapsulated in horse spleen ferritin	Pneumococcal pneumonia [15]	Enhanced anti-inflammation effect
TRAP- and PC-Gla-modified ferritin	Sepsis [65]	Reduce sepsis-induced organ injury and septic mortality
Bisdemethoxycurcumin encapsulated in human H-ferritin	Alzheimer's disease [178]	Improved drug solution and ability to cross BBB
Cyclosporine A encapsulated in human H-ferritin	Cerebral ischemia reperfusion injury [41]	Improved drug solution and ability to cross BBB
Clot-targeting peptide- and microplasmin-modified ferritin	Arterial and venous thromboses [91]	Prolonged circulation of microplasmin; thrombus clot targeted delivery
ATP encapsulated in human H-ferritin	Asthenozoospermia [52]	BTB crossing and sperm targeted delivery

KGN, kartogenin; RGD, Arg–Gly–Asp; OA, osteoarthritis; HCQ, hydroxychloroquine; Plvap/PV1, plasmalemmal vesicle-associated protein; SOD, superoxide dismutase; TRAP, thrombin receptor agonist peptide; PC-Gla, γ-carboxyglutamic acid of protein C; BBB, blood–brain barrier; ATP, adenosine triphosphate; BTB, Blood–testis barrier.

vaccines have been widely tested in infectious diseases caused by human immunodeficiency virus (HIV), influenza virus, SARS-COV-2 virus, Epstein-Barr virus (EBV) and hepatitis virus (HBV), etc.

The majority of antigens displayed on ferritin surface are in monomer (Figure 5A). Kamp et al. [181] fused the outer surface protein A (OspA) of *Borrelia* to *H. pylori* ferritin N-terminus, exposing OspA on ferritin nanoparticle surface in monomer (Figure 5B). This OspA-ferritin nanoparticle vaccine elicited high titer antibody and provided protective immunity for Lyme disease which is caused by *Borrelia* infection spread by tick bites. Ferritin-based platform for presenting peptide antigens was widely used for HIV vaccine development [184–187]. Chester et al. [188] firstly displayed HIV antigens using ferritin in 2006. A short HIV1 Tat peptide was fused to N-terminal of human L-ferritin, inducing high serum anti-Tat specific IgG activity in rats. Moin et al. [189] used ferritin to display influenza A virus's hemagglutinin (HA) subunit, eliciting cross-group protective immunity and neutralizing-antibody responses in multiple animal models. Qiao et al. [190] fused two influenza antigen epitopes, matrix protein 2 ectodomain (M2e) and CDhelix, on ferritin N-terminus in a sequential tandem format. It also induced robust humoral and cellular immune responses. Saunders et al. [191] fused sortase A-tagged SARS-COV-2 receptor binging domain (RBD) to *H. pylori* ferritin N-terminus and displayed RBD on ferritin surface, which elicited high titers of SARS-CoV-2-pseudovirus neutralizing antibodies in cynomolgus macaque. They further proved that this ferritin-based SARS-COV-2 RBD vaccine showed cross-protection for SARS-CoV and SARS-CoV-2 variants including 614G, Beta, Delta and Omicron, providing broad immunity to SARS-CoV-2 infection [192]. Ferritin antigen-displaying platform was also combined with SpyTag/Catcher technology for developing HBV subunit vaccine. PreS1 domain of HBV was fused to SpyCatcher, while SpyTag was fused to ferritin N-terminus. Through SpyTag/Catcher pair interaction, preS1 could be displayed on ferritin nanoparticle surface, which showed high level and long-lasting anti-preS1 responses and protective immunity against HBV in mice [95].

Ferritin could also display antigen in the form of dimer (Figure 5C), although it is rare. EBV infection is associated with a variety of human diseases, including malignant tumors. Unfortunately, there is still no vaccine available. Ferritin-based EBV vaccines brought new opportunities to prevent this fatal illness [182, 193–195]. Kanekiyo et al. [182] fused D123, a truncated variant of the EBV major envelop glycoprotein 350 (gp350), with *H. pylori*-bullfrog hybrid ferritin to distribute antigens evenly on ferritin nanoparticle. D123 was protruded from D123-ferritin surface and formed a dimer structure (Figure 5D), which showed stronger immunogenicity than free D123 or gp350 ectodomain in mice, eliciting neutralizing antibodies with higher titers and longer duration.

HIV envelope glycoprotein (Env) [196], influenza virus hemagglutinin (HA) membrane protein [197], and coronavirus spike protein [198, 199] share similarity in their

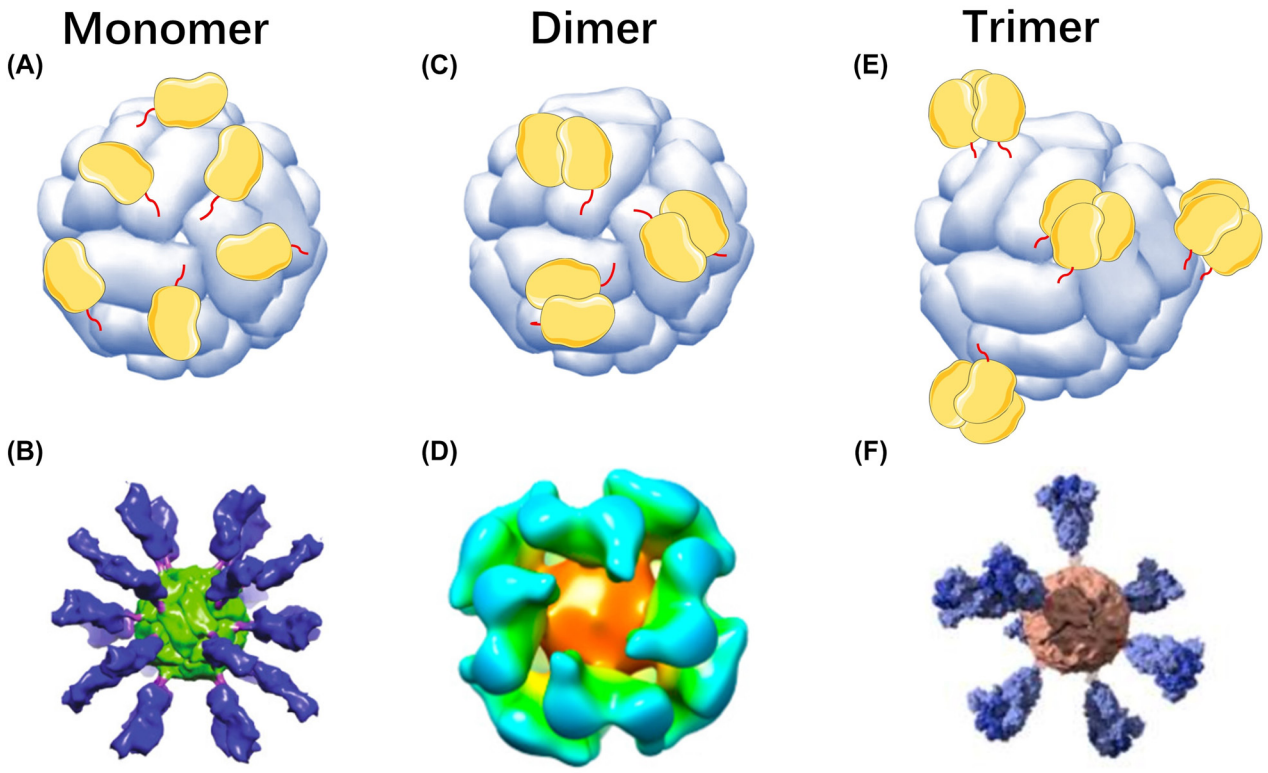

Figure 5: Ferritin-based vaccines in different forms. (A, C, E) Protein antigens were displayed on ferritin surface in monomer, dimer or trimer. The ferritins in this diagram were in light blue color (image source: Zhang et al. [180] with permission), while antigens were in yellow. (B) *B. burgdorferi* outer membrane surface lipoprotein OspA was displayed on *H. pylori* ferritin as monomer (image source: Kamp et al. [181] with permission). (D) The D123 variant of EBV envelop protein gp350 is exposed on the surface of a hybrid ferritin of bullfrog and *H. pylori* as dimer (image source: Kanekiyo et al. [182] with permission). (F) SARS-CoV-2 spike protein represented on *H. pylori* ferritin surface as trimer (image source: Powell et al. [183] with permission). OspA, outer surface protein A; *B. burgdorferi*, *Borrelia burgdorferi*; EBV, Epstein–Barr virus; *H. pylori*, *Helicobacter pylori*; gp350, EBV glycoprotein 350; D123, A truncation variant of gp350 containing the domain 1, domain 2 and domain 3, deleting most of the "mucin-like" domain of full length gp350; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

structural appearance as trimeric proteins. Most importantly, ferritin could display antigen in the form of trimer to mimic virus natural antigen forms and elicit much stronger immunoprotected antibody than antigens in monomer, indicating that ferritin could be a perfect platform for trimeric-subunit vaccine (Figure 5E). In 2013, Kanekiyo et al. [81] firstly reported ferritin-based trimeric spike strategy and tested it in influenza vaccines. They found that the distance among each Asp5 at ferritin threefold is almost identical to the distance among each trimeric influenza virus HA2 subunit. Therefore, they fused the ectodomain of HA to the Asp5 of *H. pylori* ferritin and eight trimeric HA spikes were displayed on ferritin surface. This ferritin-based influenza vaccine showed stronger efficacy in inducing protective immunity than trivalent inactivated influenza vaccine (TIV) in mice and ferrets, combined with the use of adjuvant. Later in 2016, He et al. [184] fused HIV-1 antigens V1V2 (part of gp120 of HIV), gp120 and gp140 to ferritin Asp5 separately, generating three recombinant ferritin nanoparticles, V1V2-FR, gp120-FR and gp140-FR, respectively. V1V2, gp120 and gp140 were all presented in native-like trimeric conformations. Powell et al. [183] used the trimeric spike strategy to display SARS-CoV-2 spike protein on ferritin (Figure 5F) which elicits stronger neutralizing antibody response than spike alone in mice. Other scientists also presented SARS-CoV-2 RBD on ferritin as trimer, eliciting strong immune protection in animals [97–99].

Ferritin-based vaccine is also promising for other infectious disease caused by *Neisseria gonorrhoeae* [200], rotavirus [201, 202], hepatitis C virus [203, 204], human papilloma virus (HPV) [205], and respiratory syncytial virus (RSV) [206], etc. So far, there are five ferritin-based vaccines that have started or finished phase I clinical trials (Table 5). According to the results of one trial (NCT03186781) [207], the ferritin-based H2 influenza vaccine was safe, well-tolerated and immunogenic in healthy adults and was a proof of concept for a new generation of vaccines.

Table 5: Ferritin-based vaccines in clinical trials.

Intervention	Official title	Condition or disease	National Clinical Trial (NCT) number; phase; status	First posted date; estimated or actual primary completion date	Sponsor; location country	Related publications
EBV gp350-Ferritin vaccine	Safety and immunogenicity of an Epstein–Barr virus (EBV) gp350-Ferritin nanoparticle vaccine in healthy adults with or without EBV infection	EBV infection	NCT04645147; Phase I; recruiting.	November 27, 2020; July 1, 2025	NIAID, United States	[182, 208, 209]
SpFN_1B-06-PL	SARS-COV-2-spike-ferritin-nano-particle (SpFN) vaccine with ALFQ adjuvant for prevention of COVID-19 in healthy adults	SARS-CoV-2 infection	NCT04784767; Phase I; active, not recruiting.	March 5, 2021; October 30, 2022	USAMRDC, United States	Not provided
VRC-FLUNPF081-00-VP	Influenza HA ferritin vaccine, alone or in prime-boost regimens with an influenza DNA vaccine in healthy adults	Influenza infection	NCT03186781; Phase I; completed.	June 14, 2017; September 3, 2019	NIAID, United States	[81, 207, 210, 211]
VRC-FLUNPF099-00-VP	Dose, safety, tolerability and immunogenicity of an influenza H1 stabilized stem ferritin vaccine in healthy adults	Influenza infection	NCT03814720; Phase I; completed.	January 24, 2019; April 6, 2021	NIAID, United States	[81, 212, 213]

HA, hemagglutinin; gp350, EBV major envelop glycoprotein 350; NIAID, National Institute of Allergy and Infectious Diseases; SARS-CoV-2, *severe acute respiratory syndrome coronavirus 2*; COVID-19, coronavirus disease 2019; ALFQ, ALF containing QS21 saponin; USAMRDC, United States Army Medical Research and Development Command.

Challenges and perspectives

Ferritin-based nanomedicines have made huge progress in the past decades. Ferritin delivers many drugs, including small molecules, proteins/peptides, or siRNA for diseases treatment, particular for tumor therapy. However, efforts are still needed to be made for the clinical applications of ferritin-based nanoparticles in diagnosis, treatment and prevention of many diseases.

In current pH-induced drug loading methods, the structures of ferritin and drugs may be irreversibly damaged by the acidic environment of pH 2.0. The disassembly process under acid environment influences the structure of ferritin subunits, leading to the incomplete reassembly of ferritin nanocage that holes are left on the nanocage, which leads to leakage of loaded drugs inside [69]. Working at milder condition is the main objective to improve the stability and controllability of drug loading. Gu et al. [73] substituted the six residues (^{159}GAPESG^{164}) of ferritin DE loop with 6× His, and the mutant His_6-HuHF could assemble into nanocage at pH 10.0 or Ni^+ assistance at pH 7.5, showing significantly higher loading efficiency with milder loading condition. However, this method is not suitable for loading of drugs sensitive to high pH value. To avoid the pH-induced disassembly/reassembly loading method, steric hindrance induced-assembly may be used for drug loading. Peptides responsive to other stimulation inserted into the loop between the α-helixes may form steric hindrance that influence the interface interaction of subunits, thus inhibiting the self-assembly of 24-meric nanocage. Upon adding the stimulating agents, with the cleavage of peptides, steric hindrance may be reduced, which leads to the responsive-assembly of ferritin nanocage. During this process, drugs are expected to be encapsulated into the inner cavity without irreversible damage to structure of ferritin or drugs.

The lack of systematic illustration of molecular interaction mechanism between drugs and ferritin limits the development of ferritin-based nanomedicine. Much more advanced analysis methods or techniques should be used in this field. For example, a novel potential hydrophilic pathway at twofold axis was reported which could be enlarged at high temperature for drug entry [49]. The existence of this twofold pathway was proven by amino acids mutation, but more evidence is still needed. Moreover, the interaction of non-metallic drugs with the inner surface of ferritin and the influence of drugs-ferritin interaction on ferritin secondary structure, is still not fully understood. Currently, high-resolution X-ray crystallography [18], circular dichroism [46] and molecular simulation [214] were used to elucidate the interaction between drugs and ferritin during drug loading process. To help us better understand the drug loading process, more methods,

such as Cryo-electron microscopy and surface plasmon resonance, may be applied to optimizing drug loading.

In summary, ferritin had a lot of advantages as a good drug carrier and protein displaying platform, and demonstrated great potential in targeted drug delivery and vaccine development. However, there are several issues need to be further improved. Most importantly, a non-expensive, mild and easily scaled-up drug loading method is in urgent need for ferritin-based drug delivery. In addition, the biosafety including the toxicity and immunity in the functionally modified ferritin has yet to be fully verified, and more experiments are needed. Moreover, the half-life of native human ferritin in plasma is quite short with only about 2 h, which is un-favorable for the *in vivo* efficacy of ferritin-loaded drugs in many cases, so new techniques to increase the half-life need to be developed.

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