Nanomedicine

Apoferritin applications in nanomedicine

Nanomedicine as a continuously evolving discipline is still looking for a structure with perfect properties that is usable as a multifunctional transporter. Great potential is attributed to synthetic materials such as fullerenes, porous hollow silica nanoparticles and single-wall nanotubes, among others. However, materials that are natural to the human body are more acceptable by the organism, and thus become an attractive approach in this field of research. Ferritins are proteins that naturally occur in most living organisms throughout evolution and may be a possible transporter choice. Numerous applications have demonstrated the possibilities of iron-free ferritins, called apoferritins, serving as platforms for various nanomedical purposes This article summarizes the advantages and disadvantages of these proteins and discusses their practical applications and future perspectives.

Zbynek Heger[1],
Sylvie Skalickova[1],
Ondrej Zitka[1,2],
Vojtech Adam[1,2]
& Rene Kizek*[1,2]
[1]Department of Chemistry & Biochemistry, Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic
[2]Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic
*Author for correspondence:
Tel.: +420 5 4513 3350
Fax: +420 5 4521 2044
kizek@sci.muni.cz

Ferritins (FRTs) are the major iron storage and detoxifying oligomeric proteins in most organisms, from humans through to invertebrates, plants and microorganisms [1–3]. In such different organisms, the structure of FRTs varies only slightly [4,5]. Their main role is to prevent the harmful accumulation of iron inside the organism by collecting free iron in the form of ferrihydrite phosphate ($[FeOOH]_8[FeOPO_3H_2]$) in its core for further usage of these ions as enzymatic cofactors [6–9]. In nature, the interiors of FRTs are filled with iron, but when expressed artificially in iron-free conditions, the yielded apoferritins are hollow, comprising a cavity that can be loaded with different substances, including those of an inorganic and/or organic nature [10,11].

Based on the aforementioned properties, apoferritins have attracted great interest not only because of their nanoscale nature and ability to serve as transporters, but also because of their high stability and special structure [12]. Researchers in nanotoxicology have indicated that these pharmacological

properties, as well as biodegradability, biocompatibility and nontoxicity, are crucial for transporting molecules [13–15], considering the fact that nanoscale materials have become the most rapidly developing area in the biomedical research field, particularly in the field of targeted therapy [16–18]. In this field, the apoferritins, as naturally occurring proteins, meet the requirements for targeted therapy. The aim of this article is to summarize the knowledge regarding apoferritin structure, its utilization as a material for various nanomedicine applications and, furthermore, its application as a platform for the synthesis of nanoparticles.

FRT protein superfamily

The FRT superfamily can be divided into three subfamilies: the classical FRTs; the bacterioferritins (BFRs); and the DNA-binding proteins from starved cells (DPSs). The FRT and BFR proteins are considered to be maxi-ferritins, whereas DPS proteins are considered to be mini-ferritins. These three subfamilies share the same character-

istic four-helix bundle fold [19,20]. The most significant difference between the FRT and BFR proteins is the presence of 12 heme moieties in BFRs. In addition, the DPS proteins form a smaller molecule made up of only 12 monomers with a lower iron storage capacity than the FRTs and BFRs and utilize unique ferroxidase sites [21].

Apoferritin structure

The structure of apoferritin is remarkably stable and robust, and it is able to withstand biologically extreme temperatures (up to 70°C) and a wide pH range (pH 2.0–10.0) for an appreciable period of time without significant disruption of their quaternary structure [22,23]. The native, cytosolic FRTs are proteins that are composed of two types of subunits – H-type (heavy) and L-type (light) – where we can find 53% sequence identity [24,25]. They are encoded by separate genes with nonexchanangeable functions [26,27], whereas those from plants and bacteria contain only H-type chains [28]. Twenty-four FRT subunits form a spherical cage-shaped protein shell folded in a bundle of four long parallel and antiparallel α-helices (A, B, C and D) with a fifth shorter C-terminal helix E, inclined at 60° to the major helix bundle [29]. Each subunit is formed by an individual molecule that joins its neighboring subunit through noncovalent interactions in order to form the whole molecule with a molecular mass of approximately 20 kDa occurring in octahedral (F432) symmetry [30,31]. The apoferritin structure has six twofold symmetry axes, four threefold symmetry axes and three fourfold symmetry axes. It is known that there are narrow hydrophilic channels along the threefold symmetry axes consisting of negatively charged amino acids (glutamic and aspartic acid) and hydrophobic channels along the fourfold symmetry axes [32,33]. Several channels transversing the shell facilitate inorganic or organic ions to enter and exit the protein cavity [34–36]. The protein shell forms an inner cavity with inner and outer diameters of 7–8 and 12–13 nm, respectively [29–30,37]. The inner cavity, with an 80-Å diameter, is capable of storing up to 4500 Fe(III) atoms [38,39].

Apoferritin self-assembly ability

The self-assembling ability of apoferritin is widely used by researchers in the field of nanomedicine because the protein cage may be reversibly disassociated in unfavorable environments and after a change in the environment, the conditions may be reconstituted backwards, retaining the therapeutic agent in its cavity. The same principles are also applied for the enclosure of contrast agents in imaging protocols. This natural ability is advantageous in many ways: the resulting nanoparticles form size-uniformed cavities, and thus encapsu-

lation of cargo can be highly reproducible; the encapsulation protocol is simple, based only on changes to environment conditions; and the undesired release of cargo in blood vessels is eliminated due to the absence of the required conditions. The overall scheme of the encapsulation of target molecules into apoferritin cages is shown in Figure 1. The properties of the FRT assembly were first found in natural horse spleen FRTs in 1978 [40]. The protein cage can be disassociated into all 24 subunits at low pH (2.0) and the subunits can be reconstituted backwards under the influence of higher pH [41]. As was observed using synchrotron small-angle x-ray scattering in the presence of an environment with a pH below 0.8, the disassembled subunits aggregated, which is attributed to the denaturation of the stable protein structure of FRTs [42]. The overall assembly mechanism of apoferritin was first designed by Gerl and Jaenicke using data obtained by intrinsic fluorescence, far-UV circular dichroism and glutaraldehyde cross-linking experiments [43]. The apoferritin self-assembled product was formed during a series of reactions with a mixture of partially assembled subunits, including monomers, most frequently trimers, hexamers and dodecamers [43]. It was also shown that two hexamers could be used to form a dodecamer, and two dodecamers could assemble into a 24-mer. These results led to a refined model where the 24-meric cage assembles from a dimer (M2) via tetramers (M4) and hexamers (M6) [40].

Apoferritin utilization for targeted imaging

As drug or contrast agent carriers, apoferritins could protect their cargo against degradation and prereleasing, which would cause undesired side effects. As has been described previously, apoferritins may effectively carry a cargo towards different types of tissue, as shown in Figure 2. In this area, it can be mentioned that the apoferritins may be efficiently taken up from blood by their specific receptors, SCARA5 [44,45] and TfR1. Moreover, the amount of apoferritin that is taken up can be quantitatively visualized when the protein is loaded with an MRI contrast agent, such as gadolinium and/or manganese [46,47]. Manganese–apoferritin complexes were used as highly sensitive MRI contrast agents for the detection of hepatocellular carcinoma based on manganese–apoferritin complex uptake by liver SCARA5 [48]. When injected into hepatitis B virus-transgenic mice with spontaneously developed hepatocellular carcinoma, manganese–apoferritin enabled the clear distinguishing of healthy liver tissue and tumor lesions as hyperintense and hypointense T_1-weighted magnetic resonance images. The apoferritin-encapsulated gadolinium, as a possible candidate for a new MRI contrast agent, was suggested by Makino
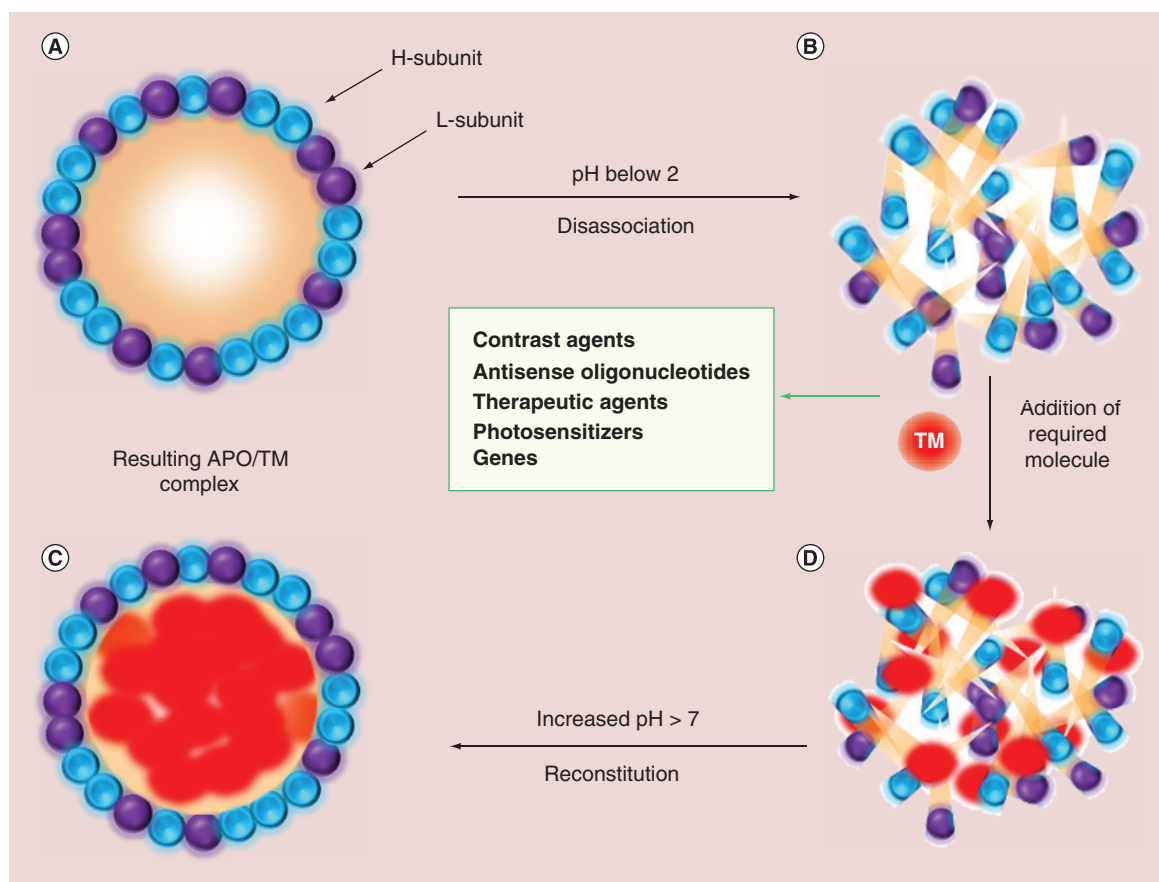
Figure 1. Overall scheme of apoferritin reversible disassociation under the influence of pH changes.
(A) Twenty-four subunits divided into H- and L-subunits form the stable apoferritin protein molecule. **(B)** Low pH (below 2.0) protein disassociates reversibly into the basic 24 subunits. **(C)** The addition of the TM required for complex formation follows. **(D)** After an increase of the pH value of solution, the protein molecule reconstitutes, thus encapsulating the target molecules in its inner cavity.
APO: Apoferritin; TM: Target molecule.

et al. [44]. Gadolinium was efficiently encapsulated into the apoferritin cavity and enhanced the T_1 relaxivity to as much as tenfold higher than the commercially used contrast agent gadolinium-DOTA. Furthermore, the *in vivo* blood clearance time of apoferritin was prolonged by its surface modification with dextran. An increased accumulation of this complex was observed mainly in the tumor region due to passive targeting via enhanced permeability and retention effect. Moreover, single-dose toxicity tests showed no serious side effects [44]. It was previously shown that the modification of apoferritin with the substrate peptides of MMP-2 and a hydrophilic polymer (PEG) may cause the aggregation of nanoparticles initiated by the action of a tumor-associated protease, MMP-2, leading to T_2 shortening on MRI [49]. Sun *et al.* demonstrated that two gold nanoclusters localized at the ferroxidase active sites of the FRT H-chain nanocomplex not only retained the intrinsic fluorescence properties of gold, but also gained enhanced intensity with a red shift and exhibited tun-

able emissions due to the coupling interaction between paired gold clusters bound in the H-chain of apoferritin [50]. Furthermore, this complex showed an organ-specific targeting ability due to the high expression of the FRT receptor SCARA5 in kidney cells, the high biocompatibility and the low cytotoxicity. Such agents are very promising for *in vitro* and *in vivo* imaging [50]. The importance of the possibility of apoferritin outer-shell functionalization was shown by Valero *et al.* [51]. In this case, apoferritin-enclosed nanomaghemite was modified with *N*-acetyl-ᴅ-glucosamine and ᴅ-mannose, and the carbohydrate-functionalized apomaghemite nanoparticles retained their recognition abilities, as demonstrated by the strong affinity with their corresponding carbohydrate-binding lectins. The *in vivo* MRI studies showed the efficiency in contrasting images, where the r_2 nuclear magnetic resonance relaxivities, as well as the precontrast and postcontrast T_2^*-weighted images, were comparable with those obtained from the commercially used Endorem® (Guerbet, Villepinte, France).
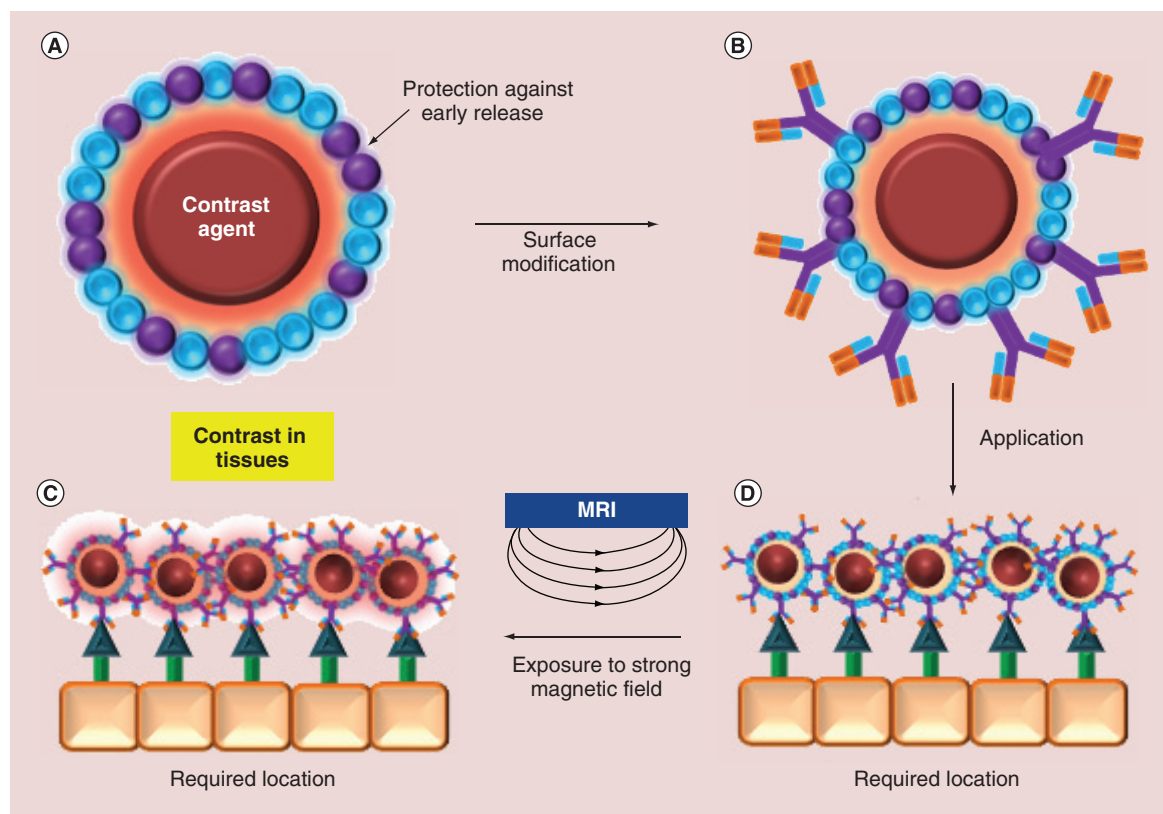
Figure 2. Apoferritin may be simply exploited as a nanotransporter, bearing various contrast agents. (A) Contrast agents may be encapsulated into the apoferritin cavity in the classical way. **(B)** After encapsulation, the surface of the apoferritin–contrast agent complex may be functionalized through antibodies or carbohydrates in order to form targeted imaging conjugates. **(C)** After application of the apoferritin–contrast agent, the complex is driven by recognition elements placed on its surface towards the required location. Moreover, the apoferritin protects the cargo against undesired degradation. **(D)** After exposure to a strong magnetic field produced by MRI, a radiofrequency signal is emitted and, subsequently, the contrast between different tissues is determined.

Apoferritins as drug nanotransporters

The first mention of the ability of FRTs to encapsulate anticancer therapeutics was published in 2005 by Simsek and Kilic in their paper entitled 'Magic ferritin: a novel chemotherapeutic encapsulation bullet' [52]. Seven years later in 2012, Kilic *et al.* formed an apoferritin complex with doxorubicin, a commonly used cytostatic drug [53]. The doxorubicin encapsulation was carried out using direct and step-wise changes of the pH of the solution from 2.5 to 7.4. It was found that up to 28 molecules of doxorubicin could be capsulated per apoferritin protein and no significant drug leakage occurred over several days' storage [53]. In the same manner, doxorubicin was encapsulated into biotinylated apoferritin whose surface was modified with streptavidin-functionalized magnetic particles [54]. This complex can be tracked by fluorescence detection and, furthermore, be applied in targeted transport using an external magnetic field. Other cytostatics, such as carboplatin, cisplatin [55,56] or daunorubicin [57], can also be encapsulated in the apoferritin cavity and the drug-loaded protein has cytotoxic effects on tumor cells (summarized in Table 1). Although apoferritin exhibits great attributes for serving as a platform for nanomedicine, the possible undesired immune response of patients still exists. The ideal nanotransporter has to go through the body undetected by immune system. Despite evidence that excessive amounts of apoferritin administered for long periods can trigger immune complex glomerulonephritis in mice [58], there is still a lack of evidence regarding immune responses in human.

In addition to its well-documented encapsulation capacity, apoferritin can also bind specifically to a variety of cell types due to the presence of FRT receptors on the surfaces of various cells [63–65]. Besides SCARA5, only one FRT receptor in human cells, TfR1, has been shown to bind both FRT (via binding with H-subunit of the protein) and transferrin [66]. The internalization of apoferritin is performed by clathrin-mediated endocytosis (also called receptor-mediated endocytosis) [67] during the acidification of endosome, thus the cargo is released gradually (Figure 3). Moreover, the functionalization of the protein surface with various

types of antibodies may offer the possibility to transport cargo towards the required site very specifically. As was shown by Cutrin *et al.*, the encapsulation of curcumin, a therapeutic with antioxidant and anti-inflammatory properties, inside the apoferritin cavity significantly increased its stability and bioavailability [47]. This complex was used to attenuate the thioacet-amide-induced hepatitis, and mice pretreated with the intraperitoneal administration of apoferritin–curcumin showed significantly attenuated hepatic injury as assessed by measuring alanine aminotransferase activity [47]. In addition, the cytostatic drug 5-fluoro-uracil (5-FU) can be sequestered into the void space of the apoferritin modified with gold in order to produce a nanoscale hybrid apoferritin modified with gold carrying 5-FU. Gold-modified apoferritin then serves as a bionanochemosensitizer, rendering tumor cells more susceptible to 5-FU by cell-cycle regulation, therefore leading to a significant decrease in the IC_{50} value of 5-FU in a human carcinoma cell line (HepG2) from 138.3 to 9.2 μM [59]. Bradshaw *et al.* proposed a complex comprising lead(II) sulfide quantum dots enclosed

in an apoferritin cage, and it was shown that after the application on colorectal carcinoma cells, they failed to recover their proliferative capacity [60]. Moreover, the generation of reactive oxygen species triggered their apoptosis. By contrast, the apoferritin–lead(II) sulfide quantum dot complex did not negatively affect non-tumor human microvessel endothelial HMEC-1 cells [60]. Genetic modification of the protein can lead to the presence of a peptide with a required sequence on the surface. Zhen and colleagues genetically modified FRT with the Cys–Asp–Cys–Arg–Gly–Asp–Cys–Phe–Cys (RGD4C) peptide, showing affinity towards tumor cells through the RGD–integrin $\alpha_v\beta_3$ interaction [10]. Doxorubicin-loaded RGD FRT nanocages exhibited longer circulation times, higher tumor uptake and tumor inhibition. In addition, these nanocages decreased cardiotoxicity compared with free doxorubicin. Apoferritin can also be fused to other proteins in order to form chimeras. With the insertion of hemagglutinin onto the interface of adjacent apoferritin subunits, the spontaneously assembly and generation of nanoparticles with immunization attributes were

Table 1. Overview of apoferritin utilization in drug delivery.				
Cargo	**Complex**	**Application**	**Apoferritin role**	**Ref.**
DOX	APO–DOX	–	Encapsulation concept	[52]
DOX	APO–DOX	–	Drug leakage elimination	[53]
DOX	MPs@APO–DOX	–	Encapsulation and surface modification	[54]
Cisplatin-; carPt	APO-cisplatin-; carPt	–	Improvement of drug toxicity profiles	[55]
Cisplatin-; oxali-; carPt	APO-cisplatin; oxali-; carPt	PC12	Enhancement of platinum-based drug uptake	[56]
Daunorubicin	APO–DNR–PLAA	–	Modification to improve complex stability	[57]
Cur; Gd	APO–Cur–Gd	Mice with thioacetamide-induced hepatitis	Enhancement of Cur and Gd stability and bioavailability	[47]
5-FU	APO–AuNP–5-FU	HepG2	Chemosensitization, decrease of drug IC_{50}	[59]
PbSQDs	APO–PbSQDs	CRCs	Platform for theranostics – imaging and treatment	[60]
X	APO–BIBA–PNIPAAm–DMIAAm	–	Surface modification to provide specifity	[61]
DOX	APOfilm–DOX	–	Controllable drug delivery and release	[62]
DOX	RGD@APO–DOX	U87-MG	Increased tumor uptake and circulation time, decreased cardiotoxicity	[10]

5-FU: 5-fluorouracil; APO: Apoferritin; APOfilm: Apoferritin mesoporous film; AuNP:Gold nanoparticle; BIBA: 2-bromo-isobutyric acid; CarPt: Carboplatin; CRC: Colorectal carcinoma cell; Cur: Curcumin; DMIAAm: 2-(dimethyl maleinimido)-*N*-ethyl-acrylamide; DNR: Daunorubicin; DOX: Doxorubicin; Gd: Gadolinium; MP: Magnetic particle; Oxali-: Oxaliplatin; PbSQD: Lead(II) sulfide quantum dot; PLAA: Poly-ʟ-aspartic acid; PNIPAAm: Poly(*N*-isopropyl acrylamide); X: No cargo defined.
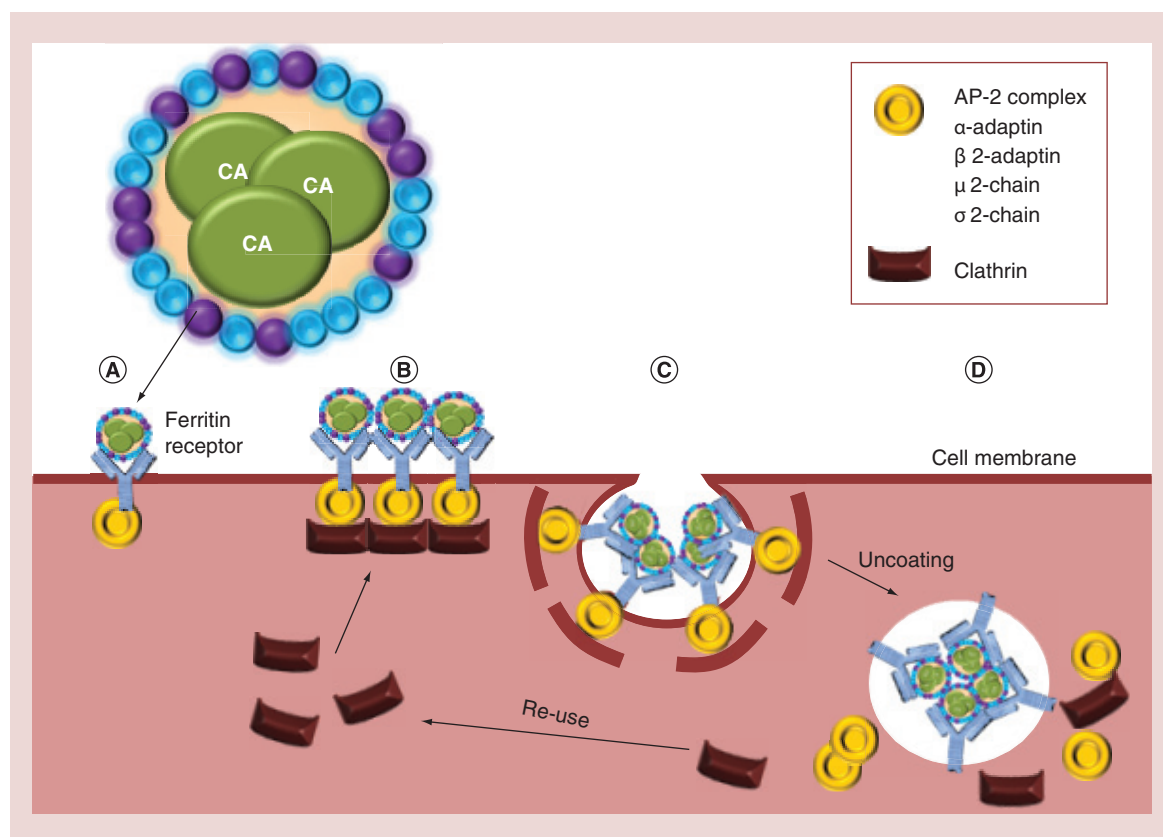
Figure 3. Apoferritin may protect chemotherapeutic agents against the tissue environment and thus significantly decrease the unwanted effects of these substances. A scheme of clathrin-mediated endocytosis is shown, demonstrating how the protein molecule is internalized into most types of cells. **(A)** Apoferritins establish binding with ferritin receptors. **(B)** After binding is established, clathrin polypeptides are attracted by the adaptor complex AP-2 and **(C)** clathrin polypeptides provide a coating of a vesicle lattice. **(D)** After the vesicle is formed, clathrin is removed and used for another purpose. In this fashion, the formed vesicle is transformed into an endosome. Due to endosome acidification, the chemotherapeutic agent may be released into the intracellular space gradually.

achieved [68]. Immunization with this nanostructure exhibited a decrease (more than tenfold) in hemagglutinin antibody titers when compared with licensed inactivated vaccine.

Bionanoparticles with the ability to form stable emulsion droplets decorated with polymer-modified apoferritin with potential to be cross-linked were prepared by grafting thermoresponsive poly(*N*-isopropyl acrylamide) and photo-crosslinkable 2-(dimethyl maleinimido)-*N*-ethyl-acrylamide to the protein surface. This structure allows the formation of capsules with thermoresponsiveness for controlled release purposes [61]. Efficient drug delivery platforms with controllable releasing speeds were constructed using mesoporous apoferritin thin films [62]. Composite nanofibrous dispersions of nanostrands and proteins were formed by assembling negatively charged proteins on the highly positively charged nanostrand surfaces. Moreover, these films also hold promise for applications in recovering dyes from dye waste waters. The structures constructed in this manner show highly

diverse possibilities for apoferritin utilization, not only in form of nanotransporters, but also for the formation of functionalized materials with potential extending beyond the boundaries of nanomedicine applications.

Apoferritins in photodynamic therapy

Another form of apoferritin utilization for medical purposes is in photodynamic therapy in cancer treatment. Photodynamic therapy is s a new therapeutic modality that is emerging as a powerful tool against malignant tumors [69]. This strategy is based on the action of photosensitizers (i.e., molecules that may accumulate preferentially inside tumor cells, where they exert a cytotoxic effect after excitation by light at appropriate wavelengths) [70]. Upon the absorption of light, the photosensitizer is promoted to an excited state and undergoes crossing with oxygen, resulting in singlet oxygen, which aggressively attacks any organic compounds, and thus become highly cytotoxic. When used as a delivery system for photosensitizers to the intracellular space, the apoferritin nanocage acts as a

unique transporter that protects loaded photosensitizers from reactive biomolecules in the cell membranes. This enables further targeting of singlet oxygen upon specific light irradiation to tumor cells only (Figure 4). As was shown by Yan and colleagues, a Methylene Blue-encapsulated apoferritin complex exhibits cytotoxic effects, as tested on MCF-7 human breast adenocarcinoma cells, when irradiated using the appropriate wavelength [71]. In addition, it was demonstrated that the encapsulation of Methylene Blue into apoferritin via the reassembly process controlled by pH is useful a tool for photodynamic therapy. When the complex was irradiated at the appropriate wavelength (633 nm), it showed a positive effect on singlet oxygen production and therefore cytotoxic effects on the MCF-7 human breast cancer cell line [72]. It was demonstrated a complex with zinc hexadecafluorophthalocyanine $ZnF_{16}Pc$ [5] to behave as a potent photosensitizer [73]. The surface of the resulting conjugate was modified with RGD4C, formed by Cys–Asp–Cys–Arg–Gly–Asp–Cys–Phe–Cys, and the complex may specifically target tumor tissue through RGD–integrin interactions. Using light irradiation, phototoxicity was induced while leaving normal tissues unaffected. Due to cancer angiogenesis, resulting in an enhanced permeability and retention effect, most types of cancers are especially active in both the uptake and accumulation of nanotransporters carrying drugs and/or photosensitisers [74] This phenomenon makes them vulnerable

to photodynamic therapy, and so the utilization of apoferritin as a photosintetizer nanotransporter offers promising prospects for the future of cancer therapy.

Apoferritins in biosensors/bioassays

Apoferritins may also be used as a part of very sensitive bioassays or biosensors. Applications of nanomaterials in electrochemical DNA biosensors and bioassays are reviewed elsewhere [75–77], and apoferritin is one of the more well-discussed nanostructures. In this field of applications, Kim *et al.* genetically engineered apoferritin by fusing GFP to its C-terminus and subsequently used this for chemical conjugation to DNA aptamers via each GFP's cysteine residue that was newly introduced through site-directed mutagenesis [78]. Furthermore, the DNA–aptamer-conjugated complexes were used as a fluorescent reporter probe in the aptamer-based 'sandwich' assay of the PDGF B-chain homodimer, which is considered to be a tumor marker. The limit of detection obtained with this bioassay was lowered to the 100 fM, and the assay sensitivity was significantly enhanced compared with standard immune-based detection. Versatile nanoparticle labels based on apoferritin were demonstrated by Liu *et al.* [79]. Their concept of using hexacyanoferrate(III) and fluorescein as model markers for loading into the cavity of apoferritin with an amino-modified DNA probe conjugated on its surface was used as a label for electrochemical DNA detection in connection with a magnetic bead-based sandwich
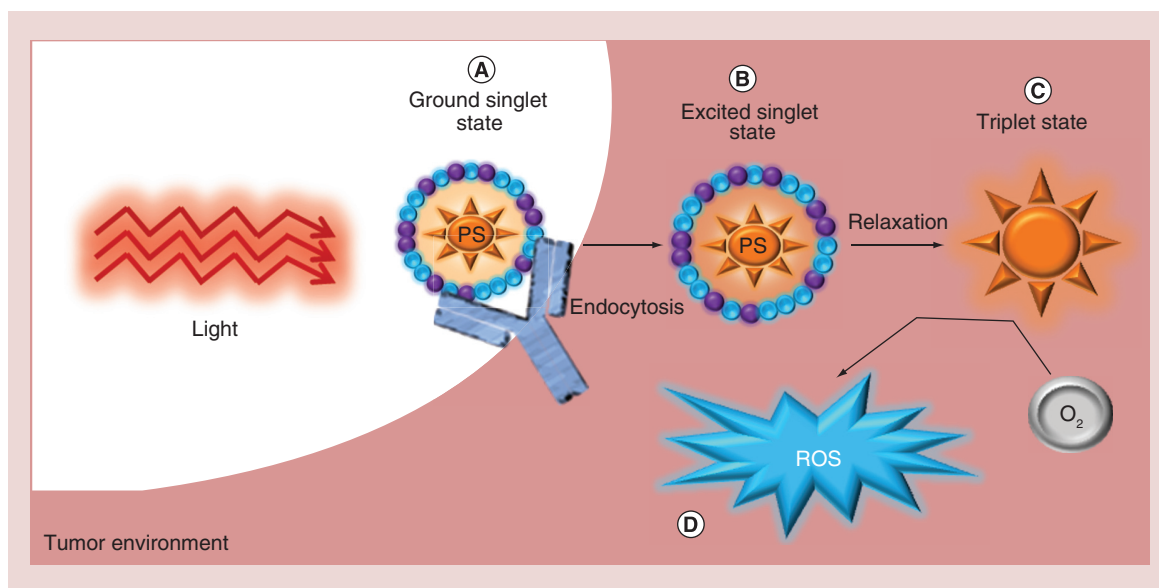

Figure 4. Apoferritins may serve a useful tool in guiding of photosensitizers to the required site of a tissue and protecting photosensitizers against the undesired effects of environment. (A) After irradiation with light, **(B)** photosensitizers absorb a photon, and subsequently, an electron is excited to the first excited singlet state. **(C)** This further relaxes to the more long-living triplet state. **(D)** The triplet-state electron interacts with molecular oxygen, leading to the formation of ROS, thereby damaging cells.
ROS: Reactive oxygen species.

hybridization assay. This method included double-hybridization events with probes linked to the biofunctionalized apoferritin and to magnetic beads, along with magnetic separation of the target DNA-linked magnetic bead–apoferritin assembly. As was mentioned in review by Pumera *et al.*, a number of metals exist that can be introduced into the apoferritin cavity and thus pave the way for different multiplexed assays [80]. Novel nanobioparticles that have been synthesized for these purposes represent a large potential for future applications, bringing new possibilities for the electrochemical biosensing of proteins or DNA.

Apoferritins as precursors for nanoparticles crafting

Because of its unique cavity structure, apoferritin has been widely used as a biotemplate for size-restricted bioinorganic nanocomposite synthesis [22,32,81–86], forming the nanoparticles with consistent size and shape, monodispersion and biocompatibility [87]. These nanocomposites may further find several applications in the field of nanomedicine, such as MRI contrast agents [88,89], as parts of various nanotransporters [90,91] or as smart theranostic platforms [92]. In the case of iron, Fe^{2+} ions are attracted by a negative charge on the outer surface surrounding the hydrophilic three-fold channels of the molecule and pass through them. Ions are subsequently condensed and oxidized at negatively charged amino acid sites on the inner surface in order to form iron oxide nanoparticles. The syntheses of Fe_3O_4–γ-Fe_2O_3, MnOOH, CoOOH, CeO_2 or Co_3O_4 typically requires the addition of oxidants, such as O_2 or H_2O_2 [22,93]. For the synthesis of Ca, Ba, Ni or Cr oxoanion compounds, the addition of an oxoanion, such as carbonate or phosphate, is necessary [94,95]. The mechanism of the permeation of positively charged ions through the channels was elucidated using x-ray crystallographic observation of apoferritin metal-binding sites [96]; however, it remains unclear how the anions enter the apoferritin cavity. Solving this issue could enhance the effectiveness of nanoparticle synthesis.

Apoferritin in gene therapy

Gene therapy includes the insertion, removal or modification of defective gene(s) for the treatment of genetically inherited diseases. The commonly used transporters for gene delivery are viral vectors, liposomes, peptides and cationic polymers [97–100]. In addition to excellent knowledge regarding the genetic nature of a disease and the specific gene sequence, it is also important to select a suitable vector. The main requirements of the gene delivery vector are the protection of delivered nucleic acid against nucleases, targeting and the ability to disrupt the endosomal membrane, thus

delivering the DNA to the nucleus [101,102]. Among the main obstacles against gene delivery vectors, aggregation, instability, toxicity and the propensity to be captured by the mononuclear phagocyte system [103] are the most significant. There is evidence of the aggregation of nonviral transporters, which could cause embolization [104]. Although the usage of apoferritin as a gene vector has not yet been published, it exhibits a few advantages; however, it is necessary to study apoferritins due to their colloidal behavior, charge, possession of electrostatic repulsion and the stability of the encapsulated DNA. Apoferritin cages possess a net negative charge at neutral pH that ensures its excellent solubility in water [105]. Due to apoferritin's outer surface positive charge, the protein may be easily modified, as was shown in the case of apoferritin with incorporated anionic ligand poly-L-aspartic acid into its structure [57]. In another study, apoferritin was modified by poly(ethylenimine) [106], which was employed for nonviral gene delivery [107,108]. The suggested method of entry of cationic gene delivery systems is by nonspecific adsorptive endocytosis followed by the clathrin-coated pit mechanism [109,110], because negatively charged nonviral vectors present on the cell membrane are able to interact with the positively charged systems.

Conclusion & future perspective

One of the main goals of nanomedicine is to create a nanocarrier that can efficiently and specifically deliver therapeutic agents to target sites in the body. Moreover, in order to enable efficient and specific delivery, a nanocarrier needs to have the ability to be easily modified. The replacement of synthetic materials, such as porous hollow silica nanoparticles, single-wall nanotubes and fullerenes, among others, with natural materials that are more acceptable to many organisms has become an attractive approach in this field of research.

Today, new insights into mechanisms of pH-sensitive vectors are being intensively studied [111–114], because pH values in tumors and other pathologically affected tissues dramatically change [115,116] and pH-sensitive vehicles, such as apoferritin, may serve as a promising tool for gene delivery systems. Apoferritin proteins may self-assemble into multisubunit, hollow, nanoscale cages with affinity towards SCARA5 and/or TfR1, and they have the potential to be modified through synthetic recognition molecules or genetically in order to form chimeric proteins or peptides on its surface. Due to their high stability, special structure and excellent nanotoxicological properties, such as biodegradability, biocompatibility and nontoxicity, apoferritins are the focus of many drug-delivery studies, synthesizing contrast agents in MRI, developing platforms for nanomaterial synthesis or bioas-

says. Particularly for nanomedical purposes, apoferritin meets the special requirement of being a widely used nanotransporter with the capability to protect its cargo against degradation. Moreover, apoferritin may eliminate the early release of its load and thus protect tissues against the adverse effects of various therapeutic agents. Importantly, the size uniformity of protein cages offers simplicity and reproducibility for cargo encapsulation. Protein nanocages also avoid random macromolecular aggregation. Apoferritin also has potential for applications in gene therapy, due to its properties of loading with a cargo and transport it to the required location. However, the lack of human trials of apoferritin means that there are insufficient data to determine whether the use of apoferritin is better than the use of traditional drugs. Despite the fact that apoferritin was previously linked with glomerulonephritis as a results of immune responses in mice [58,117], this may not be a problem when used in humane medi-

cine. In case of undesired immune responses, FRT proteins can be extracted from the patient's body, carrying out the process of iron removal and subsequent drug encapsulation. This protein, which is extracted from bodily tissue, connects the terms 'nanomedicine' and 'personalized medicine' into a powerful weapon that is applicable in fighting various diseases, including cancer.

Executive summary

Background
- Nanomaterials of natural origin are of great interest in medicine for the transporting and targeting of drugs.
- The encapsulation of drugs into these nanomaterials can also markedly decrease side effects.

Multitasking apoferritins
- With their self-assembling ability, apoferritins represent promising nanotransporters.
- Due to their ability to encapsulate various molecules, apoferritins may also be used in imaging protocols and photodynamic therapy.
- Biosensing can be considered as a potential field of application due the ability of apoferritins to interact with nucleic acids.
- Apoferritins may also be loaded with various chemicals in order to produce other nanomaterials.

Conclusion & future perspective
- Due to their excellent properties of withstanding various environmental influences, apoferritins may eliminate the early release of their load and thus protect tissues against the adverse effects of various therapeutic agents. Moreover, the cationic nature of the protein's outer surface enables simple surface modification in order to increase transporter specificity.
- There is great potential in the field of gene therapy for this material.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1   Crichton R. Intracellular iron storage and biomineralisation. In: *Iron Metabolism* (Eds). John Wiley & Sons Ltd, NY, USA, 183–222 (2009).

2   Bou-Abdallah F, Zhao G, Biasiotto G, Poli M, Arosio P, Chasteen ND. Facilitated diffusion of iron(II) and dioxygen substrates into human H-chain ferritin. A fluorescence and absorbance study employing the ferroxidase center substitution Y34W. *J. Am. Chem. Soc.* 130(52), 17801–17811 (2008).

3   Bulvik BE, Berenshtein E, Meyron-Holtz EG, Konijn AM, Chevion M. Cardiac protection by preconditioning is generated via an iron-signal sreated by proteasomal degradation of iron proteins. *PLoS ONE* 7(11), 1–9 (2012).

4   Ullrich A, Horn S. Structural investigations on differently sized monodisperse iron oxide nanoparticles synthesized by remineralization of apoferritin molecules. *J. Nanopart. Res.* 15(8), 1–9 (2013).

5   Zhen ZP, Tang W, Guo CL *et al.* Ferritin nanocages to encapsulate and deliver photosensitizers for efficient photodynamic therapy against cancer. *ACS Nano* 7(8), 6988–6996 (2013).

•• **Reports the effective usage of ferritins in photodynamic therapy.**

6   Smith JL. The physiological role of ferritin-like compounds in bacteria. *Crit. Rev. Microbiol.* 30(3), 173–185 (2004).

7   Liu XF, Theil EC. Ferritins: dynamic management of biological iron and oxygen chemistry. *Acc. Chem. Res.* 38(3), 167–175 (2005).

8   Rakshit T, Mukhopadhyay R. Tuning band gap of holoferritin by metal core reconstitution with Cu, Co, and Mn. *Langmuir* 27(16), 9681–9686 (2011).

9   Rouault TA. The role of iron regulatory proteins in mammalian iron homeostasis and disease. *Nat. Chem. Biol.* 2(8), 406–414 (2006).

10   Zhen ZP, Tang W, Chen HM *et al.* RGD-modified apoferritin nanoparticles for efficient drug delivery to tumors. *ACS Nano* 7(6), 4830–4837 (2013).

11   Hou JX, Yamada S, Kajikawa T *et al.* Role of ferritin in the cytodifferentiation of periodontal ligament cells. *Biochem. Biophys. Res. Commun.* 426(4), 643–648 (2012).

12   Zhao J, Liu ML, Zhang YY, Li HT, Lin YH, Yao SZ. Apoferritin protein nanoparticles dually labeled with aptamer and horseradish peroxidase as a sensing probe for thrombin detection. *Anal. Chim. Acta* 759, 53–60 (2013).

13   Shevchenko EV, Talapin DV, Kotov NA, O'brien S, Murray CB. Structural diversity in binary nanoparticle superlattices. *Nature* 439(7072), 55–59 (2006).

14   Roney C, Kulkarni P, Arora V *et al.* Targeted nanoparticles for drug delivery through the blood–brain barrier for Alzheimer's disease. *J. Control. Release* 108(2–3), 193–214 (2005).

15   Agnihotri SA, Mallikarjuna NN, Aminabhavi TM. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. *J. Control. Release* 100(1), 5–28 (2004).

16   Chomoucka J, Drbohlavova J, Huska D, Adam V, Kizek R, Hubalek J. Magnetic nanoparticles and targeted drug delivering. *Pharmacol. Res.* 62(2), 144–149 (2010).

17   Mundargi RC, Babu VR, Rangaswamy V, Patel P, Aminabhavi TM. Nano/micro technologies for delivering macromolecular therapeutics using poly(D, L-lactide-co-glycolide) and its derivatives. *J. Control. Release* 125(3), 193–209 (2008).

18   Soppimath KS, Aminabhavi TM, Kulkarni AR, Rudzinski WE. Biodegradable polymeric nanoparticles as drug delivery devices. *J. Control. Release* 70(1–2), 1–20 (2001).

19   Andrews SC. Iron storage in bacteria. *Adv. Microb. Physiol.* 40, 281–351 (1998).

20   Andrews SC, Robinson AK, Rodriguez-Quinones F. Bacterial iron homeostasis. *FEMS Microbiol. Rev.* 27(2–3), 215–237 (2003).

•   Provides an overview of the biology of ferritins.

21   Frolow F, Kalb AJ, Yariv J. Structure of a unique twofold symmetrical heme-binding site. *Nat. Struct. Biol.* 1(7), 453–460 (1994).

22   Douglas T, Stark VT. Nanophase cobalt oxyhydroxide mineral synthesized within the protein cage of ferritin. *Inorg. Chem.* 39(8), 1828–1830 (2000).

23   Chen G, Zhu XL, Meng FB, Yu ZG, Li GX. Apoferritin as a bionanomaterial to facilitate the electron transfer reactivity of hemoglobin and the catalytic activity towards hydrogen peroxide. *Bioelectrochemistry* 72(1), 77–80 (2008).

24   Torti FM, Torti SV. Regulation of ferritin genes and protein. *Blood* 99(10), 3505–3516 (2002).

25   Fukano H, Takahashi T, Aizawa M, Yoshimura H. Synthesis of uniform and dispersive calcium carbonate nanoparticles

in a protein cage through control of electrostatic potential. *Inorg. Chem.* 50(14), 6526–6532 (2011).

26   Caskey JH, Jones C, Miller YE, Seligman PA. Human ferritin gene is assigned to chromosome-19. *Proc. Natl Acad. Sci. USA* 80(2), 482–486 (1983).

27   Worwood M, Brook JD, Cragg SJ *et al.* Assignment of human ferritin genes to chromosomes-11 and chromosome-19q13.3–19qter. *Hum. Genet.* 69(4), 371–374 (1985).

28   Harrison PM, Arosio P. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim. Biophys. Acta* 1275(3), 161–203 (1996).

29   Uto K, Yamamoto K, Kishimoto N, Muraoka M, Aoyagi T, Yamashita I. Characterization of stable, electroactive protein cage/synthetic polymer multilayer thin films prepared by layer-by-layer assembly. *J. Nanopart. Res.* 15(4), 1–11 (2013).

30   De Val N, Declercq JP, Lim CK, Crichton RR. Structural analysis of haemin demetallation by L-chain apoferritins. *J. Inorg. Biochem.* 112, 77–84 (2012).

31   Ciasca G, Chiarpotto M, Campi G *et al.* Reconstitution of aluminium and iron core in horse spleen apoferritin. *J. Nanopart. Res.* 13(11), 6149–6155 (2011).

32   Iwahori K, Yoshizawa K, Muraoka M, Yamashita I. Fabrication of ZnSe nanoparticles in the apoferritin cavity by designing a slow chemical reaction system. *Inorg. Chem.* 44(18), 6393–6400 (2005).

••   Demonstrates the ability of apoferritins to serve as chemical reactors.

33   Suzumoto Y, Okuda M, Yamashita I. Fabrication of zinc oxide semiconductor nanoparticles in the apoferritin cavity. *Cryst. Growth Des.* 12(8), 4130–4134 (2012).

34   Galvez N, Sanchez P, Dominguez-Vera JM. Preparation of Cu and CuFe Prussian Blue derivative nanoparticles using the apoferritin cavity as nanoreactor. *Dalton Trans.* (15), 2492–2494 (2005).

35   Tian LX, Cao CQ, Pan YX. The influence of reaction temperature on biomineralization of ferrihydrite cores in human H-ferritin. *Biometals* 25(1), 193–202 (2012).

•   Provides insights into the encapsulation of magnetic particles by apoferritins.

36   Abe S, Hirata K, Ueno T *et al.* Polymerization of phenylacetylene by rhodium complexes within a discrete space of apo-ferritin. *J. Am. Chem. Soc.* 131(20), 6958–6960 (2009).

37   Liu GD, Wang J, Wu H, Lin YH. Versatile apoferritin nanoparticle labels for assay of protein. *Anal. Chem.* 78(21), 7417–7423 (2006).

38   Liu F, Du BJ, Chai Z, Zhao GH, Ren FZ, Leng XJ. Binding properties of apoferritin to nicotinamide and calcium. *Eur. Food Res. Technol.* 235(5), 893–899 (2012).

39   Uchida M, Flenniken ML, Allen M *et al.* Targeting of cancer cells with ferrimagnetic ferritin cage nanoparticles. *J. Am. Chem. Soc.* 128(51), 16626–16633 (2006).

40   Banyard SH, Stammers DK, Harrison PM. Electron-density map of apoferritin at 2.8-A resolution. *Nature* 271(5642), 282–284 (1978).

41 Dominguez-Vera JM, Colacio E. Nanoparticles of Prussian Blue ferritin: a new route for obtaining nanomaterials. *Inorg. Chem.* 42(22), 6983–6985 (2003).

42 Kim M, Rho Y, Jin KS *et al.* pH-dependent structures of ferritin and apoferritin in solution: disassembly and reassembly. *Biomacromolecules* 12(5), 1629–1640 (2011).

43 Gerl M, Jaenicke R. Mechanism of the self-assembly of apoferritin from horse spleen – cross-linking and spectroscopic analysis. *Eur. Biophys. J. Biophys. Lett.* 15(2), 103–109 (1987).

44 Makino A, Harada H, Okada T *et al.* Effective encapsulation of a new cationic gadolinium chelate into apoferritin and its evaluation as an MRI contrast agent. *Nanomed. Nanotechnol. Biol. Med.* 7(5), 638–646 (2011).

45 Li JY, Paragas N, Ned RM *et al.* Scara5 Is a ferritin receptor mediating non-transferrin iron delivery. *Dev. Cell* 16(1), 35–46 (2009).

46 Aime S, Frullano L, Crich SG. Compartmentalization of a gadolinium complex in the apoferritin cavity: a route to obtain high relaxivity contrast agents for magnetic resonance imaging. *Angew. Chem. Int. Edit.* 41(6), 1017–1019 (2002).

47 Cutrin JC, Crich SG, Burghelea D, Dastru W, Aime S. Curcumin/Gd loaded apoferritin: a novel 'theranostic' agent to prevent hepatocellular damage in toxic induced acute hepatitis. *Mol. Pharm.* 10(5), 2079–2085 (2013).

48 Crich SG, Cutrin JC, Lanzardo S *et al.* Mn-loaded apoferritin: a highly sensitive MRI imaging probe for the detection and characterization of hepatocarcinoma lesions in a transgenic mouse model. *Contrast Media Mol. Imaging* 7(3), 281–288 (2012).

49 Matsumura S, Aoki I, Saga T, Shiba K. A tumor-environment-responsive nanocarrier that evolves its surface properties upon sensing matrix metalloproteinase-2 and initiates agglomeration to enhance T-2 relaxivity for magnetic resonance imaging. *Mol. Pharm.* 8(5), 1970–1974 (2011).

50 Sun CJ, Yang H, Yuan Y *et al.* Controlling assembly of paired gold clusters within apoferritin nanoreactor for *in vivo* kidney targeting and biomedical imaging. *J. Am. Chem. Soc.* 133(22), 8617–8624 (2011).

51 Valero E, Tambalo S, Marzola P *et al.* Magnetic nanoparticles-templated assembly of protein subunits: a new platform for carbohydrate-based MRI nanoprobes. *J. Am. Chem. Soc.* 133(13), 4889–4895 (2011).

52 Simsek E, Kilic MA. Magic ferritin: a novel chemotherapeutic encapsulation bullet. *J. Magn. Magn. Mater.* 293(1), 509–513 (2005).

53 Kilic MA, Ozlu E, Calis S. A novel protein-based anticancer drug encapsulating nanosphere: apoferritin–doxorubicin complex. *J. Biomed. Nanotechnol.* 8(3), 508–514 (2012).

54 Blazkova I, Nguyen HV, Dostalova S *et al.* Apoferritin modified magnetic particles as doxorubicin carriers for anticancer drug delivery. *Int. J. Mol. Sci.* 14(7), 13391–13402 (2013).

55 Yang Z, Wang XY, Diao HJ *et al.* Encapsulation of platinum anticancer drugs by apoferritin. *Chem. Commun.* 43(33), 3453–3455 (2007).

56 Xing RM, Wang XY, Zhang CL *et al.* Characterization and cellular uptake of platinum anticancer drugs encapsulated in apoferritin. *J. Inorg. Biochem.* 103(7), 1039–1044 (2009).

57 Ma-Ham AH, Wu H, Wang J, Kang XH, Zhang YY, Lin YH. Apoferritin-based nanomedicine platform for drug delivery: equilibrium binding study of daunomycin with DNA. *J. Mater. Chem.* 21(24), 8700–8708 (2011).

58 Li M, O'Sullivan KM, Jones LK *et al.* CD100 enhances dendritic cell and CD4+ cell activation leading to pathogenetic humoral responses and immune complex glomerulonephritis. *J. Immunol.* 177(5), 3406–3412 (2006).

59 Liu XY, Wei W, Huang SJ *et al.* Bio-inspired protein–gold nanoconstruct with core–void–shell structure: beyond a chemo drug carrier. *J. Mat. Chem. B* 1(25), 3136–3143 (2013).

60 Bradshaw TD, Junor M, Patane A *et al.* Apoferritin-encapsulated PbS quantum dots significantly inhibit growth of colorectal carcinoma cells. *J. Mat. Chem. B* 1(45), 6254–6260 (2013).

61 Mougin NC, Van Rijn P, Park H, Muller AHE, Boker A. Hybrid capsules via self-assembly of thermoresponsive and interfacially active bionanoparticle–polymer conjugates. *Adv. Funct. Mater.* 21(13), 2470–2476 (2011).

•• Demonstrates apoferritins as promising aptamer sensors.

62 Huang HW, Yu Q, Peng XS, Ye ZZ. Mesoporous protein thin films for molecule delivery. *J. Mater. Chem.* 21(35), 13172–13179 (2011).

63 Moss D, Powell LW, Arosio P, Halliday JW. Effect of cell-proliferation on H-ferritin receptor expression in human T-lymphoid (MOLT-4) cells. *J. Lab. Clin. Med.* 120(2), 239–243 (1992).

64 Fargion S, Fracanzani AL, Cislaghi V, Levi S, Cappellini MD, Fiorelli G. Characteristic of the membrane-receptor for human-ferritin. *Curr. Stud. Hematol. Blood Transfus.*(58), 164–170 (1991).

65 Hulet SW, Menzies S, Connor JR. Ferritin binding in the developing mouse brain follows a pattern similar to myelination and is unaffected by the jimpy mutation. *Dev. Neurosci.* 24(2–3), 208–213 (2002).

66 Li L, Fang CJ, Ryan JC *et al.* Binding and uptake of H-ferritin are mediated by human transferrin receptor-1. *Proc. Natl Acad. Sci. USA* 107(8), 3505–3510 (2010).

67 Liu XY, Wei W, Yuan Q *et al.* Apoferritin–CeO_2 nano-truffle that has excellent artificial redox enzyme activity. *Chem. Commun.* 48(26), 3155–3157 (2012).

68 Kanekiyo M, Wei CJ, Yassine HM *et al.* Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. *Nature* 499(7456), 102–106 (2013).

69 Saboktakin MR, Tabatabaee RM. The novel polymeric systems for photodynamic therapy technique. *Int. J. Biol. Macromol.* 65, 398–414 (2014).

70 Marrelli M, Menichini G, Provenzano E, Conforti F. Applications of natural compounds in the photodynamic therapy of skin cancer. *Curr. Med. Chem.* 21(12), 1371–1390 (2014).

71 Yan F, Zhang Y, Yuan HK, Gregas MK, Vo-Dinh T. Apoferritin protein cages: a novel drug nanocarrier for

photodynamic therapy. *Chem. Commun.* 44(38), 4579–4581 (2008).

72   Yan F, Zhang Y, Kim KS, Yuan HK, Vo-Dinh T. Cellular uptake and photodynamic activity of protein nanocages containing Methylene Blue photosensitizing drug. *Photochem. Photobiol.* 86(3), 662–666 (2010).

73   Garcia AM, Alarcon E, Munoz M, Scaiano JC, Edwards AM, Lissi E. Photophysical behaviour and photodynamic activity of zinc phthalocyanines associated to liposomes. *Photochem. Photobiol. Sci.* 10(4), 507–514 (2011).

74   Liu YY, Chang Y, Yang C *et al.* Biodegradable nanoassemblies of piperlongumine display enhanced anti-angiogenesis and anti-tumor activities. *Nanoscale* 6(8), 4325–4337 (2014).

75   Mao X, Liu GD. Nanomaterial based electrochemical DNA biosensors and bioassays. *J. Biomed. Nanotechnol.* 4(4), 419–431 (2008).

76   Liu GD, Lin YH. Nanomaterial labels in electrochemical immunosensors and immunoassays. *Talanta* 74(3), 308–317 (2007).

77   Liu GD, Wang J, Wu H, Lin YY, Lin YH. Nanovehicles based bioassay labels. *Electroanalysis* 19(7–8), 777–785 (2007).

78   Kim SE, Ahn KY, Park JS *et al.* Fluorescent ferritin nanoparticles and application to the aptamer sensor. *Anal. Chem.* 83(15), 5834–5843 (2011).

79   Liu GD, Wang J, Lea SA, Lin YH. Bioassay labels based on apoferritin nanovehicles. *ChemBioChem* 7(9), 1315–1319 (2006).

80   Pumera M, Sanchez S, Ichinose I, Tang J. Electrochemical nanobiosensors. *Sens. Actuator B Chem.* 123(2), 1195–1205 (2007).

81   Okuda M, Iwahori K, Yamashita I, Yoshimura H. Fabrication of nickel and chromium nanoparticles using the protein cage of apoferritin. *Biotechnol. Bioeng.* 84(2), 187–194 (2003).

82   Ueno T, Suzuki M, Goto T, Matsumoto T, Nagayama K, Watanabe Y. Size-selective olefin hydrogenation by a Pd nanocluster provided in an apo-ferritin cage. *Angew. Chem. Int. Edit.* 43(19), 2527–2530 (2004).

83   Kramer RM, Li C, Carter DC, Stone MO, Naik RR. Engineered protein cages for nanomaterial synthesis. *J. Am. Chem. Soc.* 126(41), 13282–13286 (2004).

84   Yamashita I, Hayashi J, Hara M. Bio-template synthesis of uniform CdSe nanoparticles using cage-shaped protein, apoferritin. *Chem. Lett.* 33(9), 1158–1159 (2004).

85   Douglas T, Dickson DPE, Betteridge S, Charnock J, Garner CD, Mann S. Synthesis and structure of an iron(III) sulfide–ferritin bioinorganic nanocomposite. *Science* 269(5220), 54–57 (1995).

86   Okuda M, Kobayashi Y, Suzuki K *et al.* Self-organized inorganic nanoparticle arrays on protein lattices. *Nano Lett.* 5(5), 991–993 (2005).

87   Meldrum FC, Colfen H. Controlling mineral morphologies and structures in biological and synthetic systems. *Chem. Rev.* 108(11), 4332–4432 (2008).

88   Kim SM, Im GH, Lee DG, Lee JH, Lee WJ, Lee IS. Mn^{2+}-doped silica nanoparticles for hepatocyte-targeted detection of liver cancer in T-1-weighted MRI. *Biomaterials* 34(35), 8941–8948 (2013).

89   Kim SM, Chae MK, Yim MS *et al.* Hybrid PET/MR imaging of tumors using an oleanolic acid-conjugated nanoparticle. *Biomaterials* 34(33), 8114–8121 (2013).

90   Cui YN, Xu QX, Chow PKH, Wang DP, Wang CH. Transferrin-conjugated magnetic silica PLGA nanoparticles loaded with doxorubicin and paclitaxel for brain glioma treatment. *Biomaterials* 34(33), 8511–8520 (2013).

91   Ojer P, Neutsch L, Gabor F, Irache JM, De Cerain AL. Cytotoxicity and cell interaction studies of bioadhesive poly(anhydride) nanoparticles for oral antigen/drug delivery. *J. Biomed. Nanotechnol.* 9(11), 1891–1903 (2013).

92   Wang AF, Qi WX, Wang N *et al.* A smart nanoporous theranostic platform for simultaneous enhanced MRI and drug delivery. *Microporous Mesoporous Mat.* 180, 1–7 (2013).

93   Kang HW, Yeo J, Hwang JO *et al.* Simple ZnO nanowires patterned growth by microcontact printing for high performance field emission device. *J. Phys. Chem. C* 115(23), 11435–11441 (2011).

94   Okuda M, Suzumoto Y, Yamashita I. Bioinspired synthesis of homogenous cerium oxide nanoparticles and two- or three-dimensional nanoparticle arrays using protein supramolecules. *Cryst. Growth Des.* 11(6), 2540–2545 (2011).

95   Meldrum FC, Wade VJ, Nimmo DL, Heywood BR, Mann S. Synthesis of inorganic nanophase materials in supramolecular protein cages. *Nature* 349(6311), 684–687 (1991).

96   Toussaint L, Bertrand L, Hue L, Crichton RR, Declercq JP. High-resolution x-ray structures of human apoferritin H-chain mutants correlated with their activity and metal-binding sites. *J. Mol. Biol.* 365(2), 440–452 (2007).

97   Niidome T, Huang L. Gene therapy progress and prospects: nonviral vectors. *Gene Ther.* 9(24), 1647–1652 (2002).

98   Hosseinkhani H, He WJ, Chiang CH *et al.* Biodegradable nanoparticles for gene therapy technology. *J. Nanopart. Res.* 15(7), 1–15 (2013).

99   Chaturvedi K, Ganguly K, Kulkarni AR *et al.* Cyclodextrin-based siRNA delivery nanocarriers: a state-of-the-art review. *Expert Opin. Drug Deliv.* 8(11), 1455–1468 (2011).

100   Rudzinski WE, Aminabhavi TM. Chitosan as a carrier for targeted delivery of small interfering RNA. *Int. J. Pharm.* 399(1–2), 1–11 (2010).

101   Mahato RI. Non-viral peptide-based approaches to gene delivery. *J. Drug Target.* 7(4), 249–268 (1999).

102   Tan PH, Chan CLH, George AJT. Strategies to improve non-viral vectors – potential applications in clinical transplantation. *Expert Opin. Biol. Ther.* 6(6), 619–630 (2006).

103   Morille M, Passirani C, Vonarbourg A, Clavreul A, Benoit JP. Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. *Biomaterials* 29(24–25), 3477–3496 (2008).

104   Hsu CYM, Uludag H. Nucleic-acid based gene therapeutics: delivery challenges and modular design of nonviral gene

carriers and expression cassettes to overcome intracellular barriers for sustained targeted expression. *J. Drug Target.* 20(4), 301–328 (2012).

105 Valle-Delgado JJ, Molina-Bolivar JA, Galisteo-Gonzalez F, Galvez-Ruiz MJ, Feiler A, Rutland MW. Existence of hydration forces in the interaction between apoferritin molecules adsorbed on silica surfaces. *Langmuir* 21(21), 9544–9554 (2005).

106 Liao N, Zhuo Y, Chai YQ *et al.* Amplified electrochemiluminescent immunosensing using apoferritin-templated poly(ethylenimine) nanoparticles as co-reactant. *Chem. Commun.* 48(61), 7610–7612 (2012).

107 Gosselin MA, Guo WJ, Lee RJ. Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine. *Bioconjug. Chem.* 12(6), 989–994 (2001).

108 Fischer D, Bieber T, Li YX, Elsasser HP, Kissel T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharmacol. Res.* 16(8), 1273–1279 (1999).

109 Mcmahon HT, Boucrot E. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nat. Rev. Mol. Cell Biol.* 12(8), 517–533 (2011).

110 Mounkes LC, Zhong W, Cipres-Palacin G, Heath TD, Debs RJ. Proteoglycans mediate cationic liposome–DNA complex-based gene delivery *in vitro* and *in vivo*. *J. Biol. Chem.* 273(40), 26164–26170 (1998).

111 Gaspar VM, Marques JG, Sousa F, Louro RO, Queiroz JA, Correia IJ. Biofunctionalized nanoparticles with pH-responsive and cell penetrating blocks for gene delivery. *Nanotechnology* 24(27), 1–5 (2013).

112 Chen HP, Liu XP, Dou Y *et al.* A pH-responsive cyclodextrin-based hybrid nanosystem as a nonviral vector for gene delivery. *Biomaterials* 34(16), 4159–4172 (2013).

113 Kim HK, Thompson DH, Jang HS, Chung YJ, Van Den Bossche J. pH-responsive biodegradable assemblies containing tunable phenyl-substituted vinyl ethers for use as efficient gene delivery vehicles. *ACS Appl. Mater. Interfaces* 5(12), 5648–5658 (2013).

114 Tseng WC, Su LY, Fang TY. pH responsive PEGylation through metal affinity for gene delivery mediated by histidine-grafted polyethylenimine. *J. Biomed. Mater. Res. B Appl. Biomater.* 101B(2), 375–386 (2013).

115 Zhao QH, Qiu LY. An overview of the pharmacokinetics of polymer-based nanoassemblies and nanoparticles. *Curr. Drug Metab.* 14(8), 832–839 (2013).

116 Gerweck LE, Seetharaman K. Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer. *Cancer Res.* 56(6), 1194–1198 (1996).

117 Iskandar SS, Jennette JC. Interaction of antigen load and antibody-response in determining heterologous protein nephritogenicity in inbred mice. *Lab. Invest.* 48(6), 726–734 (1983).