CHAPTER 6

Bioengineered Ferritin Nanoprobes for Cancer Theranostics

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1 Introduction

The ideal nanoprobes for imaging or treating clinical tumors should possess the ability to efficiently load imaging agents or drugs, exhibit excellent biocompatibility and be able to specifically target tumor sites (Chauhan & Jain, 2013; Lim et al., 2015; Strebhardt & Ullrich, 2008; Tosi et al., 2016). Numerous efforts have been made to develop active tumor targeting nanoprobes in the past few decades, while a handful of these systems have been evaluated in clinical trials but none was approved for clinical use (Chen et al., 2017a; Venditto & Szoka, 2013; Wang, Billone, & Mullett, 2013). It is hard to translate chemically prepared nanoprobes from bench to bedside because their synthesis procedures are usually complicated, and they often have unknown long-term toxicity (Tosi et al., 2016). Under these circumstances, delivery of imaging agents or nanodrugs to the disease sites using biological vehicles maybe more advantageous. Endogenous self-assembling proteins are promising candidates considering they are nontoxic for the organism and therefore, safe (Karimi et al., 2017).

Because of their unique protein architecture and physicochemical properties, bioengineered ferritin nanoprobes have been extensively used as versatile nanocarriers for tumor theranostic applications (Fan, Gao, & Yan, 2013; He & Marles-Wright, 2015; Heger et al., 2014; Truffi et al., 2016; Wang et al., 2017). In recent years, the identification of the specific receptors of ferritins has not only helped us understand the physiological function of ferritins, but also prompted the use of the intrinsic tumor targeting property of ferritin nanoprobes. Taken together, these favorable properties make ferritin an ideal base of engineered nanoprobes for tumor imaging and therapy.

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2 Ferritin: Its Structure, Function, and Membrane Receptors

2.1 Structure and Function of Ferritin

Ferritin is a natural iron storage protein which plays an important role in iron homeostasis. Ferritin was first isolated from horse spleen by Laufberger in 1937, and was subsequently found in many other organisms, including humans and other mammals, plants, fungi and bacteria (Arosio, Ingrassia, & Cavadini, 2009; Theil, 1987; Wang et al., 2010). In spite of large variations in amino acid sequences from bacteria to humans, ferritin proteins essentially have the same architecture (Harrison & Arosio, 1996). The typical structure of ferritin is a 24-subunit spherical protein cage encapsulating an iron oxide core (Harrison & Arosio, 1996). Mammalian ferritins are mainly present intracellularly in the cytosol, as well as in the nuclei and mitochondria. Extracellular ferritins are found in serum as well as synovial and cerebrospinal fluids (Meyron-Holtz, Moshe-Belizowski, & Cohen, 2011). The cytosolic ferritins play important roles in iron storage and detoxification, while the physiological function of secreted ferritin is still unclear. It has been shown that elevated serum ferritin levels are linked to inflammation, angiogenesis and tumors (Knovich et al., 2009; Meyron-Holtz et al., 2011; Wang et al., 2010), and therefore, are considered a marker for these conditions.

The ferritin superfamily are spherical proteins typically composed of 24 subunits, with an outer diameter of 12 nm and an interior cavity of 8 nm diameter (Fig. 1). The interior cavity can accommodate up to 4500 Fe(III) atoms as an iron mineral core, traditionally described as ferrihydrite (Harrison & Arosio, 1996). Apoferritin refers to the iron-free form of the protein, while the iron-containing form is termed holoferritin, or simply ferritin. The apoferritin is normally composed of a mixture of ferritin H-chain and L-chain subunits, arranged with fourfold, threefold, and twofold symmetry axes, of which the threefold axes form hydrophilic channels that allow transport of metal in and out of the protein cage (Harrison & Arosio, 1996;

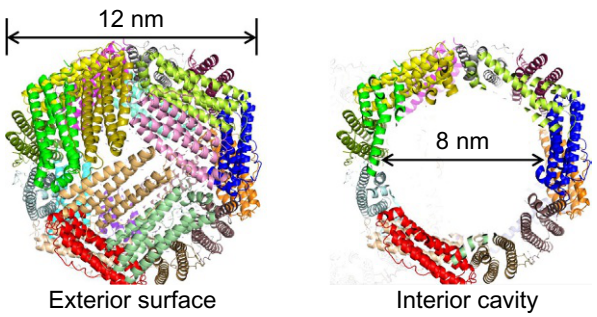

Fig. 1
Cartoon diagrams of exterior surface view of threefold symmetry axes and interior cavity of human H-ferritin (2FHA).

Theil, 1987). The H-chain is named for its initial isolation from heart, whereas the L-chain was isolated from liver (Torti & Torti, 2002). In humans, the H-chain is also heavier, with a molecular mass of 21 kDa, whereas the L-chain mass is 19 kDa (Harrison & Arosio, 1996; Theil, 1987). Therefore, the ferritin subunits are sometimes referred to as heavy (H) and light (L) ferritins, respectively.

Physiologically, H-ferritin and L-ferritin perform different functions. The H-chain with a ferroxidase center is essential for Fe(II) oxidation, while the L-chain assists in iron core formation, and lacks the catalytic center. This is the reason why recombinant L-ferritin, unlike H-ferritin, is typically iron poor. Moreover, the ratio of these two subunits in ferritin varies widely depending on tissue type, with H-chains predominant in the heart and L-chains predominant in the liver (Torti & Torti, 2002). In addition, the H/L ratio changes in inflammation and other pathological conditions.

Natural ferritin exists in both intracellular and extracellular compartments. In most tissues, ferritins are mainly present in the cytosol, nucleus, and mitochondria, and play a role in iron storage as well as iron homeostasis. Iron, a major trace element, is an integral component of many proteins and both potentially toxic and essential for life. In particular, free Fe^{2+} ions are lethal because they catalyze the formation of reactive hydroxyl radicals in oxygenated tissues by the Fenton reaction, leading to damage to DNA, lipids, and proteins (Harrison & Arosio, 1996; Torti & Torti, 2002). The balance between iron storage and utilization is maintained by the regulation of intestinal absorption of the metal from diet, along with the expression of iron transport and storage proteins, including ferritin, transferrin and the transferrin receptor. In addition to iron storage, ferritins also play an important role in iron detoxification by capturing and sequestering the intracellular labile iron pool. This protective ability of ferritin is based on the ferroxidase activity of the H-chain to catalyze the transformation of highly toxic Fe(II) to less toxic Fe(III) (Harrison & Arosio, 1996).

Ferritins in the nucleus (nuclear ferritin (Alkhateeb & Connor, 2010)) and mitochondria (mitochondrial ferritin (Drysdale et al., 2002)) protect DNA or mitochondria from iron toxicity and oxidative damage (Arosio et al., 2009). The nuclear translocation of ferritin, especially H-ferritin, is a selective energy-dependent process that does not seem to require a consensus nuclear localization signal (Thompson et al., 2002). Furthermore, the phosphorylation and *O*-glycosylation of H-ferritin seem to be involved in its nuclear translocation (Alkhateeb & Connor, 2010). Mitochondrial ferritin is an H-ferritin, with a precursor that is targeted to mitochondria by a leader sequence (Drysdale et al., 2002). Thus, H-ferritin is important for iron detoxification and cell protection. Recently, some studies have shown that cytosolic ferritins are elevated in malignant tissues; for example, cytosolic ferritin is expressed in mammary carcinomas at levels up to 10-fold higher than in benign breast tissues (Elliott et al., 1993; Weinstein et al., 1989; Werneke, Elliott, & Ledford, 1990), indicating that cytosolic ferritin may be involved in tumor progression.

Serum ferritins are predominantly composed of L chains, which have low iron content. Although several studies have reported that serum ferritin might arise from the secretion of hepatocytes, macrophages and Kupfer cells (Meyron-Holtz et al., 2011; Wang et al., 2010), the source of serum ferritins still needs to be further investigated. In addition, the physiological functions of serum ferritin as well as the identity of the L-ferritin receptor are not yet clear. Nevertheless, a significant increase in serum ferritin levels has been confirmed to be related to pathological processes, including inflammation, angiogenesis and tumor formation, implying that serum ferritins are a potential biomarker for clinical diagnosis (discussed in following sections) (Harrison & Arosio, 1996; Meyron-Holtz et al., 2011; Wang et al., 2010).

Besides their distinct architectural features, ferritins possess unique physical and chemical properties. Unlike most other proteins, which are sensitive to temperature and pH outside of the physiological range, ferritin is able to bear high temperatures of up to 75°C for 10 min and is stable in various denaturants, including urea or guanidinium chloride. These unique features are due to the large numbers of salt bridges and hydrogen bonds formed between subunits of ferritin (Harrison & Arosio, 1996; Santambrogio et al., 1992). Interestingly, the assembly of ferritin, despite its rigidness under physiological conditions, is pH-dependent (Kang et al., 2008). The ferritin architecture breaks down to subunits in extreme acidic (pH 2–3) or basic (pH 10–12) environments and reconstructs, almost completely, when pH returns back to the physiological range (Belletti et al., 2017; Kang et al., 2008; Santambrogio et al., 1992; Truffi et al., 2016; Uchida et al., 2010). These unique properties make ferritin an ideal and powerful nanoplatform to construct multifunctional nanoprobes for imaging and drug delivery.

2.2 Ferritins and Their Membrane Receptors

Several research groups had already reported that human ferritin is selectively taken up by tumor cells as early as in the 1960s (Caulfield, 1963; Easty, Yarnell, & Andrews, 1964; Ryser, Caulfield, & Aub, 1962). Fargion et al. then reported in the 1980s that human H-ferritin specifically binds to a protein of ~100 kDa molecular weight on tumor cells (Fargion et al., 1988). However, it was not until 2010 that transferrin receptor 1 (TfR1) was identified as the human H-ferritin receptor by Seaman's group using expression cloning (Li et al., 2010), following their earlier identification in 2005 of the mouse T cell immunoglobulin-domain and mucin domain-2 (TIM-2) as the H-ferritin specific receptor (Table 1) (Chen et al., 2005). They found that TfR1 binds specifically to human H-ferritin with little or no binding to L-ferritin. Furthermore, after binding to TfR1 on the cell surface, human H-ferritin enters both endosomes and lysosomes. The finding that TfR1 can bind to H-ferritin as well as transferrin suggests that this dual receptor function may coordinate the use of iron through these iron-binding molecules. Soon after the identification of the H-ferritin receptor, our group reported that human H-ferritins target TfR1 on both tumor cells and tumor tissues from clinical samples (Fan et al., 2012).

Table 1: Ferritins and their membrane receptors

Ferritins	Membrane Receptors	Years	References
Mouse H-ferritin	T cell immunoglobulin-domain and mucin domain-2 (Mouse TIM2)	2005	Chen et al. (2005)
Mouse L-ferritin	Scavenger receptor class A member 5 (Mouse Scara5)	2009	Li et al. (2009)
Human H-ferritin	Transferrin receptor 1 (Human TfR1)	2010	Li et al. (2010)
Human L-ferritin	–	–	–

Because serum ferritin changes with physiological or pathological process, it is suggested that the assumed receptors in liver for both H-ferritin and L-ferritin may serve to regulate the levels of serum ferritin (Moss et al., 1992). A putative ferritin receptor was purified from human liver, which showed higher affinity for L-ferritin and lacked the specificity for human H-ferritin (Adams, Powell, & Halliday, 1988; Moss et al., 1992). Although the molecular weight of this putative receptor is consistent with the glycosylated TIM receptors, a human ortholog for mouse TIM-2 (receptor for mouse H-ferritin) has not been identified yet, and this receptor still remains putative.

The scavenger receptor Class A member 5 (Scara5) has already been identified as the receptor for mouse L-ferritin on mouse embryo kidney cells (Table 1) (Li et al., 2009). While human orthologs of Scara5 exist, no direct evidence has demonstrated that human L-ferritin binds to human Scara5. Finding the receptors for L-ferritin is fundamental for us to better understand the relationships between serum ferritin (iron homeostasis) and physical conditions or pathological processes. Because no receptors for human L-ferritin have been found, this area is well worth further study.

2.3 Cross-Interaction of Ferritins and Their Membrane Receptors From Different Species

Horse spleen ferritin (HosFn) has been widely used as a nanocarrier in nanomedicine due to its commercial availability and reasonable cost. Thus, cross-interaction studies of ferritins and ferritin membrane receptors in different species mainly concentrate on HosFn, while the human ferritin nanoprobes do not have such an issue in pharmaceutical use.

HosFn is nearly 92% (22L/2H) composed of L subunits (Harrison, 1986; Sun et al., 2011a), thus, it is typically regarded as L-ferritin by many researchers (Conti et al., 2016; Geninatti Crich et al., 2015; Mendes-Jorge et al., 2014; Sun et al., 2011a). Li et al. mentioned in their research that they observed the direct interaction of HosFn with human TfR1 (the receptor of human H-ferritin) (Table 2), but they did not present the detailed data (Li et al., 2010). Mendes-Jorge et al. reported that HosFn binds to mouse endothelial cells via Scara5 (mouse L-ferritin receptor) by observation of the colocalization of HosFn and Scara5 on mouse cells (Table 2) (Mendes-Jorge et al., 2014). Little else is known about the endogenous receptors of HosFn in humans.

Table 2: Cross-interaction of HosFn and ferritin membrane receptors from different species

Ferritins	Interaction with Receptors	Years	References
Horse spleen ferritin (22L/2H)	Human TfR1	2010	Li et al. (2010)
	Mouse Scara5	2014	Mendes-Jorge et al. (2014)
		2016	Conti et al. (2016)
	Human Scara5?	2015	Geninatti Crich et al. (2015)

Interestingly, Crich and colleagues reported that horse spleen ferritin can be effectively internalized by both mouse (Conti et al., 2016) and human breast cancer cells (Geninatti Crich et al., 2015). Crich and colleagues further demonstrated that HosFn is specifically taken up by mouse breast cancer cells via binding Scara5, which is confirmed by the siRNA knock-down of mouse Scara5 on mouse breast cancer cells reducing the uptake of HosFn. This finding is consistent with the report of Mendes-Jorge et al. (2014). Together, these evidences indicate that HosFn (22 L of 24 subunits) shares (cross-interacts with) the same receptor as mouse L-ferritin.

For human breast cancer cells, Crich and colleagues deduced that HosFn binds to tumor cells through Scara 5, based on the fact that the human breast cancer cells also express Scara5 (Geninatti Crich et al., 2015), the human ortholog protein of mouse Scara5. So far, this theory still lacks evidence that directly proves the interaction of HosFn and human Scara5 protein. Thus, as shown in Table 2, this interaction is still to be confirmed.

In summary, HosFn can cross interact with the receptor of human H-ferritin—human TfR1, and the receptor of mouse L-ferritin—mouse Scara5 (Fan, Zhou, & Yan, 2017). Other interactions still need to be explored.

3 Natural Human Ferritins as Prognostic Tumor Markers

3.1 Secretory Ferritins

Although the physiological function and source of secretory ferritins are still unknown, numerous evidence indicate that secretory ferritin is involved in pathological processes. For instance, a low concentration of serum ferritin indicates iron deficiency (e.g., anemia) and a high serum ferritin indicates iron overload (e.g., hemochromatosis) (Knovich et al., 2009; Wang et al., 2010). In addition, elevated serum ferritin is also found in inflammation, infection, and liver diseases (Arosio et al., 2009; Knovich et al., 2009; Wang et al., 2010). There is increasing evidence that serum ferritin levels are elevated in many malignancies, which attracted widespread attention considering that serum ferritin can be used as a tumor biomarker. For instance, serum ferritin is elevated in breast cancer patients (Jezequel et al., 2012; Knovich et al., 2009; Wang et al., 2010). Serum ferritin is also used as a biomarker for relapse of malignant diseases. Matzner et al. reported that serum ferritin was markedly

increased in all relapsed cases of acute leukemia (Matzner, Konijn, & Hershko, 1980). In all cases, remission was associated with the normalization of serum ferritin levels. These correlations suggest that serum ferritin may be useful in the initial clinical evaluation and in the assessment of response to therapy in patients with acute leukemia and malignant lymphoma (Matzner et al., 1980). Jain et al. recently reported that elevated serum ferritin is predictive of inferior survival in patients with acute leukemia and may be an early marker of an underlying systemic pathological inflammation (Jain et al., 2016). Szymendera et al. and Volpino et al. found that measuring serum ferritin levels is a useful clinical indicator in patients with testicular germ-cell tumors (Szymendera et al., 1985) and lung cancer (Volpino et al., 1984), respectively. Moreover, it has been found that melanoma cells can secrete ferritin, which contributes to the progression of melanoma (Gray, Arosio, & Hersey, 2003). The H/L ratio of serum ferritin also varies in pathological conditions (Cazzola et al., 1985). Under normal physiological conditions, serum ferritin is predominantly composed of L-chains. However, in many malignant conditions, the ratio of H/L in serum ferritin is increased (Arosio et al., 2009; Knovich et al., 2009; Meyron-Holtz et al., 2011; Wang et al., 2010).

Besides serum levels, secretory ferritin levels in cerebrospinal fluid (CSF) have also been found to increase during malignant infiltration of the central nervous system, and have been studied for their ability to serve as a biomarker for the diagnosis of brain malignancies (de Almeida et al., 2008; Meyron-Holtz et al., 2011). Intriguingly, exhaled ferritin has been reported as a potential biomarker for lung cancer (Carpagnano et al., 2012).

3.2 Tissue Ferritins

A number of studies have shown that tissue ferritins are highly expressed in tumorigenic cell lines as well as in some malignant tissues from patients. Although mechanisms underlying these changes are still unclear, these studies reveal that tissue ferritin, especially H-ferritin, may play an important role in malignancy (Arosio et al., 2009; Knovich et al., 2009; Meyron-Holtz et al., 2011; Wang et al., 2010). Importantly, recent reports demonstrated that H-ferritin exerts a negative control on the formation of ovarian cancer stem cells. Moreover, the change of expression levels of H-ferritin may play a role in regulating the amount of cancer stem cells generated, and therefore the formation of metastasis and relapse after chemotherapy (Lobello et al., 2016). Consistent with this report, Rosager et al. found that low levels of H-ferritin-positive tumor cells were associated with poor survival in astrocytic brain tumors (Rosager et al., 2017). Thus, H-ferritin may behave as a prognostic factor for different types of cancers, and it also could be used as a potential cancer therapy target.

Intriguingly, several recent studies also demonstrated that human L-ferritin is a potential prognostic marker in different kinds of cancers. In breast cancer, Jezequel et al. demonstrated that L-ferritin expression is a potential node-negative breast cancer prognostic marker (Jezequel et al., 2012). In gastric cancer, Zhang et al. found that L-ferritin protein was

significantly higher in gastric tissues than in adjacent tissues. In addition, the associations between L-ferritin expression and depth of tumor invasion, differentiation grade, lymph node metastasis and TNM (Tumor, Nodes, Metastasis) stage were significant (Zhang, Chen, & Xu, 2017). This work demonstrated that L-ferritin might be used as a prognostic molecular biomarker in gastric cancer patients and may be a potential novel therapeutic target for gastric cancer. Similarly with the report of Zhang et al., Rosager et al. found that high amounts of L-ferritin positive microglia/macrophages had a negative prognostic value for astrocytic brain tumors (Rosager et al., 2017). These reports indicate that, besides assisting with iron formation, L-ferritin may play an important role in the tumorigenesis and progress of cancers. This novel function of L-ferritin in tumors is worth further exploration.

In conclusion, human ferritins themselves in tissues or secretions can act as prognostic markers for tumor theranostics.

4 Tumor Targeting Strategies of Ferritin Nanoprobes

In general, tumor targeting strategies of nanoprobes can be divided into passive and active targeting. Passive targeting of nanoprobes mainly depend on the enhanced permeability and retention (EPR) effects due to the disordered blood and lymph vessels systems in tumor tissues (Greish, 2010). The EPR effects of nanoprobes rely on their size distribution (Ngoune et al., 2016). Because the sizes of the different bioengineered ferritins are typically around 12–20 nm, bioengineered ferritin nanoprobes should all have similar passive tumor targeting abilities. Thus, here we focus on active targeting strategies using ferritin nanoprobes.

4.1 Indirect Targeting of Bioengineered Ferritin Nanoprobes Against Tumors

Engineered nanoparticles have been extensively used to develop nanoprobes that provide diagnostic, therapeutic, and prognostic information about the status of tumors (Cheng et al., 2012; Xie, Lee, & Chen, 2010). Nanoprobes developed for these purposes are typically modified with targeting ligands, such as antibodies, peptides, or small molecules, to enhance their tumor targeting capability (Shi et al., 2016; Wilhelm et al., 2016). Before the cell membrane receptors of ferritin were identified, the tumor targeting strategies of ferritin nanoprobes were bioengineering and chemical modification. Due to ferritin nanoprobes being proteins encoded by genes, it is convenient to manipulate the functionalization of ferritins via gene engineering (Fan et al., 2013). In addition, both lysine and cysteine residues exposed on the surface of ferritin nanocages can be used to effectively conjugate with chemical groups via cross-linking with *N*-hydroxysuccinimide (NHS) ester or maleimide groups (Truffi et al., 2016). Thus, ferritin nanocages can be either genetically or chemically modified to construct active tumor targeting nanoprobes (Fig. 2) (Wang et al., 2017).

Most of the bioengineered ferritin nanoprobes choose human H-ferritin as the structural motif of the nanoprobe (Table 3). Several studies have genetically functionalized the H-ferritin

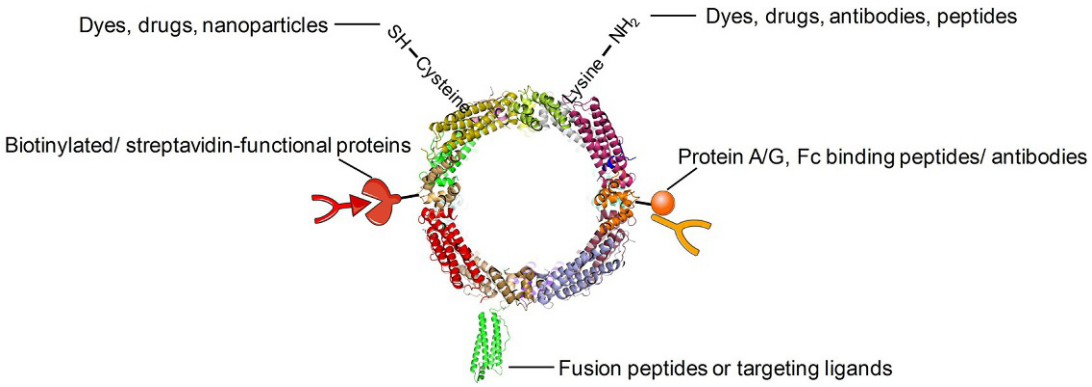

Fig. 2
Illustration of strategies to provide surface functionalization of ferritin nanoprobes by chemical or genetic modification.

Table 3: Tumor targeting moieties of engineered ferritin nanoprobes

Ferritin Types in Engineered Nanoprobes	Targeting Moieties	Targeting Receptors	Tumor Cell Types	References
Human H-ferritin	RGD-4C (CDCRGDCFC) peptide	$\alpha_v\beta_3$ integrins	Human melanoma cell, C32; Human glioma cell, U87MG; Human epithelial lung cancer cell, A549; mouse breast cancer cell, 4T1	Huang et al. (2017a, 2014), Li et al. (2012b), Lin et al. (2011a), Uchida et al. (2006), and Zhen et al. (2013a, 2013b, 2014)
Human H-ferritin	EGF	EGFR	Human breast cancer cell, MDA-MB-231 and MCF-7	Li et al. (2012a)
Human H-ferritin	EGF-derived peptide	EGFR	Human breast cancer cell, MDA-MB-468	Lee et al. (2015)
Human H-ferritin	VNTANST peptide	Vimentin	Mouse melanoma cell, B16F10	Lee et al. (2015)
Human H-ferritin	α-Melanocyte-stimulating hormone peptide (MSH)	Melanocortin receptor	Mouse melanoma cell, B16F10	Fantechi et al. (2014) and Vannucci et al. (2012)
Human H-ferritin	Tumor targeting proapoptotic peptide $CGKRK(KLAKLAK)_2$	p32-mediated internalization	Human breast cancer cell, MDA-MB-231, and human promyelocytic leukemia cell, HL60	Kim et al. (2016a)
Human H-ferritin	Human IgG-specific repebody + Cetuximab/ Trastuzumab	EGRF/HER2	Human breast cancer cell, MDA-MB-468, and SK-BR-3	Kim et al. (2017)

Continued

Table 3: Tumor targeting moieties of engineered ferritin nanoprobes—cont'd

Ferritin Types in Engineered Nanoprobes	Targeting Moieties	Targeting Receptors	Tumor Cell Types	References
Human H-ferritin	Mouse anti CSPG4 Ab	Melanoma-specific antigen CSPG4	Human melanoma cell, Colo 38	Falvo et al. (2013)
Human H-ferritin	Protein G + mouse antihuman-CLDN4	Claudin-4	Human pancreatic cancer cell, Capan-1	Hwang et al. (2013)
Human H-ferritin	FAP-specific single chain variable fragment (scFv)	Carcinoma-associated fibroblasts	Mouse breast cancer cell, 4T1	Zhen et al. (2016)
Human L-ferritin	RGD (GRGDSP) peptide	$\alpha_v\beta_3$ integrins	Human glioma cell, U87MG, and fibrosarcoma cell, HT1080	Kim et al. (2016b)
Horse spleen ferritin	DRGETGPAC peptide	Carcinoma-associated fibroblasts	Human prostate cancer cell, PC-3, and prostate cancer-associated fibroblasts	Ji et al. (2013)
Horse spleen ferritin	Anti-PSMA antibodies	Prostate specific membrane antigen (PSMA)	Human prostate cancer cell, LNCaP	Dostalova et al. (2016)
Horse spleen ferritin	C3d peptide	NCAM	Tumor-derived endothelial cells	Crich et al. (2006)
P. furiosus ferritin	Fc-binding peptide + Herceptin	HER2	Human breast cancer cell, SKBR3	Kang et al. (2012)
P. furiosus ferritin	Fc-binding peptide + rabbit antifolate receptor Ab	Folate receptor	Human oral epithelial cancer cell, KB	Kang et al. (2012)
Dps, a bacterial ferritin	RGD (CDCRGDCFC) peptide	$\alpha_v\beta_3$ integrins	Human glioma cell, U87MG	Ahn et al. (2014)

protein cage with specific ligands such as peptides (e.g., RGD) (Uchida et al., 2006), growth factors (e.g., EGF) (Li et al., 2012a), and antibody-binding proteins (e.g., protein G) (Hwang et al., 2013) to improve its targeting capability. For instance, Douglas's group genetically modified human H-ferritin with RGD-4C (CDCRGDCFC) peptide that can specifically target tumor angiogenesis via binding to $\alpha_v\beta_3$ integrin molecules on the vascular endothelium (Kitagawa et al., 2012; Uchida et al., 2006). Furthermore, many groups demonstrated that the RGD-4C modified H-ferritin nanoprobe bound to many types of tumor cells, including human amelanotic melanoma (Uchida et al., 2006), glioblastoma (Lin et al., 2011a; Zhen et al., 2013a, 2013b), lung adenocarcinoma cells (Li et al., 2012b), and mouse breast cancer cells (Huang et al., 2017a, 2014; Zhen et al., 2014). Currently, the RGD modification is the most-used tumor targeting strategy in bioengineered ferritin nanoprobes. Another ligand, a matrix

metalloproteinase (MMP) protease substrate, was also introduced onto the exterior surface of the H-ferritin nanocage, and could be cleaved by MMP enriched in the tumor (Lin et al., 2011b). As a matter of fact, any ligand, as long as it is able to target tumor markers, could be used for modification of ferritin nanoprobes, such as melanocyte-stimulating hormone (MSH) and its receptor for mouse melanoma cells (Fantechi et al., 2014; Vannucci et al., 2012), or epidermal growth factor (EGF), EGF-derived peptides and its receptor EGFR for human breast cancer cells (Lee et al., 2015; Li et al., 2012a). In addition, some newly identified peptides, including VNTANST peptide for Vimentin in mouse melanoma cells (Lee et al., 2015), and CGKRK$(KLAKLAK)_2$ peptide for proapoptotic tumor targeting in human breast cancer and promyelocytic leukemia cells (Kim et al., 2016a) have already been genetically modified on the surface of H-ferritin nanoprobes to target tumor cells. An antibody is a powerful tool for tumor targeting. The antibody Fc binding protein-protein G (Hwang et al., 2013), or human IgG-specific binding repebody (Kim et al., 2017) were also genetically introduced to the exterior of H-ferritin nanoprobes, which allowed antibody-functionalized ferritin nanoprobes to target human breast cancer cells or pancreatic cancer cells. These antibody-binding ferritin nanoprobes also possess the potential to bind therapeutic antibodies to treat cancer cells.

Besides genetic modification, chemical conjugation provides useful approaches for functionalization of ferritin nanoprobes. Falvo et al. directly conjugated a monoclonal antibody to the surface of the H-ferritin nanocage to enable the ferritin nanoprobes to specifically target human melanoma cells (Falvo et al., 2013). A fibroblast activation protein-α specific single chain variable fragment (scFv) was conjugated onto the exterior of the ferritin nanocage to target carcinoma-associated fibroblasts of mouse breast cancer (Zhen et al., 2016). In addition to the use of single-ligand modified magnetoferritin, Chen's group recently generated chimeric ferritin nanocages for multiple function loading and multimodal imaging by combination of chemical modification and genetic engineering to visualize tumors with high resolution and sensitivity (Lin et al., 2011a).

Human L-ferritin is another choice for the structural motif of bioengineered ferritin nanoprobes. Kim et al. genetically modified RGD peptide (GRGDSP) onto the exterior of human L-ferritin to target human glioma and fibrosarcoma cells (Kim et al., 2016b). However, compared with human H-ferritin, L-ferritin is less frequently used in tumor targeting ferritin nanoprobes.

Horse spleen ferritin (HosFn) is also employed to develop tumor targeting nanoprobes. Because HosFn is a commercially available natural ferritin, the targeting nanoprobes based on HosFn are typically modified by chemical conjugation. For instance, DRGETGPAC peptide was chemically conjugated onto the surface of HosFn to target the human prostate cancer cell, PC-3, and prostate cancer-associated fibroblasts (Ji et al., 2013). In addition, biotin was chemically introduced to the exterior of HosFn to construct a universal

ligand/antibody-binding nanoprobe based on the biotin-streptavidin interaction (Li & Mann, 2004). Biotin-C3d peptide was successfully loaded on the biotin-HosFn nanoprobe through streptavidin, thus making a nanoprobe to specifically target tumor-derived endothelial cells (Crich et al., 2006). With the assistance of gold nanoparticles, HosFn was modified with antibody-binding peptides. Anti-PSMA antibodies were then loaded on the surface of the HosFn-Au-peptide nanoprobe to make this system specifically target human prostate cancer cells (Dostalova et al., 2016).

Recently, a novel kind of ferritin isolated from *Pyrococcus furiosus* (optimal living condition at 100°C) was reported (Tatur, Hagen, & Matias, 2007). With a similar structure to human ferritin, the *P. furiosus* ferritin has been proved to have better thermal stability than human ferritins. Thus, *P. furiosus* ferritin is another candidate for tumor targeting nanoprobes. The Fc-binding peptide was also genetically introduced into the exterior of *P. furiosus* ferritin and allowed the ferritin nanoprobes to target human breast cancer cells (with anti-Her2 antibody) and human oral epithelial cancer cells (with antifolate receptor antibody) (Kang et al., 2012). Because of the high stability of *P. furiosus* ferritin, in vitro applications based on this kind of ferritin may be more suitable.

DNA-binding proteins from starved cells (Dps) are 12-mer bacterial ferritins that protect DNA from oxidative stress, and have been implicated in bacterial survival and virulence. Like other ferritins, Dps proteins have a hollow spherical cage-like structure (Arnold, Zhou, & Barton, 2016). Genetic modification of the Dps with RGD peptide (GRGDSP) is another way to construct tumor targeting nanoprobes. Ahn et al. reported that RGD-modified Dps was able to specifically target human glioma cells (Ahn et al., 2014). Considering its relatively smaller size, the Dps-based tumor targeting nanoprobe may possess advantages (e.g., being easily excreted) in tumor imaging applications (Chakravarty, Goel, & Cai, 2014).

4.2 Direct Targeting of Ferritin Nanoprobes Against Tumors

Because the finding that TfR1 is the membrane receptor of human H-ferritin was reported, the intrinsic tumor targeting property of ferritin nanoprobes has drawn increasing attention from nanomedicine researchers. This finding brings us new insight into the physiological functions of ferritin and allows its application as a powerful nanoplatform for cancer diagnosis and therapy. As shown in Table 4, recent studies tend to use the intrinsic tumor targeting property of ferritin nanoprobes, instead of genetically or chemically modified ferritin nanoprobes.

Seaman's group demonstrated that after binding to TfR1 on the cell surface, the H-ferritin-TfR1 complex is internalized, and transported to endosomes and lysosomes (Li et al., 2010). The TfR1 (also named CD71) is a type II transmembrane glycoprotein, which forms a homodimer on the surface of cells (Daniels et al., 2006a). TfR1 was originally identified as the receptor for transferrin (Tf). The Tf-TfR1 regulated iron uptake pathway is the most important route for cellular iron uptake. In addition, TfR1 is also involved in regulating cell

Table 4: Tumor targeting strategies via ferritin-receptor interactions

Ferritin Types	Ferritin Receptors	Tumor Types	References
Human H-ferritin	Human TfR1	Human clinical tumor tissues: hepatocellular carcinoma, lung squamous cell carcinoma, colonic adenocarcinoma, cervical squamous cell carcinoma, prostate adenocarcinoma, ovarian serous papillary carcinoma, breast ductal carcinoma, thymic carcinoma, esophagus squamous cell carcinoma.	Fan et al. (2012)
Human H-ferritin	Human TfR1	Human hepatocellular carcinoma cell, SMMC-7721; human ovarian cancer cell, SKOV-3; human colon cancer cell, HT-29, HCT-116; human breast cancer cell, MDA-MB-231, MDA-MB-468; human melanoma cell, A375; human pancreatic cancer cell, Panc-1, CFPAC-1; human gastric cancer cell, MGC-803; human squamous cell carcinomas of the head and neck cell, FaDu, A-253, Detroit 562	Cai et al. (2015), Cao et al. (2014), Damiani et al. (2017), Fan et al. (2012), Lei et al. (2016), Liang et al. (2014), Pandolfi et al. (2017), and Tongwei et al. (2017)
Human H-ferritin	Mouse receptor (*need to be identified*)	Mouse breast cancer cell, 4T1; mouse lung cancer cell, 3LL	Huang et al. (2017b) and Mazzucchelli et al. (2016)
Horse spleen ferritin	Mouse Scara5 or human TfR1	Mouse breast cancer cell, TUBO; mouse glioma cell, C6; human hepatocellular carcinoma cell, HepG2; human breast cancer cell, MCF-7, MDA-MB-231; human cervical cancer cell, Hela; human prostate cancer cell, LNCaP; human colon cancer cell, HT-29	Chen et al. (2017b, 2017c), Conti et al. (2016), Dostalova et al. (2017), Geninatti Crich et al. (2015), Ghosh et al. (2016), Li et al. (2016a), Liu et al. (2011, 2012), Turino et al. (2017), Yan et al. (2008), and Yang et al. (2015)
Rat H-ferritin	Human receptor (*need to be identified*)	Human glioblastoma cell, U87MG;	Wang et al. (2016)
Pig pancreas ferritin	Human receptor (*need to be identified*)	Human gastric cancer cells, BGC823; human cervical cancer cell, Hela	Ji, Huang, and Huang (2012)
Pig spleen ferritin	Human receptor (*need to be identified*)	Human hepatocellular carcinoma cell, HepG2; human cervical cancer cell, Hela; human breast cancer cell, MCF-7	Liu et al. (2013)

growth (Neckers & Trepel, 1986; O'Donnell et al., 2006). It has been shown that expression of TfR1 in cancer cells may be up to 100-fold higher than in normal cells (Daniels et al., 2006a, 2006b). This might be due to rapidly proliferating cancer cells requiring more iron. The fact that TfR1 is overexpressed in a variety of malignancies and is efficiently internalized makes it an excellent target for tumor diagnosis and treatment (Daniels et al., 2006b).

The strategy of targeting TfR1 for tumor imaging and therapy has already been summarized by many excellent reviews (Daniels et al., 2006a, 2006b, 2012; Fan et al., 2013). Monoclonal antibodies to TfR1 and its natural ligand transferrin have been successfully used to target malignant cells, either alone or carrying various cytotoxic or imaging agents (Daniels et al., 2006b, 2012). For instance, transferrin (Tf)-conjugated diphtheria toxin, termed Tf-CRM107, has been applied in treatment of brain tumors and has been approved for phase III clinical trials (Laske, Youle, & Oldfield, 1997; Weaver & Laske, 2003). Anti-TfR1 antibody-conjugated ricin A chain, termed 454A12-RTA, is currently in phase II clinical trials (Daniels et al., 2012).

Instead of using transferrin and antibodies to target TfR1 (Daniels et al., 2006b), we have previously demonstrated the use of H-ferritin nanoprobes as a new ligand, and successfully targeted TfR1 on different tumor tissues. We screened 474 clinical samples and found that H-ferritin specifically binds to the nine most common human solid tumors, including liver, lung, colon, cervical, ovarian, prostate, breast, and thymus cancers (Fan et al., 2012). Furthermore, it has also been shown that TfR1 can be used as a prognosis indicator in breast cancer (Yang et al., 2001), leukemia (Das Gupta & Shah, 1990; Habeshaw et al., 1983), lung cancer (Kondo et al., 1990), and bladder cancer (Seymour et al., 1987). Thus, H-ferritin nanoprobes possess intrinsic broad-spectrum tumor targeting properties.

Consistent with our clinical tissue results, H-ferritin has been verified to specifically target human hepatocellular carcinoma cells, ovarian cancer cells, colon cancer cells, breast cancer cells, melanoma cells, pancreatic cancer cells, gastric cancer cells, and squamous cell carcinomas of the head and neck, of which all the tumor cells expressed high levels of TfR1 (Cai et al., 2015; Cao et al., 2014; Damiani et al., 2017; Fan et al., 2012; Lei et al., 2016; Liang et al., 2014; Pandolfi et al., 2017; Tongwei et al., 2017). Intriguingly, Sakamoto et al. recently demonstrated that H-ferritin is preferentially incorporated by TfR1-positive cells in a threshold-dependent manner, whereas there is no threshold for transferrin uptake (Sakamoto et al., 2015). This finding renders H-ferritin a more unique tumor targeting property than other ligands or antibodies of TfR1.

Interestingly, several studies recently reported that human H-ferritin also exhibits specific binding to mouse breast cancer cells (Mazzucchelli et al., 2016) and mouse lung cancer cells (Huang et al., 2017b). Because the receptor of mouse H-ferritin has been identified as TIM-2, according to the homological analysis, the potential receptor for human H-ferritin in mouse cancer cells may be TIM-2, but not mouse TfR1 (Huang et al., 2017b). However, this hypothesis needs to be further confirmed.

As we mentioned in Section 2, the commercially available horse spleen ferritin is extensively used in ferritin nanoprobes for tumor theranostics. After the intrinsic tumor targeting property of human H-ferritin was reported, the targeting property of HosFn was also investigated. To date, HosFn has been reported to specifically target mouse breast cancer (Conti et al., 2016)

and glioma cells (Chen et al., 2017b), and human hepatocellular carcinoma cells (Liu et al., 2011, 2012; Yang et al., 2015), breast cancer cells (Conti et al., 2016; Geninatti Crich et al., 2015; Ghosh et al., 2016; Li et al., 2016a; Turino et al., 2017; Yan et al., 2008), cervical cancer cells (Chen et al., 2017c), prostate cancer cells (Dostalova et al., 2017), and colon cancer cells (Yang et al., 2015). To the best of our knowledge, HosFn specifically targets mouse cancer cells via cross-interacting with mouse Scara5 (Li et al., 2009), the receptor of mouse L-ferritin, while HosFn recognizes human cancer cells via binding to TfR1 (Li et al., 2010), the receptor of human H-ferritin.

Unexpectedly, some reports demonstrated that ferritins from other species also specifically bind to human cancer cells. For instance, Wang et al. found that rat H-ferritin targets human glioma cells (Wang et al., 2016). Ji et al. demonstrated that pig pancreatic ferritin specifically recognizes human gastric cancer cells and cervical cancer cells (Ji et al., 2012). Liu et al. reported that pig spleen ferritin specifically binds to human hepatocellular carcinoma cells, cervical cancer cells, and breast cancer cells (Liu et al., 2013). These results indicate that there are potential receptors in human cancer cells for these different ferritins. Considering the highly conserved structure of ferritins from different species and absolutely different receptors of human H-ferritin and mouse H-ferritin, it is necessary to systematically study the cross-interactions of ferritins and their reported receptors.

Taken together, the intrinsic tumor targeting property of natural ferritins will most likely be the key strategy to designing tumor targeting ferritin nanoprobes in the future.

5 Ferritin Nanoprobes for Tumor Imaging

With the emergence of nanotechnology, ferritins can be conveniently mineralized or synthesized to produce many kinds of nanoparticles without disruption of the integrity of the protein shells (Fan et al., 2013; Kashanian et al., 2012; Meldrum et al., 1991; Meldrum, Heywood, & Mann, 1992; Sun et al., 2011b; Suzumoto, Okuda, & Yamashita, 2012). By employing recombinant human H-ferritin as a template, Douglas's group biomimetically synthesized a type of magnetic nanoparticle in 2006 (Uchida et al., 2006). This engineered ferritin molecule contains an iron core in the form of magnetite (Fe_3O_4), which is different from the natural ferritin iron core of ferrihydrite (Fe_2O_3) (Cao et al., 2010; Uchida et al., 2006). This engineered ferritin, which has the same architecture as natural H-ferritin, is termed magnetoferritin. Because of the unique architecture, the ferritin nanoprobe provides an ideal nanoplatform for multifunctional loading to enhance the functionality of its surface (e.g., to target tumors), and metal cations can be encapsulated in the interior (e.g., contrast imaging probes) (Fig. 2).

Because the magnetoferritin nanoprobe is a biomimetic product, this means that natural ferritin nanoparticles exist in the human body. It is possible to directly use endogenous ferritin

as the reporter to monitor disease progression in vivo with MRI rather than administering foreign premade ferritin nanoparticles (Cohen et al., 2009; Gilad et al., 2007). Kim et al. and Choia et al. have developed a model which allows tumor cells overexpressing H-ferritin to use ferritin as a reporter for MRI. This model could be used for in vivo tumor imaging and monitoring of tumor metastasis in lymph nodes (Choi et al., 2012; Kim et al., 2010). Lu et al. designed an endogenous ferritin reporter gene for magnetic resonance imaging (MRI) with a tumor-specific promoter (AFP-promoter) to monitor the in vivo tumor targeting of peptide-functionalized superparamagnetic iron oxide (Lu et al., 2017). The advantage of using ferritin as a reporter for MRI is that it does not require an exogenous contrast agent to be delivered to the targeted tumor area and makes it possible to carry out long-term in vivo imaging. However, the level of ferritin expression during tumor development needs to be investigated. Compared with magnetoferritin nanoparticles, the limitation of using endogenous ferritin is its inherent low sensitivity.

Magnetoferritin nanoparticles consist of iron nanocrystals. The superparamagnetism of magnetoferritin makes it an ideal contrast agent for magnetic resonance imaging (MRI) for tumor diagnosis. MRI can provide high spatial resolution and functional anatomic and physiological information with simultaneous noninvasive imaging. Normally, use of magnetoferritin nanoparticles for imaging results in reduced signal intensity in T_2-weighted MRI, which is also called T_2* signal loss (Uchida et al., 2006, 2008). The tumor-bearing area displays weakened intensity in MRI when using ferritin nanoparticles as the contrast agent compared to MRI of normal tissue. This negative change makes it difficult to acquire enough accurate information for tumor diagnosis.

Therefore, ferritin nanoprobe-based multimodality imaging is used, which combines MRI with other imaging modalities, such as fluorescence imaging or positron emission tomography (PET). Based on the special architecture of ferritin nanoprobes, two modification strategies could allow the performance of multimodal imaging in a single examination. One is introduction of functional groups onto the exterior surface of the nanoparticles by chemical or genetic modification of ferritin. For example, a fluorescent dye can be covalently conjugated onto H-ferritin (Uchida et al., 2006), and GFP could be fused into H-ferritin by genetic engineering (Li et al., 2012b). However, fluorescence imaging may not have sufficient signal intensity during noninvasive detection, especially when tumors are located in deep tissue, because the light could not effectively penetrate the skin and deep tissues even using in situ exposure (Terashima et al., 2011). Recently, our group developed a novel ferritin nanoprobe to acquire both MRI and nuclear imaging in a single dose via chemically conjugating magnetoferritin with ^{125}I. This strategically engineered magnetoferritin nanoprobe can image tumors with high sensitivity and specificity using SPECT and MRI in living mice after a single intravenous injection, thus enabling simultaneous functional and morphological tumor imaging without reliance on

multiple injections (Zhao et al., 2016). Novel fluorescence imaging technology is also needed to overcome the limitations of MRI based on magnetoferritin. Chen's group has developed near-infrared fluorescence imaging (NIRF) by conjugation of Cy 5.5/ZW800/Alexa 750/IRdye800 dye (Li et al., 2012a; Lin et al., 2011a, 2011b; Zhen et al., 2013a, 2013b, 2016) onto H-ferritin for in vivo imaging of tumor xenografted mice either with NIRF only or combining it with PET for multimodal imaging (Lee et al., 2011; Lin et al., 2011a).

Another route is entrapping functional contrast agents into the core of the nanoparticles by regulating the disassembly/reassembly process under pH control (Huang et al., 2014). Gadolinium (Gd(III)) chelates and Mn^{II} could be loaded into the core of ferritin nanoprobes for in vivo tumor MRI with enhanced T1-weighted signal because of high R1 relaxivity (Aime, Frullano, & Crich, 2002; Crich et al., 2006; Kalman, Geninatti-Crich, & Aime, 2010; Szabo et al., 2012). Radioisotopes are another potential contrast agent that can be loaded into the core of the nanoparticles for multimodal tumor imaging. Chen's group loaded ^{64}Cu into the core of H-ferritin nanocages to carry out in vivo PET for tumor imaging by combining PET with NIRF imaging in a single examination (Lin et al., 2011a). In addition, Huang et al. loaded IR820, a photoacoustic imaging dye, into the cavity of H-ferritin nanocages to perform the in vivo PAI/NIRF multimodal imaging (Huang et al., 2014). The generality of modification and functionalization makes ferritin nanoparticles a promising nanoplatform to achieve multimodal imaging in translational cancer diagnosis.

Recently, our group developed magnetoferritin as a dual-functional reagent, allowing simultaneous targeting and visualization of tumors. Based on this finding, we developed a novel type of immunochemistry (IHC) technique for tumor detection. After verifying the interaction between H-ferritin and its receptor TfR1 in clinical tissue specimens, we found that H-ferritin specifically recognizes nine types of tumor tissues (Fan et al., 2012). In addition, we found that magnetoferritin nanoprobes have intrinsic peroxidase-like activity (Fan et al., 2012; Gao et al., 2007). Both the mineral phase composition and the size of the iron core encapsulated in ferritin protein shells determine the peroxidase activity of the magnetoferritin (Cai et al., 2015; Fan et al., 2012). Biomimetically synthesized magnetoferritin nanoparticles with mineral cores consisting of magnetite or maghemite exhibited a much higher peroxidase activity when compared with natural holoferritin. Thus, magnetoferritin nanoparticles can catalyze peroxidase substrates to produce the same color reaction as peroxidase enzymes. For instance, magnetoferritin nanoparticles react with TMB to produce a blue color and DAB to produce a brown precipitate. Furthermore, Zhang et al. reported that cobalt-doped magnetoferritin nanoprobes can further enhance the peroxidase-like activity of magnetoferritin, thus improving the sensitivity of the magnetoferritin-based IHC method for tumor detection (Tongwei et al., 2017).

With architectures similar to human H-ferritin, other types of ferritins are also extensively employed to develop multimodal imaging nanoprobes for tumor imaging. For horse spleen ferritin, Gd-HPDO3A/Mn^{II}/Melanin-Fe^{3+}/^{64}Cu were loaded into the cavity of HosFn for MRI/PAI/PET multimodal imaging via regulating the disassembly/reassembly process under pH control (Conti et al., 2016; Geninatti Crich et al., 2015; Kalman et al., 2010; Szabo et al., 2012; Yang et al., 2015). FAM was chemically conjugated onto the exterior surface of HosFn for NIRF imaging (Crich et al., 2006; Ji et al., 2013). CuS and ^{64}Cu were loaded into the cavity of a rat H-ferritin nanoprobe for PAI/PET imaging (Wang et al., 2016). For Dps, Cy5.5 dye was conjugated onto the surface of the protein cage for NIRF tumor imaging (Ahn et al., 2014).

As shown in Table 5, the unique architecture and physicochemical properties of ferritin nanoprobes have made it easy to construct multimode imaging nanoprobes for NIRF/MRI/SPECT/PET/PAI or IHC for tumor imaging in the last decades (Fig. 3). Combining optical, photoacoustic and nuclear imaging, and tumor targeting strategies could improve and refine the intraoperative tumor imaging, to accurately excise the tumor lesion with clear margins, and provide targeted theranostics tools for cancer diagnosis and therapy (Fig. 4).

Table 5: Ferritin nanoprobes and their applications in tumor imaging

Ferritin Nanoprobes	Encapsulation/Labeling Imaging Reagents	Imaging Methods	References
Human-H-ferritin	Fe_3O_4 (magnetite/maghemite)/$Co_xFe_{3-x}O_4$	IHC	Cai et al. (2015), Fan et al. (2012), and Tongwei et al. (2017)
Human-H-ferritin	Cy5.5 Fe_3O_4 (magnetite/maghemite) ^{125}I	NIRF MRI SPECT	Cao et al. (2014), Liang et al. (2014), Mazzucchelli et al. (2016), and Uchida et al. (2008)
Surface-modified human H-ferritin	Cy 5.5/ZW800/Alexa 750/IRdye800 ^{64}Cu	NIRF PET	Li et al. (2012a), Lin et al. (2011a, 2011b), and Zhen et al. (2013a, 2013b, 2016)
Surface-modified human H-ferritin	Fe_3O_4 (magnetite/maghemite)	MRI	Li et al. (2012b)
Surface-modified human H-ferritin	IR820	NIRF/PAI	Huang et al. (2014)
Horse spleen ferritin	Gd-HPDO3A/Mn^{II} Melanin/Fe^{3+} ^{64}Cu	MRI PAI PET	Conti et al. (2016), Geninatti Crich et al. (2015), Kalman et al. (2010), Szabo et al. (2012), and Yang et al. (2015)
Surface-modified horse spleen ferritin	FAM Gd-HPDO3A	NIRF MRI	Crich et al. (2006) and Ji et al. (2013)
Rat H-ferritin	CuS ^{64}Cu	PAI PET	Wang et al. (2016)
Surface-modified *Escherichia coli* DNA-binding protein	Cy 5.5	NIRF	Ahn et al. (2014)

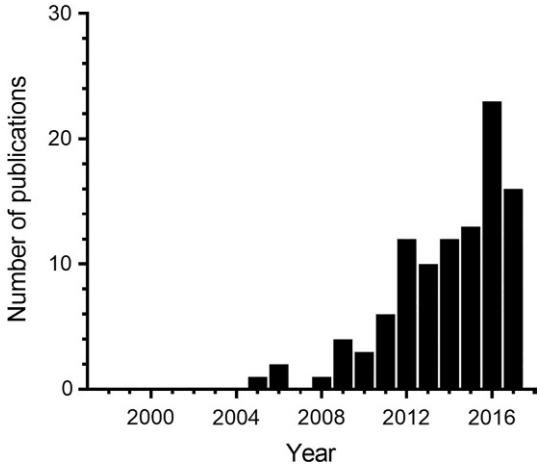

Fig. 3
Number of published papers on ferritin nanoprobes for tumor imaging by the end of September 2017. The data is based on Web of Science.

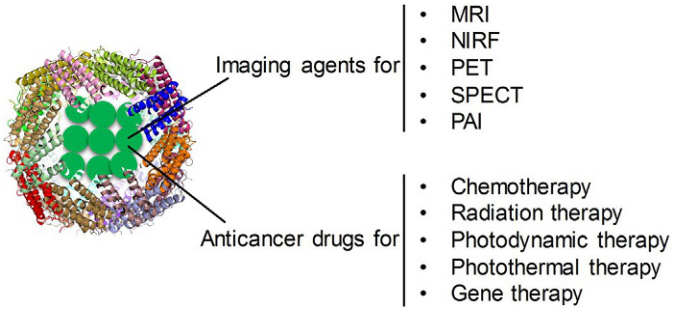

Fig. 4
Encapsulation cargos inside of ferritin nanoprobes for tumor theranostics.

6 Ferritin Nanocarriers for Tumor Therapy

In the last decades, plenty of studies have begun to design ferritin nanoprobes as carriers to deliver drugs for therapeutic purposes (Fig. 5). Instead of a magnetite core, apoferritin nanoparticles can be used to encapsulate chemical drugs in the interior cavity by manipulating disassembly/reassembly of the nanoprobe under pH control or urea concentration control (Kilic, Ozlu, & Calis, 2012; Liang et al., 2014; Simsek & Kilic, 2005). The first anticancer drug encapsulation inside a horse spleen ferritin nanocage was cisplatin and carboplatin, reported by Guo's group, with each ferritin encapsulating around 30 cisplatin molecules and 15 carboplatin molecules (Yang et al., 2007). Functional studies showed that ferritin with entrapped cisplatin could induce apoptosis of cancer cells (Falvo et al., 2013; Ji et al., 2012). However, Doxorubicin

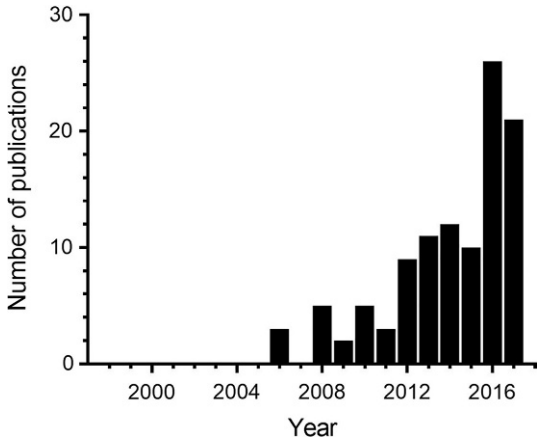

Fig. 5
Number of published papers on ferritin nanoprobes for tumor therapy by the end of September, 2017. The data is based on Web of Science.

(Dox) encapsulation in ferritin nanocages represents the most extensively studied system for tumor-targeted drug delivery (Table 6).

Dox is a widely used clinical anticancer drug for several types of solid tumors, while its use is dose-limited due to its severe side effects. The initial Dox-loading strategy was employing a disassembly/reassembly process under pH control by using HosFn, and the maximum loading was 28 Dox per ferritin molecule, but the yield was very low (Kilic et al., 2012). After analyzing the structure and physicochemical properties of human H-ferritin, we developed, for the first time, a urea-based Dox-loading strategy, improved the loading efficiency of HFn-Dox and evaluated the antitumor activity of HFn-Dox in vivo in three different human tumor xenograft tumor models (Liang et al., 2014). Following our finding, Lei et al. and Mazzucchelli et al. also developed HFn-Dox and tested the antitumor activity in cell lines and tumor models (Lei et al., 2016; Mazzucchelli et al., 2016). Zhen et al. demonstrated that Dox precomplexed with Cu(II) can be effectively loaded into ferritin nanocages, and ferritin-Cu-Dox exhibits significant antitumor activity in vitro and in vivo (Zhen et al., 2013a). However, this heavy-metal dependent drug loading strategy is hard to translate.

Recently, Ceci's group reported that fusion of human H-ferritin with proline, serine, and alanine elements and chemical modification with PEG significantly improve the Dox-loading efficiency (up to 90 Dox per ferritin nanocage) (Table 6) (Falvo et al., 2016). They also demonstrated that this modified ferritin drug delivery system exhibits significant antitumor activity in human fibrosarcoma xenograft model (Fracasso et al., 2016) and head and neck cancer xenograft model (Damiani et al., 2017); however, this modification also brings new challenges to translational research, and the researchers underestimated the potential impact of additional amino acid sequences on biosafety and the in vivo response (Tosi et al., 2016).

Table 6: Ferritin nanocarriers and their antitumor effects

Ferritin Nanoprobes	Encapsulation Drugs	Encapsulating Methods	Loading Efficiency (Drug/Ferritin or wt%)	Treatment Details and Tumor Growth Inhibition (TGI)	References
Horse spleen ferritin	Doxorubicin (anticancer drug)	Disassembly at pH 2.5, then reassembly at pH 7	28 molecules	–	Kilic et al. (2012)
Human H-ferritin	Doxorubicin	Disassembly at 8 M urea, then gradual reassembly from 7 M urea to PBS	33 molecules	Single dose of 20 mg Dox (eqv.)/kg; ~90% TGI for human colon cancer xenograft; ~86.67% TGI for human melanoma xenograft; ~75% TGI for human breast cancer xenograft model	Liang et al. (2014)
Human H-ferritin	Doxorubicin	Disassembly at 8 M urea, then gradual reassembly from 7 M urea to PBS	32.5 molecules	–	Lei et al. (2016)
Human H-ferritin	Doxorubicin	Disassembly at 8 M urea, then gradual reassembly from 7 M urea to PBS	29 molecules	1.24 mg/kg of Dox (eqv.) were i.v. injected every 4 days for 3 weeks; 75% TGI for mouse breast cancer	Mazzucchelli et al. (2016)
Surface-modified human H-ferritin	Doxorubicin	Precomplexation with Cu(II). then incubation with ferritin nanoprobes	73.41 wt%	5 mg Dox (eqv.)/kg) were i.v. injected every 2 days for 2 weeks; 89.6% TGI for human glioma xenograft model	Zhen et al. (2013a)
Surface-modified human H-ferritin	Doxorubicin	Disassembly at pH 2, then reassembly at pH 8.0	90 molecules	5 mg/kg of Dox (eqv.) were i.v. injected twice a week for two weeks; ~90.9% TGI for human fibrosarcoma xenograft model	Falvo et al. (2016) and Fracasso et al. (2016)
Surface-modified human H-ferritin	Doxorubicin	Disassembly at pH 2, then reassembly at pH 8.0	86 molecules	5 mg/kg of Dox (eqv.) were i.v. injected five times twice a week; ~88.88% TGI for human head and neck cancer xenograft model	Damiani et al. (2017)
Surface-modified human H-ferritin	Doxorubicin	Chemical conjugation	88 molecules	Single dose of 0.25 mg/kg Dox (eqv.) was administered intratracheally; 40-fold decrease of tumor BLI signal for mouse lung cancer model	Huang et al. (2017b)
Horse spleen ferritin	Methylene blue (PDT drug)	Disassembly at pH 2, then reassembly at pH 7	1 molecule	–	Yan et al. (2008, 2010)
Horse spleen ferritin	Daunomycin (anticancer drug)	Loading drug at pH 5, assistance with poly-L-aspartic acid	0.2 molecule	–	Ma-Ham et al. (2011)

Continued

Table 6: Ferritin nanocarriers and their antitumor effects—cont'd

Ferritin Nanoprobes	Encapsulation Drugs	Encapsulating Methods	Loading Efficiency (Drug/Ferritin or wt%)	Treatment Details and Tumor Growth Inhibition (TGI)	References
Surface-modified human H-ferritin	ZnF16Pc (PDT drug)	Incubation in PBS	60 wt%	i.v. injection of 1.5 mg ZnF16Pc (eqv.)/kg, with irradiation of 671 nm laser, 0.3 W/cm^2 for 15 min; 83.64% TGI of human glioma xenograft model; 82.65% TGI for mouse breast cancer model with folate receptor target; 85.9% TGI of mouse breast cancer model in combination with Doxil (10 mg/kg); 88.60% TGI for mouse breast cancer model in combination with FAP targeting	Zhen et al. (2013b, 2014, 2015, 2016)
Surface-modified human H-ferritin	IR820 (PTT drug)	Disassembly at pH 2, then reassembly at pH 7.4	17.32 wt%	0.4 mg IR820 (eqv.) with irradiation of 808 nm laser, 0.5–1 W/cm^2 for 10 min; 100% tumor elimination of mouse breast cancer model	Huang et al., 2014
Surface-modified human H-ferritin	Sinoporphyrin sodium (PDT and PTT drug)	Disassembly at pH 2, then reassembly at pH 7.4	66.67 wt%	8 mg/kg of drug (eqv.) was injected i.v., then exposed to a 630-nm laser at a laser dose of 75 J; 100% tumor ablation for mouse breast cancer model	Huang et al. (2017a)
Horse spleen ferritin	Cisplatin (anticancer drug)	Disassembly at pH 2, then reassembly at pH 7	30 molecules	–	Yang et al. (2007)
Horse spleen ferritin	Cisplatin	Disassembly at pH 13, then reassembly at pH 7	20–55 molecules	–	Pontillo et al. (2016)
Pig pancreas ferritin	Cisplatin	Disassembly at pH 13, then reassembly at pH 7	11.26 molecules	–	Ji et al. (2012)
Antibody-conjugated human H-ferritin	Cisplatin	Disassembly at pH 2.2, then reassembly at pH 7.5	50 molecules	3 mg cisplatin (eqv.)/kg, were i.v. injected twice. 64.29% TGI of human melanoma xenograft model	Falvo et al. (2013)
Horse spleen ferritin	Carboplatin	Disassembly at pH 2, then reassembly at pH 7	15 molecules	–	Yang et al. (2007)
Human H-ferritin	Curcumin (anticancer drug)	Disassembly at pH 12.5, then reassembly at pH 7.4	90 molecules	–	Pandolfi et al. (2017)

Human H-ferritin	Curcumin	Disassembly at pH 2, then reassembly at pH 7.5	14.7 molecules	–	Chen et al. (2014b)
Truncated human H-ferritin	Curcumin	Disassembly at pH 4.0, then reassembly at pH 7.5	14 molecules	–	Chen et al. (2016)
Horse spleen ferritin	Curcumin	Disassembly at pH 2, then reassembly at pH 7	9.6 molecules	10 mg Curcumin (eqv.)/kg of were i.v. injected every 3 days for 6 weeks; 60% of mouse breast cancer response to the treatment	Conti et al. (2016), Cutrin et al. (2013), and Geninatti Crich et al. (2015)
Horse spleen ferritin	Gold-based anticancer drugs (Auoxo3/Auoxo4/ Au_2phen)	Disassembly at pH 13, then reassembly at pH 7.4	210 Auoxo3 192 Auoxo4 216 Au_2phen	–	Ferraro et al. (2016) and Monti et al. (2017)
Human H-ferritin	Atropine (anticancer drug)	Disassembly at 8 M urea, then gradual reassembly from 7 M urea to PBS	46.7 molecules	0.30–0.45 mg/kg of atropine (eqv.) were i.v. injected twice a week for 4 weeks; ~86.67% TGI for human pancreatic cancer xenograft model.	Lei et al. (2016)
Human H-ferritin	β-Carotene (anticancer drug)	Disassembly at pH 11, then reassembly at pH 7.5	12.4 molecules	–	Chen et al., 2014a
Horse spleen ferritin	^{235}U (anticancer radiation reagent)	Incubation in PBS	~800 molecules	–	Hainfeld (1992)
Pig spleen ferritin	5-Fluorouracil (anticancer drug)	Incubation with gold nanoparticle loaded ferritin nanoprobes	45.5 molecules	–	Liu et al. (2013)
Human H-ferritin	siRNA	Disassembly at pH 2, then reassembly at pH 8.5	0.2 molecule	–	Li et al. (2016b)
Human H-ferritin	Gefitinib	Incubation in Tris buffer (pH 8.0)	~10 molecules	–	Kuruppu et al. (2015)

In addition to encapsulating chemical drugs in its cavity, the exterior surface is another platform to load protein drugs, such as antibodies, toxins, and peptides, by genetic engineering or chemical conjugation. Interestingly, Huang et al. reported that a human H-ferritin nanoprobe can penetrate airway mucus and tumor tissue to treat lung cancers. Employing chemical conjugation, Huang et al. loaded up to 88 Dox on the surface of H-ferritin, and further modified it with PEG. This airway mucus-penetrating ferritin delivery system exhibits excellent antilung cancer activity (Huang et al., 2017b).

Besides Dox, Daunomycin was successfully loaded inside of horse spleen ferritin with about 0.2 molecules per nanocage by a poly-L-aspartic acid-assisted loading strategy, although its antitumor activity was absent (Ma-Ham et al., 2011). Other types of anticancer drugs, such as curcumin, have been effectively loaded inside of human H-ferritin (Pandolfi et al., 2017), truncated human H-ferritin (Chen et al., 2016), or HosFn (Conti et al., 2016) via disassembly/reassembly of the nanoprobes under pH control. The significant antitumor activity of HosFn-curcumin was demonstrated in a mouse breast cancer model (Conti et al., 2016; Cutrin et al., 2013; Geninatti Crich et al., 2015). Anticancer gold(III) compounds, such as Auoxo3, Auoxo4, and Au_2phen, were effectively encapsulated within the HosFn nanocages and exhibited significant cytotoxic effects on different human cancer cells (Ferraro et al., 2016; Monti et al., 2017). β-carotene was loaded inside of human H-ferritin via a pH control loading strategy, but with no antitumor activity (Chen et al., 2014a). Atropine was abundantly encapsulated inside of the human H-ferritin nanocage by a urea loading strategy, and exhibited excellent antitumor activity in a human pancreatic cancer xenograft model (Lei et al., 2016). 5-Fluorouracil was successfully encapsulated inside of pig spleen ferritin with the assistance of gold nanoparticles, and Gefitinib was loaded inside of human H-ferritin via incubation in Tris buffer. Both of them lack in vivo antitumor studies (Kuruppu et al., 2015; Liu et al., 2013). siRNA was also successfully encapsulated inside of the human H-ferritin nanocage by a pH control loading strategy, while the antitumor activity of HFn-siRNA was only tested in tumor cells in vitro (Li et al., 2016b). This report paves the way for future application of ferritin nanoprobes in gene therapy, but more efforts are needed in this field.

With the unique property of storing metal ions, ferritin-based nanocages could be exploited to deliver radioisotopes for increased loading efficiency and improving pharmacokinetics in vivo. Hainfeld reported that ^{235}U can be effectively loaded (~800 molecules per nanocage) inside of HosFn via incubation in PBS, and the ^{235}U-HosFn nanocages are able to kill tumor cells (Hainfeld, 1992). The clinically used isotopes, such as ^{90}yttrium and ^{177}lutetium, are also able to use this ferritin delivery system to improve the tumor targeting properties and in vivo behaviors.

The drugs used for photodynamic therapy (PDT) or photothermal therapy (PTT), such as Methylene blue, ZnF16Pc, IR820 and Sinoporphyrin sodium, typically have high immunogenicity, nonbiodegradability, long-term toxicity, and poor pharmacokinetics

(Huang et al., 2014; Yan et al., 2008, 2010). Ferritin nanoprobes provide an ideal nanocarrier for PDT and PTT drug delivery. Employing RGD-modified human H-ferritin, Xie's group demonstrated that ZnF16Pc was effectively encapsulated inside of the ferritin nanocage via incubation and exhibits excellent PDT effects in treating a human glioma xenograft model, combining with Doxil to treat a mouse breast cancer model, or treating a mouse breast cancer model in combination with FAP targeting (Zhen et al., 2013b, 2014, 2015, 2016). Huang et al. reported that IR820 could be abundantly encapsulated inside of the surface-modified human H-ferritin via a pH control loading strategy, and the IR820-ferritin nanoprobes exhibit 100% tumor elimination in a mouse breast cancer model (Huang et al., 2014). Recently, Liu's group developed surface-modified human H-ferritin as the nanocarrier of Sinoporphyrin sodium by a pH control loading strategy, and demonstrated that the PDT/PTT treatments exhibit 100% tumor ablation in a mouse breast cancer model (Huang et al., 2017a).

In summary, taking advantage of the disassembly/reassembly of the ferritin nanoprobe under pH control or urea concentration control, anticancer drugs (e.g., Dox, Daunomycin, Cisplatin, Carboplatin, Curcumin, Atropine, β-carotene, 5-fluorouracil and Gefitinib), radioisotopes (^{235}U), siRNA and PDT/PTT drugs (e.g., Methylene blue, ZnF16Pc, IR820 and Sinoporphyrin sodium) have been successfully loaded inside ferritin nanocages, and some of them have been evaluated for antitumor activity in tumor models (Fig. 4). It will be interesting to use this nanoplatform to combine multimodal imaging and targeted drug delivery for cancer diagnosis and therapy.

7 Conclusions and Prospects

Genetically or chemically conjugating targeting motifs onto the surface of ferritin is the major strategy to improve ferritin nanoprobe tumor homing. While the strategy has been demonstrated to be very promising, it also brings drawbacks that make the bioengineered nanoprobes hard to translate from bench to bedside. These modifications may alter the nanoprobes' immunogenicity upon entering the body, significantly reduce the yield of ferritin nanoprobes and increase the complexity of purification of ferritin nanoprobes (Liang et al., 2014; Uchida et al., 2006; Zhao et al., 2016). Thus, the surface-modified ferritin nanoprobes may not be the best choice for the translational studies of ferritin-based tumor theranostics.

The identification of ferritin receptors, especially the reporting of TfR1 as the receptor of human H-ferritin, reveals its intrinsic tumor targeting property (Fan et al., 2012; Li et al., 2010). This intrinsic property, together with its unique architecture, excellent biocompatibility and reversible disassembly/reassembly ability, makes ferritins an ideal nanocarrier for tumor theranostics. Combined with other numerous favorable features of ferritin such as stability, homogeneity, and ease of scale-up, ferritin nanoprobes are promising candidates for translation to clinical use. However, we point out that different ferritins from different species

do not share the same receptors. So, when employing ferritin as a nanocarrier to develop an antidisease system, we must clearly recognize which receptor is suitable. Because HosFn is cheap and conveniently supplied, it still has potential for research in the near future. For now, the cross-interaction receptors of HosFn are human TfR1 and mouse Scara5, and other interactions are yet to be confirmed.

Ideally, human ferritins have more advantages in comparison with HosFn, for example, they are of human origin, which means that they have better biocompatibility and no immunogenic reaction. The receptors of human ferritins are more comprehensive, especially H-ferritin, of which the receptor is TfR1, a well-known tumor biomarker in tumor therapy. Moreover, lab production of recombinant human ferritins is straightforward, elementary, robust, and convenient. Thus, we recommend using human ferritin as the nanocarrier for translational studies.

Dealing with the potential toxicity and immune response of the human body to ferritin nanoprobes is important for its translational application in clinical imaging and therapy. Revealing the physiological and pathological functions of ferritin will help us to better understand the potential effects of its use in the human body. Although numerous new findings have provided us with insights to better understand this protein (i.e., human H-ferritin recognizes TfR1; serum ferritin levels correlate with tumor levels), the novel and unexpected functions of ferritin continue to be revealed, and further study of this interesting molecule is warranted. For example, the issue of the source of secretory ferritin and identification of human L-ferritin receptors; the mechanism and function of ferritin in its involvement in many pathological processes; and the exact binding site of the human H-ferritin/TfR1 interaction are aspects that will be important to unravel (Fan et al., 2013). In addition, the influence of human ferritin nanoprobes on iron homeostasis, the biodistribution and clearance of these nanoprobes after entering the human body are the main concern for the current translational studies of ferritin nanoprobes. As ferritin nanoprobes have been recognized as an ideal nanoplatform because of their physiological features and bioengineering versatility, further advances in their clinical applications for tumor diagnosis, imaging, and therapy are expected.

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