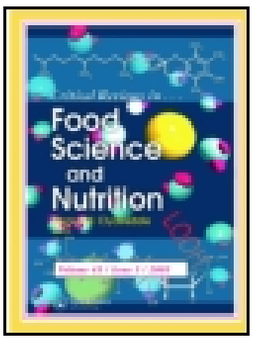

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Ferritin cage for encapsulation and delivery of bioactive nutrients: From structure, property to applications

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Abstract

Ferritin is a class of naturally occurring iron storage proteins, which is distributed widely in animal, plant, and bacteria. It usually consists of 24 subunits that form a hollow protein shell with high symmetry. One holoferritin molecule can store up to 4500 iron atom within its inner cavity, and it becomes apoferritin upon removal of iron from the cavity. Recently, scientists have subverted these nature functions and used reversibly self-assembled property of apo ferritin cage controlled by pH for the encapsulation and deliver of bioactive nutrients or anticancer drug. In all

these cases, the ferritin cages shield their cargo from the influence of external conditions and provide a controlled microenvironment. More importantly, upon encapsulation, ferritin shell greatly improved the water-solubility, thermal stability, photo stability, cellular uptake activity of these small bioactive compounds. This review aims to highlight recent advances in applications of ferritin cage as a novel vehicle in the field of food science and nutrition. Future outlooks are highlighted with the aim to suggest a research line to follow for further studies.

Keywords: Ferritin, Shell-like structure, Self-assembly property, Bioactive nutrients, Encapsulation and delivery

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INTRODUCTION

Ferritin is widely distributed in animal, plant and bacteria except for yeast. Ferritin is a specific class of iron storage and detoxification proteins, and one ferritin molecule can accumulate up to ~4500 iron atoms within protein inner cavity. Ferritin usually has 24 subunits that arranged in 432 symmetry to form a hollow protein shell (outside diameter 12 nm, inside diameter 8 nm) (Figure 1A). The native iron oxide particles can be removed from holoferritin protein cage by reduction of Fe(III) and subsequent chelation of Fe(II), resulting in the formation of apoferritin having an empty, intact protein cage (Harrison and Arosio, P. 1996; Zhao 2010). The apoferritin shell is remarkably stable due to tight packing, and it can withstand heating to 80 °C for 10 min (Stefanini et al., 1996). So far, among all known shell-like proteins, ferritin has been the most widely used as either a biotemplate to synthesize a variety of inorganic and organic nanomaterials or a vehicle to bioactive nutrients or drug. All obtained nanoparticles with ferritin were found to have the same and narrow size distribution (Iwahori et al., 2005; Li et al., 2007). Ferritin encapsulation and delivery has several advantages over other systems. First, ferritin encapsulated nanoparticles are homogeneous, and have nearly the same and narrow size as ~12 nm. Second, by using the simply reversible dissociation and reassembling characteristic of apoferritin at different pH values, small molecules can be easily and effectively encapsulated within the inner cavity of ferritin without the use of any organic solvents. Third, ferritins are

present, under physiological conditions, both inside cells and, although at low levels (around 20 μg/L), in the bloodstream, where they are stable and soluble. Fourth, ferritins can be easily functionalized by genetic and/or chemical conjugation involving one of the many chemical groups present on the protein external surface. For preparation of various inorganic nanoparticles by ferritin, please refer to recent review articles (Uchida et al., 2010; Yamashita et al., 2010). The purpose of this review is to present an overview of our current knowledge of structure, self-assembly property, and applications of ferritin. We also discuss the challenges for ferritins as a novel vehicle for encapsulation and delivery of bioactive nutrients.

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STRUCTURE OF FERRITIN

General Features of Ferritin Structure

Since ferritin was discovered in 1937 by the French scientist Victor Laufberg, who isolated a new protein from horse spleen that contained over 20% by dry weight of iron (Laufberg, 1937), great progress has been made in the ferritin field. All this progress is tightly associated with increasing understanding of ferritin structure. The structure of ferritin is highly conserved in all living kingdom, although its roles in the regulation of iron trafficking vary substantially (Harrison and Arosio, 1996). All ferritins have a shell-like structure, which usually consists of 24 subunits. The shape of the ferritin subunit is cylindrical, with a length of 5 nm and a width of 2.5 nm (Figure 1A, d). Each subunit is composed of a four-α-helix bundle containing two

antiparallel helix pairs (A, B and C, D) that are connected by a long nonhelical stretch of 18 residues (the BC-loop) between B and C helices. The N-terminus, the loop BC, and the helices A and C form the outside of the molecule, while helices B and D face the inside surface. A break in the hydrogen bonding at His 136 causes a kink which separates the D helix into two parts. This D helix kink occurs at a position where three subunits come together near the 3-fold axis, allowing a channel to form. A fifth short helix (E helix) lies at one end of the bundle at about 60° to its axis. The E helix exists around the four-fold intersubunit symmetry axes of the protein shell (Sun et al., 1993; Crichton et al., 1996; Harrison and Arosio, 1996; Chasteen and Harrison, 1999; Masuda et al., 2010).

Although the structure of ferritin is source-dependent, there are two structural features in common among all reported ferritin. First, ferritin is rich in acidic residues such as Glu and Asp which are mainly distributed on its inner surface, resulting in its relatively low isoelectric point (*pI*) ranging 5.0–6.0. Consequently, ferritin has high negative charge density on its interior surface, while the net charge of its exterior surface is close to zero or slightly positive at pH 7.0. Second, one ferritin molecule has eight 3-fold and six 4-fold channels with pore sizes between 0.3–0.5 nm (Figure 2), which connect the inner cavity to the solution. Each 3-fold channel is composed of amino acid residues contributed from the C-terminus of helix C and N-terminal of helix D. With the 4-fold channel, four E helices of ferritin lie roughly parallel. Through these channels, metal ions and small organic molecules with positive charge can diffuse into the cavity

due to electrostatic attraction (Zhao, 2010). However, large molecules cannot enter into the cavity because of steric hindrance.

Generally, all ferritin cage architectures have three chemically distinct interfaces: the interior surface, the exterior surface, and the interface between subunits (Uchida et al., 2010). These three interfaces can be manipulated in order to impart functionality by design. By combining both chemical and genetic alteration of the subunits, one can simultaneously impart novel function to different surfaces of the cage. Remarkably, regions not directly involved in the assembly process of the ferritin cage architectures are generally amenable to modifications without loss of the cage-like architecture. An achievable goal is now the modification of ferritin cage to achieve multiple functionalities in a single architecture in order to direct cage assembly, encapsulation of a bioactive nutrient or drug, targeting to a specific surface or cell, and assembly into hierarchical structures.

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Structure of Animal Ferritin

In vertebrates, ferritins usually consist of two types of subunits, H (heavy) and L (light), with apparent molecular weights of 21 and 19.5 kDa, respectively. The two have about 55% identity in their amino acid sequence. The H-subunit contains a dinuclear ferroxidase center necessary for iron uptake and for fast oxidation of ferrous iron by oxygen. It consists of A and B iron-binding sites of conserved amino acid ligands His65, Glu27, Glu107, and Glu62. H-bonding

residues Gln141 and Tyr34 are near the B-site (Chasteen and Harrison, 1999; Crichton and Declercq, 2010). Differently, the L-subunit lacks such ferroxidase center but contains a putative nucleation site important for slower iron oxidation and mineralization (Crichton et al., 1996). The subunit structure of horse L-chain ferritin was shown in Figure 1B, a. Ferritins from lower vertebrates, such as bullfrogs and fish, contain a third subunit type, named the H′ chain, which harbors the residues forming both the ferroxidase center and the nucleation site (Dickey et al., 1987; Harrison and Arosio, 1996).

Most known mammalian ferritins are cytosolic heteropolymeric proteins. However, a new type of ferritin has been recently identified in human, mouse, and rat as a homopolymer of 24 identical subunits that is located inside the matrix of mitochondria. Human mitochondria ferritin (MtF) is encoded by an intronless gene on chromosome 5q23.1. It has a high degree of sequence homology (79%) with human H-chain ferritin (HuHF) (Levi et al., 2001). The precursor protein has a long N-terminal leader sequence for mitochondria export. The protein was shown to be specifically targeted to the mitochondria and processed into a functional ferritin with iron incorporation activity. The residues of the ferroxidase center are all conserved with this specific ferritin. Expression of this protein in transfected HeLa cells showed MtF to be functionally active in incorporating iron, apparently even more efficient than the cytosolic ferritins composed of H- and L-chains (Corsi et al., 2002).

Structure of Plant Ferritin

Compared with animal ferritin, plant ferritin exhibits distinctive structural features. First, only the H-type subunit has been identified in phytoferritin thus far, and it shares ~40% sequence identity with the animal H-subunit (Laulhère and Briat, 1993; Masuda et al., 2001). Second, the H-type subunit in mature phytoferritin contains a specific extension peptide (EP) at its N-terminal sequence (Figure 1B, b). In the case of soybean seed ferritin (SSF), each EP domain is composed of ~30 amino acid residues (Liao et al, 2014). The crystal structure of recombinant H-4 SSF shows that the EP is located on the exterior surface of protein and stabilizes the entire oligomeric conformation of phytoferritin by its interaction with a neighboring subunit on the shell surface (Masuda et al., 2010). Recent studies of our group reveal the role of the EP during iron oxidative deposition in phytoferritin as the second binding and ferroxidase center that contributes to mineralization of the iron core at high iron loading of ferritin (> 48 irons/shell) (Li et al., 2009). On the other hand, the EP exhibits a significant serine protease-like activity (Fu et al., 2010), which is responsible for protein autodegradation during seed germination (Lobréaux and Briat, 1991; van Wuytswinkel et al., 1995). Associated with the degradation is faster iron release from ferritin to meet the requirements of seedling growth (Fu et al., 2010; Yang et al., 2010). Third, naturally occurring phytoferritin are usually a heteropolymer composed of two different H-type subunits. For example, ferritin from soybean and pea seeds consists of H-1 and H-2 subunits sharing ~80% amino acid sequence identity (Liao et al., 2014; Zhao, 2010). The

fourth, each 4-fold channel in plant ferritin is lined with eight histidines and thus is more hydrophilic when compared to those in animal ferritin, which are mainly rich in leucines and more hydrophobic (Masuda et al., 2001). Recent our studies demonstrate that, different from animal ferritin, both the 4-fold and 3-fold hydrophilic channels of plant ferritin are necessary for iron diffusion into the inner cavity, followed by oxidation (Lv et al., 2014). Finally, the pore size of the 4-fold channels of mature soybean seed ferritin (mSSF), which consists of H-1 and H-2 subunits with an identical ratio, appears to be larger because all E-helices from 12 of H-1 subunits have been deleted (H-1△E) after assembly with H-2 analogues into protein shell (Masuda et al., 2001). Such increase in the pore size around the 4-fold axes may endow this ferritin with new property. Indeed, our studies have demonstrated that recombinant heteropolymeric SSF nanocages can self-assemble into 1D array of linear chains and 2D square arrays in the absence and presence of urea, respectively, through channel-directed electrostatic interactions with poly(α, L-lysine) at pH 7.0 (Yang et al., 2014a; Yang et al., 2014b). This strategy might present a simple and new principle for fabricating protein hierarchical architectures.

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Structure of bacterial Ferritin

In contrast to mammalian and plant ferritins, bacterial ferritins tend to be homopolymers with subunits which are more similar to H chains than to L chains, particularly in that they

possess the carboxylate-bridged di-iron centers. So far, three classes of ferritin have been found in prokaryotes. The first class is bacterial ferritins, designated FTNs, similar to mammalian H chain ferritins with a ferroxidase center. The second class of ferritins was named as the BFRs, bacterioferritins (Figure 1B, c), which contain heme bound at the interface between two subunits as well as a ferroxidase center (Le Brun et al., 2010).

The third class of ferritins from bacteria is called Dps (DNA-binding protein from starved cells), which is expressed in bacteria and accumulated to high levels prior to conditions of oxidative or nutritional stress, making the cell in stationary phase more resistant to H_2O_2 than actively growing cells (Chiancone and Ceci, 2010; Frenkiel-Krispin et al., 2001; Martinez and Kolter, 1997). Dps has a shell-like structure of 3:2 tetrahedral symmetry assembled from 12 identical subunits with a central cavity measuring ~45 Å in diameter. Each Dps subunit is folded into a four-helix bundle through hydrophobic interactions (Figure 1B, d). The 3:2 symmetry of the assembled protein leads to two inequivalent environments along the three-fold axes. One corresponds to the three-fold interactions found in the 24mer ferritins having 4:3:2 octahedral symmetry and involves the N-terminal end of the subunit while the other three-fold interaction takes place at the C-terminal end of Dps but is not typical of the ferritins (Grant et al., 1998; Ilari et al., 2000).

REVERSIBLE SELF-ASSEMBLY PROPERTY OF FERRITIN

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Living systems utilize proteins as building block to construct a large variety of self-assembled nanoscale architectures such as ferritin (Schreiber et al., 2009). X-ray diffraction analysis into the assembly mechanism of horse spleen apoferritin suggests that stable dimers act as assembly intermediates (Banyard et al., 1978). Furthermore, analytical ultracentrifugation of this protein at different pH values demonstrates the high stability of dimers in solution and also suggests that assembly proceeds from dimers to tetramers and octamers (Stefanini et al., 1987). The overall assembly mechanism of horse spleen apoferritn was first proposed by Gerl and Jaenicke (Gerl and Jaenicke, 1987) as following:

$$24M_1^* \rightarrow 24M_1 \leftrightarrow 8M_1 + 8M_2 \leftrightarrow 8M_3 \leftrightarrow (4M_6) \leftrightarrow 2M_{12} \rightarrow M_{24}$$

This mechanism was proposed based on the observation that the completely self-assembled product was formed through a series of concentration dependent association reactions involving a mixture of partially assembled subunits. In this mechanism, M_1^* is the unfolded monomer, M_i are intermediates with i folded monomers, and M_{24} is the completely self-assembled ferritin. To date, a detailed mechanism by which ferritin self assembles remains unknown. Apoferritin is ferritin that lacks the iron core, and usually consists of 24 identical or different polypeptide subunits that interact to form a roughly spherical molecule of 12 nm diameter (Toussaint et al., 2007). The horse speen apoferritin shell is remarkably stable because of tight packing (Stefanini et al., 1996). Despite the rigid architecture, the association among the ferritin subunits occurs either in an acidic environment or upon treatment with the high concentration of guanidine

hydrochloride (GdnHCl) (Kang et al., 2008; Lin et al., 2011; Santambrogio et al., 1992). Complete dissociation occurs only at pH 2.0 or pH 11.0 (Kim et al., 2011; Zhang et al., 2014). The dissociation and reassembly characteristic of different apoferritin at different pH values provides a convenient route for preparing nanocomposites loaded with small molecules (Chen et al., 2014; Zhang et al., 2014). Figure 3 describes the process involved in the encapsulation of the small bioactive molecules into apoferritin during its reassembly. In this case, the protein nanocage of apoferritin is dissociated into individual subunits at pH ~2.0 or 11.0 (Chen et al., 2014; Zhang et al., 2014), and the subunits reconstitute into a cage-like structure at pH 7.5. During the reassembly process, these small molecules were entrapped within the inner cavity of the protein. The encapsulated molecules will be retained within the apoferritin shell because their size is larger than the pore size of the protein channels (3–4 Å) (Harrison and Arosio, P. 1996; Masuda et al., 2010). Such encapsulation approach provides an alternative route for large molecules which are not available by diffusing through the protein channels into the inner cavity of apoferritin. It has to be mentioned that this procedure is not suitable for those molecules which are unstable at pH 2.0 or 11.0.

APPLICATION OF FERRITIN IN THE FIELD OF FOOD SCIENCE AND NUTRITION

Recently, animal and plant ferritin cages have received great attention in the field of food science and human nutrition. Scientists have used the reversible disassembly and reassembly

property of ferritin cages controlled by pH to trap various bioactive nutrients. This step is easy, simple, and environment friendly. Upon encapsulation, the water-solubility, thermal stability, photo stability, and cellular uptake activity of these trapped small molecules were pronouncedly improved as compared to these small molecules alone. In addition, ferritin shell can also prevent these molecules from the interference of other components occurring in foodstuffs.

Carotenoids are a class of natural pigments mainly found in fruits and vegetables that typically have 40-carbon molecules and multiple conjugated double bonds (Qian et al., 2012). Recently, there is a considerable interest in carotenoids such as β-carotene because of a number of potential health benefits. For example, it has been proposed as a cancer prevention agent, ulcer inhibitor, life extender, and heart attack inhibitor (Colditz et al., 1985; Cutler, 1984; Gerster, 1993; Kritchevsky, 1999; von Lintig, 2010). Nevertheless, its utilization is currently limited mostly because of its poor water-solubility and instability. As a result, β-carotene has to be dissolved in oils or dispersed in other suitable matrices before they can be utilized in foods (Soares and Craft, 1992). Moreover, β-carotene is also highly prone to chemical degradation during food processing and storage due to various environmental effects, such as common thermal stress (Tai and Chen, 2000). Therefore, it is very important to improve the water-solubility and thermal stability of β-carotene.

Recently, β-carotene has been successfully encapsulated within ferritin nanocages by taking advantages of the reversible dissociation and reassembly characteristic of apoferritin at different

pH values. Each ferritin nanocage contains 12.4 molecules of β-carotene within its inner cavity in average (Chen et al., 2014a). Compared to lipid-soluble β-carotene, these β-carotene loaded apoferritin nanocomposites are water-soluble. More importantly, the thermal stability of β-carotene encapsulated within protein cages was pronouncedly improved with respect to free β-carotene (Chen et al., 2014a). All these new properties might facilitate its application in food industry.

Curcumin [(E,E)-1,7-bis(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-ione] is a hydrophobic polyphenol derived from the rhizome of turmeric (Anderson et al., 2000). It is a yellow pigment and has been extensively used in food and chemical industries as coloring, flavoring and preservative (Bhawana et al., 2011). Increasing interests in this dietary polyphenol have been received in recent years due to its vast array of beneficial pharmacological effects (Aggarwal et al., 2003; Pizzo et al., 2010). Though having multiple medicinal benefits and extremely high safety profile (Strimpakos and Sharma, 2008), wide applications of this promising molecule in food and chemical industries have been hindered by its poor water solubility (estimated to be 11 ng/mL), short biological half-life, and low bioavailability following oral administration (Anand et al., 2007). By using a similar method, curcumin was likewise trapped within the inner cavity of rHuHF with an average ratio of 14.7/1. Compared with free curcumin which exhibits a maximal absorption at ~425 nm, the curcumin-loaded apoferritin sample has a maximal absorption at around 400 nm, resulting in a blue shift by 25 nm (Figure 4),

indicating that a strong interaction occurs between trapped curcumin molecules and amino acid residues located on the inner surface of apoferritin (Chen et al., 2014b). Compared with apoferritin alone (Fig. 4, inset: picture a), the curcumin-containing ferritin solution has a yellow color (Fig. 4, inset: picture b). As expected, curcumin is water-insoluble (Fig. 4, inset: picture c). Upon encapsulation of curcumin by ferritin, its thermal stability was found to be greatly improved. For example, only 29.2% of free curcumin was left after being heated for 4.5 min at 60 °C, whereas 94.5% of the total was kept upon encapsulation. Moreover, the photo-stability of curcumin was also largely improved by ferritin encapsulation (Chen et al., 2014b).

The above mentioned improvement in the stability of curcumin or β-carotene molecules within ferritin nanocages was believed to be attribute to a dual protective action of ferritin, namely, physical and chemical protection. Apoferritin would be not denatured when heated at 80 °C for 10 min (Stefanini et al., 1996), so its spheroidal crust may act as a physical barrier that isolates temperature and light, while preventing any prooxidants in the aqueous phase from contacting with the molecules locked within the cavity. This corresponds to the physically protective effect of ferritin. The chemical protection of ferritin may derive from cysteine residues and other functional groups of ferritin, which might serve as effective antioxidants. In addition, ferritin may form molecular complexes with these small organic compounds through hydrophobic interactions or van der Waals interactions or hydrogen bonds which may help to stabilize these molecules inside ferritin.

Anthocyanins are a major group of water-soluble plant pigments that are responsible for fruit and flower coloration either by themselves or in conjunction with other phytochemicals. Besides anthocyanin esthetic contributions, considerable attention has been drawn to their potential health promoting properties (Lila, 2004; Zafra-Stone et al., 2007; Ziberna et al., 2012). The presence of an oxonium ion makes the anthocyanins particularly susceptible to nucleophilic attack by various oxidants (Jackman et al., 1987). Additionally, anthocyanins are easily affected by a number of reactions occurring in food products, and the major problem associated with the storage of anthocyanins is their instability caused by temperature, oxygen, light, and some enzymes during processing and storage of the commodity. Therefore, many efforts have been invested in developing techniques for protecting anthocyanins against damage by the above-mentioned factors. For example, a certain degree of pigment stabilization may be conferred by acylation with various organic acids, copigmentation, self-association, and/or metal chelation (Francis, 1989; Jackman et al., 1987). However, the anthocyanin-protective effects of these methods are still very limited. To improve the thermal and photo stability of cyanidin-3-O-glucoside (C3G) molecules, C3G has been encapsulated within the cavity of apo recombinant soybean seed H-2 subunit ferritin (rH-2) with a C3G/protein ratio of 37.5 to 1. As expected, such encapsulation increased both thermal and photo stability of C3G molecules by a factor of about 2. More importantly, Caco-2 cell monolayer absorption and adhesion analyses showed that C3G molecules encapsulated within apoferritin nanocages were more efficient in

transport as compared to free C3G (Zhang et al., 2014).

Rutin is a common dietary flavonoid with important pharmacological activities. However, its application in the food industry is limited mainly because of its poor water-solubility which is often associated with low and variable bioavailability and short biological half-life (Mauludin et al., 2009). To increase its water solubility, recently, the rutin molecules has been likewise encapsulated within apo rH-2 cages with a rutin/protein molar ratio of 30.1 to 1, and the encapsulation and loading efficiency were 25.1% (w/w) and 3.29% (w/w), respectively. In vitro experiments of rutin release demonstrated that the entrapment of rutin was effective, with more than 75% (w/w) still encapsulated in the ferritin cage after storage for 15 days. Furthermore, the thermal and UV radiation stability of ferritin trapped rutin was greatly improved due to the encapsulation as compared to free rutin. Additionally, the antioxidant activity of these ferritin composites was partly retained as compared to free rutin molecules (Yang et al., 2015).

Calcium is an essential nutrient required for critical biological functions such as nerve conduction, muscle contraction, mitosis, blood coagulation, and structural support of the skeleton. Dairy products are a good source of bioavailable calcium (Perales et al., 2006), however they are not suitable for strict vegetarians. Moreover, in the less developed countries such as China and India, especially in rural areas, the consumption of milk is much lower than that in developed ones (Weaver et al., 2009). Fortification of food with Ca-enriched nutrients has traditionally not been taken into account with regard to its possible negative interactions with other nutrients. For

example, calcium ions have been reported to have a negative effect on iron and zinc uptake (Lönnerdal, 1997; Lynch, 2000), because DMT1 located in the small intestinal is a common receptor for these divalent metal ions (Hallberg et al., 1991). Also, dietary factors such as tannins and oxalate greatly inhibit calcium uptake (Perales et al., 2005). Recently, a new class of soluble and edible Ca–protein complexes were synthesized, and in these new ferritin complexes, ~140 calcium ions were encapsulated within a ferritin nanocage. Cell experimental results found that these new complexes have the following advantages over traditional Ca complexes: (1) several factors such as tannic acid (TA), oxalic acid (OA), and zinc ions almost have no effect on calcium uptake from these new complexes, in which they usually have a strong inhibitory effect on calcium absorption; (2) the complexes could be absorbed by Caco-2 cells in a newly TfR-1 involved pathway different from a known DMT1-mediated one for divalent ions, and therefore calcium ions encapsulated within ferritin do not interfere with absorption of other divalent ion minerals (Li et al., 2014). All these findings demonstrate that ferritin as a nanoplatform plays an important role in the field of food science and nutrition.

FERRITIN AS A VEHICLE FOR DRUG ENCAPSULATION AND DELIVERY

Cisplatin (CDDP) and carboplatin (CBDCA) are two major anticancer drugs used worldwide for the treatment of various malignancies (Wang and Lippard, 2005). . However, their clinical applications are largely restricted by both high toxicity and severe tumor resistance. To

decrease the toxicity of CDDP and CBDCA, Guo's research group has reported a novel strategy for the delivery of platinum drugs by encapsulating each of them in the cavity of apo horse spleen ferritin. The encapsulation was achieved through manipulating the pH-dependent unfolding–refolding process of apoferritin at pH 2.0 and 7.4, respectively, in saturated drug solution (Yang et al., 2007). This represents the first report for drug encapsulation by ferritin. *In vitro* assays on the rat pheochromocytoma cell line show that ferritin–cisplatin inhibits the cells in a slow but sustaining mode and the cellular uptake of platinum is enhanced by ferritin. Ferritin–carboplatin and ferritin–oxaliplatin complexes only exhibit a marginal cytotoxicity towards this cell line under similar concentrations (Xing et al., 2009).

To enhance the selectivity of cisplatin-loaded ferritin, an average of three molecules of monoclonal antibody (mAb) Ep1 to the human melanoma-specific antigen CSPG4 were conjugated to a single ferritin cage encapsulating about 50 cisplatin molecules. These synthesized ferritin composites can specifically bind to a $CSPG4^+$ melanoma cell line, but not to a $CSPG4^-$ breast carcinoma cell line. As compared to the cisplatin-containing ferritin nanoparticle alone, the mAb-derivatized ferritin-cisplatin nanoparticles exhibited a 25-fold higher activity for melanoma cell line (Falvo et al., 2013).

Another strategy for increasing the selectivity of ferritin for tumor is to couple epidermal growth factor (EGF) on the outer surface of ferritin molecules by genetic modification (Li et al., 2012). Up-regulation of the epidermal growth factor receptor (EGFR) is found in many types of

cancer (Gschwind et al, 2004). Thus, since EGF is the EGFR ligand, it could be used as a probe molecule. Therefore, ferritin nanoparticles with EGF on their surfaces could provide a method for targeted intracellular delivery of chemotherapeutic drugs by these nanoparticles. As expected, these nanoparticles can specifically bind to and then be taken up by breast cancer MCF-7 cells and MDA-MB-231 cells, but not normal breast epithelial MCF-10A cells. In contrast, binding and absorption of non-targeted ferritin-based nanoparticles to breast cancer cells are negligible (Li et al., 2012).

Human H chain ferritin receptor has been identified to be TfR-1 (Li et al., 2010). Ferritin enters the endosome or lysosome by endocytosis. Based on the fact that TfR1 is highly expressed on human cancer cells, recently ferritin cages was loaded with doxorubicin (Dox) for tumor-specific drug delivery by using the intrinsic tumor-targeting properties of H-type ferritin (Liang et al., 2014). It was found that Dox-loaded ferritin nanoparticles were specifically bound and subsequently internalized into HT-29 tumor cells via interaction with overexpressed TfR1 and released Dox in the lysosomes. In vivo in the mice bearing HT-29 tumors, Dox-loaded ferritin nanoparticles exhibited more than 10-fold higher intratumoral drug concentration than free Dox and significantly inhibited tumor growth after a single-dose injection. Since the ferritin nanocarrier has well-defined morphology and does not need any ligand modification or property modulation, it can be easily produced with high purity and yield (Liang et al., 2014). All these unique properties make the ferritin nanocage without modification an ideal vehicle for efficient

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antitumor drug delivery.

Recently, surface-modified ferritin, a protein-based nanoparticle, has been reported to serve as an efficient photosensitizer delivery vehicle (Zhen et al., 2013). It was found that RGD4C-modified ferritins can encapsulate a large amount of zinc hexadecafluorophthalocyanine ($ZnF_{16}Pc$), a potent but rather hydrophobic photosensitizer, and selectively deliver it to tumors to induce efficient PDT against cancer. However, the exact mechanism behind the heavy loading, especially the mechanism by which large molecules $ZnF_{16}Pc$ diffuse into the inner cavity, is still unclear because the size of $ZnF_{16}Pc$ molecules are much greater than that of ferritin channels. Ferritin has been also reported to encapsulate another traditional photosensitizer, methylene blue (MB) by the above mentioned pH-controlled reversible process. The MB-containing nanocomposites were taken up by the MCF-7 cells within an hour of mixing (Yan et al., 2010). Moreover, a NIR dye called new cyanine green, IR820 has been reported to be loaded within the cavity of ferritin, and resulting ferritin composites has strong absorbance in the NIR region, due to the strong interaction between IR820 dye and ferritin, for photoacoustic/fluorescence multimodal imaging-guided photothermal therapy (Huang et al., 2014).

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CHALLENGES FOR FERRITIN AS A VIHICLE FOR ECAPSULATION AND DELIVERY OF BIOACTIVE NUTRIENTS OR DRUG

A human ferritin receptor has been identified to be transferrin receptor-1 (TfR-1; Li et al.,

2010). It was observed that TfR-1 is specific for H ferritin but not for L ferritin. Interestingly, transferrin only partially inhibited H ferritin binding to TfR-1 indicating the ferritin and transferrin-binding sites do not overlap. Ferritin enters the endosome or lysosome by endocytosis. Since H-type subunits in phytoferritin only share ~40% sequence identity with the animal H-subunit, and contain 24 extra EP domains, so future work is needed to investigate whether or not TfR-1 is also suitable for plant ferritin. Another study indicated that the existence of a specific ferritin receptor in Caco-2 cells and inhibitors of endocytosis reduced iron uptake by the cells. The results support a model of soybean seed ferritin endocytosis through the apical cell membrane by an AP2-mediated endocytic pathway, followed by protein cage degradation (San Martin et al., 2008). If ferritin can escape from the stomach, it could be absorbed by Caco-2 cells in the gut by an AP2- or TfR-1-mediated endocytic pathway, corresponding to the second pathway (Kalgaonkar and Lönnerdal, 2009).

The digestive stability of ferritin has been a major interest when developing ferritin iron as potential iron supplements. If ferritin cannot survive meal preparation and gastrointestinal digestion to a significant extent, any ferritin absorption pathway would contribute little to human iron nutrition. However, recent report found that ferritin is not stable in the stomach, and most of ferritin molecules can be degraded by pepsin at pH 2.0 in this region (Hoppler et al., 2008). Although most in vitro digestion studies have used a pH of 2 for the pepsin digestion because this has been assumed to be the pH of adult stomach (Engle-Stone et al., 2005; Glahn et al., 2002;

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Yun et al., 2004), several recent studies on stomach aspirates from adult human subjects show that the postprandial pH is around 4, even up to 3 h after a meal (Kalantzi et al., 2006; Simonian et al., 2005). Therefore, it is crucial for ferritin as a novel class of vehicle for encapsulation and delivery of bioactive nutrients to improve its stability in the staomach at moderately low pH. Phytoferritin is usually composed of H-1 (26.5 kDa) and H-2 (28.0-kDa) subunits. Previous studies showed that H-2 is more resistant toward proteolysis compared with H-1 (Masuda et al., 2001). It has been established that pea seed ferritin (PSF) contains more H-2 subunits than SSF (Li et al., 2009). Therefore, it is possible that the ferritin consisting of either higher amounts of H-2 subunits or only H-2 subunits likely shields more ferritin from digestion. Recently, by using the self-assembly properties of ferritin, a series of soybean seed ferritins were prepared, and their digestive stability were evaluated *in vitro* at pH 4.0. Results showed that the digestive stability of recombinant soybean seed ferritin depends on their H-2/H-1 ratios; namely, ferritin with a higher H-2/H-1 ratio exhibits a stronger digestive stability at pH 4.0 (Lv et al., 2015). Moreover, an alternative strategy to improve the stability of phytoferritin has been reported; namely, binding of proanthocyanidins (PAs) to phytoferritin could prevent protein from degradation by the protease(s) (Deng et al., 2011). Such binding can significantly inhibit the degradation of SSF by the protease(s) in simulated gastric fluid (SGF) at pH 4.0. The condition of pH 4.0 may reflect that of the stomach contents of infants and young children (Deng et al., 2011), so PAs might be used as a natural food additive to prevent SSF from degradation by pepsin in their stomach.

Similarly, the stability of SSF in simulated intestinal fluid (SIF) was also increased upon treatment with PAs. These findings raise the possibility that the bioaccessibility of phytoferritin to the gastrointestinal tract was improved in the presence of PAs (Deng et al., 2011). Moreover, it has been reported that tannic acid and epigallocatechin gallate (EGCG) can induce ferritin association (Li et al., 2012; Wang et al., 2014). The hydrogen bond and hydrophobic interaction may be two main factors responsible for the interaction between them. It was also found that ferritin association induced by these small molecules can further improve the digestive stability of ferritin in *vitro* at pH 4.0, but the evidence *in vivo* has been lacking.

A reduced form of nicotinamide-adenine dinucleotide (NADH) is also widely distributed in foodstuffs. Recent studies have demonstrated that NADH molecules bind on the surface of ferritin shell close to the 4-fold channel of pea seed ferritin (PSF), which is 1.58 nm from the tryptophan residues calculated by fluorescence resonance energy transfer (Lv et al., 2013). The interaction between them has been ascribed to van der Waals interactions or hydrogen bonds, as suggested by isothermal titration calorimetry (ITC) measurement. Unfortunately, the inhibitory activity of NADH against ferritin degradation by pepsin has not been reported.

Recent studies have assessed the effect of cooking and skim milk on ferritin stability *in vivo* (Lv et al., 2015). Suckling rat pups were used to evaluate the digestive stability of HoSF in the presence of skim milk *in vivo*. It was found that horse spleen ferritin (HoSF) was not detected in the gut of rat pups after 1 h of digestion. However, HoSF was detectable in the intestine of the rat

pups. Different from *in vitro* digestion, heat treatment affected the digestive stability of HoSF *in vivo* by leading to reduced amounts of HoSF in intestine. Moreover, the presence of a food matrix (skim milk) may play a protective role for HoSF during digestion. This view is consistent with a previous study showing that casein, α-lactalbumin and β-lactoglobulin in skim milk were detectable *in vivo* even after 4 h of digestion (Miranda et al., 1983). The protective function of skim milk for ferritin might stem from the interactions between HoSF and molecules in skim milk, which can stabilize the ferritin structure. However, the mechanism behind this remains unknown. Similar results were obtained for HoSF mixed with skim milk and heated in boiling water for 30 min before digestion. Further, even after 2 h of digestion, HoSF was still detectable in the intestine of rat pups. Thus, it appears that most of HoSF can survive digestion in vivo, and ingestion of ferritin (HoSF) together with other proteins (skim milk) may improve its digestive stability.

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CONCLUSIONS AND PERSPECTIVES

The structure of ferritin is source-dependent, but the shell-like structure is highly conserved among all known ferritins. Using ferritin shell-like structure in combination with its disassembly and reassembly property controlled by pH has proven to be highly interesting for the encapsulation and deliver of bioactive nutrient or anticancer drug due to its safety, high symmetry, solubility and stability, monodispersity, and ease of genetic and chemical

manipulation. Upon ferritin encapsulation, the water-solubility, thermal stability, photo stability, and cellular uptake activity of these small bioactive compounds were markedly improved. All these findings have demonstrated that ferritin represents a novel alternative dietary source for encapsulation of bioactive nutrients. However, no single protein-cage architecture has all the requisite properties of size, shape, chemical/thermal stability, and functional-group presentation. One approach to solving this problem is to find more naturally occurring ferritin-like cages or non-native ferritin cages with a different size or shape by genetic or chemical modification. Moreover, the stability of ferritin needs to be improved to escape from gastric digestion. Although many combinations between proteins and bioactive nutrients or drug have been achieved, both the yields and stability of the resulting ferritin and bioactive compound complexes were not satisfactory, therefore finding improved synthetic procedures with respect to protein stability, and scalability still is a continuing process.

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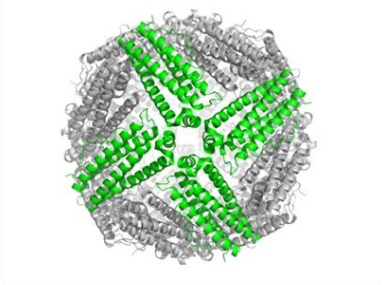
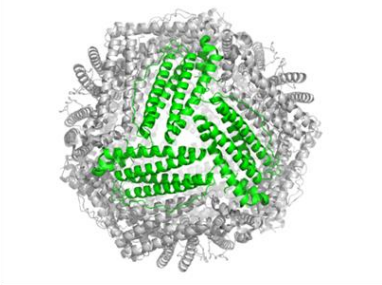
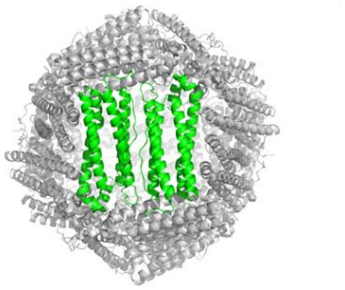
(A)



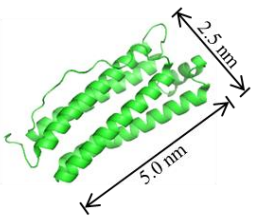
a. 4-Fold axis



b. 3-Fold axis

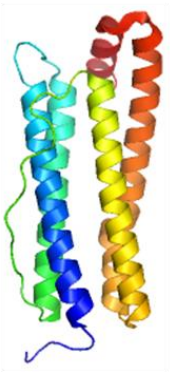


c. 2-Fold axis

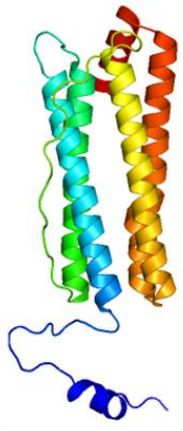


d. Ferritin subunit

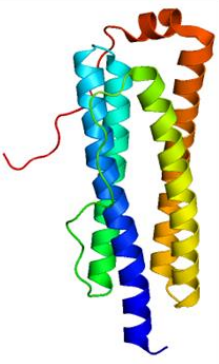
(B)



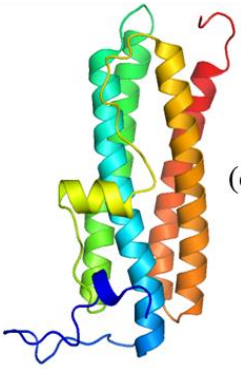
(a)



(b)



(c)



(d)

Figure 1 (A) Ferritin shell with views down the (a) 4-fold, (b) 3-fold and (c) 2-fold axes (channels) of the human H-chain ferritin shell, and the structure of its subunit (d). (B) The subunit structure of horse L-chain ferritin (a), soybean seed H-4 ferritin (b), haem-containing bacterial ferritin (BFR) (c), and Dps protein (d).

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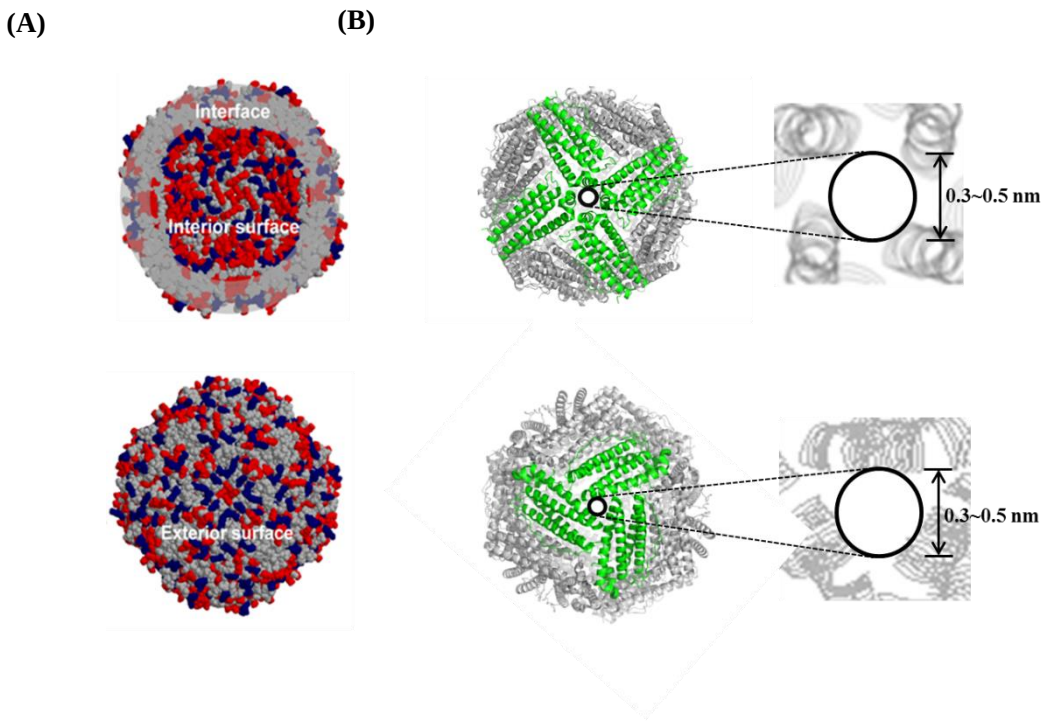

Figure 2 (A) Ferritin has high negative charge (red) density on its interior surface, while the net charge of its exterior surface is close to zero or slightly positive at pH 7.0. (B) The porn size of ferritin 3-fold and six 4-fold channels is between 0.3–0.5 nm. Figure was re-drawn according to Yang et al., 2014a.

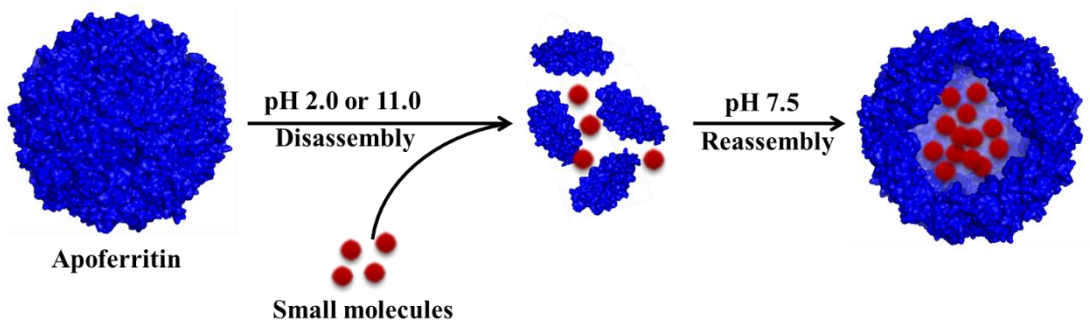

Figure 3 Encapsulation of small bioactive compounds within apoferritin by taking advantage of its reversible disassembly and reassembly characteristic controlled by pH.

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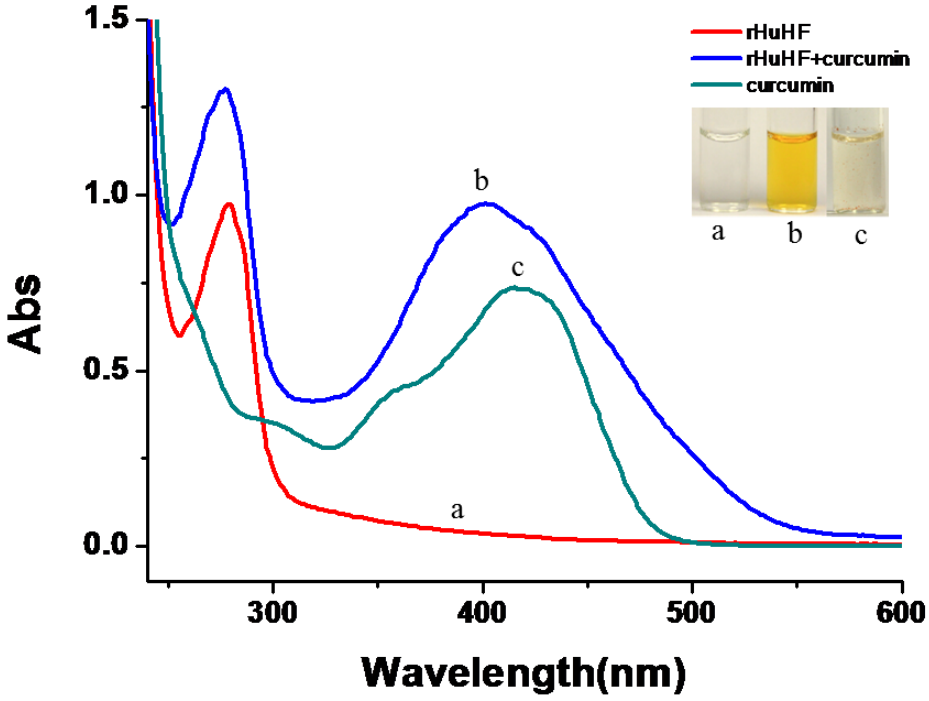

Figure 4 The water-solubility of curcumin was greatly improved upon encapsulation within the inner cavity of rHuHF. Inset: Pictures of rHuHF alone (a), rHuHF-encapsulated curcumin (b), and free curcumin simply mixed with deionized water (c). *Source*: Chen et al., 2014b.

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