




## Review

# Ferritins as natural and artificial nanozymes for theranostics

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Received: 2019.08.31; Accepted: 2019.09.23; Published: 2020.01.01

## Abstract

Nanozymes are a class of nanomaterials with intrinsic enzyme-like characteristics which overcome the limitations of natural enzymes such as high cost, low stability and difficulty to large scale preparation. Nanozymes combine the advantages of chemical catalysts and natural enzymes together, and have exhibited great potential in biomedical applications. However, the size controllable synthesis and targeting modifications of nanozymes are still challenging. Here, we introduce ferritin nanozymes to solve these problems. Ferritins are natural nanozymes which exhibit intrinsic enzyme-like activities (e.g. ferroxidase, peroxidase). In addition, by biomimetically synthesizing nanozymes inside the ferritin protein shells, artificial ferritin nanozymes are introduced, which possess the advantages of versatile self-assembly ferritin nanocage and enzymatic activity of nanozymes. Ferritin nanozymes provide a new horizon for the development of nanozyme in disease targeted theranostics research. The emergence of ferritin nanozyme also inspires us to learn from the natural nanostructures to optimize or rationally design nanozymes. In this review, the intrinsic enzyme-like activities of ferritin and bioengineered synthesis of ferritin nanozyme were summarized. After that, the applications of ferritin nanozymes were covered. Finally, the advantages, challenges and future research directions of advanced ferritin nanozymes for biomedical research were discussed.

Key words: Ferritins, nanozymes, theranostics

## 1. Introduction

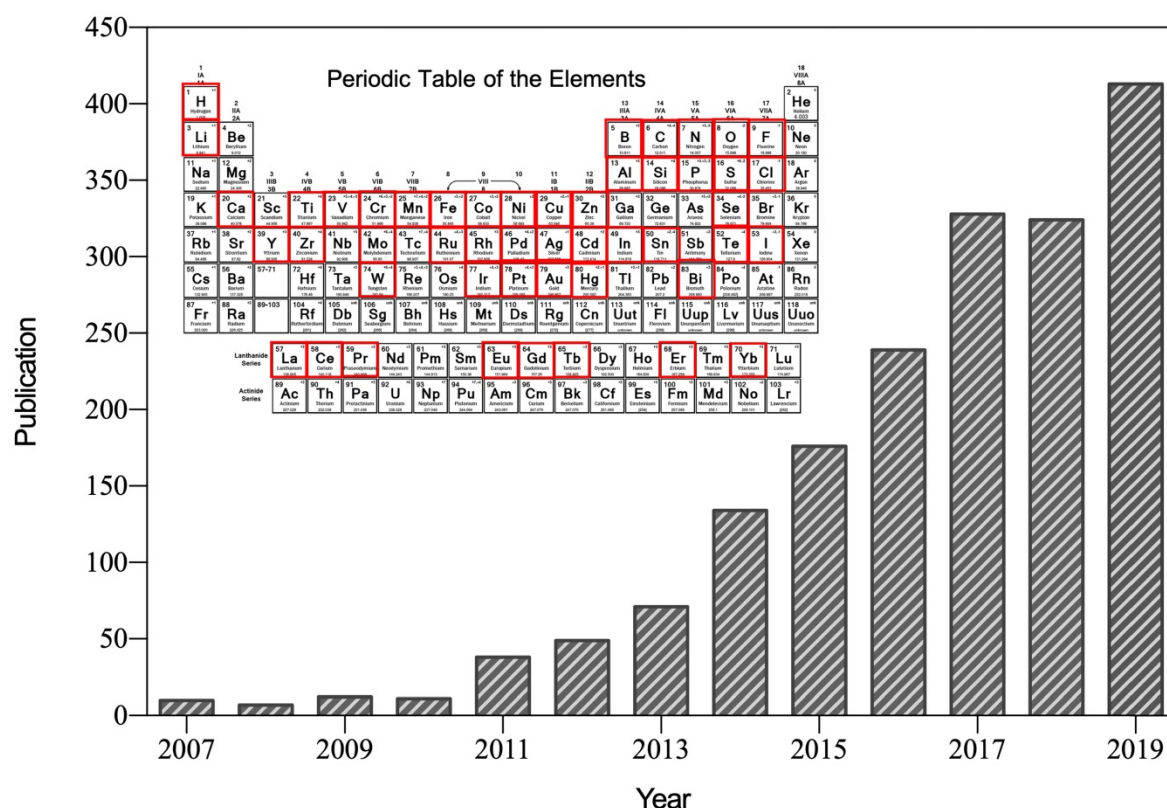
After the first reported suggesting that ferromagnetic (Fe<sub>3</sub>O<sub>4</sub>) nanoparticles possess intrinsic peroxidase-like activity in 2007 [1], nanozyme was specifically referred to the nanomaterials with intrinsic enzyme-like characteristics (Figure 1). Nanozymes can catalyze the substrates of natural enzymes under mild conditions, and possess similar catalytic capability and reaction kinetics as natural enzymes [2]. Compared with natural enzymes, nanozymes possess higher catalytic stability, more economic and ease of large-scale preparation [2, 3]. In addition, nanozymes possess inherent nanomaterial

properties, such as light, magnetism, heat, and so on, which are able to be designed to suitable for specific biological microenvironment and external stimulations [4]. The superiorities of nanozymes over natural enzymes attracted many scientists devoting to the research filed of nanozyme. Emerging studies have since then revealed that various nanomaterials (such as metal and metallic oxide nanoparticles, carbon nanoparticles, metal-organic frameworks (MOFs), and so on) exhibit enzyme-like activities [5]. Nanozyme is regarded as a new material and next-generation artificial enzyme, and the concept of

nanozyme is well accepted now. More than 350 research laboratories from 30 countries have been working on nanozymes, and more than 540 types of nanozymes synthesized from 49 elements have been reported (Figure 1). By bridging the nanotechnology and biology, nanozymes have emerged as novel promising agents for disease theranostic strategies [6].

However, size-controllable synthesis and targeting modifications of nanozymes are still challenging in the theranostic applications. Typically, the enzyme-like activity of nanozymes is size-dependent. The smaller size is, the larger specific surface area of nanozymes is, and the higher relative enzyme-like activity of nanozymes is [1]. Thus, size-controllable synthesis of nanozymes is important for stabilizing the theranostic effect of nanozymes. Moreover, when nanozymes are used for disease-targeted theranostics, nanozymes are typically chemically modified with targeting motifs, such as antibodies [7]. This chemical coupling procedure is practicable under the premise of that nanozymes possess amidogen, carboxyl or sulfhydryl on its surface. However, some nanozymes (such as Prussian blue) are not suitable for chemical group modification [8]. In addition, these chemical conjugates require complicated multi-step chemical reactions and expensive reagents [9]. Thus, targeting modifications of nanozymes need new strategies.

The emergence of ferritin nanozymes provides a promising strategy to solve these challenges. Ferritin, an iron storage protein nanocage, is a natural nanomaterial composed of organic and inorganic components, which possesses constant nanosized cavity and intrinsic enzyme-like activities for biomineralization. Therefore, ferritin can be used as nanozyme carrier or reactor for size-controllable synthesis of nanozymes. Moreover, ferritin nanocages possess both passive and active tumor targeting abilities, which have been widely used for tumor targeted theranostics [10, 11]. In recent works, we identified that ferritin can target tumor tissues by recognizing transferrin receptor 1 (TfR1) which is highly expressed in tumors [9, 12-15]. Taking the advantage of biomineralization process of ferritin, we synthesized different types of metallic oxide nanozymes in the cavity of ferritin with a confined size [12, 16, 17]. Thus, ferritin nanozyme can be used for tumor targeted theranostics. Through genetic or chemical approaches, we modified ferritin nanocages with targeting moieties to endow different targeting abilities of ferritin nanozymes [16]. Ferritin nanozymes possess the advantages of uniform size, modifiable targeting ability, good compatibility, thus ferritin nanozyme is a superior strategy for size-controllable synthesis and targeting modifications of nanozymes.



**Figure 1.** Number of published papers on nanozyme research included by the Web of Science by the end of August 2019. Until now, more than 49 elements have been employed to synthesize nanozyme.

Here, in this review, we systematically introduced the concept and development of ferritin nanozyme, as well as the recent research progress of ferritin nanozyme. In detail, we summarized the natural enzyme-like activities of ferritin, ferritin as a nanoreactor to fabricate various nanozymes with a confined size and endow nanozymes with different targeting abilities, and the disease-targeted biomedical applications of ferritin nanozymes. Finally, the challenges and perspectives are discussed for future exploration in this field. We expect to find the rules for the synthesis of different kinds of nanozymes inside the ferritin nanocage through learning the way of biomineralization process of ferritin. Moreover, we expect to design new kinds of ferritin nanozymes to better adapt the complicated biological microenvironment and intelligent response to specific stimulations.

## 2. Ferritins as natural nanozymes

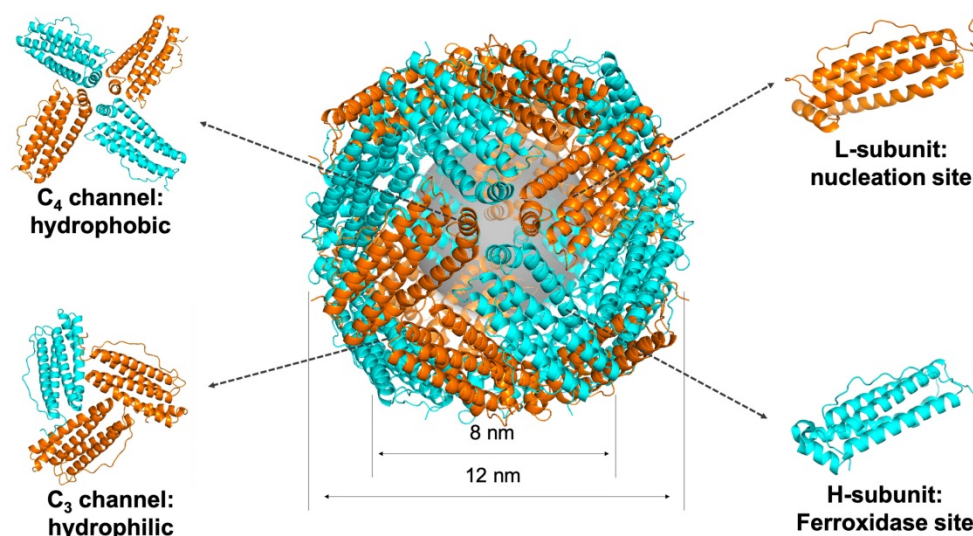
### 2.1 The structure of ferritin

Ferritin is a spherical iron storage protein which is widely existed in organisms. In 1937, ferritin was first isolated from horse spleen [18], since then, ferritin has been found in humans, animals, plants, fungi and bacteria [19]. The amino acid sequence of ferritin from different species varies a lot, while the protein structure keeps consistent [20]. Naturally, 24 ferritin subunits self-assembled into a spherical protein cage, with inner and outer diameter of 8 and 12 nm, respectively (Figure 2). Ferritin protein cage encapsulates up to 4500  $\text{Fe}^{3+}$  atoms into its cavity in the form of iron core. The iron core of ferritin consists of a ferric-oxyhydroxide-phosphate complex [21]. Typically, the phosphate contained in the complex is

dissolved when it is isolated out of the protein cage by heat or denaturants treatment, and the remaining product is ferrihydrite,  $5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$  [22]. After removing the mineral core located in the cavity, we can get the ferritin protein shell, called apoferritin.

Each subunit of ferritin consists of a bundle of four long helices, a short helix, and a long loop [20]. The N-terminal of each subunit exposes on the outer surface of ferritin nanocage, while the C-terminal folds into the inner cavity [19]. The ferritin nanocage possesses six  $\text{C}_4$  channels and eight  $\text{C}_3$  channels, which form the symmetrical structure of ferritin protein cage [22] (Figure 2). The  $\text{C}_3$  channels show hydrophilic property, allow metal ions, like  $\text{Fe}^{2+}$  ion, and  $\text{H}_2\text{O}$  molecule to enter the cavity. The  $\text{C}_4$  channels show hydrophobic property, allowing  $\text{O}_2$  or small organic molecules to enter the cavity [20].

Ferritin subunits self-assembled into a spherical shell through the hydrophobic interaction [20]. There are many hydrogen bonds and salt bridges within the subunits [23], so that ferritins stay stable even at high temperature environment and tolerate many denaturants, such as guanidine hydrochloride and urea. Under the condition of high concentration of guanidine hydrochloride (6M) or urea (8M), ferritin protein shell is disassembled. After removing these denaturants, ferritin subunits will re-assemble into a protein shell [24]. Under the environment of extreme acid (pH 2-3) or extreme alkaline (pH 10-12), ferritin protein shell disassembles. When it recovers to the physiological environment, ferritin subunits will also re-assemble into a protein shell [25]. These characteristics of ferritin make it a useful platform as nanocarrier or nanoreactor for biomedical applications.



**Figure 2.** The structure of ferritin. Ferritin is a 24-subunit oligomer with a combination of H-subunit (ferroxidase site) and L-subunit (nucleation site) that self-assemble into a spherical symmetrical protein shell, with inner and outer diameter of 8 and 12 nm, respectively. Eight hydrophilic channels and six hydrophobic channels are formed on the protein shell. Ferrihydrite core is synthesized in the cavity (PDB entry 5N27).

Mammalian ferritins, like human ferritin, typically consist of two different types of subunits, H-subunit and L-subunit (Figure 2). The molecular weights of H-subunit and L-subunit are 21 kDa and 19 kDa, respectively. There are evidences that H-subunits and L-subunits have cooperative roles in the iron-storage mechanism of human ferritin [26].

## 2.2 Ferritin protein shell shows ferroxidase-like activity

In fact, ferritin as a natural nanomaterial is also a kind of nanozyme, which possesses intrinsic ferroxidase-like activity. In 1973, it was firstly found that horse spleen apoferritin (HSAF) possesses catalytic activity, which catalyzes the oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  with molecular  $\text{O}_2$  as electron acceptor where other proteins have no such effect [27]. Soon afterwards, it was reported that ferritin H-subunit contains a ferroxidase site [28], which catalyzes the oxidation of ferrous ion into ferric iron rapidly, whereas the L-subunit doesn't have. L-subunit facilitates the iron oxidation at ferroxidase site and promotes oxidation on the mineral surface once the iron binding capacity of the ferroxidase site was exceeded, thus L-subunit facilitates the nucleation of ferrihydrite [29]. Thereby, in mammal ferritin, H-subunit rich ferritins exhibit fast iron incorporation rate, which can rapid catalyze the oxidation of toxic  $\text{Fe}^{2+}$  into nontoxic  $\text{Fe}^{3+}$ , are suitable for high metabolic activity tissues, such as heart and brain. Whereas L-subunit rich ferritins exhibit lower iron incorporation rate, but store more iron. This characteristic is found in iron-rich tissues, such as liver and spleen.

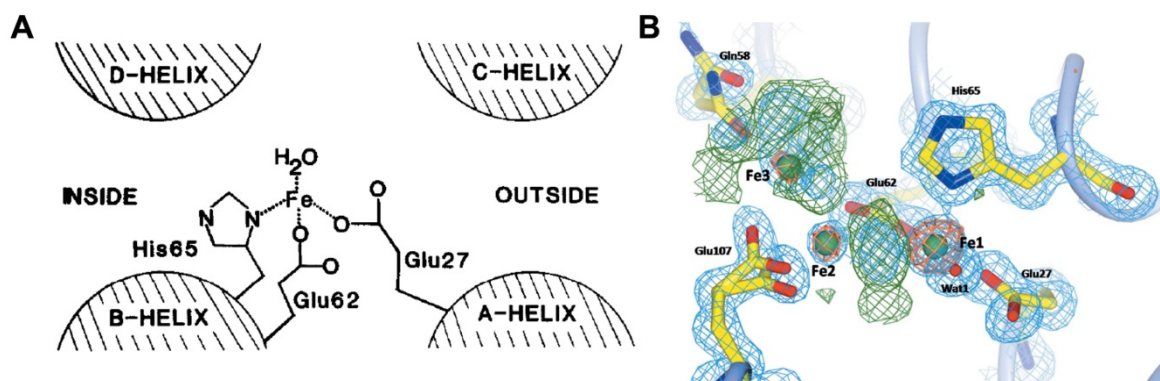
Owing to the high ferroxidase-like activity of ferritin H-subunit, the iron-core formation within ferritin is much faster than the formation of ferric oxyhydroxide under protein-free conditions [20]. The ferroxidase site of human H-subunit mainly consists of three amino acid residues (Glu 27, Glu 62, His 65)

[30] (Figure 3 A). These three residues are conserved in most known H-subunit sequences from different species, but are absent in L-subunit sequences [19]. It is reported that ferritin H-subunit lost its ferroxidase-like activity after mutation of two amino acid residues, E62K and H65G (using amino acids of L-subunits to replace H-subunits on 62 and 65) [30]. Later on, in 1996, Harrison and Arosio presented a di-iron ferroxidase center model, which indicated that there were two iron bind sites ( $\text{Fe}_A$  and  $\text{Fe}_B$ ) in the ferroxidase center [20], and they showed that the inner surface amino acid residues Glu 62, Glu 107, Tyr 34, Gln 141 formed the second iron bind site ( $\text{Fe}_B$ ) in the ferroxidase center [31]. Furthermore, it was reported that Gln 58 may also possess iron bind ability [32] (Figure 3B). Moreover, the inner surface Glu residues of human ferritin H-subunit located nearby the ferroxidase site contribute to the deposition of iron core, which is supported by the results that mutation of both ferroxidase site and inner surface Glu residues in human ferritin H-subunit resulted in completely disappearance of iron oxidation and nucleation [33].

Ferritin isolated from microorganisms, such as bacterial ferritins, is similar to that of human ferritin H-subunit, with subunit molecular weight around 20 kDa [19]. These ferritins also possess ferroxidase site like human ferritin H-subunit, which can catalyze the oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  and store iron in the cavity of ferritin [34].

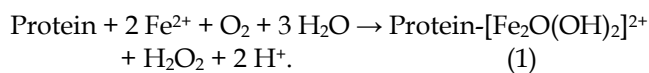
The possible procedure of iron-core formation inside the ferritin nanocage contains 4 steps [20, 22, 28, 29, 31, 35, 36].

1.  $\text{Fe}^{2+}$  ion traversing the ferritin protein shell. Due to the hydrophilic property of  $\text{C}_3$  channels on the surface of ferritin nanocage,  $\text{Fe}^{2+}$  ions can freely enter the cavity of ferritin nanocage through  $\text{C}_3$  channels and then move to the ferroxidase site.

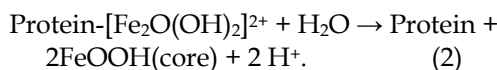


**Figure 3.** The ferroxidase site of ferritin H-subunit. (A) The proposed ferroxidase center in recombinant H-subunit ferritin. Reprinted with permission from [30]. Copyright 2015 Federation of European Biochemical Societies. (B) Electron density in the Human ferritin H-subunit oxidoreductase site after 1 min of free diffusion of iron into the crystals. Reprinted with permission from [32]. Copyright 2015 International Union of Crystallography.

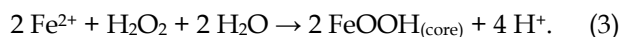
2.  $\text{Fe}^{2+}$  ion oxidation. After reaching to the ferroxidase site of ferritin H-subunits,  $\text{Fe}^{2+}$  ions bind to the ferroxidase catalytic site, then oxidation occurred in the presence of  $\text{O}_2$  resulting in the generation of  $\text{H}_2\text{O}_2$  [36].



3. The oxidized  $\text{Fe}^{3+}$  atoms move to the nucleation site of the cavity, hydrolyze and form the mineral core [35].



4. Once the iron core nuclei have reached 100 or more  $\text{Fe}^{3+}$  atoms,  $\text{Fe}^{2+}$  ions directly oxidize and deposit on the surface of the mineral core in the presence of  $\text{H}_2\text{O}_2$  [35]. So that, along with the consumption of  $\text{Fe}^{2+}$  ions and  $\text{H}_2\text{O}_2$ , the size of the mineral core increases constantly. Finally, a stable ferrihydrite mineral core is formed under the confined size of the ferritin cavity.

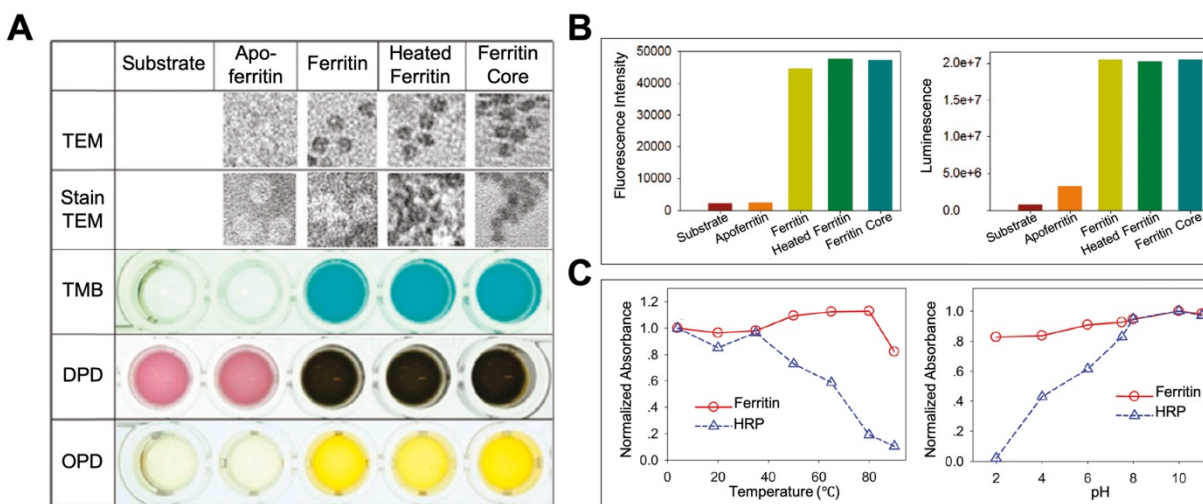


### 2.3 Natural isolated ferritins show other enzyme-like activities

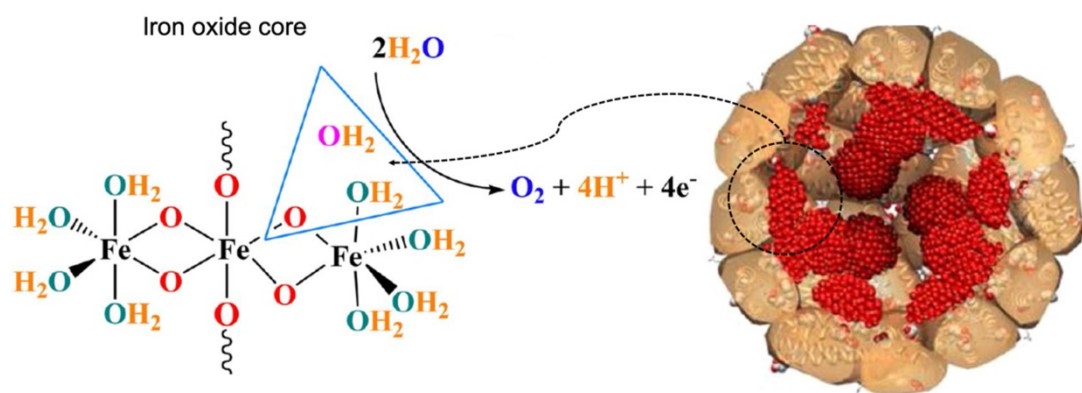
By exerting its ferroxidase-like activity, ferritin plays an important role in antioxidation and detoxification metabolisms [31]. Some studies also showed that except for the iron binding and ferroxidase-like activity, ferritin also showed peroxidase-like activity [37-41], which may endow ferritin more functions in modulating the redox metabolism. In 1997, Arapova, G. S. *et al* first found that horse spleen ferritin possesses intrinsic peroxidase activity, which catalyzes the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB), ortho-tolidine,

and ortho-phenylenediamine (PDA) in the presence of  $\text{H}_2\text{O}_2$  at 20 °C [37]. In 2011, Tang *et al* also confirmed this phenomenon by proving that horse spleen ferritin catalyzes the oxidation of TMB, o-phenylenediamine (OPD), N,N-dimethyl-p-phenylenediamine (DPD) (Figure 4 A), p-hydroxyphenylpropionic acid (p-HPPA), and luminol (Figure 4 B) in the presence of  $\text{H}_2\text{O}_2$  at 50 °C for 15 minutes [41]. They found that horse spleen ferritin still exhibits high peroxidase activity even at high temperature (over 80 °C) or extreme acid environment (pH 2.0). The thermal stability and pH tolerance of the peroxidase activity of horse spleen ferritin are superior to those of horseradish peroxidase (HRP) (Figure 4 C). In 2012, Violetta Borelli *et al* reported that ferruginous bodies, of which human lung ferritin is the main proteinaceous component, possess peroxidase-like activity [40]. These studies indicate that natural ferritins isolated from different spices exhibit intrinsic peroxidase activity [37-41].

Interestingly, Arapova, G. S. *et al* showed that the peroxidase activity of ferritin was decreased after thermal inactivation and detergents treatment, which indicated that the peroxidase activity of ferritin was derived from ferritin protein shell [37]. Whereas, Tang *et al* showed that the peroxidase-like activity of horse spleen ferritin was derived from its ferric nanocore but not the ferritin protein shell [41]. Which component of ferritin, iron core or protein shell, is responsible for the peroxidase activity needs further clarification. These catalytic activities of ferritin, derived from whether protein shell or iron core, inspired us to utilize ferritin as a nanoreactor or nanocarrier for biomedical applications by exerting its catalytic activities.



**Figure 4.** The peroxidase activity of ferritin. (A) TEM images of ferritin samples and the analysis of its peroxidase-like activity using TMB, DPD, and OPD as substrates at 50 °C. (B) Analysis of peroxidase-like activity of ferritin using p-HPPA (left) and luminol (right) as substrates. (C) The comparison of thermal stability (left) and pH tolerance (right) of peroxidase activity between ferritin and HRP. Reprinted with permission from [41]. Copyright 2011 American Chemical Society.



**Figure 5.** Water oxidation catalyzed by the iron oxide core of horse spleen ferritin. Reprinted with permission from [42]. Copyright 2019 Springer Nature.

Apart from ferroxidase- and peroxidase-like activities, ferritin also exhibits other types of catalytic activities. Recently, it was reported that horse spleen ferritin was an iron-based catalyst which catalyzed water oxidation and released molecular oxygen under alkaline environment (pH 11.0) [42] (Figure 5). The authors declared that iron oxide core of horse spleen ferritin responded for this catalytic activity. When iron inside the horse spleen ferritin nanocage was consumed by bacteria, its water oxidation activity was reduced.

Horse spleen ferritin was also reported being a photocatalyst which catalyzes the synthesis of gold nanoparticle on the surface of horse spleen ferritin. The authors regarded that the outer surface of horse spleen ferritin possessed a putative nucleation site which binds  $\text{Au}^{3+}$  ions [43]. The ferrihydrite mineral core of horse spleen ferritin possesses semi-conductor capacity [43]. Photochemical excitation of the ferrihydrite core transfers electrons across the horse spleen ferritin protein shell to photoreduce  $\text{AuCl}_4^-$  to form gold nanoparticles in the putative surface nucleation site of horse spleen ferritin [43].

### 3. Ferritins as artificial nanozymes

#### 3.1 Ferritin as a template to synthesis inorganic metal-based nanomaterials

Due to the unique fixed nanosized cavity structure and eight negatively charged hydrophilic channels on the protein shell,  $\text{Fe}^{2+}$  and other positively charged metal ions can enter the cavity of ferritin protein shell through hydrophilic channels freely. Then these metal ions can be catalyzed to form metallic oxide core at the ferroxidase site of the cavity. Taking advantages of these characteristics of ferritin, it would be an efficient approach to synthesize inorganic metal-based nanomaterials in a size-controllable manner by using ferritin as a template (Table 1).

A series of inorganic metallic oxide nanoparticles have been reported to be synthesized in the 8 nm cavity of ferritin by using ferritin as a nanoreactor (Table 1), including  $\text{Fe}_3\text{O}_4$  [12, 44, 45],  $\text{MnOOH}$  [46, 47],  $\text{In}_2\text{O}_3$  [48],  $\text{TiO}_2$  [49],  $\text{EuOOH}$  [49],  $\text{Co}_3\text{O}_4$  [16, 50],  $\text{Ni}(\text{OH})_3$  [51],  $\text{Cr}(\text{OH})_3$  [51],  $\text{FeS}$  [52],  $\text{CdS}$  [53],  $\text{PbS}$  [54],  $\text{ZnSe}$  [55],  $\text{FePO}_4$  [56] nanoparticles. The additional oxidizing agents, such as  $\text{H}_2\text{O}_2$  accelerates the oxidation of metal ions, and additional  $\text{NaOH}$  promotes the mineralization and nucleation.

Moreover, some metal ions can bind to the inner surface of ferritin nanocage through the electrostatic interaction with hydrophilic channels. In the presence of some reducing agents, such as ascorbic acid, these metal ions were reduced into metal atoms, and then aggregated into metal nanoparticles in the cavity of ferritin. Through this way, some metal or metal alloy nanoparticles have been synthesized in ferritin nanocages (Table 1), such as  $\text{Au}$  [57],  $\text{Ag}$  [58],  $\text{Cu}$  [58],  $\text{Pd}$  [59],  $\text{Co}$  [58, 60],  $\text{Ni}$  [58, 60],  $\text{Pt}$  [61],  $\text{CoPt}$  [62],  $\text{AuPd}$  [63].

When metal ions bind to the cavity of ferritin nanocages and phosphates are added to the solution, metal phosphates nanoparticles can be synthesized in the cavity of ferritin nanocages through the phosphorylation process. By this way, silver phosphate nanoparticles were synthesized in the cavity of HSAF nanocages with a controlled size [64].

Compared with other methods for the synthesis of nanoparticles, using ferritin as a nanoreactor to synthesize nanoparticles in its cavity has several advantages [48, 52]. (1) Uniform size. Owing to the confined size of inner cavity and the accurate catalysis of protein shell, the biomineralization of nanomaterials inside ferritin was controllable and efficient. The size of the synthesized nanoparticles was confined under 8 nm and uniform. (2) Monodisperse and good biocompatibility. The synthesized nanoparticles were nanocaged by ferritin protein shell, thus the monodisperse state, good biocompatibility and low biotoxicity of ferritin protein

shell shall not be affected. (3) The nanoparticles were synthesized inside the ferritin nanocage under near room temperature and mild environment, which differentiates from the normal chemical synthesis typically needing high temperature, high pressure and extreme acid or alkaline environment. (4) Ease of surface functional modifications. Through genetic or chemical approaches, functional motifs, such as targeting molecules, therapeutic drugs, can be easily modified onto ferritin nanocages. Thus, we can endow nanoparticles synthesized inside the ferritin nanocages with specific functions by surface functional modifications of ferritin protein shell.

### 3.2 Ferritin as a nanoreactor to synthesize nanozymes

Due to the superiority of ferritin nanocage as a nanoreactor to synthesize nanomaterials with controllable size, some studies employed ferritin to synthesize nanozymes in its protein cavity by different methods (Table 2) (Figure 6).

Metal ions oxidation (Figure 6 A) or reduction (Figure 6 B) in the cavity of ferritin was the most common method to synthesize ferritin nanozymes. Through two-step reduction reactions, some alloy metal-based ferritin nanozymes were also synthesized (Figure 6 C). Moreover, by directly oxidizing metal ions on the surface of ferrihydrite core in the cavity of ferritin, ferritin nanozymes were synthesized (Figure 6 D). In addition, through a pH-dependent disassembly-reassembly procedure of ferritin, some nanozymes were incorporated into the cavity of ferritin (Figure 6 E). In addition, some metal ions bound on the outer surface of ferritin protein cages. After the addition of reducing agents, metal nanozymes were synthesized on the outer surface of ferritin (Figure 6 F). Taken together, these ferritin nanozymes synthesized in different methods showed different kinds of enzyme-like activities and exerted different functions in applications.

**Table 1.** Summary of inorganic metal-based nanomaterials synthesized in ferritin.

Synthetic Methods	Mineral Core	Ferritin Type	Core Size	Reference	
Oxidation	Fe <sub>3</sub> O <sub>4</sub>	Recombinant Human HFn	4.7 ± 0.8 nm	[12]	
			5.6 ± 0.9 nm	[44]	
	MnOOH	Horse Spleen Apoferritin	5.9 ± 1.3 nm	[45]	
		Horse Spleen Apoferritin	7.15 - 7.80 nm	[46]	
		Recombinant Human HFn			
		Recombinant Human LFn			
		Horse Spleen Apoferritin	6.7 ± 1.7 nm	[46]	
	In <sub>2</sub> O <sub>3</sub>	Horse Spleen Apoferritin	6.6 ± 0.5 nm	[48]	
	TiO <sub>2</sub>	Horse Spleen Apoferritin	5.7 ± 1 nm	[49]	
	EuOOH	Horse Spleen Apoferritin	5.7 ± 1 nm		
	Co <sub>3</sub> O <sub>4</sub>	Horse Spleen Apoferritin	6 nm	[50]	
		Recombinant Horse liver LFn	6 nm		
		pFFn	5.7 ± 1.0 nm	[16]	
	Ni(OH) <sub>3</sub>	Horse Spleen Apoferritin	7 nm	[51]	
		Recombinant Horse liver LFn			
	Cr(OH) <sub>3</sub>	Horse Spleen Apoferritin	7 nm		
		Recombinant Horse liver LFn			
		FeS	Horse Spleen Apoferritin	7.8 nm	[52]
		CdS	Horse Spleen Apoferritin	4.0 ± 1.2 nm	[53]
		PbS	Horse Spleen Apoferritin	5 ± 2 nm	[54]
	ZnSe	Horse Spleen Apoferritin	3 nm	[55]	
	FePO <sub>4</sub>	Horse Spleen Apoferritin	8 nm	[56]	
Reduction	Au	Horse Spleen Apoferritin	6.3 ± 0.8 nm	[57]	
		Ag	4.0 ± 0.5 nm	[58]	
		Cu	3.0 ± 0.5 nm	[58]	
		Pd	2.0 ± 0.3 nm	[59]	
		Co	3.0 ± 0.5 nm	[58]	
			3.5 nm	[60]	
		Ni	Horse Spleen Apoferritin	3.5 ± 0.5 nm	[58]
			3 nm	[60]	
		Pt	Pig Spleen Apoferritin	4.3 ± 0.9 nm	[61]
		CoPt	Horse Spleen Apoferritin	4.1 nm	[62]
		AuPd	Recombinant Horse liver LFn	2.4 ± 0.3 nm	[63]
		Phosphatization	Silver Phosphate NPs	Horse Spleen Apoferritin	-

**Table 2.** Summary of ferritin nanozymes.

Ferritin Type	Nanozyme Core	Enzyme-like activity type	Nanozyme Core Size	Synthetic Methods	Applications	Reference
Recombinant Human LFn	Pt NPs	Ferroxidase	-	Reduction	Regulates iron homeostasis	[65]
Horse Spleen Apoferritin	Au NPs	Enhanced Ferroxidase Activity	3.03 ± 0.57 nm 4.21 ± 0.93 nm 4.84 ± 1.14 nm 3.34 ± 0.56 nm 2.81 ± 0.49 nm	Reduction	Improve <i>in vivo</i> iron uptake	[66]
	Ag NPs		2.37 ± 0.29 nm 3.70 ± 0.56 nm 5.92 ± 0.94 nm 5.69 ± 1.08 nm 2.71 ± 0.47 nm			
	Pt NPs		2.34 ± 0.33 nm 3.24 ± 0.56 nm 4.69 ± 0.84 nm 5.32 ± 1.06 nm 5.79 ± 1.04 nm			[67]
Horse Spleen Apoferritin	Fe <sub>3</sub> O <sub>4</sub> /γ-Fe <sub>2</sub> O <sub>3</sub>	Peroxidase	3.21 nm 6.83 nm	Oxidation	Detection of H <sub>2</sub> O <sub>2</sub>	[68]
Recombinant Human HFn	Fe <sub>3</sub> O <sub>4</sub>	Peroxidase	4.7 ± 0.8 nm 5.35 ± 0.95 nm 4.3 nm	Oxidation	Tumor diagnosis	[12] [69] [17]
Recombinant Human HFn	Co <sub>x</sub> Fe <sub>3-x</sub> O <sub>4</sub>	Peroxidase	5.53 ± 0.94 nm 5.54 ± 1.08 nm 5.81 ± 0.92 nm	Oxidation	Tumor diagnosis	[69]
pfFn	Co <sub>3</sub> O <sub>4</sub>	Peroxidase	5.7 ± 1.0 nm	Oxidation	Tumor diagnosis	[16]
Horse Spleen Ferritin	Prussian blue NPs	Peroxidase	-	Oxidation/ Deposition	Glucose Detection	[70]
Horse Spleen Apoferritin	Au clusters	Peroxidase	2 nm	Reduction	Glucose Detection	[71]
Horse Spleen Apoferritin	Fe atoms	Peroxidase	16 Fe atoms	Reduction	Glucose, Cholesterol, and Alcohol Detection	[72]
Horse Spleen Apoferritin	Fe-Pt NPs Fe-Pd NPs Fe-Rh NPs	Peroxidase	6 nm	Two-step Reduction		
pfFn	Ag NPs Pd NPs	Peroxidase	4.7 ± 1.59 nm 4.21 ± 1.39 nm	Reduction on the outer surface	Discoloration of nitrophenol and diazo dyes	[73]
pfFn	Pd Nanoclusters	Oxidase	5 ± 1 nm	Reduction	Catalyze the aerobic oxidation of alcohols	[74]
Horse Spleen Apoferritin	Nano-CeO <sub>2</sub>	SOD	4.5 nm	pH dependent Disassembly-Reassembly	Scavenging of intracellular ROS	[75]
Horse Spleen Apoferritin	Pt NP	Catalase Peroxidase	1.87 ± 0.40 nm	Reduction	pH, temperature dependent activity	[76]
Horse Spleen Apoferritin	Pt NPs	Catalase	2 nm	Reduction	Scavenging of intracellular ROS	[77]
Horse Spleen Apoferritin	Pt-Au alloy NPs	SOD	3 nm	Reduction	ROS	[78]
Horse Spleen Apoferritin	Au-Ag alloy NPs	SOD Catalase Peroxidase	-	Reduction	Cytoprotecting	[79]
Horse Spleen Apoferritin	Au-Ag alloy NPs	Catalyze the reduction of 4-nitrophenol	6.3 ± 1.0 nm 6.1 ± 0.7 nm 6.0 ± 0.8 nm 5.8 ± 0.8 nm 5.6 ± 0.6 nm	Reduction	Reduction of 4-nitrophenol	[80]
Recombinant Human HFn	Nitrogen-doped Porous Carbon Nanospheres (N-PCNSs)	Oxidase Peroxidase Catalase SOD	100 ± 10 nm	Chemical Coupling	Tumor therapy	[81]
Horse Spleen Apoferritin	[Cp*Ir(biot-p-L)Cl] Sav	Artificial Transfer Hydrogenase	4.5 nm · 5.5 nm · 5.1 nm	pH dependent Disassembly-Reassembly	Reduction of cyclic imines	[82]
Recombinant horse LFn	[Rh(nbd)Cl] <sub>2</sub>	Artificial metalloenzyme	-	Inner cavity binding site immobilization	Polymerization of phenylacetylene	[83]

### 3.2.1 Ferritin nanozymes with ferroxidase-like activity

Since the ferritin L-subunit lacks ferroxidase site, the recombinant L-subunit ferritin (LFn) doesn't possess the iron storage ability. In 2014, L. Li *et al* reported that recombinant human LFn can obtain ferroxidase-like activity by synthesizing Pt nanoparticles in its cavity through a reduction process

[65] (Figure 6 B). The synthesized LFn-Pt ferritin nanozymes can catalyze the oxidation of Fe<sup>2+</sup> ions and acquire the iron storage ability, thus regulating the iron homeostasis in a cellular environment [65].

In addition, ferritin nanozymes can be used for enhancing ferroxidase-like activity of ferritin. Through a reduction process, Au, Ag, Pt nanoparticles were synthesized in the cavity of HSAF. The obtained



HSAF-Au [66], HSAF-Ag [66], HSAF-Pt [67] ferritin nanozymes showed enhanced ferroxidase-like activity than that of HSAF. A series of experiments have showed that these nanozymes improved the iron uptake ability of ferritin *in vivo* [66, 67].

### 3.2.2 Ferritin nanozymes with peroxidase-like activity

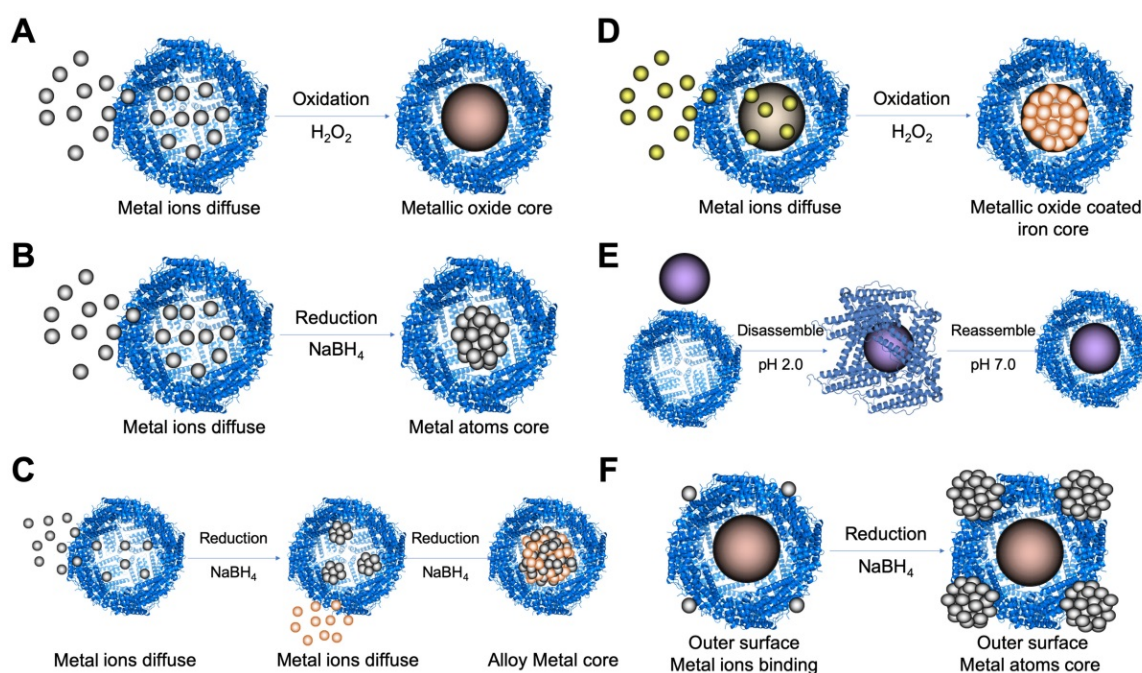
Some types of ferritin nanozymes were reported to show peroxidase-like activity. After the discovery of the peroxidase-like activity of  $\text{Fe}_3\text{O}_4$  nanoparticles [1], our group realized that the biomimetically mineralized  $\text{Fe}_3\text{O}_4$  core in the cavity of ferritin nanocages may also show peroxidase-like activity. Later, we synthesized  $\text{Fe}_3\text{O}_4$  core ( $4.7 \pm 0.8$  nm) in the cavity of recombinant human H-subunit ferritin (HFn) [12]. This HFn- $\text{Fe}_3\text{O}_4$  nanozyme was tested for tumor diagnosis by exerting its peroxidase-like activity [12].

Since then, many metal oxide nanoparticles, such as  $\text{Fe}_3\text{O}_4$  [17, 68, 69],  $\text{Co}_x\text{Fe}_{3-x}\text{O}_4$  [69],  $\text{Co}_3\text{O}_4$  [16] were reported to be biomimetically mineralized and deposited in the cavity of ferritin nanocages and showed peroxidase-like activity. By loading  $\text{Fe}^{2+}$  and  $\text{Co}^{2+}$  ions simultaneously, a series of  $\text{Co}_x\text{Fe}_{3-x}\text{O}_4$  mineral cores were formed in the cavity of HFn [69]. The size of these  $\text{Co}_x\text{Fe}_{3-x}\text{O}_4$  mineral cores were controlled around 6 nm with narrow distribution [69]. And these synthesized HFn- $\text{Co}_x\text{Fe}_{3-x}\text{O}_4$  ferritin nanozymes showed higher peroxidase-like activity than that of HFn- $\text{Fe}_3\text{O}_4$  nanozymes [69].

Furthermore, one of our works showed that

$\text{Co}_3\text{O}_4$  cores were formed in the cavity of *pyrococcus furiosus* ferritin (pfFn) by mimicking the iron loading process of ferritin [16] (Figure 6 A). We compared the catalytic activity of pfFn- $\text{Co}_3\text{O}_4$  and pfFn- $\text{Fe}_3\text{O}_4$  ferritin nanozymes by calculating the  $K_{\text{cat}}/K_M$ , which reflects the catalytic efficiency of an enzyme for a given substrate. The as-prepared pfFn- $\text{Co}_3\text{O}_4$  ferritin nanozymes showed much higher peroxidase-like activity than that of pfFn- $\text{Fe}_3\text{O}_4$  ferritin nanozymes, as the  $K_{\text{cat}}/K_M$  of pfFn- $\text{Co}_3\text{O}_4$  ferritin nanozymes was 20-fold higher than that of pfFn- $\text{Fe}_3\text{O}_4$  ferritin nanozymes for substrates TMB and  $\text{H}_2\text{O}_2$ , respectively [16].

Lucia Melnikova *et al* reported that  $\text{Fe}_3\text{O}_4/\gamma\text{-Fe}_2\text{O}_3$  nanozymes were biomimetically synthesized in the cavity of HSAF [68]. HSAF- $\text{Fe}_3\text{O}_4/\gamma\text{-Fe}_2\text{O}_3$  possesses peroxidase-like activity which catalyzes the decomposition of  $\text{H}_2\text{O}_2$  in the presence of *N,N*-diethyl-*p*-phenylenediamine sulfate (DPD) substrate [68]. By increasing the loading amount of  $\text{Fe}^{2+}$  ions, the size of  $\text{Fe}_3\text{O}_4/\gamma\text{-Fe}_2\text{O}_3$  magnetic core formed inside HSAF increased from 3.21 nm to 6.83 nm [68]. The authors showed that there was a linear dependence between peroxidase-like activity of HSAF- $\text{Fe}_3\text{O}_4/\gamma\text{-Fe}_2\text{O}_3$  and the size of  $\text{Fe}_3\text{O}_4/\gamma\text{-Fe}_2\text{O}_3$  magnetic core [68]. Apoferritin exhibits seldom peroxidase-like activity [68], which is consistent with the report of Tang *et al* [41].



**Figure 6.** The synthetic strategies of ferritin nanozymes. (A) Oxidation-based biomineralization of ferritin nanozymes. (B) Reduction-based synthesis of ferritin nanozymes. (C) Two-step reduction-based synthesis of ferritin nanozymes. (D) Oxidation and deposition-based synthesis of ferritin nanozymes. (E) pH-dependent disassembly-reassembly based synthesis of ferritin nanozymes. (F) Outer surface reduction-based synthesis of ferritin nanozymes. (PDB entry 5N27).

It was reported that  $[\text{Fe}(\text{CN})_6]^{4-}$  ions reacted with  $\text{Fe}^{3+}$  ions on the surface of iron oxide core of horse spleen ferritin can form Prussian Blue nanoparticles (PBNPs) on the surface of ferrihydrite within the ferritin cavity under acid environment (pH 3.0) [70] (Figure 6 D). Horse spleen ferritin-PBNPs ferritin nanozymes showed peroxidase-like activity similar with that of Prussian Blue coated  $\text{Fe}_2\text{O}_3$  magnetic nanoparticles, which catalyzes the peroxidase substrates TMB and 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to give a color reaction [70]. The peroxidase-like activity of horse spleen ferritin-PBNPs nanozymes is pH, temperature dependent and is well fits with the typical Michaelis-Menten kinetics for  $\text{H}_2\text{O}_2$ , TMB and ABTS substrates, respectively [70]. Horse spleen ferritin-PBNPs ferritin nanozymes exhibited high affinity and sensitivity to  $\text{H}_2\text{O}_2$  when ABTS was used as substrate, which makes horse spleen ferritin-PBNPs ferritin nanozyme a sensitive agent for glucose detection [70].

By loading metal ions into the cavity of ferritin, followed by a reduction process, some metal nanoparticles, such as Au clusters [71], Fe atoms [72] and Fe-Pt, Fe-Pd, Fe-Rh alloy nanoparticles (two-step reduction) [72] (Figure 6 C), were synthesized within the cavity of HSAF and showed peroxidase-like activity. HSAF-Au ferritin nanozyme possessed a 2 nm Au cluster, and exhibited higher peroxidase activity than that of natural enzyme under acidic pH and high temperature (over 60 °C) [71].

Wei Zhang *et al* reported that when  $\text{Fe}^{2+}$  ions bound to the inner cavity of HSAF, after adding reductant, 16 Fe atoms were reduced in the cavity of HSAF [72]. This HSAF-Fe ferritin nanozyme exhibited peroxidase-like activity and was used for the detection of  $\text{H}_2\text{O}_2$  [72]. By continuing loading other metal ions, such as Pt, Rh and Pd, mineral cores (Fe-Pt, Fe-Rh, Fe-Pd) were formed in the cavity of HSAF with narrow size distribution (around 6 nm) [72] (Figure 6 C). The synthesized HSAF-Fe-Pt, HSAF-Fe-Rh and HSAF-Fe-Pd ferritin nanozymes showed much higher peroxidase-like activity than that of HSAF-Fe ferritin nanozymes [72].

Some kinds of noble metal ions also anchored the outer surface of ferritin nanocages, thus formed non-coated metal nanoparticles possess enzymatic activity. For example, Marie Peskova *et al* reported that Ag and Pd ions bound to the outer surface of *Pyrococcus furiosus* ferritin (pfFn) [73]. By controlling the amount of metal ions and reductants in the reaction system, non-coated Ag and Pd nanoparticles were synthesized on the outer surface of pfFn with a confined size distribution ( $4.7 \pm 1.59$  nm for Ag,  $4.21 \pm 1.39$  nm for Pd) [73] (Figure 6 F). The synthesized

pfFn-Ag and pfFn-Pd ferritin nanozymes possess peroxidase-like activity and their enzymatic activity was impacted by detergents [73]. The peroxidase-like activity of pfFn-Ag ferritin nanozymes was only detected in the presence of sodium dodecyl sulfate (SDS), while peroxidase-like activity of pfFn-Pd ferritin nanozymes was enhanced by SDS and other anionic detergents [73].

### 3.2.3 Ferritin nanozymes with oxidase-like activity

S. Kanbak-Aksu *et al* reported that Pt nanoclusters with a size distribution of  $5 \pm 1$  nm were synthesized within the cavity of pfFn through a reduction process [74] (Figure 6 B). The synthesized pfFn-Pt ferritin nanozymes showed oxidase-like activity which catalase the aerobic oxidation of alcohols in the presence of  $\text{O}_2$  [74]. The excellent thermal stability and high hydrophilicity of pfFn makes pfFn-Pt ferritin nanozyme a reliable catalyst for aerobic oxidation of alcohols at high temperatures or other harsh environments.

### 3.2.4 Ferritin nanozymes with superoxide dismutase- (SOD-) like activity

Taken advantages of the pH dependent disassembly-reassembly feature of ferritin, some nanozymes, such as nano- $\text{CeO}_2$  [75], can be encapsulated in the cavity of ferritin nanocages. Under acid condition, pH 2.0, ferritin nanocages disassembled into subunits, once the pH return to physiological condition, ferritin subunits reassembled into a protein nanocage. Thus,  $\text{CeO}_2$  nanoparticles can be encapsulated into the cavity of HSAF nanocages during the reassembly process [75] (Figure 6 E). HSAF- $\text{CeO}_2$  ferritin nanozymes exhibited higher SOD-like activity which scavenging reactive oxygen species (ROS) than that of  $\text{CeO}_2$  nanozymes compared under the same concentration of Ce atom.

### 3.2.5 Ferritin nanozymes with multi-enzyme-like activities

Some kinds of ferritin nanozymes showed multi-enzyme-like activities. For example, Jia Fan *et al* reported that a  $1.87 \pm 0.40$  nm Pt nanoparticle was synthesized within the cavity of HSAF by reduction [76] (Figure 6 B). HSAF-Pt ferritin nanozymes exhibit peroxidase and catalase activities in a pH and temperature dependent manner [76]. In acidic pH, HSAF-Pt ferritin nanozymes mainly showed peroxidase-like activity, and the optimum pH was 4.0, same as that of natural HRP. In neutral and alkaline pH, HSAF-Pt ferritin nanozyme mainly showed catalase-like activity, and the catalase-like activity enhanced with the increase of pH (from pH 7.0 to pH 12.0) [76]. Both the peroxidase- and catalase-like

activities of HSAF-Pt ferritin nanozymes were maintained even when the temperature was over 60 °C [76]. Moreover, when the temperature increased from 20 °C to 80 °C, the catalase-like activity of HSAF-Pt ferritin nanozymes increased gradually, while the peroxidase-like activity of HSAF-Pt ferritin nanozymes decreased gradually [76]. In addition, the authors showed that HSAF alone, which is a protein shell without iron, did not possess peroxidase-like activity [76], indicating that the peroxidase-like activity comes from the inner Pt nanoparticles, not the ferritin protein shell. This conclusion is consistent with the report by Tang et al in 2011 [41].

Moreover, Lianbing Zhang *et al* showed that HSAF-Pt ferritin nanozymes exhibited both catalase- and SOD-like activities under pH 7.4 physiological environment [77]. The catalase- and SOD-like activities of HSAF-Pt ferritin nanozymes cooperated together to eliminate intercellular ROS. Later on, they synthesized a 3 nm PtAu alloy nanoparticle inside the HSAF by reduction, and they showed that HSAF-PtAu ferritin nanozymes possess higher catalase- and SOD-like activities than that of HSAF-Pt ferritin nanozymes [78].

Au-Ag alloy nanoparticles were also reported to be synthesized in the cavity of HSAF by reduction [79, 80]. Changing the ratio of Au atoms / Ag atoms in the reaction system did not affect the uniform size of Au-Ag alloy nanoparticles (around 6 nm) inside the HSAF [80]. The synthesized HSAF-AuAg ferritin nanozymes showed catalytic activity on the reduction of 4-nitrophenol [80]. Soon afterwards, it was reported that HSAF-AuAg ferritin nanozymes exhibited peroxidase-, catalase-, and SOD-like activities simultaneously, which was used for cytoprotecting [79]. The authors showed that these three kinds of enzyme-like activities mainly generated from Au-Ag alloy nanoparticles, while the HSAF protein shell accelerates the electron transfer and promote the catalytic process [79].

Kelong Fan *et al* reported that Nitrogen-doped Porous Carbon Nanospheres (N-PCNSs) possess four enzyme-like activities (oxidase, peroxidase, catalase and SOD) [81]. By chemical coupling recombinant Human HFn nanocages on the outer surface of N-PCNSs nanozymes ( $100 \pm 10$  nm), a ferritin nanozyme (HFn-N-PCNSs) was synthesized [81].

### 3.2.6 Ferritin nanozymes with other enzyme-like activities

Some artificial metalloenzymes were also reported to be encapsulated or in site synthesized in the cavity of ferritin nanocages. For example, Artificial metalloenzyme  $[\text{Cp}^*\text{Ir}(\text{biot-p-L})\text{Cl}]$  Sav (with a size distribution of 4.5 nm · 5.5 nm · 5.1 nm)

was encapsulated in the cavity of HSAF by pH dependent disassembly-reassembly method [82]. This ferritin nanozyme possesses artificial hydrogenase activity, which catalyzes the reduction of cyclic imines into nontoxic product [82].

Another work showed that artificial metalloenzyme  $[\text{Rh}(\text{nbd})\text{Cl}]_2$  can be in site immobilized in the inner cavity of recombinant horse LFn [83]. This ferritin nanozyme possesses catalytic activity for polymerization of phenylacetylene [83].

These works suggested that ferritin nanozyme may be an ideal strategy for protecting artificial metalloenzymes from the harsh environment and *in vivo* delivery of artificial metalloenzymes.

## 4. Applications of ferritin nanozymes

### 4.1 Biosensing

#### 4.1.1 H<sub>2</sub>O<sub>2</sub> detection

HSAF-Fe<sub>3</sub>O<sub>4</sub>/γ-Fe<sub>2</sub>O<sub>3</sub> ferritin nanozyme was used for H<sub>2</sub>O<sub>2</sub> detection by exerting its peroxidase-like activity [68]. HSAF-Fe<sub>3</sub>O<sub>4</sub>/γ-Fe<sub>2</sub>O<sub>3</sub> catalyzes the oxidation of substrate DPD to the radical cation DPD<sup>+</sup> in the presence of H<sub>2</sub>O<sub>2</sub>. DPD<sup>+</sup> produces a stable purple color with peak absorption at 551 nm, the amount of H<sub>2</sub>O<sub>2</sub> added in the reaction system determined this absorption. By plotting the standard curve between the increase of absorption value per minute (dA/min) and H<sub>2</sub>O<sub>2</sub> concentration, the concentration of H<sub>2</sub>O<sub>2</sub> can be determined in the range of 5.8-88.2 mM [68]. Since the oxide TMB produced a blue color with peak absorption at 652 nm, HSAF-Fe ferritin nanozymes were reported to use TMB as substrate to detect H<sub>2</sub>O<sub>2</sub> [72].

#### 4.1.2 Glucose detection

The catalytic oxidation of glucose by oxygen in the presence of glucose oxidase (GOx) produces H<sub>2</sub>O<sub>2</sub>. Determining the concentration of H<sub>2</sub>O<sub>2</sub> produced in this reaction indirectly reflects the concentration of glucose. Ferritin nanozymes possessing peroxidase-like activity, such as HSAF-PBNPs [70], HSAF-Au [71], HSAF-Fe [72], were used for glucose detection in this way. Due to the high affinity of HSAF-PBNPs to H<sub>2</sub>O<sub>2</sub>, the glucose can be detected as low as 0.39 μM with the linear range of 0.39 μM to 6.25 μM [70]. HSAF-PBNPs based glucose detect system was more sensitive than that of naked GO-COOH (1 μM) and Fe<sub>3</sub>O<sub>4</sub> (5 μM) nanozymes [70], indicating the superiority of ferritin nanozymes over naked nanozymes in biosensing.

#### 4.1.3 Enzyme-linked immunosorbent assay (ELISA)

HSAF-PBNPs ferritin nanozymes were also used for ELISA to detect anti-horse spleen ferritin

antibodies [70]. The positive results also indicated that the loading of nanozymes does not affect the targeting ability of ferritin protein shell.

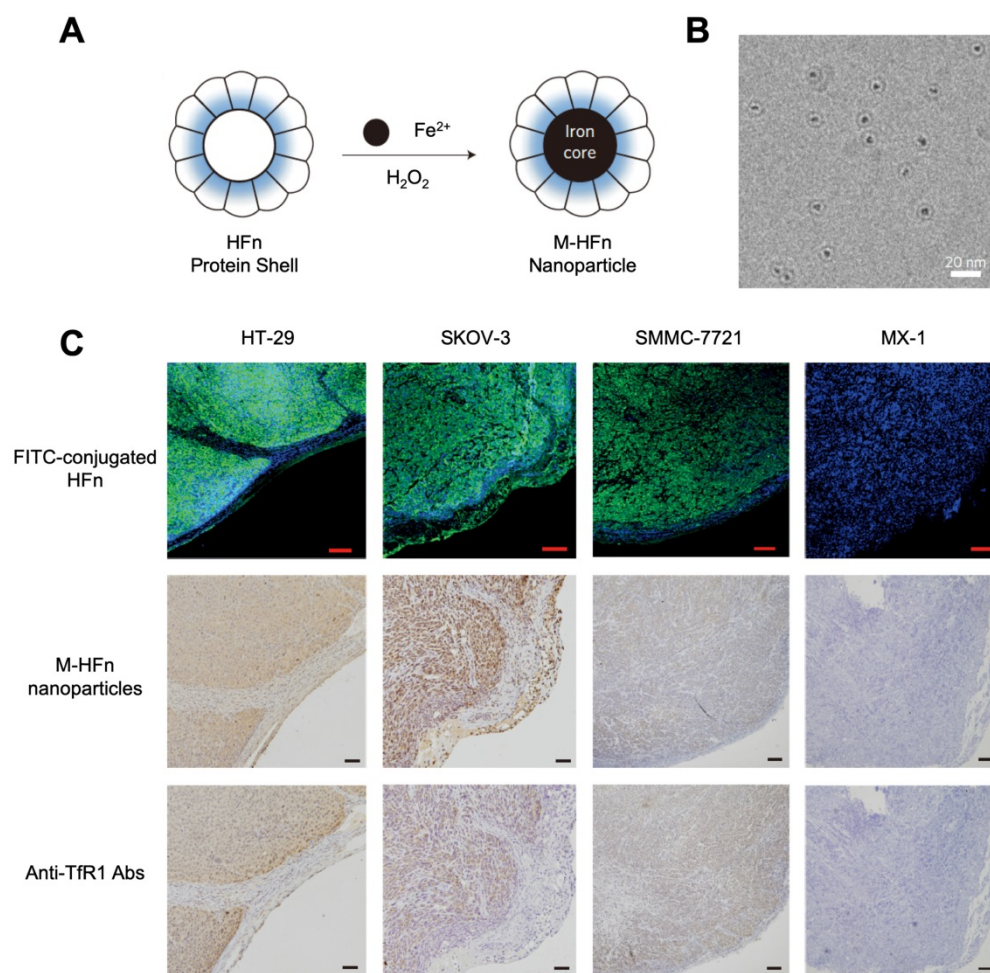
## 4.2 Disease diagnosis

### 4.2.1 Tumor diagnosis

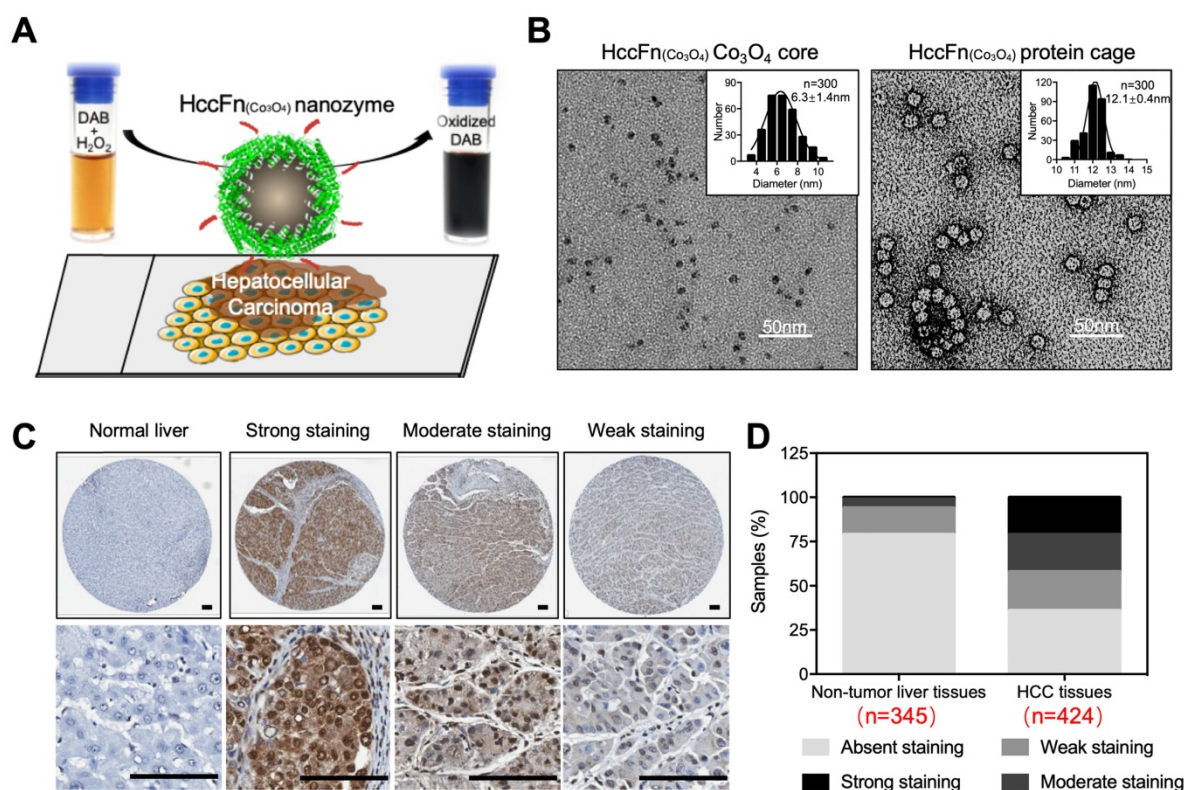
In 2012, for the first time, Kelong Fan *et al* reported that HF<sub>n</sub>-Fe<sub>3</sub>O<sub>4</sub> ferritin nanozyme was a dual-function agent for targeting and visualizing tumor tissues [12] (Figure 7). Fe<sub>3</sub>O<sub>4</sub> nanoparticles were biomimetically synthesized in the cavity of recombinant human HF<sub>n</sub>. HF<sub>n</sub> specifically binds to tumor cells which overexpress transferrin receptor 1 (TfR1) [9, 12-15], while the Fe<sub>3</sub>O<sub>4</sub> core exerts peroxidase-like activity to catalyze the oxidation of substrate di-azo-aminobenzene (DAB) in the presence of H<sub>2</sub>O<sub>2</sub> to give a color reaction. Ferritin endows nanozyme tumor targeting ability. This dual-function of HF<sub>n</sub>-Fe<sub>3</sub>O<sub>4</sub> ferritin nanozyme makes it a one-step agent for rapid pathological diagnosis of tumor tissues. HF<sub>n</sub>-Fe<sub>3</sub>O<sub>4</sub> ferritin nanozyme possesses

advantages (such as time-saving, low cost, ease to operate et al) over traditional HRP-conjugated antibodies for tumor diagnosis. The authors examined 474 clinical specimens from patients with 9 types of tumors, and they verified that ferritin nanozymes can distinguish tumor cells from normal cells with a sensitivity of 98% and specificity of 95%.

In 2017, Tongwei Zhang *et al* reported that by substituting HF<sub>n</sub>-Fe<sub>3</sub>O<sub>4</sub> into cobalt-doped HF<sub>n</sub>-Co<sub>x</sub>Fe<sub>3-x</sub>O<sub>4</sub> ferritin nanozyme, its peroxidase-like activity was enhanced [69]. The authors synthesized a series of Co<sub>x</sub>Fe<sub>3-x</sub>O<sub>4</sub> cores with different cobalt-doping ratios (0, 20%, 40%, 60%) inside the HF<sub>n</sub> protein shell. They found that the peroxidase-like activity of HF<sub>n</sub>-Co<sub>x</sub>Fe<sub>3-x</sub>O<sub>4</sub> ferritin nanozymes was positively correlated with the cobalt-doping ratio of Co<sub>x</sub>Fe<sub>3-x</sub>O<sub>4</sub> core. The peroxidase-like activity of ferritin nanozymes relied on its mineral core. Enhanced peroxidase-like activity of HF<sub>n</sub>-Co<sub>x</sub>Fe<sub>3-x</sub>O<sub>4</sub> ferritin nanozymes made it a more sensitive agent for tumor diagnosis.



**Figure 7.** (A) The synthesis process of HF<sub>n</sub>-Fe<sub>3</sub>O<sub>4</sub> ferritin nanozymes. (B) Cryo TEM image of HF<sub>n</sub>-Fe<sub>3</sub>O<sub>4</sub> ferritin nanozymes. Scale bar = 20 nm (C) HF<sub>n</sub>-Fe<sub>3</sub>O<sub>4</sub> ferritin nanozymes target and visualize xenografted human tumor tissues. Human colorectal cancer cell line HT-29, human ovarian carcinoma cell line SKOV-3, hepatocellular carcinoma cell line SMMC-7721 are TfR1-positive cells, human breast cancer cell line MX-1 are TfR1-negative cells. In FITC-conjugated HF<sub>n</sub> group, green signal represented FITC, blue signal represented DAPI-stained nuclei. In M-HFn and Anti-TfR1 Abs groups, the tissues were stained by DAB (yellowish-brown signal), and then counterstained by hematoxylin (light blue signal). Reprinted with permission from [12]. Copyright 2012 Springer Nature.



**Figure 8.** HccFn-Co<sub>3</sub>O<sub>4</sub> ferritin nanozyme-based HCC diagnosis. (A) Schematic diagram of HccFn-Co<sub>3</sub>O<sub>4</sub> ferritin nanozyme-based HCC diagnosis. (B) Transmission electron microscope (TEM) images of Co<sub>3</sub>O<sub>4</sub> core and protein cage of HccFn-Co<sub>3</sub>O<sub>4</sub> ferritin nanozyme. The size distribution of respond nanoparticles were shown on the top right corner. Scale bar = 50 nm. (C) HccFn-Co<sub>3</sub>O<sub>4</sub> ferritin nanozyme based staining in tissue microarrays derived from HCC patients. The normal liver tissues and HCC tissues were stained by DAB (yellowish-brown signal), and then counterstained by hematoxylin (light blue signal). (D) Staining analysis of HccFn-Co<sub>3</sub>O<sub>4</sub> ferritin nanozyme to non-tumor liver tissues and HCC tissues. Reprinted with permission from [16]. Copyright 2019 American Chemical Society.

In 2019, Bing Jiang *et al* reported that Co<sub>3</sub>O<sub>4</sub> core was biomimetically synthesized in the cavity of hepatocellular carcinoma (HCC) targeted peptide (SP94) displayed pfFn (HccFn) nanocages [16]. The synthesized ferritin nanozyme was named HccFn-Co<sub>3</sub>O<sub>4</sub>. HccFn ferritin nanocages possess HCC-targeted ability, which is enabled by the display of HCC cell-specific peptide SP94 on the outer surface of pfFn through a genetic engineering approach. The peroxidase-like activity of Co<sub>3</sub>O<sub>4</sub> core catalyzes the substrate to make color reaction to visualize HCC tumor tissues. Due to the apparent higher peroxidase-like activity of Co<sub>3</sub>O<sub>4</sub> core than Fe<sub>3</sub>O<sub>4</sub> core (20-fold higher catalytic efficiency) and HCC targeted ability of HccFn, HccFn-Co<sub>3</sub>O<sub>4</sub> ferritin nanozymes showed high sensitivity for HCC-specific diagnosis (Figure 8). The authors examined 424 HCC specimens and verified that HccFn-Co<sub>3</sub>O<sub>4</sub> ferritin nanozymes could distinguish HCC tissues from normal liver tissues with a sensitivity of 63.5% and specificity of 79.1%, which was comparable with that of clinically used HCC specific marker alpha fetoprotein (AFP). Moreover, the pathological analysis indicated that HccFn-Co<sub>3</sub>O<sub>4</sub> ferritin nanozymes staining result was a potential predictor of prognosis in HCC patients. Staining intensity was

positively correlated to tumor differentiation degree ( $P = 0.0246$ ), tumor invasion ( $P < 0.0001$ ) and negatively correlated with overall survival ( $P = 0.0084$ ) of HCC patients.

#### 4.2.2 Cardiovascular disease diagnosis

HF<sub>n</sub>-Fe<sub>3</sub>O<sub>4</sub> ferritin nanozymes were further explored to be used for pathological identification of high-risk and ruptured atherosclerotic plaques in humans [17]. The authors showed that HF<sub>n</sub> could intrinsically recognize plaque-infiltrated active macrophages through the interaction with TfR1. Plaque-infiltrated active macrophages drive atherosclerotic plaque progression and rupture and are significantly associated with the plaque vulnerability, thus HF<sub>n</sub>-Fe<sub>3</sub>O<sub>4</sub> ferritin nanozymes specifically stain unstable ruptured plaque (Figure 9). Stable plaque seldom exists plaque-infiltrated active macrophages, thus HF<sub>n</sub>-Fe<sub>3</sub>O<sub>4</sub> ferritin nanozymes did not stain stable plaque.

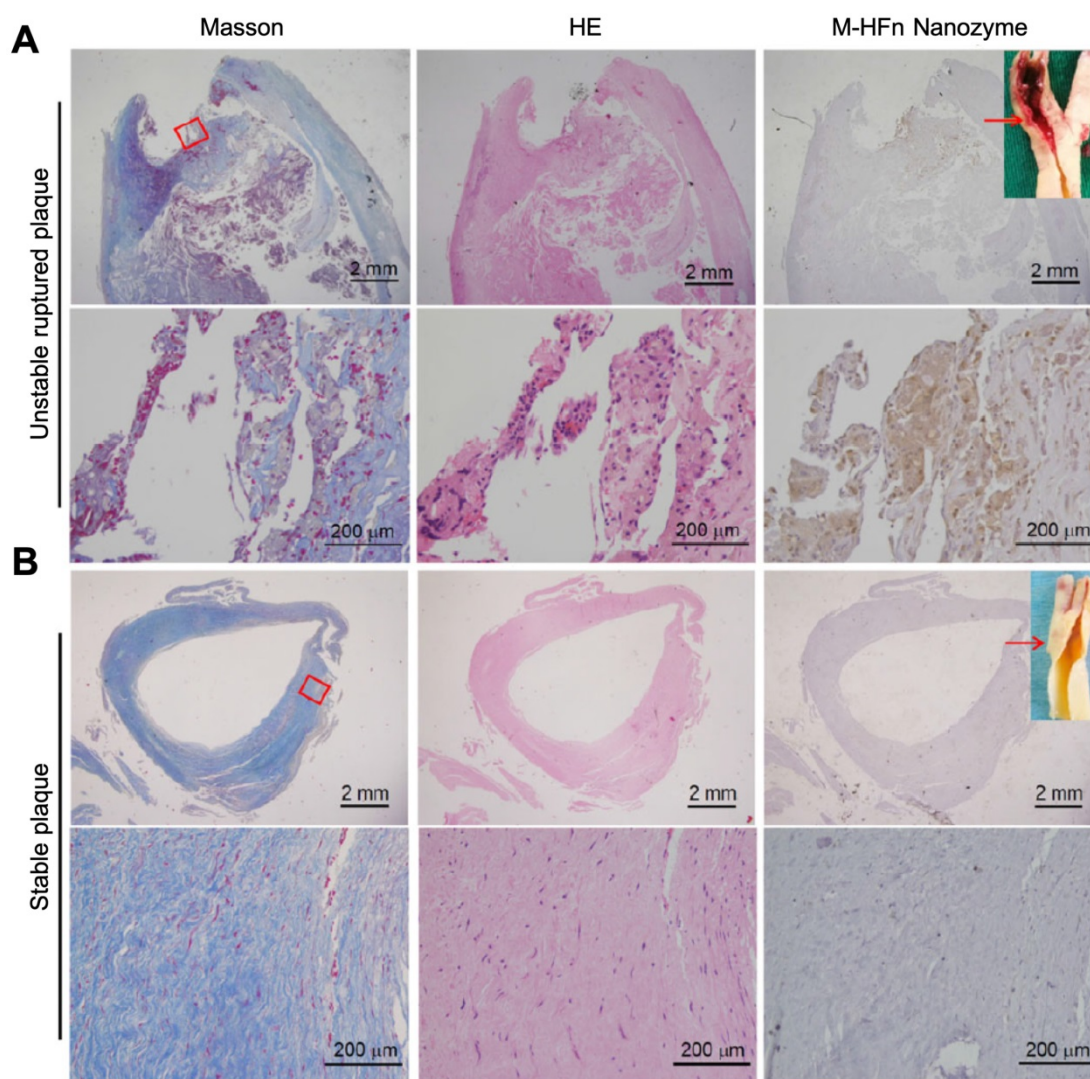
#### 4.3 Cancer Therapy

Ferritin has been widely used as a nanocarrier to deliver various drugs for cancer therapy. Due to its unique self-assemble ability, hollow cavity capable of encapsulating drugs, and an outer surface that can be

modified genetically and chemically for targeting, ferritin has recently emerged as a promising drug delivery carrier [84]. Many chemotherapeutic drugs and small molecules, such as doxorubicin [85], cisplatin [86], curcumin [87], gefitinib [88], siRNA [89], have been encapsulated into ferritin for cancer therapy. However, by exerting the enzyme-like activities of ferritin nanozymes to kill tumor cells was rarely being reported. As described below, we introduce a ferritin nanozyme which combines the tumor targeting ability of ferritin with the multi-enzyme-like activities of nanozyme to treat tumor.

In 2018, Kelong Fan *et al* reported that HFfn-conjugated Nitrogen-doped Porous Carbon Nanospheres (HFfn-N-PCNSs ferritin nanozymes) possess four enzyme-like activities (oxidase, peroxidase, catalase and SOD), which are responsible for ROS regulation [81]. Due to the tumor targeting

and lysosome localization ability of HFfn and intracellular ROS generation ability of N-PCNSs nanozymes under acidic pH, HFfn-N-PCNSs ferritin nanozymes were evaluated for tumor targeted catalytic therapy [81]. HFfn specifically bound to the TfR1 overexpressed on the surface of tumor cells, then HFfn was endocytosed into lysosomes where the pH environment is acidic. Once the HFfn-N-PCNSs ferritin nanozymes localized to the acidic pH environment of tumor lysosomes, it mainly displayed peroxidase- and oxidase-like activities. These two enzyme-like activities allow it to exert ROS mobilization including (1) transferring oxygen to free radicals accompanied with O<sub>2</sub> consumption, and (2) catalyzing H<sub>2</sub>O<sub>2</sub> into free radicals, which thus synergistically burst ROS level and induce cell damage. The excess ROS generated in tumor cells caused tumor regression (Figure 10).



**Figure 9.** HFfn-Fe<sub>3</sub>O<sub>4</sub> ferritin nanozymes specifically stain unstable and ruptured plaques [17]. Carotid sections with unstable ruptured plaque (A) and stable plaque (B) were attained by Masson (for collagen distribution identification), HE and HFfn-Fe<sub>3</sub>O<sub>4</sub> ferritin nanozymes (stained by DAB, and then counterstained by hematoxylin). Arrows indicate the histological section position. Reprinted with permission from [17]. Copyright 2019 Tsinghua University Press and Springer-Verlag GmbH Germany, part of Springer Nature.

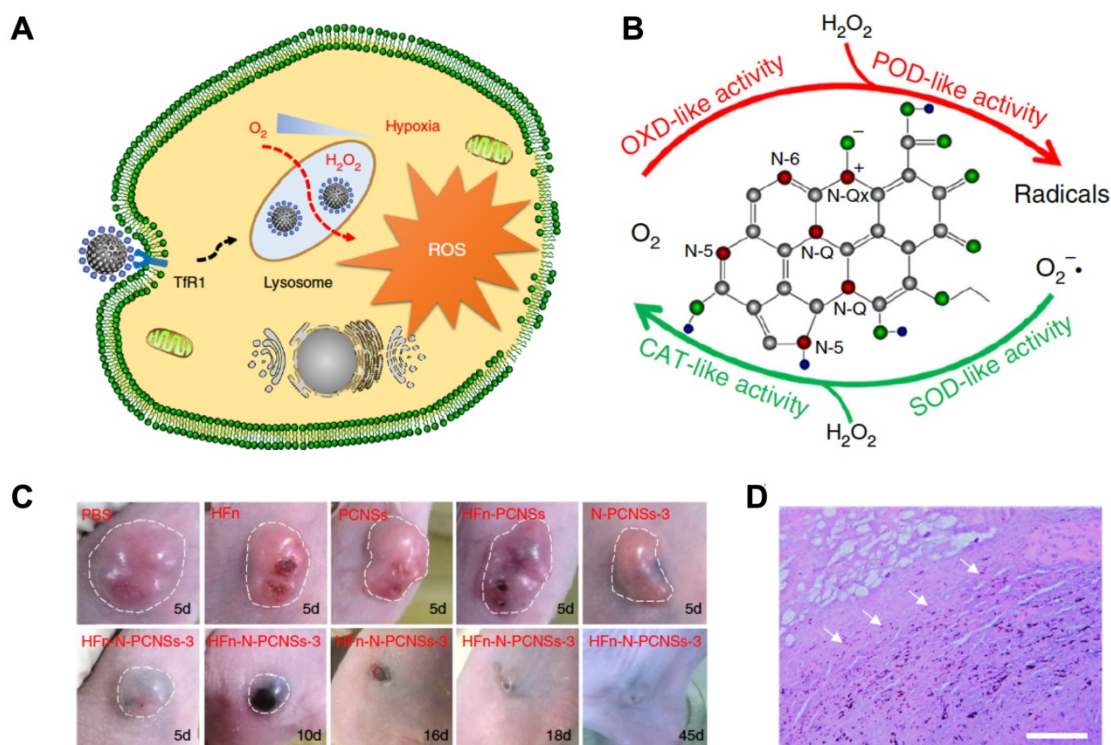
#### 4.4 Antioxidation and cell protection

Ferritin nanozymes were used for antioxidation and cell protection *in vivo* by exerting its enzyme-like activities to eliminate ROS. In 2010, Lianbing Zhang *et al* reported that HSAF-Pt ferritin nanozymes exhibited catalase- and SOD-like activities under pH 7.4 physiological environment [77]. When incubating with Caco-2 cells stressed with 5 mM  $H_2O_2$ , HSAF-Pt ferritin nanozymes protected cells from ROS damages and significantly increase the cell viability (Figure 11 A). Interestingly, the HSAF also showed cell protection ability, which indicated that apoferritin may also possess the ability to eliminate ROS. Later on, they found that HSAF-PtAu ferritin nanozymes exhibited higher catalase- and SOD-like activities than that of HSAF-Pt, and showed better effect on cell protection [78]. The authors also supposed that HSAF possesses a receptor on cell surface. Through receptor-mediated endocytosis, ferritin nanozymes may enter into cells and exert enzyme-like activities to eliminate intracellular ROS (Figure 11 B). This hypothesis was later verified, as the authors found that HSAF-Pt and HSAF-PtAu ferritin nanozymes significantly reduced the intracellular ROS of Caco-2 cells stressed with 2 mM  $H_2O_2$ . After the treatment of endocytosis inhibitor chlorpromazine, the

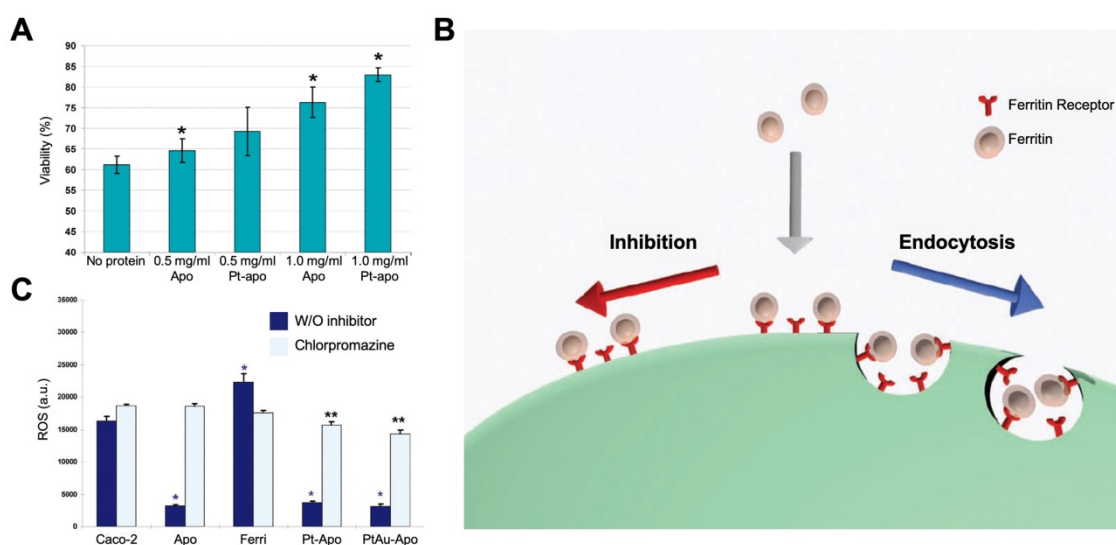
intracellular ROS of Caco-2 cells was significantly elevated (Figure 11 C).

Moreover, HSAF-Au-Ag ferritin nanozymes were shown to possess triple-enzyme like activities (SOD, catalase, peroxidase) which scavenge  $O_2^-$  and reduce  $H_2O_2$  to eliminate intracellular ROS [79]. HSAF-Au-Ag ferritin nanozymes have pH-dependent peroxidase- or catalase-like activities. In acidic pH environment of lysosome, it would be able to scavenge intracellular  $H_2O_2$  by exerting its peroxidase-like activity. However, in other parts of cytoplasm (near neutral pH), HSAF-Au-Ag ferritin nanozymes mainly show catalase- and SOD-like activities and scavenge intracellular  $O_2^-$  and  $H_2O_2$ .

Xiangyou Liu *et al* also reported that HSAF- $CeO_2$  ferritin nanozymes possess SOD-like activity which scavenge  $O_2^-$  to scavenge ROS [75]. The authors declared that HSAF possesses a receptor on cell surface which mediated the active cellular uptake of HSAF- $CeO_2$  ferritin nanozymes. This viewpoint corresponded to that of Lianbing Zhang *et al* reported [78]. Due to the improved biocompatibility and active endocytosis process initiated by HSAF protein cages, HSAF- $CeO_2$  ferritin nanozymes significantly scavenge the intracellular ROS of hepatoma carcinoma cell HepG2.



**Figure 10.** HFn-N-PCNSs ferritin nanozymes-based cancer therapy. (A) Schematic diagram for HFn-N-PCNSs ferritin nanozymes induced tumor cell death. (B) HFn-N-PCNSs ferritin nanozymes show four enzyme-like activities. (C) Tumor response to HFn-N-PCNSs ferritin nanozymes treatment. (D) Accumulation of HFn-N-PCNSs ferritin nanozymes in tumor tissues. White arrows indicate the location of HFn-N-PCNSs ferritin nanozymes. Reprinted with permission from [81]. Copyright 2018 Springer Nature.



**Figure 11.** (A) Effects of treatment with HSAF-Pt ferritin nanozymes on the viability of Caco-2 cells stressed with 5 mM  $H_2O_2$ . Reprinted with permission from [77]. Copyright 2010 American Chemical Society. (B) The binding and cellular uptake of ferritin nanozymes via receptor-mediated endocytosis. This endocytosis was blocked by inhibitors. Reprinted with permission from [78]. Copyright 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (C) Effects of treatment with HSAF-Pt and HSAF-PtAu ferritin nanozymes on the ROS generation of Caco-2 cells stressed with 2 mM  $H_2O_2$ . Chlorpromazine was used to inhibit endocytosis of ferritin. Reprinted with permission from [78]. Copyright 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Although the above works did not clarify what the ferritin receptors are, many works have reported the receptors of different types of ferritins. The receptor of HSAF was reported to be mouse Scara5 by many groups, same as that of mouse LFn [90-93]. The receptor of human HF<sub>n</sub> was reported to be human TfR1 [9, 12-15], while the receptor of the mouse HF<sub>n</sub> was mouse TIM-2 [94-96]. In addition, human HF<sub>n</sub> also recognized mouse TIM-2 due to a cross reaction [90, 97]. Moreover, it was recently reported that human oligodendrocytes took up human HF<sub>n</sub> to meet iron requirement via the human Tim-1 receptor [98]. The receptor of human LFn in the human body is still unknown. If the ferritin was only recognized by the corresponding receptor and entered into cells through receptor-mediated endocytosis, so that ferritin nanozymes exert its ROS regulation function.

#### 4.5 Other applications

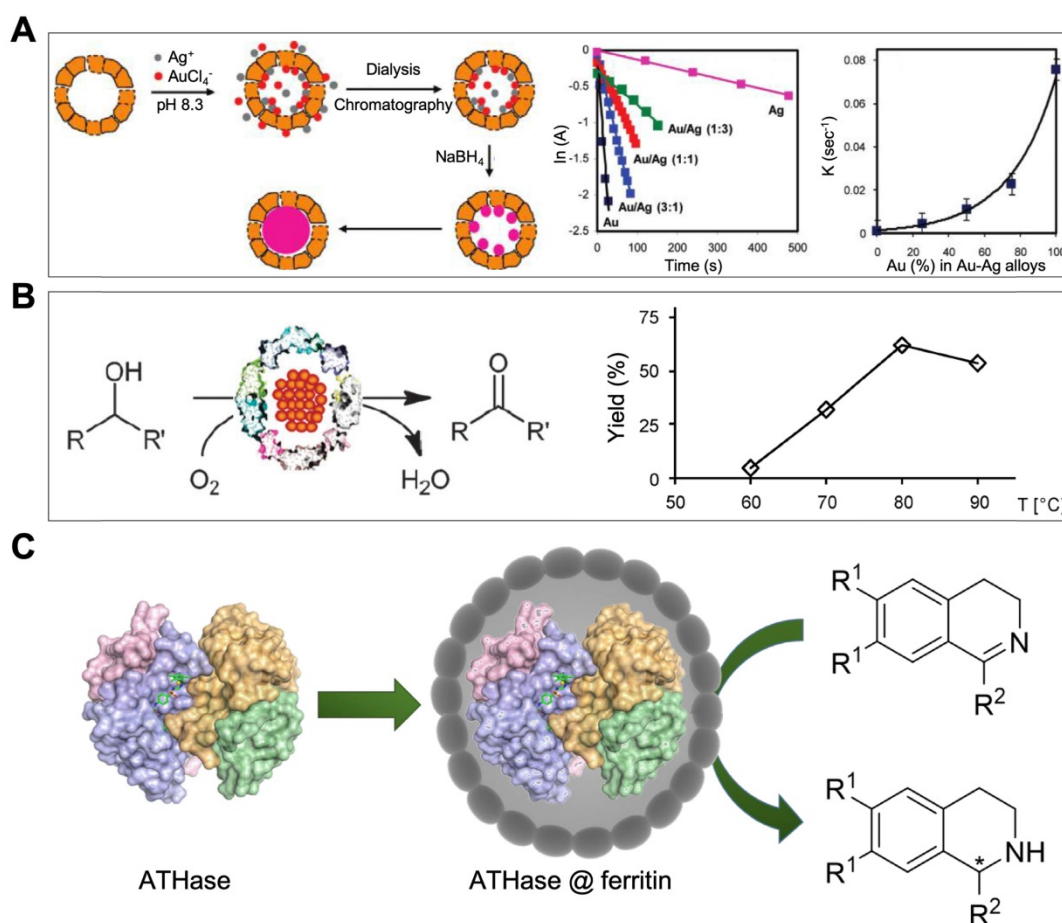
Ferritin nanozymes were also used for decontaminations. The catalytic activities of ferritin nanozymes can oxidase or reduce organic reagents which are harmful to human bodies or environment. By encapsulating Au-Ag alloy nanoparticles inside the HSAF, HSAF-Au-Ag ferritin nanozymes were synthesized [80]. HSAF-Au-Ag ferritin nanozymes catalyzed the reduction of 4-nitrophenol efficiently. This catalytic reaction rate was positively correlated with the Au/Ag ratio of Au-Ag alloy nanoparticles (Figure 12 A), indicating the catalytic activity mainly derives from Au nanoparticles.

In addition, pfFn-Pd ferritin nanozymes were reported to possess oxidase-like activity which catalyze the aerobic oxidation of alcohols [74] (Figure 12 B). This optimum reaction temperature was around 80 °C. At this temperature, most of natural enzymes were soon denatured, while pfFn-Pd ferritin nanozymes still kept high stability and catalytic activity due to the excellent thermal stability of pfFn. pfFn-Pd ferritin nanozymes also catalyzed the discoloration of diazo dyes and reduction of p-nitrophenol [73].

Moreover, pfFn nanozymes were also used for removing phosphate and arsenate from aqueous solution [99]. The pfFn mineral iron cores have strong absorption ability for phosphate and arsenate. Specially, during or after the iron oxidation process catalyzed by pfFn nanozymes and with incorporating phosphate in the reaction system, a ferric-oxyhydroxide-phosphate complex was synthesized inside the cavity of pfFn. pfFn nanozymes removed these oxo anions from aqueous solution to residual pM levels, demonstrating a great potential to be used for sewage treatment.

Moreover, an artificial transfer hydrogenase [Cp\*Ir(biot-p-L)Cl] Sav was also encapsulated into the cavity of HSAF [82]. This synthesized ATHase@ferritin ferritin nanozyme catalyzed the reduction of cyclic imines for detoxification (Figure 12 C). HSAF provides protective protein shell for precious metal-based artificial enzymes to defend the complicated physiological environment and possibly deliver it to targeted site *in vivo*.





**Figure 12.** (A) HSAF-Au-Ag ferritin nanozymes catalyze the reduction of 4-nitrophenol. The catalytic reaction rate is positively correlated with the Au/Ag ratio of Au-Ag alloy nanoparticles. Reprinted with permission from [80]. Copyright 2010 American Chemical Society. (B) pFn-Pd ferritin nanozymes catalyze the aerobic oxidation of alcohols. Reprinted with permission from [74]. Copyright 2012 Royal Society of Chemistry. (C) ATHase@ferritin ferritin nanozymes catalyze the reduction of cyclic imines. Reprinted with permission from [82]. Copyright 2018 Royal Society of Chemistry.

## 5. Conclusion and perspectives

Since the first discovery of the peroxidase-like activity of  $\text{Fe}_3\text{O}_4$  nanoparticles, nanozyme has been investigated for over a decade. Hundreds of new nanozymes have been developed and applied for biosensing, environmental protection, anti-biofouling, imaging and theranostics. However, the size-controllable synthesis and targeting modifications of nanozymes are still challenging in this field.

The emerging of ferritin nanozymes provides an effective way to address these challenges. Ferritins are natural nanozymes which exhibit multi enzyme-like activities (e.g. ferroxidase, peroxidase). In addition, ferritins are artificial nanozymes which serve as nanoreactors to synthesize nanozymes in its cavity. Compared with other types of nanozymes, ferritin nanozymes possess following prominent advantages:

1) Size-controllable synthesis of nanozymes. Ferritin possesses uniform protein shell and natural synthesis ferric oxide (ferrihydrite) nanoparticles within its protein cavity. Learning the way of

biomineralization process of ferritin, we may synthesize different types of metal or metallic oxide nanozymes in the cavity of ferritin with a confined size (less than 8 nm). In addition, the size of nanozymes will be controlled by controlling the amount of metal ions added to the reaction system.

2) Targeting modification of nanozymes. Through genetic or chemical approaches, we can readily modify ferritin nanocages with targeting moieties. Combining targeting modification of ferritin nanocages with nanozyme encapsulated in ferritin inner cavity, we can easily realize targeting modifications of nanozymes. In addition, the size of ferritin nanocage is 12 nm, which is an ideal size for overcoming the physiological barriers posed by the tumor environment and passively target the tumor site. Thus, ferritin nanozymes possess passive tumor targeting ability. Moreover, human HFn can specifically target tumor cells via binding to its receptor TfR1. Some studies reveal that human HFn traverses the blood brain barrier (BBB) [97, 100], thus it could be used for brain tumor treatment [97, 101]. This characteristic endows ferritin nanozymes natural

tumor targeting and BBB traversing abilities. Moreover, some nanozymes show pH dependent multi-enzyme-like activities. Under different pH environment *in vivo*, nanozymes may exert distinct functions (generate ROS or eliminate ROS) [102]. Through the targeting delivery of ferritin, nanozymes may actively target specific environment and exert special functions.

3) High biocompatibility and stability. Ferritin, as a natural protein, shows excellent biocompatibility for *in vivo* applications. Due to the structure superiority, ferritin exhibits high stability under robust environment, and it can resist high temperature and denaturants. In addition, benefiting from surrounding ferritin protein as a protective layer, the nanozyme is shielded from nonspecific biomacromolecules cellular binding, or unwanted sequestration leading to inactivation, or nonspecific binding in biological environments. Moreover, ferritin nanozymes may avoid nanozymes from forming unwanted protein corona in circulation *in vivo*.

4) Reduce toxicity and immune response caused by nanozymes *in vivo*. Using ferritin derived from human species to synthesized nanozymes may significantly reduce the immune response and toxicity caused by nanozymes during *in vivo* biomedical applications. Ferritin derived from human body does not contain any potential toxic elements that would activate inflammatory or immunological response. Nanozymes may avoid from immune response when shelled by ferritin nanocages. In addition, when nanocaged by ferritin, some toxic nanozymes, like noble metal based nanozymes, may be avoid of the contact with the main organs and thus reduce its toxicity.

5) Selectivity of ferritin nanozymes for substrates. The low substrate specificity of nanozymes is a major barrier between nanozyme and natural enzyme. Creating ferritin nanozymes by adding specific substrate or microenvironment targeting ability to ferritin nanocages may make up this barrier.

There are still challenges for ferritin nanozymes in biomedical applications.

1) Limited types of nanozymes can be synthesized in ferritin nanocages. Learning the way of biomineralization process of ferritin, Fe<sub>3</sub>O<sub>4</sub> and Co<sub>3</sub>O<sub>4</sub> nanozymes were synthesized in the cavity of ferritin. Exploring to load other kinds of metal oxide nanoparticles, such as Mn<sub>3</sub>O<sub>4</sub>, CuO, etc, and exhibiting different kinds of enzyme-like activities may further expand the biomedical applications of ferritin nanozymes.

2) Robust strategies to control the uniformity and size of ferritin nanozymes are needed. Controlling the amounts of metal ions and the adding rate of oxidants

or reductants in the reaction system may efficiently control the uniformity and the size of ferritin nanozymes. However, the operating technique is still immature; a robust ferritin nanozyme synthesis system needs to be established.

3) Limited applications of ferritin nanozymes. Ferritin nanozymes have been used for biosensing, antibacterial and disease theranostics. However, recent reported ferritin nanozymes are seldom used for *in vivo* applications, especially for therapy applications. How to better combine the superiority of ferritin and nanozymes to create new ferritin nanozymes for effective theranostics needs further exploration.

In addition, ferritin nanozymes also inspire us to use other natural nanostructures (*e.g.* magnetosome, viral capsid) to create functional nanozymes for diseases targeted theranostics. Importantly, ferritin nanozymes provide a way for us to learn from nature to optimize and design nanozymes, thus solving problems in the field of nanozyme and nanobiology.

Taken together, ferritin nanozyme is a promising agent for disease-targeted theranostics. How to better control the size of nanozymes inside the ferritin protein cage and synthesize nanozymes with high activity and selectivity still need further exploration. Taking advantage of the BBB traversing ability of ferritin and ROS scavenging ability of nanozymes, ferritin nanozymes are expected to be used for brain disorders therapy (*e.g.* stroke, Parkinson's disease). Combining the characteristics of the tumor microenvironment (*e.g.* acidic pH, hypoxia, tumor stem cells) with the modification of ferritin nanocage may help us to create novel types of ferritin nanozymes for more effective tumor therapy.

## Abbreviations

HSAF: Horse spleen apoferritin; HFn: Recombinant H-subunit ferritin; LFn: Recombinant L-subunit ferritin; pfFn: *Pyrococcus furiosus* ferritin.

## Acknowledgements

This work was financially supported by the Strategic Priority Research Program (No. XDB29040101), the National Natural Science Foundation of China (No. 31900981, 31871005, 31530026), Chinese Academy of Sciences under Grant No. YJKYYQ20180048, the Key Research Program of Frontier Sciences (No. QYZDY-SSW-SMC013), Chinese Academy of Sciences and National Key Research and Development Program of China (No. 2017YFA0205501), Youth Innovation Promotion Association CAS (2019093), and the Grant for International Joint Research Project of the Institute of Medical Science, the University of Tokyo.

## Competing Interests

The authors have declared that no competing interest exists.

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