Ferritin self-assembly, structure, function, and biotechnological applications

Vsevolod V. Sudarev[1], Sofya M. Dolotova[1,2], Siarhei Bukhalovich[1], Sergey V. Bazhenov[1,3], Yury L. Ryzhykau[1,4], Vladimir N. Uversky[5], Nikolay A. Bondarev[1], Stepan D. Osipov[1], Anatolii E. Mikhailov[1], Daria D. Kuklina[1], Tatiana N. Murugova[4], Ilya V. Manukhov[1,3,6], Andrey V. Rogachev[1,4], Valentin I. Gordeliy[7,8,9], Ivan Yu. Gushchin[1], Alexander I. Kuklin[1,4], Alexey V. Vlasov[1,4*]

[1]Research Center for Molecular Mechanisms of Aging and Age-Related Diseases, Moscow Institute of Physics and Technology, 141700 Dolgoprudny, Russia

[2]Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry RAS, 117997 Moscow, Russia

[3]HSE University, 101000 Moscow, Russia

[4]Frank Laboratory of Neutron Physics, Joint Institute for Nuclear Research, 141980 Dubna, Russia

[5]Department of Molecular Medicine and Byrd Alzheimer's Research Institute, Morsani College of Medicine, University of South Florida, Tampa, Florida, United States of America

[6]Laboratory of Microbiology, Moscow State University of Food Production, Volokolamskoe Highway 11, 125080 Moscow, Russia

[7]Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, 52425 Jülich, Germany

[8]JuStruct: Jülich Center for Structural Biology, Forschungszentrum Jülich, 52428 Jülich, Germany

[9]Institut de Biologie Structurale Jean-Pierre Ebel, Université Grenoble Alpes–Commissariat à l'Energie Atomique et aux Energies Alternatives–CNRS, F-38027 Grenoble, France

*To whom correspondence shall be addressed. Email: aleksei.vlasov@phystech.edu

Abstract

Ferritin is a vital protein complex responsible for storing iron in almost all living organisms. It plays a crucial role in various metabolic pathways, inflammation processes, stress response, and pathogenesis of cancer and neurodegenerative diseases. In this review we discuss the role of ferritin in diseases, cellular iron regulation, its structural features, and its role in biotechnology. We also show that molecular mechanisms of ferritin self-assembly are key for a number of biotechnological and pharmaceutical applications. The assembly pathways strongly depend on the interface context of ferritin monomers and the stability of its different intermediate oligomers. To date, several schemes of self-assembly kinetics have been proposed. Here, we compare different self-assembly mechanisms and discuss the possibility of self-assembly control by switching between deadlock intermediate states.

1. Introduction

Ferritin is a globular protein complex, which is present in a wide range of living organisms[1]. It is responsible for oxidizing and storing iron. Typically, ferritin consists of 24 homologous subunits and each subunit carries an active center converting Fe^{2+} to ferrihydrite[2]. These subunits spontaneously assemble into a full-fledged globule with a cavity for storing iron[3].

1.1. Short historical retrospective

Ferritin was first isolated from horse spleen in 1937 by Victor Laufberger[4]. Due to the fact that the protein contained more than 20% (w/w) of iron, it was called "ferritin" (from the Latin "ferratus"), i.e. "associated with iron"[5]. From 1942 to 1946 the group of Leonor Michaelis and Sam Granik published a series of papers on ferritin[6]. Researchers showed that it consists of a protein coat called “apoferritin” and an inorganic mineral core[7], including up to 4000 iron atoms[8]. Under the influence of low pH, the protein globule disassembles and detaches its inorganic core[7]. In 1943, the ferritin iron-storing functions and its involvement in iron metabolism were shown[9]. In humans, ferritin is an iron-storage and regulator of iron metabolism.

New types of ferritin-like proteins have been discovered since 1943[10]. The diversity of ferritins and ferritin-like proteins in distant lineages and from a common ancestor has shown the important role of these proteins in iron acquisition and the cell life cycle[1]. These ferritins have significant differences in amino acid sequences and structural organization, but the main function of iron storage is retained. Ferritin genes can be represented either by gene duplication or by separate gene inheritance, which

makes the protein globule naturally “customizable” in terms of regulation of iron metabolism in different organisms. The biological function of ferritin in various organisms has been discussed in a number of reviews[2,6,11–14].

Due to its globular structure and stability in a wide range of conditions, ferritin (in particular, its protein shell "apoferritin") has become a model object of structural biology. The first X-ray study of the protein was carried out in 1943, which showed the similarity of the structures of apoferritin and ferritin[15]. Since the mid-1950s, globular water-soluble protein complexes such as myoglobin[16] and ferritin have become a subject of interest for structural biology. The first low-resolution structural models of ferritin were obtained in the 1960s[17–19] (Figure 1A). In the 1970s, the presence of ferritin channels was shown (Figure 1B) and various hypotheses for the mechanisms of iron uptake were proposed[20]. In 1978, the first high-resolution structure (2.8 Å, Figure 1C, D) of a ferritin (from horse spleen) was obtained[21]. The first high-resolution structure (2.4 Å, Figure 1E) for human H-chain ferritin was obtained in 1991[22] by X-ray diffraction (XRD). Obtaining various structures of ferritin with high resolution is still the focus of current research[23–26] (Figure 1F). In addition, due to the symmetry of its structure, ferritin remains an important model object for structural studies[27–30].

In terms of biomedical applications, there is an active search for ferritin receptors[3,24]. New mechanisms of ferritin regulation are being studied[3]. With regard to functional studies, an immunoradiometric assay was developed in 1972 to determine the level of ferritin in human serum[31]. Its serum concentration has been found to correlate with the volume of iron stores in the body[31]. In humans, ferritin concentration changes in various metabolic disorders, and the protein has been found to be associated with inflammation[3,32,33] and infections[34]. The ferritin test is now becoming a routine medical test[35]. The protein becomes a marker of iron deficiency anemia[36], metabolic syndrome and diabetes[37,38], coronary heart disease[39], atherosclerosis[40], and other diseases[41–43]. The participation of iron and ferritin in oncological processes is also being actively studied[44]. At the same time, the determination of the level of serum ferritin remains an important method of medical analysis (in particular, with a new coronavirus infection[45,46]).

During the last decade, ferritin and ferritin-like proteins have been considered not only as markers of diseases, but also find application in medicine for the production of recombinant vaccines and drug delivery. In 2013, the first ferritin-based self-assembled influenza vaccine was developed[47]. Ferritin subunits combine with the viral protein

hemagglutinin to form a globular protein particle. This vaccine has been shown to be highly effective.

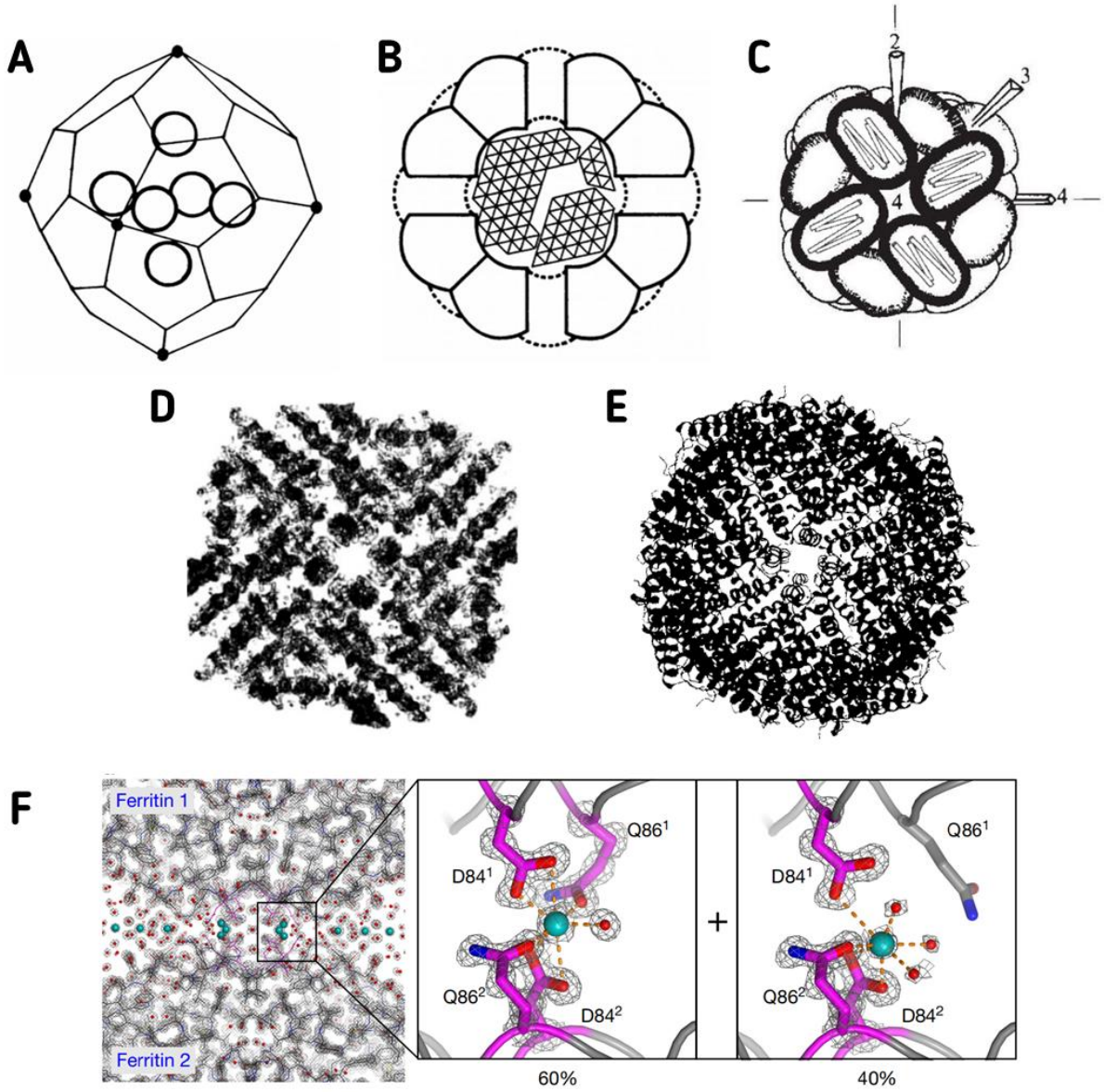


Figure 1. Evolution of ferritin models. **A** One of the first models of ferritin structure[17] (1960). **B** The model, which reflects the presence of channels in the protein envelope[20] (1974). **C** A schematic model according to the first high-resolution data for ferritin (from horse spleen, 2.8 Å), the shapes of separate subunits are represented (1978, adapted from the paper[21]). **D** A complete slice through the apoferritin electron density map[21] (1978). **E** The first high-resolution model (2.4 Å) of a human H-chain ferritin[22] (1991, PDB ID: 1FHA). **F** Ferritin structure with the best obtained resolution[30] (1.06 Å, human H-chain, 2018). All pictures are reprinted from corresponding papers.

The final keystone of ferritin studies is molecular mechanisms of its self-assembly. Deep understanding of these mechanisms will allow creating a number of ferritin-based tools towards the efficient production of chimeric recombinant self-assembling protein complexes with the desired properties.

1.2. Iron metabolism in human body

In order to understand the importance of ferritin in the human body we first have to understand the role of iron. Here, we introduce a more detailed description of metabolism of this micronutrient.

Iron is an important micronutrient, since its inclusion is necessary for proper functioning of globins (heme-containing proteins[48]), in particular, hemoglobin, the main oxygen carrier in humans[49]. Iron is also a part of other important proteins, involved, for example, in cellular respiration and proliferation. Naturally, iron is difficult to consume (oxides or trivalent forms). In cells, iron is found in two main forms: soluble ferrous iron (Fe^{2+}) and oxidized poorly soluble ferric iron (Fe^{3+}). Fe^{2+} is toxic, since it participates in the Fenton reaction[50] ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$), during which reactive oxygen species (ROS) and free radicals are formed; therefore, its concentration must be strictly regulated in living organisms. To cope with the problems of iron storage and toxicity of Fe^{2+} cells use ferritin. Taking into account the fact that iron is poorly excreted from the body (mainly with dying skin or with bleeding[51]), it becomes very important to control the processes of its assimilation and storage.

Iron is supplied with food either in the form associated with hemes, or in the form of inorganic compounds, containing Fe^{3+}. In the intestine, under the influence of low pH by gastric acid and ferroreductases (especially duodenal and upper jejunum cytochrome B[52]), ferric iron is reduced to ferrous Fe^{2+} (Figure 2) and in this form enters the enterocytes (intestinal mucosa cells) through divalent metal transporter 1 (DMT1). Iron in hemes is well-absorbed, independent of duodenal pH, and it directly enters cells by an unknown mechanism[53], where it releases Fe^{2+} by heme oxygenase of the first type[54]. Ferrous iron binds to the membrane transporter ferroportin, then it is oxidized by hephaestin to Fe^{3+} (possibly with the help of its homologue ceruloplasmin at plasma-side) and is released into the blood plasma[52,55]. Fe^{3+} also enters the bloodstream from macrophages which absorb old erythrocytes[53,55,56]. The iron is additionally replenished by processing from senescent red blood cells.

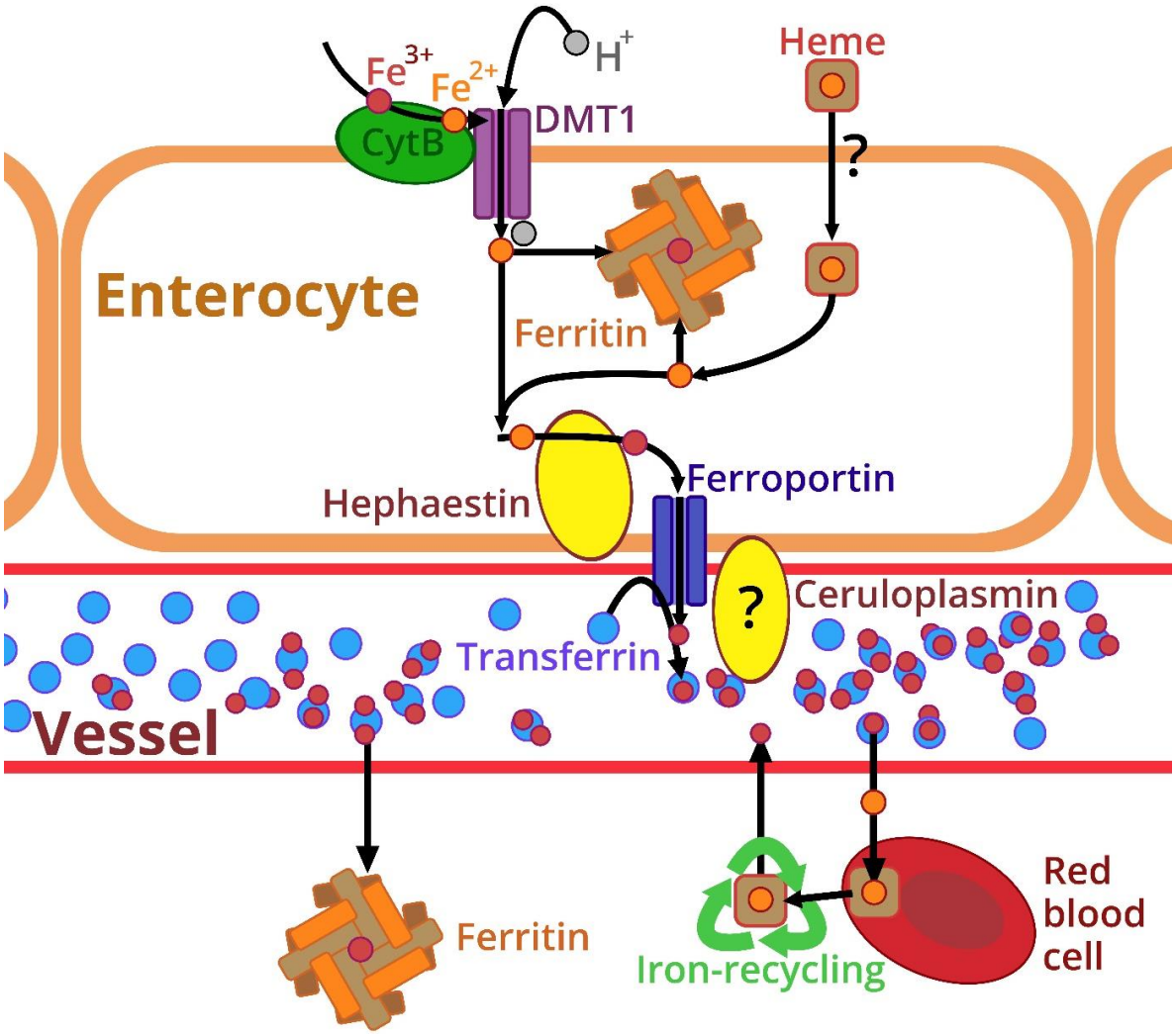


Figure 2. A scheme of iron metabolism in the human body. Hemes and ferric iron ions (red dots) are absorbed with food. The ferric iron is reduced to a ferrous form (orange dots) by cytochrome B and enters an enterocyte (intestine epithelial cell). The ferrous iron is oxidized to a ferric form by hephaestin and excreted into the blood through ferroportin. The blood iron is additionally replenished by processing from senescent red blood cells. In the blood two ferric ions bind one molecule of a transferrin (blue circles). Transferrin is a transport protein, which transports iron in blood vessels to other cells (mostly in bone marrow, spleen, and liver). In cells iron is often stored in ferritin. For more detailed description of this process see section "Biological role of ferritin".

In blood, two Fe^{3+} ions bind to the protein transferrin[57], which transports iron to the cells of spleen and liver (10–20%), and the bone marrow, where erythropoiesis occurs (about 75% of the transported iron)[53]. Iron, contained in transferrin, is less than a thousandth of the whole iron pool in the body. Iron-bound transferrin can interact with the transferrin receptor (TfR). After binding with two transferrins, the receptor is internalized in the endosome[57] (Figure 3). The pH inside the endosome decreases, which leads to release of Fe^{3+} ions from transferrin and assembly of DMT1 on the

endosomal membrane[58]. Ferroreductase Steap3 reduces iron to a ferrous form and it enters the cytosol through DMT1[58].

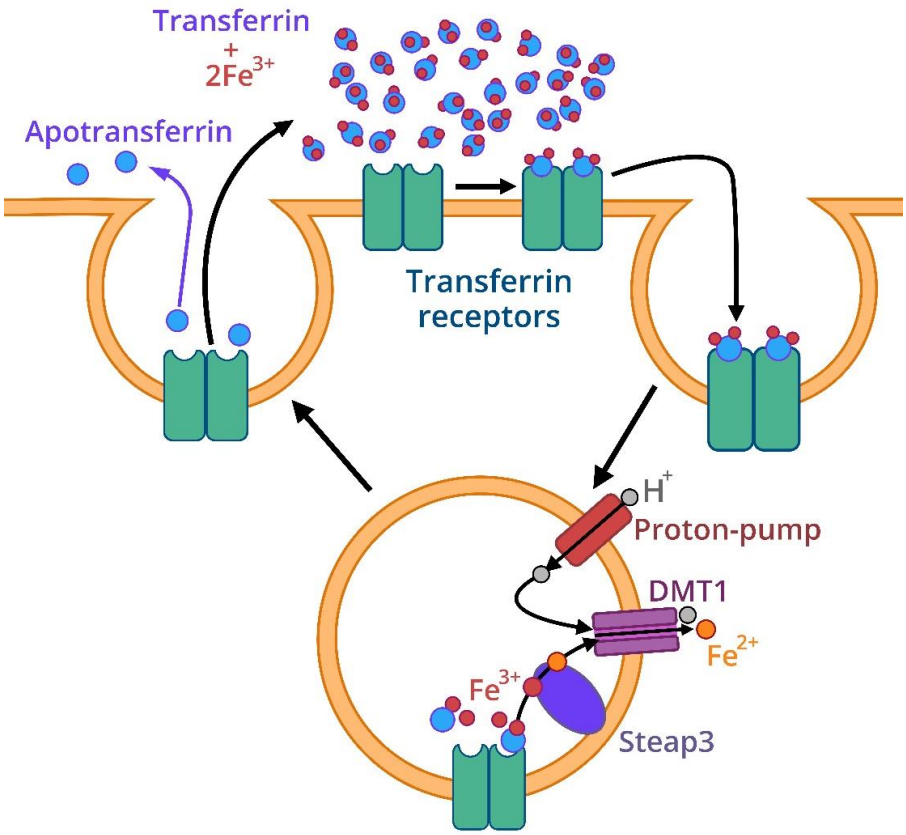


Figure 3. Transferrin recycling process. An iron-bound transferrin interacts with the transferrin receptor (TfR, in the middle of the figure). After binding with two transferrins the receptor is internalized in the endosome (on the right side of the figure). The pH inside the endosome decreases (bottom part of the picture), which leads to the release of ferric ions (red dots) from transferrin and the assembly of DMT1 on endosomal membrane. Ferroreductase Steap3 reduces iron to a ferrous form (orange dots). Ferrous ions are released into the cytosol through DMT1. Apotransferrin molecules and internalized TfRs are recycled after exocytosis. Adapted from the paper[57].

The released iron is used in the further cellular metabolism. In the bone marrow iron is consumed for the synthesis of new hemoglobin molecules[53]. In liver cells, iron is mainly stored in ferritins. The ferrous iron is trapped by ferritin, oxidized inside the protein, and crystallized[2]. Ferritin performs a similar storage function in enterocytes, bone marrow cells, macrophages, and other tissues[59].

The main regulatory mechanism of the iron concentration in blood is realized at the stage of the metal passage through ferroportins. Hepcidin, a peptide hormone that is mostly secreted by hepatocytes, inactivates ferroportins and triggers their endocytosis[53], which leads to a decrease in the concentration of free Fe^{3+} in the blood. Accordingly, hepcidin level has a significant effect on iron metabolism. A large number

of genetic diseases of disturbed iron homeostasis is associated with mutations that cause abnormal production of this hormone. There are many different ways of hepcidin regulation. Its biosynthesis is stimulated by an increase in the level of Fe^{3+} in cells and blood, and also by the action of interleukin 6 and bacterial lipopolysaccharides (LPS)[60]. Several proteins and likely vitamin D are also able to regulate this important hormone. Hepcidin has also been related to severe cases of anemia[61].

Unfortunately, heavy divalent metals (in particular, Pb, Mn, Cu, Co, and Zn) can pass through the metabolic pathway of iron. They compete for binding with the DMT1 receptor[62] and other proteins involved in the synthesis of new hemoglobin molecules, exhibiting their toxicity[53,63].

Although ferritin is an intracellular protein, it is also found in human serum. Serum ferritin level rises sharply during acute inflammation[32]. The mechanisms of its release from the cell are poorly understood[64]. It has been shown that ferritin can bind to TfR1[24] and some other receptors[3,59], however, a unique ferritin receptor has not been found[3]. It is possible that ferritin is released into the extracellular space from liver cells and macrophages through vesicular secretion[3]. The function of serum ferritin has not yet been determined. There is some evidence that it might be connected with signaling, since ferritin has a great influence on the activity and functioning of macrophages - active participants in inflammatory processes[3,44].

This review considers iron metabolism focusing on heme iron. There are also other sources of iron, e.g., sulfur and mono/binuclear iron compounds; however, they are out of the scope of this paper.

1.3. Biological roles of ferritin

1.3.1. Ferritin function

A human consumes about 10-20 mg of iron per day from food, but only 1-2 mg is absorbed by the body; the same amount is excreted from the body daily[53], and most of the absorbed iron enters the recycling cycles. Although the main function of ferritin was considered to be mainly the storage of iron, it also has other functions[65]. Modern studies showed that ferritin is a regulator of iron homeostasis and an antioxidant, and its main role is regulation of iron toxicity and ROS[2,59].

Ferritin and other ferritin superfamily proteins (such as bacterioferritin) catalyze oxidation of Fe^{2+} to Fe^{3+} and store the Fe^{3+} product as a ferrihydrite-like mineral core. This way, the protection of cells against the toxic products of spontaneous iron oxidation (e.g., ROS) is realized. In order to oxidize Fe^{2+} the protein uses molecular oxygen or hydrogen peroxide. The storage cavity of the protein, where oxidation takes place, is separated from the surrounding solvent by a protein shell with a thickness of 2 nm. The

iron-storage function of ferritin has a central role in cellular iron homeostasis. For example, diatoms with increased ferritin expression rates have advantage in areas of prolonged iron limitation with pulsed iron inputs. They are able to survive under harsh iron-deficient conditions due to accumulation of iron during iron-rich periods[66]. Moreover, in some phytoplanktonic organisms, ferritin is optimized for iron buffering rather than storage. *Ostreococcus tauri* ferritin is translated during the night and degrades soon after transition to light when the activity of iron-dependent enzymes for nitrogen fixation and photosynthesis is maximal[67]. In addition to its iron-storage function, a ferritin could have other biological roles[68] such as involvement in immunity, autoimmunity[69], and lipid metabolism[70].

DNA binding protein from starved cells (DPS), along with ferritin and bacterioferritin, belong to a ferritin family[71–73]. DPS can oxidize and store iron and has spherical shape, but there are some differences: it consists of 12 identical subunits only and it binds DNA (however, some natural DPS miss the N-terminal DNA-binding domain[74,75]). The main cellular role of these proteins is defense of DNA against oxidative damage; it is achieved by either oxidizing iron to prevent the formation of oxidative radicals or by forming DPS-DNA complexes to physically protect DNA[76,77]. In one paper[78] it was proven using mutagenesis that both activities are vital in different stress conditions.

Some ferritins are able to release iron from ferrihydrite core. Iron release mechanisms differ in eukaryotes and bacteria. In particular, eukaryotic ferritins release iron in lysosomes[79], while bacterioferritins contain a heme molecule (bound covalently to methionine groups). Heme carries electrons which reduce Fe^{3+} to releasable soluble form Fe^{2+}. Often, this mechanism of iron release is enhanced by proteins that either use it by themselves or can carry the iron to the target structure (different reductases, for example ferredoxin – Bfd[80]). Ferritin is known to be an iron hub, where processes of iron release and binding occurs via the 3-fold channels. The rate of iron release can be increased in the presence of one electron reducing agents (such as NAD) and some proteins[81–83].

24-meric ferritins have conserved active sites for iron oxidation in the inner surface of separate subunits (see section “Structure and function”), whereas for DPS such sites are located at the interface between different subunits[81]. The formation of the iron reservoir occurs in several stages, including the nucleation and subsequent growth. Initially, Fe^{2+} binds to catalytic sites with trivalent form production, then moves to the emerging nucleus[81]. Further, the process proceeds in a simplified condition due to the additional deposition of ions on the nucleus. Oxidation of iron occurs with the participation of oxygen in 24-meric proteins and peroxide in DPS with the formation of

intermediate compounds[81]. The process in 24-meric proteins releases peroxide, which can be similarly used by the protein[81].

1.3.2. Regulation of ferritin expression

Organisms from all three Domains (Bacteria, Archaea, and Eukarya) produce ferritin and regulate its biosynthesis. Ferritin expression is regulated by several factors, including iron ion concentration, oxidative stress, and starvation.

In *Escherichia coli* cells *ftnA* gene expression (the gene encoding 24-meric bacterial ferritin, see section “Diversity of ferritins and ferritin-like proteins”) is mostly regulated by iron-dependent transcriptional activator Fur complexed with Fe^{2+} (Fur competes with H-NS for binding at sites upstream from the *ftnA* promoter which in turn leads to derepression of transcription)[84]. Also, there is indirect evidence that in *E. coli ftnA* expression could be induced by oxidative stress in an OxyR-dependent manner[85], while in the obligate anaerobe *Bacteroides fragilis ftnA* expression is strongly regulated by redox[86]. Biosynthesis of DPS (another member of ferritin superfamily) is mostly activated by OxyR in response to oxidative stress[87]. The amplitude of this induction is high enough that the promoters of *dps* in gram-negative and its homologue *mrgA* in gram-positive bacteria can be used to construct biosensors for detecting oxidative stress[88–90].

Additionally, genes of ferritin superfamily members (ferritin FtnA, haem-containing bacterioferritin Bfr, and DNA binding protein DPS, see section “Diversity of ferritins and ferritin-like proteins”) are regulated by stationary phase factors[84,87,91]. It was shown that *E. coli* master-regulator protein CsrA binds with high affinity and specificity to *bfr* and *dps* mRNAs and inhibits their translation, while it modestly activates *ftnA* expression[91]. For the *dps* gene, it was shown that its induction at stationary phase is provided by an increase in transcription from the promoter recognized by σ38[87]. In the logarithm phase, this promoter is repressed by the Fis protein in complex with σ70[92].

In eukaryotic cells ferritin expression regulation is much more complex, depends on many factors, and realized on all levels: transcriptional, translational, and posttranslational. The iron concentration is one of the main regulatory factors. At low cellular iron concentration regulatory protein binds to a stem-loop structure at 5’-UTR of ferritin-coding mRNA and prevents translation[93]. As soon as cellular iron levels increase, the regulatory protein is displaced from its binding site, so that translation of ferritin mRNA can take place. In plants, iron-dependent transcriptional activation of ferritin expression is described[94,95]. In animals, in response to oxidative stress, ferritin gene expression is induced via both transcriptional activation and translational

derepression[96]. In mammals ferritin expression is regulated even by hormones, growth factors, and second messengers like cAMP[97].

In human cells, iron-regulatory elements (IRE) / the iron regulatory protein (IRP) system regulate translation of multiple proteins that are involved in iron metabolism[98], including ferritin (Figure 4A). IREs are special sites in mRNA sequences of regulated proteins. IRP is a protein comprising an iron-sulfur cluster which binds to IRE under certain conditions. The binding of IRP to IRE alters the stability of the regulated mRNA, which leads to a change in the level of gene expression. In the case of the ferritin gene, IRE is located in the 5'-untranslated region of mRNA. Therefore, binding of IRP interferes with the ribosome binding to mRNA and leads to a decrease of ferritin level[2]. In some other proteins (for example, TfR1), the IRE is located in the 3'-terminus of the mRNA. In this case, binding to IRP stabilizes the molecule and increases protein expression[2,98].

There are two types of IRP proteins: IRP1 and IRP2. IRP1 changes its conformation and ceases to bind IRE at high Fe^{2+} concentration[98]. The concentration of IRP2 in the cell is controlled by the FBXL5 protein complex. The complex becomes unstable and degrades in proteasomes at low iron concentrations, while at high concentrations it ubiquitinates IRP2, which transforms the protein into an inoperative conformation and ultimately leads to its proteolysis[98]. Thus, at high iron concentration, both IRP proteins cannot bind to IRE and perform their regulatory function (Figure 4A). It leads to an increase of ferritin concentration in the cell. This ferritin is able to efficiently absorb free iron, which leads to a decrease of the concentration of this micronutrient and, thus, stabilization of the binding between IRP and IRE. In this case, the expression of ferritin decreases.

Another mechanism of ferritin regulation is its degradation via interaction with a special protein NCOA4[99] (Figure 4B). NCOA4 binds to ferritin and leads it to the autophagosome, where it is further degraded. NCOA4 is itself sensitive to the iron concentration in the cell. Its conformation changes at high concentration of iron and the protein forms a stable bond with the ubiquitin ligase HERC2. Ubiquitination leads to NCOA4 degradation[100]. Thus, similar to the IRE / IRP mechanism, feedback occurs (Figure 4B). NCOA4 releases ferritin at high concentration of Fe^{2+}, then ferritin collects free iron and reduces its concentration until NCOA4 again becomes able to bind to ferritin and lead it to degradation. This interaction makes the NCOA4-ferritin pair a sensitive cellular iron sensor, which maintains the homeostasis of this micronutrient in cells.

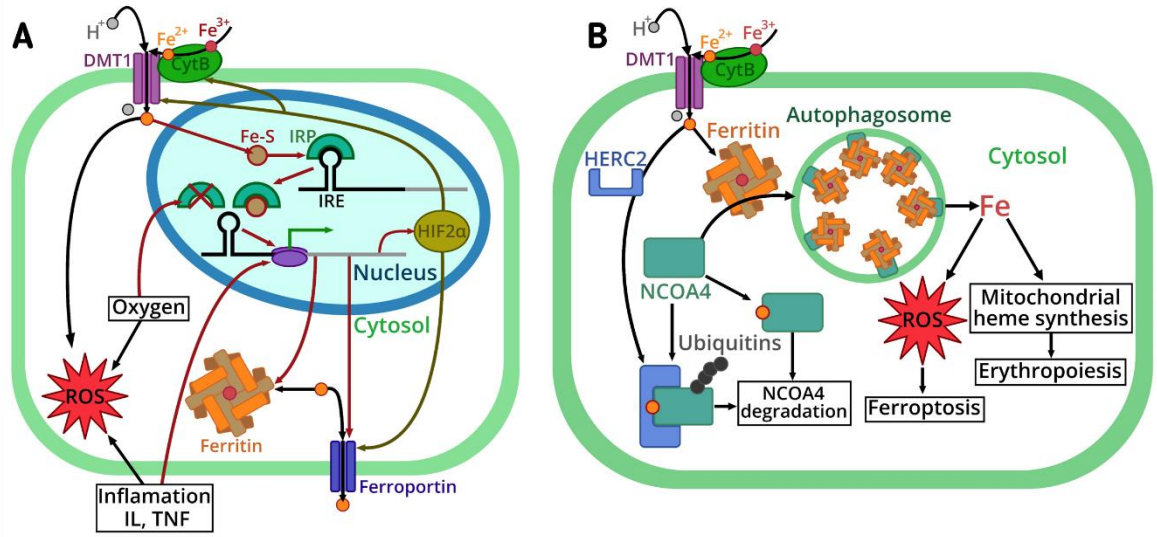


Figure 4. Ferritin metabolism in eukaryotic cell. **A** IRE / IRP regulation of ferritin. Binding of IRPs to IREs in the nucleus (green round rectangle) prevents ferritin expression. With an increase of iron in the cell, IRPs cannot bind to the DNA regulatory region of the IRE. This leads to the binding of the ribosome with the following expression of ferritin and other iron metabolism proteins, such as ferroportin and HIF2α. Stress conditions (such as inflammation) can also indirectly regulate expression[111]. Ferritin (together with ferroportins) accumulate excess of iron (orange dots). HIF2α transcriptionally regulates the expression of ferroportin, DMT1 and Duodenal CytB (DCytB). In case of iron deficiency, DMT1 and DCytB are expressed; conversely, in excess of iron, ferroportin is expressed. **B** NCOA4-mediated regulation of ferritin. At low iron concentration NCOA4 binds ferritin, leading the protein to the autophagosome degradation along with iron release. This leads to an increase of available intracellular iron (labile iron pool), as well as an increase of ROS. Iron can be used to create hemes, iron-sulfur clusters, in erythropoiesis, etc. This mechanism of ferritin degradation (ferritinophagy) occurs in the iron-dependent pathway of cell death (ferroptosis). In addition to basal autophagy degradation of NCOA4, there is another iron-mediated degradation pathway. With an excess of iron, a HERC2 (regulatory protein) binds NCOA4 leading to its ubiquitin-mediated degradation.

The process of ferritin degradation is called ferritinophagy[101,102] and can lead to iron-regulated cell death – ferroptosis (see section “Ferritin and human diseases”). During the degradation of ferritin, the stored iron is released in a ferrous form by an unknown mechanism, possibly similar to the mechanism of transferrin endocytosis[2,64] (Figure 4B). This leads to an increase of available intracellular iron, which is usually attached to small molecules (labile iron pool)[82,103]. The labile iron can be used to create hemes, iron-sulfur clusters, in erythropoiesis, etc[103]. In case of iron deficiency, ferritin can become the source of iron in the cell, although such amount of excreted free iron might be dangerous[101,102]. Probably, ferritin can alter the labile iron pool in a more accurate way by passive reduction of its iron core with consequent release through its

channels[82,101]. Thus, the balance between the labile iron pool and ferritin, iron uptake, iron protein utilization, and iron release is realized.

Ferritin is regulated both at the transcriptional stage (IRPs) and at the post-translational level (NCOA4). Regulation processes might also take place at the step of self-assembly, especially taking into account the fact that subunit composition affects protein functionality; however, it requires further research.

Apparently, complexity and diversity of mechanisms of expression regulation of the ferritin superfamily genes in both pro- and eukaryotic cells are associated with the high importance of coordinating iron metabolism with a number of external conditions.

1.3.3. Ferritin and human diseases

Abnormal amounts of ferritin in human blood are often associated with the presence of various illnesses, such as inflammation[32,33], infection in upper respiratory tract[34,43], or autoimmune diseases[104]. Its synthesis is activated by many pro-inflammatory factors. Interleukin-1β (IL-1β), IL-2, IL-6, and TNFα activate ferritin production along the pathway of the transcription factor NF-κB[3,59]. A binding site for this factor in the promoter region of the ferritin gene increases the level of ferritin transcription[59,105] (Figure 4A). Transcription of ferritin L-chains increases during oxidative stress (which can be caused by inflammation) in a similar way, but through other enhancers[2]. Such mechanisms of ferritin regulation might be associated with the increase of the serum ferritin during inflammation, which consists mainly of L-chains[2], and can occur even with iron deficiency. Other proinflammatory factors, such as interferon gamma and bacterial lipopolysaccharides (LPS), trigger cascades that suppress the IRP / IRE mechanism, and thus indirectly increase ferritin concentrations[3] (Figure 4A). Various infections, such as tuberculosis[106], malaria[107], and sepsis[108,109] cause an inflammatory increase of ferritin.

Ferritin is also involved in the maintenance of inflammation. It is required by macrophages for normal functioning and rapid response to infectious stimuli (synthesis of NO, IL-6, and IL-1β)[3,110]. In general, during inflammation, ferritin is activated similarly to proinflammatory cytokines[105] and protects cells from inflammation-induced oxidative stress[2,3].

A number of studies note the connection between changes in ferritin concentration and cancer. Recently, it was reported that ferritin is involved in carcinogenesis[3,44]. Many solid tumors are formed by infiltration of macrophages, one of the few cell types that secrete ferritin[44]. This confers significant benefits in terms of immune protection, protection from iron-mediated damage and hypoxia, increased proliferation and angiogenesis, and decreases the level of the inflammatory response[111]. Furthermore, ferritin plays a significant role in the resistance of cells to hypoxia, which is a typical but

challenging condition of tumor cells. Ferritin stabilizes hypoxia factor 1α (HIF1α), which is very important for the metabolism of cancer cells[44]. Ferritin absorbs iron and the substrate (ROS) of prolyl hydroxylase, which inhibits HIF1α[2,112].

Hypoxia also increases the synthesis of ferritin L-chains, which can stimulate the epithelial-mesenchymal transition in gliomas. This transition leads to severe diseases and increases fatal outcomes[113]. This is confirmed by the fact that the concentration of L-ferritin is increased in many malignant tumors[113]. Knockdown of H-ferritin makes cancer cells more resistant to treatment and activates the synthesis of ROS, which leads to an increase in mutagenesis[114,115]. For these reasons, some cancer treatment approaches need to be modified. A number of drugs kill cancer cells through the creation of cell-lethal doses of ROS. However, such an approach does not work in the case of ferritin overexpression by tumor tissue.

In addition, genetic disorders in iron metabolism cause neurodegenerative diseases where ferritin mediates these processes. Disturbances in the regulation of ferritinophagy and ferroptosis are associated with the development of neurodegenerative diseases and carcinogenesis[100]. Mutations of ferritin or disproportionate expression of its L-subunit lead to improper iron detoxification. This form of iron is already detrimental to brain cells[116,117].

Unbound iron acts as a strong oxidizing agent, producing ROS, which may cause cell damage. A relatively small amount of unbound iron (for example, in some iron metabolic disorders) can lead to formation of cancer[118]. With an increase in iron concentration, oxidizing activity determines the mechanism of ferroptosis, iron-mediated cell death. It is characterized by oxidation of lipids and is accompanied by morphological and functional degradation of mitochondria. At the same time, oxidizing properties of free iron are regulated by inflammatory cytokines[44]. This is needed to induce inflammation and quite important for immune response. Such regulation is needed to induce inflammation and is quite important for immune response. Hence there is a fine balance between oncotransformation and immunity, which is strongly dependent on iron metabolism and regulation. Ferritin plays the main role in this balancing, performing its protective function for cells.

The storage of iron has long been considered as the main function of ferritin, but new evidence suggests that its role in cell metabolism is much more diverse. Firstly, ferritin is an active regulator of free cellular iron concentration[2]. There is a feedback loop between the concentrations of iron and ferritin in the cell, since the protein concentration is tightly regulated (at the post-transcriptional and degradation levels) by the current concentration of Fe^{2+}, and, at the same time, ferritin affects the concentration of available iron. Secondly, the protein has a protective antioxidant function, and it significantly

increases the viability of cells. An increase in ferritin concentration during inflammation and infections reduces toxic free iron and enhances the protection of cells against oxidative stress which occurs during inflammation[3].

Finally, there is a possibility that regulatory dysfunctions might influence the self-assembly processes and shift an H- L-subunit equilibrium in a subunit composition of ferritins, therefore leading to iron metabolic disorders. Consequently, studying how regulation processes influence self-assembly of large protein complexes may help improve techniques for therapeutic applications.

2. Structure and function

2.1. Structure – function interplay in ferritins

Ferritin forms a cage with six four-fold channels and eight three-fold channels, which are the place where 4 and 3 subunits meet together, respectively (see detailed description below). These channels have a small circumference (ranging from ~0.2 nm to ~0.5 nm) that varies along the axial direction of the globule. To date, both the 3-fold and 4-fold channels are known to be responsible for Fe^{2+} uptake, and the 3-fold channel is the main entry gate[119–121]. After entering the cage, Fe^{2+} is transferred to a dinuclear metal ion binding site that is called the ferroxidase center (FOC)[119,121], where the oxidation occurs, and after that Fe^{3+} is pushed to the ferritin cavity by the next Fe^{2+} [122]. Inside the protein cage, Fe^{3+} forms a ferrihydrite-like mineral core[123]. Amino acids comprising FOC are highly conservative among a number of clades of ferritins (even in BFRs, which belongs to different protein subfamily[1], see section “Diversity of ferritins and ferritin-like proteins”), although the location of the active site is diverse in different proteins[124].

A common feature of all ferritins is structural organization of separate subunits. Each ferritin subunit forms a bundle of four α-helices, called A, B, C, and D respectively (Figure 5A). The second (B) and the third (C) helices are connected by a long loop. Most proteins also have an additional short helix E on the C-terminus, placed at an acute angle from the bundle[2] (Figure 5A). Such structural organization is even more conservative than protein amino acid sequences[125]. Thus, human H-ferritin and *E. coli* ferritin A share only about 20% of similarity in amino acid sequences, being structurally similar (Figure 5B). All ferritins in native cells form stable globular quaternary structure and are almost never found as separate ferritin subunits[2]. Subunits of different types from one organism can form stable heteropolymeric globules[126–129]. There is a number of known point mutations and truncations in different parts of ferritin (e.g. the human H-ferritin subunits and *E. coli* ferritin A subunits (N-, C-terminal, the loop between the B and C helices[130])), which might lead to disruption of self-assembly for some species. These facts emphasize the conservatism and functional significance of the self-assembly process.

Typical ferritins (FTNs, see section “Diversity of ferritins and ferritin-like proteins”) share other common features between each other. Their globule consists of twenty-four protein monomers. Its outer radius is ~6 nm, and inner is ~4 nm (the thickness of the protein layer is ~2 nm). As we previously stated, the ferritin globule is very stable. It has 4-3-2 symmetry and 4-fold and 3-fold symmetry axes pierce corresponding channels in the shell (six 4-fold and eight 3-fold, respectively). Four C-terminal regions of separate subunits interact with each other, forming one 4-fold channel (Figure 5C). There are also 3-fold channels, each of which comprises three separate subunits. A disordered N-

terminus region might make a contribution in this interaction. In animal ferritins, 4-fold channels are hydrophobic and likely responsible for oxygen uptake inside the protein globule[59]. Conversely, 3-fold channels are hydrophilic and responsible for iron uptake[59]. Such differentiation of functions is not conservative among other proteins: for example, channels of *E. coli* ferritin exhibit both hydrophobic and hydrophilic properties[131]. 2-fold symmetry axes are formed on the interaction surfaces between two monomeric subunits. Active center amino acids (Figure 5A) are also more conserved among typical ferritins than among all ferritin-like proteins.

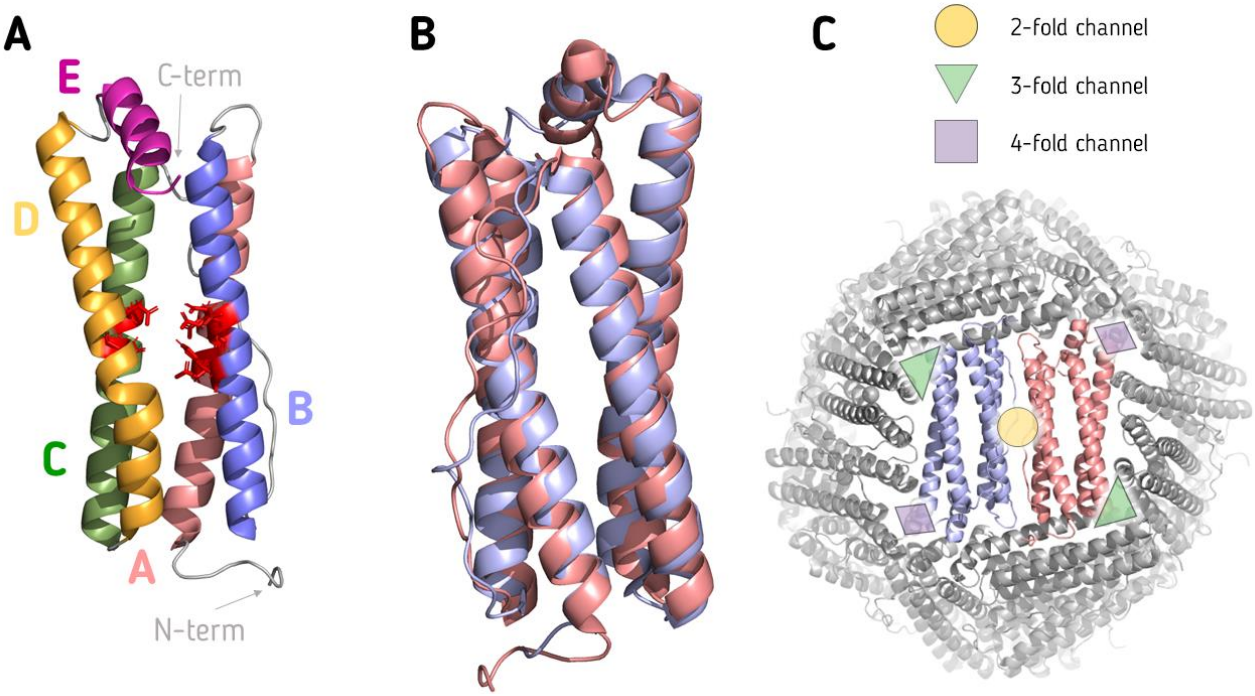


Figure 5. High-resolution ferritin structures: common features of different ferritins. **A** Human H-chain ferritin monomer (PDB ID: 7A6A[29]). Different helices are named from 'A' to 'E'. Active center amino acids are highlighted using red color. **B** Alignment of human H-chain ferritin monomer with *E. coli* ferritin (PDB ID: 6B8F[30], 2Y3Q[276]). **C** Human H-chain ferritin complex with highlighted channels and symmetry axes (PDB ID: 7A6A[29]).

Human H-ferritin active center consists of six amino acids: Glu-61, Glu-62, His-65, Glu-107, Gln-141, and Glu-27[81,132] (Figure 6A). Ferrous ions Fe^{2+} enter the protein through 3-fold channels, interacting with conservative acidic amino acids[132] (they act as temporary sites for ion landing), and then move along other amino acid residues to the active center[121]. The active center has two sites of iron oxidation (Figure 6B). Then, two Fe^{3+} ions interact with ROS, which form during oxidizing reactions, and form a single peroxo complex[50,81]. This complex moves to the site of mineralization, where it is slowly hydrolyzed and stored as a ferrihydrite. In this case, ROS are neutralized along with the protons release.

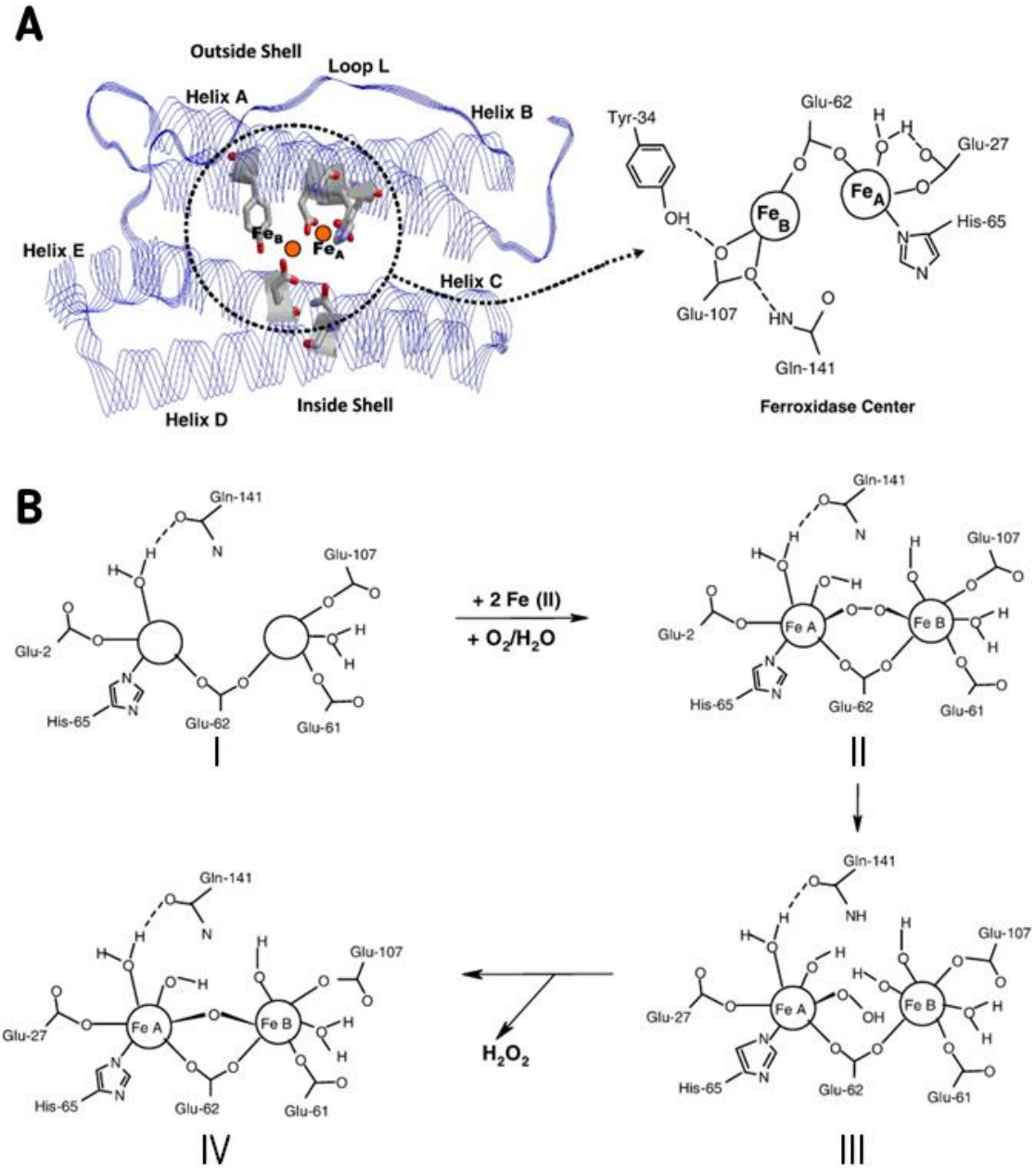


Figure 6. A mechanism of iron oxidation in the active center of ferritin subunit. **A** General overview of an active site. On the right side a diagram of dinuclear ferroxidase center is shown. **B** Ferroxidase center reaction intermediates in H-chain ferritin. The first step (**I**) is apoprotein, the second (**II**) is μ-peroxo di-iron(III) complex, the third one (**III**) is μ-hydroperoxo di-iron(III) complex, and the last one (**IV**) is μ-oxo di-iron(III) complex. Adapted from[77].

The mechanisms of iron mineralization are not well-studied, but their effect on protein functionality is notable. In mammals, including humans, there are two types of monomeric protein chains: heavy (H-chain, ~21 kDa), and light (L-chain, ~19 kDa)[126,133]. Only the H-subunits of 24-meric ferritin are able to catalytically oxidize Fe^{2+}. In bacteria and archaea, 24-subunit ferritin is a homopolymer. Each of its subunits is catalytically active as H-ferritin subunits from eukaryotes[95]. There is also M “middle” subunit ferritins found in some eukaryotes, whose function is similar to H-subunits[134].

The H-chain has a ferroxidase activity (it contains an active ferroxidase site), while the L-chain has a more efficient site of iron mineralization[135]. Amino acids of this site are highly conservative among such subunits[1]. The formation of heterodimers from H- and L-subunits is more prevalent than the formation of homodimers during ferritin self-assembly processes[136]. H- and L-subunits ratio affects protein function and the shape of the mineral core[137]. Namely, a complex rich in L-chains releases iron slowly (because they accelerate mineralization), while a complex rich in H-chains provides rapid uptake and release of metal ions. In this regard, different tissues contain ferritin isoforms with different subunit ratios. Nevertheless, non-iron-storing serum ferritin[138], and iron-storing ferritins in liver cells[139] mainly consists of L-subunits. Simultaneously, ferritin rich in H-subunit is found in brain and heart cells, which actively use iron[139].

Although homopolymeric L-ferritin does not contain ferroxidase sites, it is still capable of notable iron accumulation under certain conditions[140]. L-chains may contribute to the process of iron oxidation by transporting electrons through the protein shell[141]. In general, L-chain seems to be a late evolutionary adaptation[1,141]. It is important for optimization of iron storage by the whole complex and acts as a regulator of protein ferroxidase activity. A self-assembly might modulate the ferroxidase activity[119]. There are proteins which self-assembly is disturbed by point mutations. Therefore, regulation of self-assembly might allow one to control ferroxidase activity and iron metabolism in cells. It is important since the intermediate products of iron oxidation reactions are toxic to the cell. Therefore, these processes must be isolated from the cytosol, and ferritin provides «compartmentalization» - a cavity in which the processes of iron oxidation and product mineralization take place[2].

Ferritin has many distinctive features in its inner organization, such as a globular shape, 4 helix–bundle structure of subunit, ion-specific channels, 4-3-2 symmetry, and self-assembly. During evolution it became a precise machine for storing iron. Its activity depends on the overall structural organization of a protein, subunit structure and composition, and mechanisms of self-assembly. The role of self-assembly processes here might be extremely important and needs to be investigated further.

2.2. Diversity of ferritins and ferritin-like proteins

Ferritins are highly conserved proteins that are widely distributed in various species from archaea to humans[1,72,142]. They are also found in viruses and mycoplasma[1,124]. The ferritin family includes ferritin itself, bacterioferritin, and DNA-binding mini-ferritin DPS. Sometimes ferritin from plants is considered as a separate member of the family and is called phytoferritin[143]. In ref.[1], a phylogenetic analysis of

known ferritins and ferritin-like proteins (Figure 7) was carried out and suggestions were made about the evolution of ferritins.

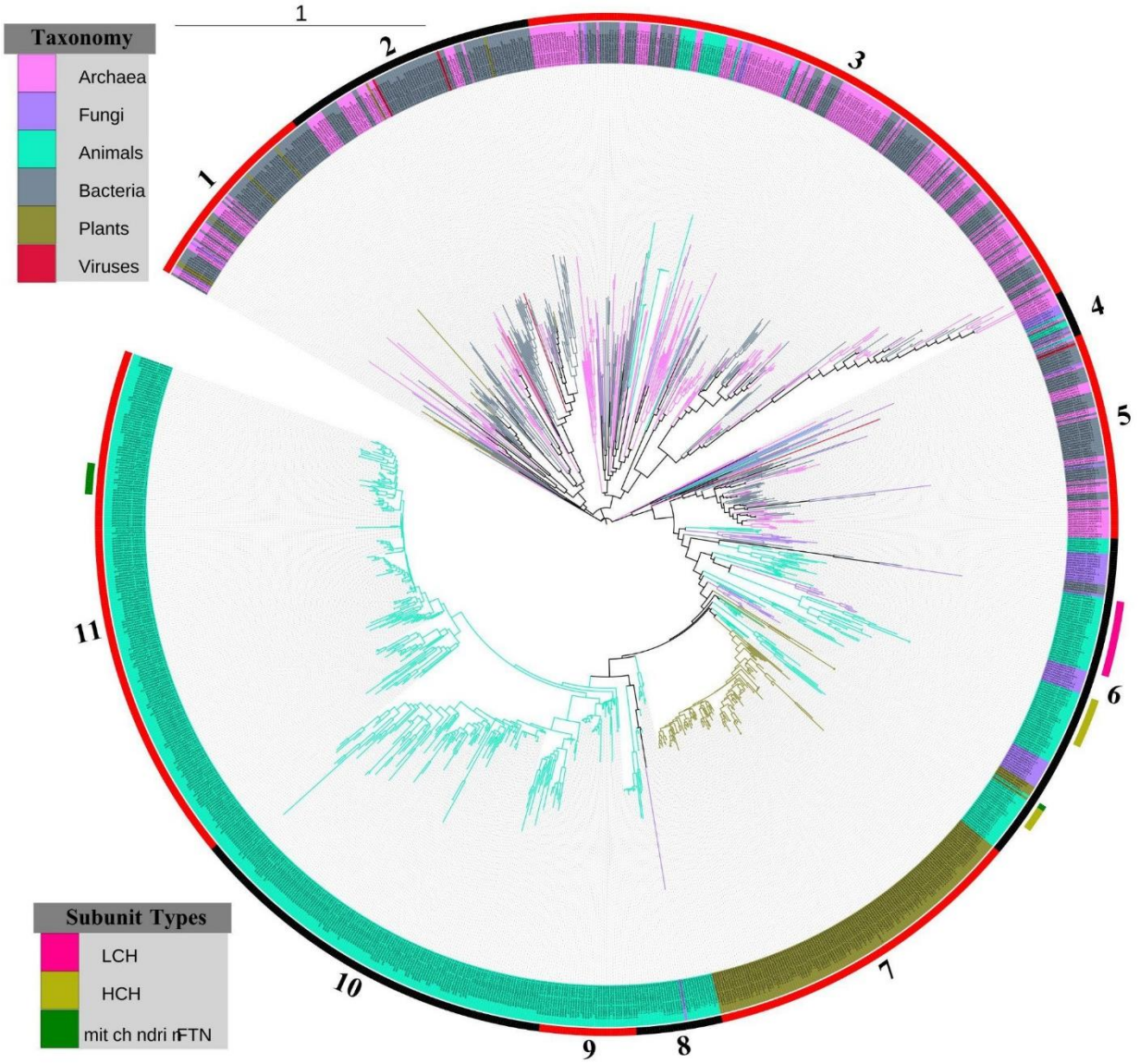


Figure 7. Phylogenetic tree of researched[1] ferritins. The inner circle is the phylogenetic tree derived from the consensus sequences of ferritins. The upper left legend shows their taxonomic groups with corresponding colors in the branches and middle circle. The scattered colored curves outside the corresponding taxons show their subunit types, as indicated in the lower left legend. The branch length is indicated as a scale bar. Reprinted from ref.[1]

The most ancient divergence in ferritin family evolution is likely the divergence of typical ferritin (Figure 7, clades 5-11), which is presented in all Kingdoms, from the other ferritins, which are presented in prokaryotes predominantly (Figure 7, clades 1-3). Let's look closer at clades 1 and 2 with exclusive bacterial ferritin variants: bacterioferritin (BFR) and DPS, correspondingly. They have significant structural distinctions from typical ferritins.

BFR is heme-containing ferritin. The quaternary structure of bacterioferritin is equivalent to that of ferritin, however, with a heme group between pairs of subunits. Heme is not essential for 24-mer formation[144], and it is not important for Fe oxidation[131]. Its main function is electron transfer across the protein cage, which supports Fe^{3+} reduction and subsequent release[80,145]. This fact together with evidence that ferritin expression is induced during anaerobic respiration[146] might indicate the involvement of ferritin into this process. The BFR active center is predicted to act as a gated iron pore for simultaneous passing and oxidizing iron[131].

DPS differs even in general structural organization (mentioned above): it forms a 12-meric globular structure, although the size of its monomer subunits does not differ much. Also, DPS have positively charged N-terminus, which enables binding to negatively charged DNA[147]. Due to this ability, DPS serves for DNA protection from oxidation damage in a cell[78]. Ferritins from some archaea and bacteria inherited intermediate properties both from DPS and other ferritins[148–150].

The clade 3 includes a rather interesting group of proteins called rubrerythrin, members of ferritin superfamily. They are not typical ferritins since they do not form a sphere, but they still have homology to the four typical ferritin alpha-helices and a conservative diiron site. These proteins even perform ferroxidase activity in the presence of O_2[151]. The key feature of rubrerythrin is its ability to reduce peroxide to 2 water molecules in anaerobic conditions[151]. Some homologues of this protein are present in eukaryotes, but not very widely, which seems to be a result of horizontal gene transfer[1]. Clade 4 includes divergent proteins with atypical sequences and couldn't be assigned to one specific enzymatic group.

The other clades (Figure 7, clades 5-11) present a subfamily of typical ferritins (FTN). Clades 10 and 11 contain L- and H-chains of animal ferritins. H-chains are catalytically active and broadly spread among animal species. L-chain is presented typically in mammals, underlying the evolutionary and functional uniqueness of these subunits. L-chain doesn't have a ferroxidase activity but takes place in electron transfer during iron oxidation and reduction in ferritin[141,152]. The clade 9 contains a rather specific M-chain ferritin; the key feature of this ferritin is that it is evolutionarily closer to L-chain, but has a ferroxidase function like H-chain[133]. Animal-like ferritins from bacteria form clade 5. According to the phylogenetic tree, they are closer to the presumptive common ancestor of all ferritins than animal ferritins. Ferritin A from *E. coli* (one of the most studied ferritins) belongs to this type of subunits. Plant phytoferritins unite in the 7th clade, having a number of specific structural features[143]. While phytoferritin forms a traditional 24-meric sphere, each subunit contains extension peptide (EP) at its N-terminal extremity[153]. The EP is located on the exterior surface of protein, which was recently found to act as a

second ferroxidase center for iron-binding and oxidation, and regulate iron release during the germination and early growth of seedlings[143].

Interestingly, only a few bacterial species, including *Escherichia coli*, *Klebsiella pneumonia*, and *Salmonella enterica*, have a ferritin-like protein designated ferritin B (*ftnB* gene) that shares 33% homology with FtnA and is thought to serve as a facile cellular reservoir of Fe^{2+}, since it does not have conserved residues of the ferroxidase domain[154,155].

Protection of mitochondria from oxidative stress and, partially, iron homeostasis of the cell are driven by special mitochondrial ferritin (MF) which was found in animals and insects[2,156]. Mitochondrial ferritins from different species are homologous to their corresponding H-chains and also similar to each other[1]. A structural feature of MF is the presence of a mitochondrial signal, which directs the synthesized precursor protein into the mitochondria and is cleaved during post translational modifications[2].

One more class of ferritin-like proteins is enapsulins - large globular proteins, whose form-factor is similar to ferritins (Figure 8). A protein globule consists of 60 to 180 monomers and contains a large inner cavity[157]. The encapsulin subunits by themselves do not have ferroxidase activity, but internal encapsulin ferritin acts as a ferroxidase in the complex. As well as ferritin, encapsulins are a potential tool for nanotechnology applications[157]. Encapsulin can contain up to 30,000 iron atoms (compared to about 4,000 for ordinary ferritin), but its biological functions are not completely clear[157]. Recently, it has been suggested that encapsulins might be iron-containing organelles of prokaryotes[158].

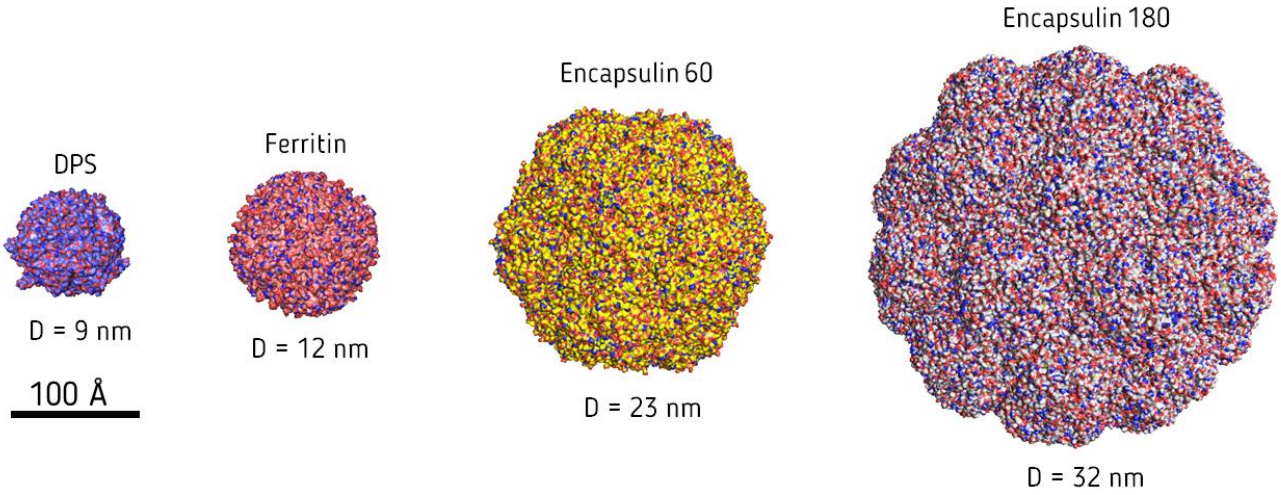


Figure 8. The size scale of ferritin-like complexes. From left to right: DPS from *Thermosynechococcus elongatus* (PDB ID: 2C41[277]), ferritin from *Homo sapiens* (PDB ID: 7A6A[29]), encapsulin 60 (PDB ID: 6I9G[278]) from *Mycolicibacterium hassiacum*, encapsulin 180 (PDB ID: 4PT2[158]) from *Myxococcus xanthus*.

Many organisms have a large number of ferritin gene copies, either from different clades or similar ones. For example, one study[1] claims that the genome of the western lowland gorilla contains 25 copies of the H-ferritin gene. According to the same study[1] humans have four genes, at least three of which are functional. However, there are no experimental evidence as to how many of them are pseudogenes. Mice and most mammals also have pseudogenes for H and L chains[12,159,160]. While organisms which are sensitive to iron overload bear little number of ferritin genes[161], such multiple duplication might be connected with active iron metabolism and absorption. *E. coli* simultaneously produces DPS, BFR, and FTN[131]. Interestingly, unlike in other eukaryotes, ferritins are the least spread (present only in one copy or missing e.g. in yeasts) in fungi, that have other mechanisms for iron absorption and storage[162]. Some fungal species with more than one ferritin gene are intracellular parasites[1,2]. Ferritin genes are also essential for reproduction and normal physiology of other parasites[163], indicating the specific role of this protein in parasitism.

Different ferritins in living organisms from all kingdoms and ramification of ferritin phylogenetic tree might indicate high importance of ferritins and evidence of their functional variety for regulation of iron metabolism.

2.3. Functional intrinsic disorder in ferritins

Often, the multifunctionality of proteins is related to their structural heterogeneity rooted in the phenomenon of intrinsic disorder. In fact, it was indicated that any protein (including the most ordered ones) exists as a highly dynamic conformational ensemble representing a complex assembly of regions/segments with different levels of (dis)order, such as foldons, unfoldons, semi-foldons, inducible foldons, inducible morphing foldons, and non-foldons, which are involved in constant conformational exchange and structural interconversion[164–166]. Such highly dynamic and complex structural mosaic defines protein functionality in terms of the structure-function continuum model, where the remarkable multifunctionality and binding promiscuity is derived from the structural heterogeneity and conformational plasticity of proteins[167–169].

Until quite recently, this protein intrinsic disorder phenomenon was mostly ignored by the scientific community, as protein functionality was traditionally considered within the classic lock-and-key model, where a unique protein function is defined by a unique protein structure encoded by a unique amino acid sequence. The situation was changed at the turn of the century[170–173], when it was recognized that the intrinsically disordered proteins (IDPs) and proteins with intrinsically disordered regions (IDRs) represent a new important (and highly populated) realm within the protein universe, and that protein functionality is critically dependent on both order and disorder[167–169].

Therefore, consideration of ferritins from this angle can provide some interesting clues pertaining to the multifunctionality of these proteins.

To gain some insights into this exciting possibility, we looked at the intrinsic disorder status of several ferritins originating from a bacterium, a plant, and a human using a set of commonly used disorder predictors. This analysis included a bacterial ferritin from *Mycobacterium tuberculosis* (BFRB_MYCTU, UniProt ID: P9WNE5, 181 residue-long), four ferritins from the *Arabidopsis* chloroplasts FRI1_ARATH, FRI2_ARATH, FRI3_ARATH, and FRI4_ARATH (UniProt IDs: Q39101 (255 residues), Q9SRL5 (253 residues), Q9LYN2 (259 residues), and Q9S756 (259 residues), respectively), and three human ferritins, ferritin light chain (FTL, UniProt ID: P02792, 175 residue-long), ferritin heavy chain (FTH1, UniProt ID: P02794, 183 residue-long), and ferritin mitochondrial (FTMT, UniProt ID: Q8N4E7, 242 residue-long), and its results are shown in Figure 9, which represents aligned disorder profiles generated for ferritins by PONDR® VSL2, which is a commonly used predictor evaluating intrinsic disorder predisposition of target proteins based solely on their amino acid sequences. Note that this analysis was conducted for the precursor proteins, which in case of ferritins from the *Arabidopsis* chloroplasts and human mitochondrial ferritin contain N-terminally located transit peptide, which ranges in length from 45 to 57 residues.

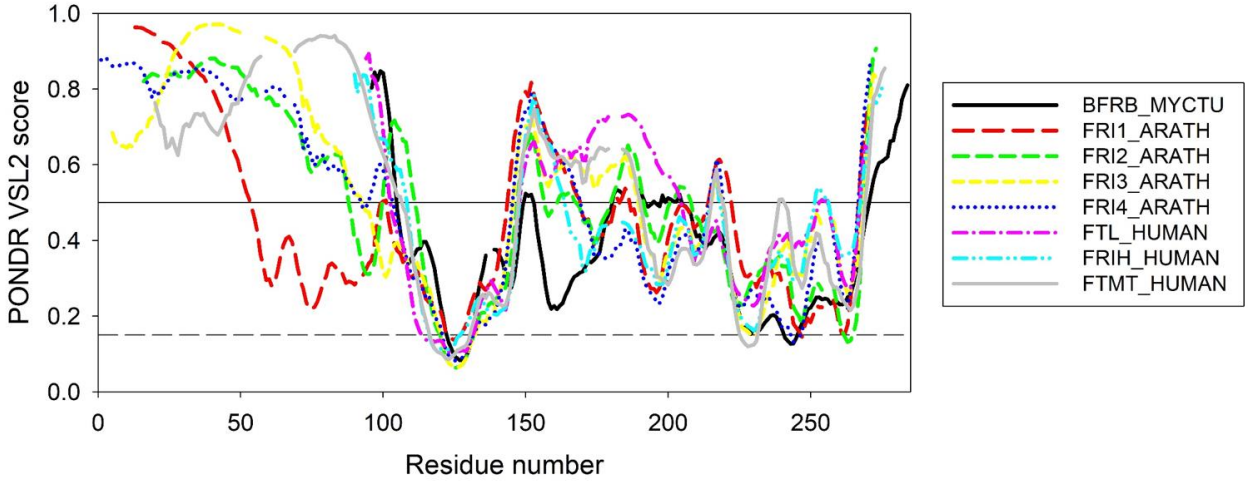
This analysis revealed that all ferritins are expected to carry intrinsically disordered regions. Surprisingly, despite the fact that the analyzed proteins, in general, are characterized by low sequence identity (see bottom part of Figure 9), significant parts of their disorder profiles showed noticeable similarity (especially in their C-terminal regions). Based on the increasing levels of their intrinsic disorder content (percent of predicted intrinsically disordered residues, PPIDR), ferritins can be arranged in the following order:

BFRB_MYCTU (22.1%) < FTL (27.3%) < FTH1 (28.4%) < FRI1_ARATH (33.3%) < FRI2_ARATH (48.2%) < FRI4_ARATH (48.7%) < FRI3_ARATH (51.7%) < FTMT (52.9%).

Based on the criteria commonly used for the classification of proteins as highly ordered, moderately disordered, and highly disordered if their PPIDR<10%, 10%≤PPIDR<30%, and PPIDR≥30%, respectively[174], “short” ferritins belong to the group of moderately disordered proteins, whereas all “long” ferritins are highly disordered proteins.

It is clear that “long” ferritins (four ferritins from the *Arabidopsis* chloroplasts and a human mitochondrial ferritin) differ from “short” ferritins (bacterial ferritin from *Mycobacterium tuberculosis* and human light and heavy chain ferritins) by the presence of mostly disordered N-terminal tails (Figure 9). This is further illustrated by Figure 10

representing 3D structures modeled for these eight ferritins by AlphaFold, which is a novel deep learning algorithm that generate highly accurate predictions of protein structures based on the incorporation of the physical and biological knowledge about protein structure[175,176]. In agreement with their disorder profiles, “short” ferritins and C-terminal regions of “long” ferritins show remarkable structural similarity, whereas N-terminal tails of “long” ferritins are highly disordered.



Percent Identity Matrix created by CLUSTAL 2.1

		1	2	3	4	5	6	7	8
1:	sp|P9WNE5|BFRB_MYCTU	100.00	22.56	22.75	27.54	25.45	16.97	22.49	21.89
2:	sp|Q39101|FRI1_ARATH	22.56	100.00	68.33	63.11	66.94	40.00	49.43	38.46
3:	sp|Q9SRL5|FRI2_ARATH	22.75	68.33	100.00	68.70	70.25	38.37	46.89	36.61
4:	sp|Q9LYN2|FRI3_ARATH	27.54	63.11	68.70	100.00	78.74	42.44	48.02	39.56
5:	sp|Q9S756|FRI4_ARATH	25.45	66.94	70.25	78.74	100.00	40.35	50.86	38.74
6:	sp|P02792|FRIL_HUMAN	16.97	40.00	38.37	42.44	40.35	100.00	55.43	53.71
7:	sp|P02794|FRIH_HUMAN	22.49	49.43	46.89	48.02	50.86	55.43	100.00	79.23
8:	sp|Q8N4E7|FTMT_HUMAN	21.89	38.46	36.61	39.56	38.74	53.71	79.23	100.00

Figure 9. Intrinsic disorder predisposition of illustrative examples of ferritins from bacteria, plants, and animals. Disorder profiles are generated by PONDR® VSL2[279], which, based on the results of recent Critical Assessment of protein Intrinsic Disorder prediction (CAID) experiment[280], was recognized as one of the most accurate disorder predictors. Breaks in the curves correspond to the gaps in the multiple sequence alignments. A threshold of 0.5 was used to identify disordered residues and regions in query proteins, whereas residues/regions with disorder scores ranging from 0.15 to 0.5 are considered rigid.

It is expected that intrinsic disorder can play a role in the functionality of ferritins. In fact, based on their PONDR® VSL2-based predicted disorder scores (PDS), residues of human FTMT involved in binding of iron ions are expected to be either disordered (Glu122 (PDS=0.716) and His125 (PDS=0.687)) or flexible Glu167 (PDS=0.297) and Gln (PDS=0.321)), with only Glu87 (PDS=0.132) being located within the ordered region. Similarly, five residues responsible for the coordination of iron ions (i.e., located within the target distance + 0.75 Å from the metal ions) are located within the disordered

(Ser119, PDS=0.624; Glu122, PDS=0.716; Thr123, PDS=0.747; and Leu205, PDS=0.508) or flexible regions (Gln201, PDS=0.321), and the remaining three iron coordinating residues (Leu88, PDS=0.115; Phe192, PDS=0.136; and Thr195, PDS=0.122) are located within short ordered segments flanked by disordered or flexible regions.

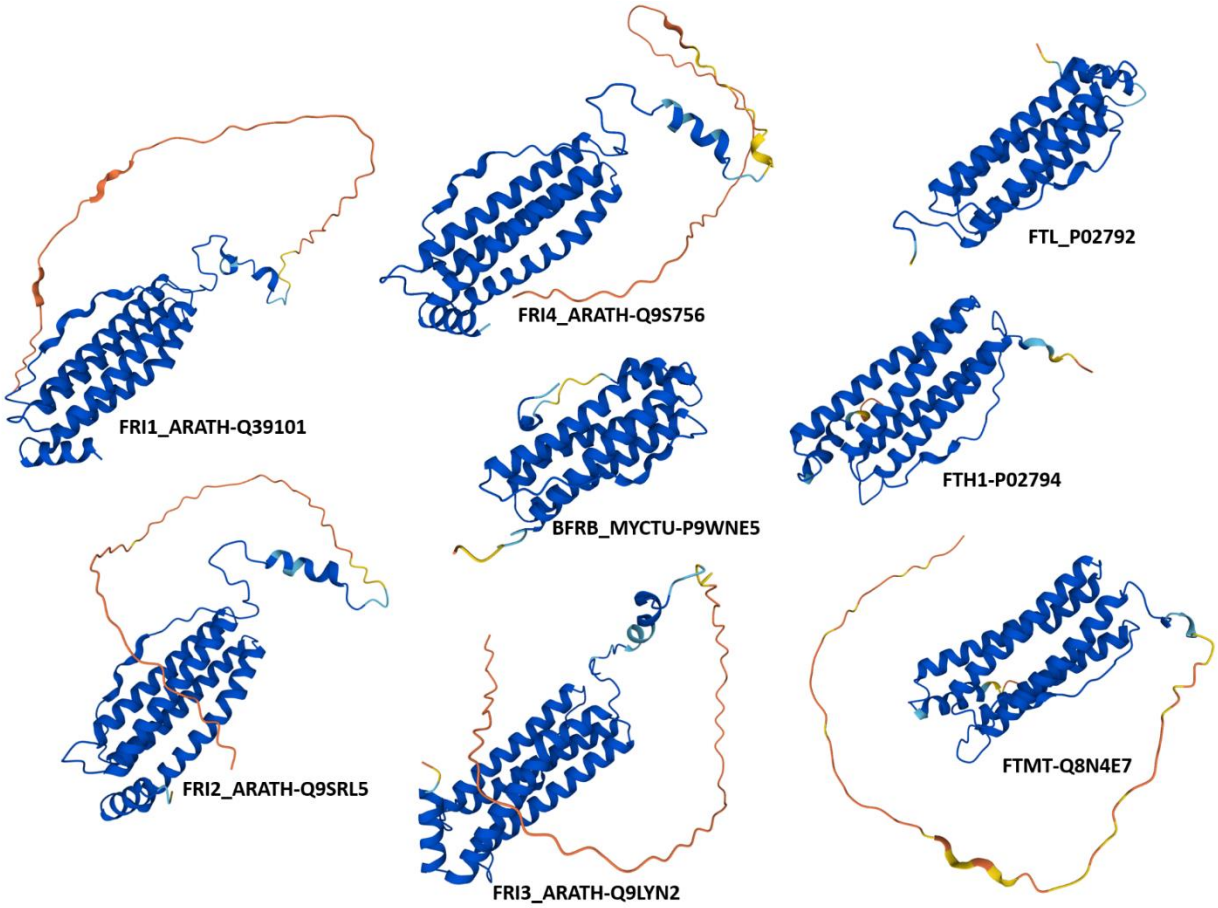


Figure 10. 3D structure models generated for ferritins by AlphaFold, which produces a per-residue confidence score (pLDDT) between 0 and 100 that can be used for the evaluation of model confidence. Presented structures are colored based on the model confidence, where blue and cyan colors correspond to very high (pLDDT > 90) and confident (90 > pLDDT > 70) predictions, respectively, whereas yellow and orange segments correspond to structural elements predicted with low (70 > pLDDT > 50) and very low confidence (pLDDT < 50). It is expected that regions with low pLDDT may be unstructured in isolation.

Furthermore, intrinsically disordered N-terminal arms of “long” ferritins contain translocation signals; i.e., mitochondrion transit peptide (residues 1-49) in human FTMT or chloroplast transit peptides in *Arabidopsis* FRI1 (residues 1-48), FRI2 (residues 1-45), FRI3 (residues 1-49), and FRI4 (residues 1-57), respectively. Furthermore, even without transit peptides, “long” ferritins are noticeably longer than their “short” counterparts (e.g., FRI1 (207 residues), FRI2 (208 residues), FRI3 (210 residues), FRI4

(202 residues) and FTMT (193 residues)), with the “extra” residues being preferentially concentrated within the disordered N-terminal extensions. Often, disordered regions are utilized by proteins for formation of homooligomers and for interaction with binding partners. In line with these observations for other proteins, STRING-based (Search Tool for the Retrieval of Interacting Genes[177–179]) analysis of eight ferritins revealed that all of them are promiscuous binders. In fact, these proteins are located within the dense protein-protein interaction networks that include 52, 174, 118, 133, 143, 90, 116, and 58 partners for BFRB_MYCTU, FRI1_ARATH, FRI2_ARATH, FRI3_ARATH, FRI4_ARATH, FTL_HUMAN, FTH_HUMAN, and FTMT_HUMAN, respectively (note, in this analysis, the medium confidence of 0.4 was used for minimum required interaction score).

To further illustrate this high level of intractability, Figure 11 shows STRING-generated disorder profiles for the least (BFRB_MYCTU) and most connected ferritins (FRI1_ARATH). In the PPI network centered at the Bacterioferritin BfrB from *Mycobacterium tuberculosis* (strain ATCC 25618 /H37Rv), there are 52 proteins involved in 242 interactions. Therefore, on average, each protein in this network interacts with at least 9 partners (note that BfrB located at the center of this network interacts with at least 51 partners) (see Figure 11A). PPI enrichment p-value of the resulting network is <1.0e-16, indicating that proteins involved in this network have more interactions among themselves than what would be expected for a random set of proteins of the same size and degree distribution drawn from the genome. The five most enriched biological processes ascribed to proteins from this network (in terms of Gene Ontology, GO) include Removal of superoxide radicals (GO:0019430), Intracellular sequestering of iron ion (GO:0006880), Reactive oxygen species metabolic process (GO:0072593), Cellular response to superoxide (GO:0071451), and Chaperone-mediated protein folding (GO:0061077).

The five most enriched molecular functions of these 52 proteins are Ferroxidase activity (GO:0004322), Protein folding chaperone (GO:0044183), Antioxidant activity (GO:0016209), Unfolded protein binding (GO:0051082), and Oxidoreductase activity, acting on a sulfur group of donors (GO:0016667). The five most enriched cellular components, where these 52 proteins can be found include Capsule (GO:0042603), Host cell surface (GO:0044228), Host cell endoplasmic reticulum (GO:0044165), Host intracellular membrane-bounded organelle (GO:0033648), and Host cell cytoplasm part (GO:0033655). Figure 11B shows densely connected PPI network generated for chloroplastic Ferritin-1 from *Arabidopsis thaliana* (AtFer1), where 174 proteins are engaged in 2,476 interactions.

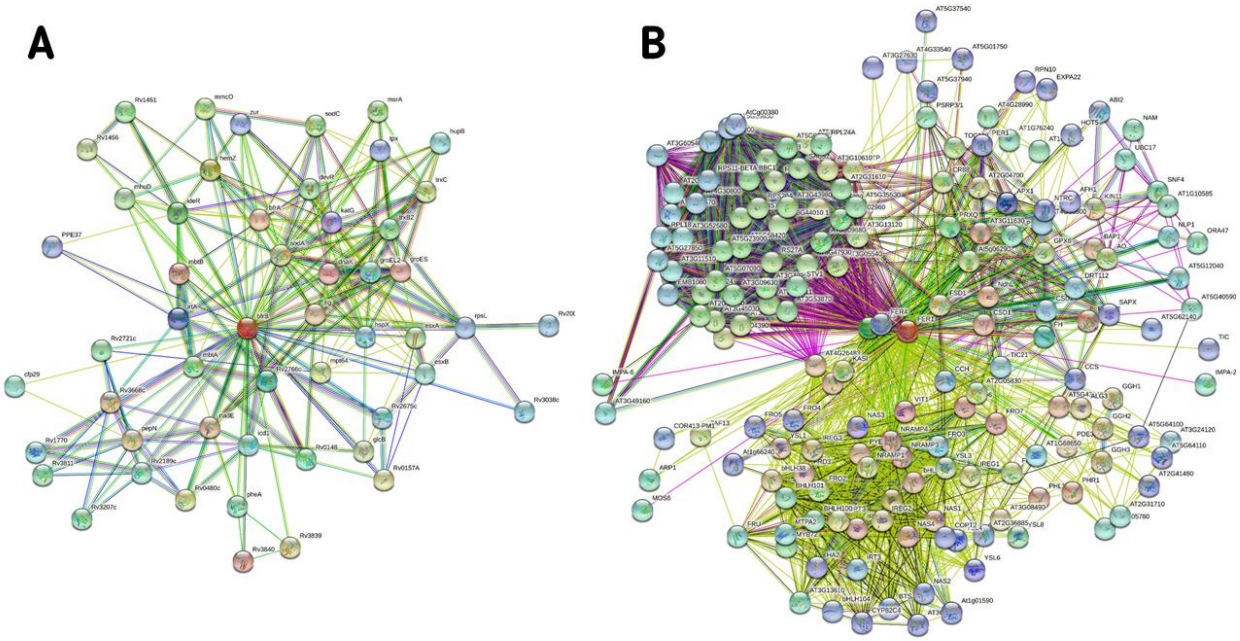


Figure 11. Protein-protein interaction networks generated for **A** BFRB_MYCTU and **B** FRI1_ARATH. These networks represent proteins and their predicted or known functional associations and use seven types of evidence, which are indicated by the differently colored lines: a green line represents neighborhood evidence; a red line – the presence of fusion evidence; a purple line – experimental evidence; a blue line – co-occurrence evidence; a light blue line – database evidence; a yellow line – text mining evidence; and a black line – co-expression evidence[178].

On average, each protein in this PPI interacts with almost 29 partners (AtFer1 interacts with 173 partners), and PPI enrichment p-value of this network is <1.0e-16. The most enriched biological processes, in which proteins from this network take part, include Nicotianamine biosynthetic process (GO:0030418), Positive regulation of translational fidelity (GO:0045903), Nickel cation transport (GO:0015675), Cobalt ion transport (GO:0006824), and Cellular response to iron ion starvation (GO:0010106); their most common molecular functions are Ferric-chelate reductase activity (GO:0000293), Nicotianamine synthase activity (GO:0030410), Gamma-glutamyl-peptidase activity (GO:0034722), Iron-nicotianamine transmembrane transporter activity (GO:0051980), and mRNA 5-UTR binding GO:0048027; and five most enriched cellular components include Cytosolic small ribosomal subunit (GO:0022627), Small ribosomal subunit (GO:0015935), Ribosomal subunit (GO:0044391), Polysomal ribosome (GO:0042788), and Cytosolic ribosome (GO:0022626). Therefore, these functional analyses supported an important idea that ferritins can be engaged in a very broad spectrum of functional processes either directly or via their numerous binding partner

3. Applications

Ferritin and ferritin-like family proteins are widely used in structural studies and nanotechnology. Here, we mention several of them (Figure 12); more applications can be found in different reviews[180–183].

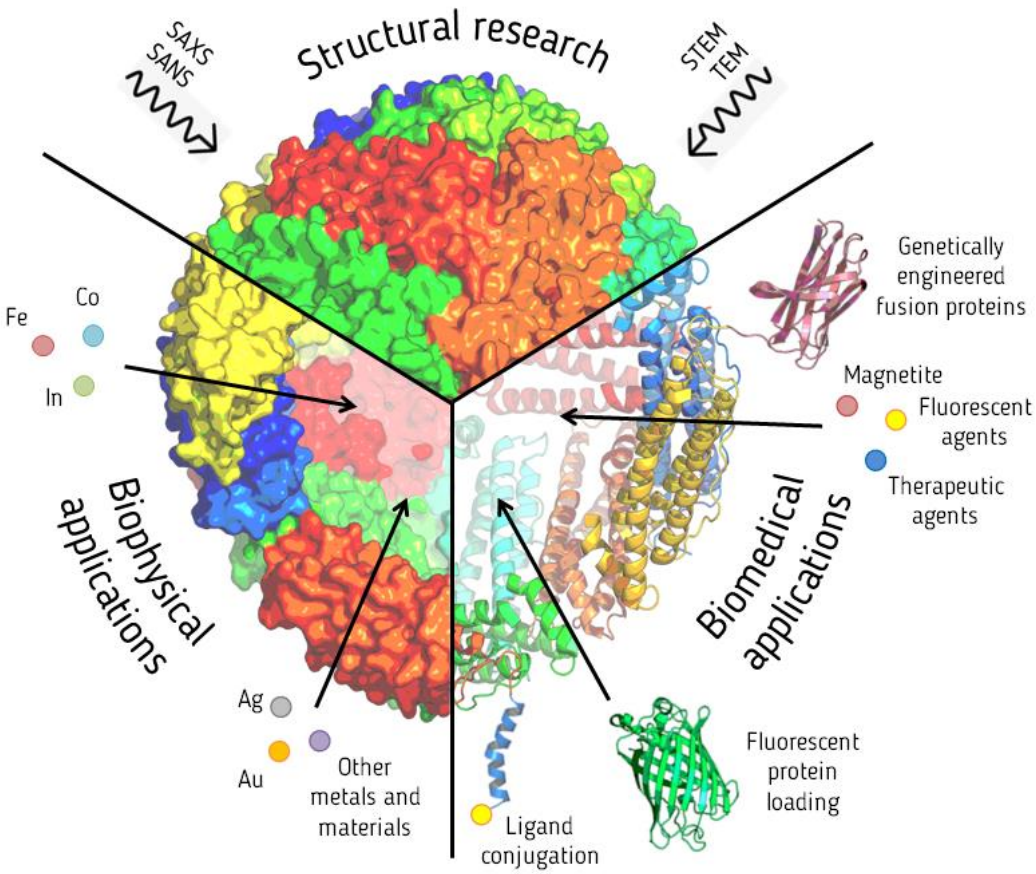


Figure 12. An overview of applications of ferritin. Structural studies: ferritin as a good model object for SAS and electron microscopy. Biophysical applications: ferritin is used in synthesis of nanoparticles from different metals and materials for magnetic resonance imaging, electron cryotomography, etc. Biomedical applications: ferritin is used as a drug carrier, contrasting agent both *in vitro* and *in vivo* and for vaccine production.

3.1. (Apo)Ferritin as a model for structural studies

Since 1991, many structural studies of ferritins have been carried out, and today the PDB database contains more than 1,500 ferritin structures. Most of them were obtained by X-ray diffraction (XRD) method. The structure of ferritin with the highest resolution (1.06 A) was obtained by Zhang et al. in 2018 by XRD[30]. In recent years the number of structures obtained by electron microscopy is growing.

Table 1. List of ferritin structures with maximal resolution

Source	PDB code	Resolution, Å	Method	Year
Ferritin				
Homo sapiens	1FHA	2.40	XRD	1991
Homo sapiens	6B8F	1.06	XRD	2019
Equus caballus	6JEE, 2V2P, 6HJT	1.3, 1.33, 1.15	XRD	2019, 2018, 2012
Escherichia coli	4CVR	1.1	XRD	2014
Mus musculus	1LB3 (L-chain) 7A4M (H-chain)	1.21 1.22	XRD CryoEM	2003 2020
Lithobates catesbeianus	4LQH (native M-ferritin)	1.16	XRD	2013
Helicobacter pilory	3BVF	1.5	XRD	2009
Archaeoglobus fulgidus	1S3Q	2.10	XRD	2005
Pyrococcus furiosus	2JD6	2.75	XRD	2007
Glycine max	3A68	1.8	XRD	2010
Encapsulin				
Thermotoga maritima	7KQ5	2.00	CryoEM	2021
DPS				
Pyrococcus furiosus	7STW	2.37	CryoEM	2021
Staphylococcus aureus	2D5K	1.85	XRD	2014

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In plants, ferritins are found in plastids and have a conservative structure[1,2] (see section “Diversity of ferritins and ferritin-like proteins”). Few plant ferritin structures have been described in literature. One of them is the structure of ferritin from *Glycine max* (soy) with a resolution of 1.80 A, described in paper[184]. A feature of ferritin from this organism is an additional domain at the N-terminus, consisting of about 30 amino acids, which provides surprisingly high thermal stability of the complex (106°C)[185], while removal of this domain decreases thermostability down to 88°C (Figure 13A). Addition of this domain to human ferritin significantly increases its thermal stability (from 82 to 103°C).

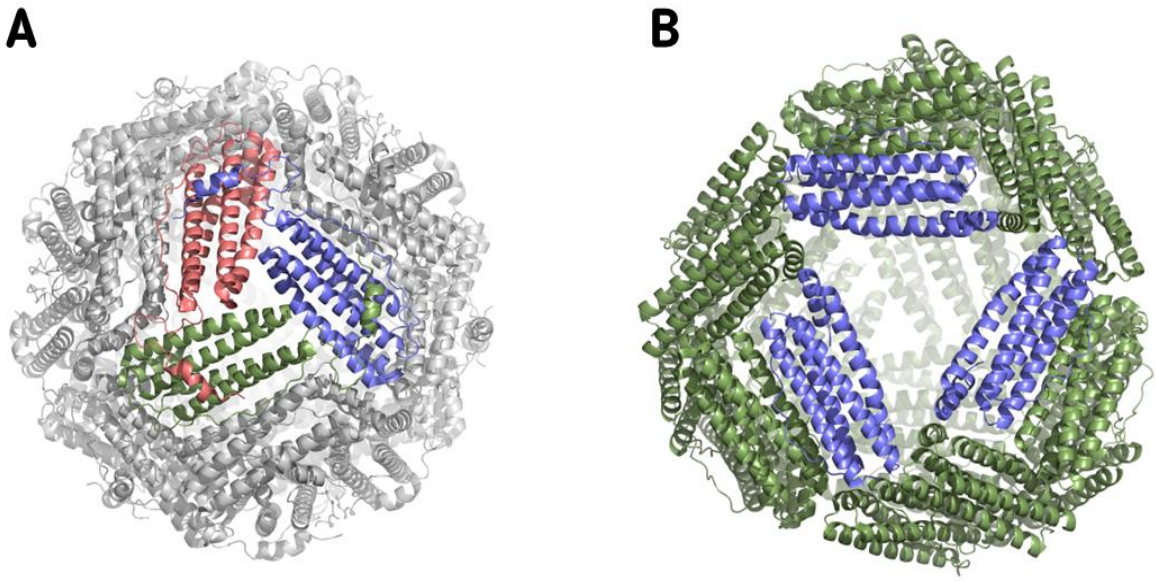


Figure 13. High-resolution structures of ferritins with unusual structural features. **A** Ferritin from *Glycine max* (PDB ID: 3A68[184]). The E-helix of one subunit covers the neighboring subunit, causing extrathermophility of the complex. **B** Ferritin from *Archaeoglobus fulgidus* (PDB ID: 1S3Q[186]) has four large triangular pores.

Ferritins from organisms of the Archaea superkingdom have been studied much less than those from eukaryotes and prokaryotes, but even the existing structures are sufficient to underscore the diversity of structures within the superkingdom. Ferritin from the hyperthermophilic archaeon *Archaeoglobus fulgidus* has a unique structure (Figure 13B) (PDB ID: 1S3Q)[186]. Unlike most ferritins, whose 24 subunits form 4-fold channels and have 432-symmetry, AfFtn has atypical tetrameric structure with four large triangular pores. Iron ions, however, do not pass through these pores due to the positive charge located at the vertices of the triangle[186,187]. Such a tetrahedral structure is not usual for archaeal ferritins. For example, ferritin from *Pyrococcus furiosus* (PDB ID: 2JD6) forms a standard globule with 432-symmetry[188].

Ferritin is a convenient object for structural studies[29]. The shape of the ferritin and apoferritin can be easily modeled by filled sphere or spherical shell, which facilitates the work by small-angle X-ray or neutron scattering (SAXS/SANS) methods[27,189–194]. Despite the availability of the high-resolution apoferritin structure, SAXS studies of ferritin can be used for different tasks described below[195].

SAXS/SANS methods can be used to check the final assembly of ferritins or chimeric recombinant protein complexes based on ferritin, such as recombinant vaccines consisting of chimeras of ferritin and antigen, as well as nanoparticles for drug delivery[196–198]. In addition, SAS studies of large protein complexes with a known structure of their separate domains can shed light on the structure of the whole protein complex[199–201].

For the same reasons, (apo)ferritin can be used to check limitations of SANS and SAXS methods. For example, the series of papers[27,190,191,193,202–204] shows how to obtain maximum amount of structural information using apoferritin as a model object. In the work[204] it was shown how to obtain high-quality SAXS data using size-exclusion chromatography (SEC) and dynamic light scattering, ferritin was used as one of the model objects. In one study[27] the authors increase signal to noise ratio together with elimination of a signal from higher oligomeric species (which might be present in the sample) by combining of SEC-SAXS and Extrapolation to infinite dilution (EID) methods. In another work[203] apoferritin was used as a model object for novel SEC-SAXS laboratory setup. Murugova et al.[191] compared structural parameters of ferritin obtained with the help of synchrotron beamline[205] and SAXS instrument. These works demonstrate the application of apoferritin for testing and optimization of SAXS instruments, which is widely applicable in structural biology[197,206,207].

Potentially, apoferritin can be applied to calibration of SAS instruments in addition to standard calibration procedures[208–210]. Apoferritin SAS curve I(q) contains a number of specific maxima. They can be used for validation of primary data treatment algorithms, for example, for two-detector systems[190,211]. Self-consistency of small- and wide-angle X-ray scattering data can be checked by apoferritin I(q) maxima (e.g., SASBDB[212] ID: SASDA82, SASDCD8).

Having a large number of iron atoms inside the ferritin, for example, can provide a good contrast in STEM and TEM methods. It can also be used for studying initial stages of crystal formation using tomography[28].

In addition, due to the fact that ferritin can be crystallized relatively easily, it might be used to develop new crystallization methods. One study[30] describes the preparation of a crystal-hydrogel hybrid, which can be reversibly expanded to 180% of the initial volume, maintaining a periodic structure.

In general, the wide biological diversity and high conservatism of ferritins emphasize their importance in the basic mechanisms of cell life. It would be interesting to obtain intermediates of ferritin (a monomeric subunit or different oligomers smaller than the whole globule) and clarify the question whether different ferritins undergo different mechanisms of self-assembly.

3.2. Nanotechnological applications

The ability to form a stable quaternary structure makes ferritin a unique tool in nanotechnology. Currently ferritin-like superfamily proteins are actively used to create nanoparticles and nanodevices.

Ferritin can absorb not only iron, but also other metals and compounds. For example, during the formation of a ferrihydrite core, phosphate can also be adsorbed from the solution[213]. Based on this property, it is possible to purify water from the phosphates using ferritin[214]. This approach is more accessible than other chemical and biological methods, so there is a wide range of possible applications[71].

There are many methods of nanoparticle synthesis[215]. The main disadvantage of such methods is size heterogeneity, but there are a number of methods for obtaining homogeneous nanoparticles[216]. Metal uptake in the ferritin cavity solves this problem because the limited size of the cavity does not allow further growth of nanoparticles. For obtaining nanoparticles with a desired size different ferritins might be used[217] (Figure 8).

In nature, ferritin forms a core of ferrihydrite[59]. It is also possible to artificially synthesize nanoparticles from different compounds (Mn, Co, Ni, Cr, Pd, Ag, In, etc.), semiconductors (CdS, CdSe, ZnSe), and other materials[217]. The synthesis of nanoparticles from divalent cations does not need manipulations with the protein[71]. However, additional changes are required for monovalent and trivalent cations. For instance, Cys residues were relocated for the synthesis of gold nanoparticles[218]. Recently, a complex two-step reduction strategy for gold ions was developed for native ferritin from horse spleen[218].

There are several methods to eliminate a protein shell after nanoparticle synthesis, for example, by heating and UV treatment[219]. The obtained nanoparticles can be employed as memory gates, contrast agents for electron cryotomography, magnetic resonance imaging (MRI)[71], two-dimensional crystals[217], catalysts for carbon nanotube growth, metal induced lateral crystallization, charge storage nodes of floating gate memory, nanometer-scale etching masks[219], and many other tools.

3.3. Biomedical applications

Nanoparticles from metal compounds are widely used in biotechnology, e.g. in diagnostics and treatment of diseases including cancer[220]. Recently different ferritins became a focus for “green” technologies of nanoparticle synthesis. However, size limitations should be taken into account depending on application[221]; therefore, a required protein from the whole ferritin-like superfamily should be chosen carefully.

As previously stated, ferritins are widely used in metal nanoparticles synthesis. Such nanoparticles are used as theranostic agents for cancer treatment[222], possess antimicrobial properties (silver nanoparticles)[223], and applied as biomedical tools in many other cases[224]. Magnetic nanoparticles are also used to control their biodistribution *in vivo* by MRI methods[225].

Recently, an interest to protein nanoparticles increased because i) they are biodegradable and biocompatible, ii) they have prolonged circulation time for passive targeting, iii) antibodies and ligands can be easily conjugated using biochemical methods for active targeting, and iv) proteins are amphiphilic that makes them more flexible in interactions with conjugated objects and environment[226]. Nanoparticle synthesis methods (thermal denaturation, coacervation/desolvation, etc.) have some disadvantages such as big nanoparticle size and size diversity[227,228]. Ferritin-assisted synthesis avoids it due to the fixed size of the protein.

Ferritin-like proteins are excellent platforms for multi-modal nanoparticles development[181] (Figure 12). They combine properties from different materials and perform several functions simultaneously. For instance, ferritin can be loaded by magnetite or fluorescent dyes[182] and be detected by magnetic resonance or fluorescence imaging. These methods are used to control the nanoparticle distribution *in vivo* during active targeting research[181].

Multi-modal nanoparticles for disease treatment require extra manipulations with protein and its surface. The cheapest and most useful methods of nanoparticle synthesis require minimal external interference in the process. Thus, ferritin self-assembly may be a very helpful tool for convenient production of protein nanoparticles.

Loading drugs into ferritin is carried out in three ways, mainly passive loading, pH-dependent disassembly-reassembly, and disassembly-reassembly using chaotropic agents[229]. The first method is the most intuitive but not optimal, because only small molecules can be easily encapsulated. Disassembly-reassembly can solve this problem, but the main disadvantage is probable irreversible damage and aggregation of nanoparticles. Using stabilizing agents such as glycerol-acetate and tris-base buffer helps recover nanoparticles. Understanding the interaction ways and self-assembly mechanisms can help reduce corresponding aggregation and precipitation.

Functionalization of nanoparticle surfaces provides the possibility of active targeting of nanoparticles. It can be achieved with chemical modifications by small molecules[180], chemical conjugation of ligands to nanoparticle's surface[230], and genetically engineered fusion proteins connecting ferritin and monomers[229]. Currently, there are many studies on ferritin fusion with different peptides. However, chimeric fusion affects the properties of self-assembly and stability of protein globule, limiting the size of the attached peptide. GFP and RFP were successfully used as bound proteins[231,232]. This approach was also successfully used to produce chimeric vaccines[47] (see section "Vaccines"). This method is the most challenging because it can become a universal platform for drug delivery and vaccine production[229]. However, currently the usage of ferritin in biomedical applications (especially chimeric fusion) is restricted by non-complete understanding of the self-assembly process.

Ferritins are good carriers of different small-molecules; therefore, the processes of self-assembly are key for successful development of delivery systems. Although there is no information about protein self-assembly control during the preparation of such carriers in literature, the understanding of these mechanisms may bring a breakthrough in drug-delivery industry.

3.4. Vaccines

As was previously stated, another important biotechnological application of ferritin is vaccine production. Due to the oligomerization of this protein, it is possible to provide multimeric antigen presentation. In addition, ferritin has 3-fold channels, which allow the formation of a trimeric antigen on the surface of the protein globule[233].

The first example of a ferritin-based vaccine was designed in 2013 (Figure 14) against influenza viruses[47]. The hemagglutinin was fused to the H-ferritin subunit and formed a triple domain structure. The authors claimed self-assembly of the nanoparticles from a chimeric protein HA-ferritin (Figure 14A). The HA-ferritin nanoparticles (Figure 14B), are a molecule of ferritin with trimers of HA protein just in front of the ferritin 3-fold channels. The HA trimers are the same as the viral spikes. Immunization with this vaccine caused 10x antibody response than a clinical inactivated vaccine. This article opened the door to a new type of powerful vaccine.

More ferritin-based vaccines were developed later, including influenza vaccine[47], HIV vaccine[234],vaccine against Epstein-Barr virus[235], and HCV[236] (Figure 15). In 2014, a promising improvement of nanoparticle vaccine technology based on SpyTag/SpyCatcher[237] was investigated[238]. Four years later, the same group presented application of this technology to ferritin for tumor vaccine development[239].

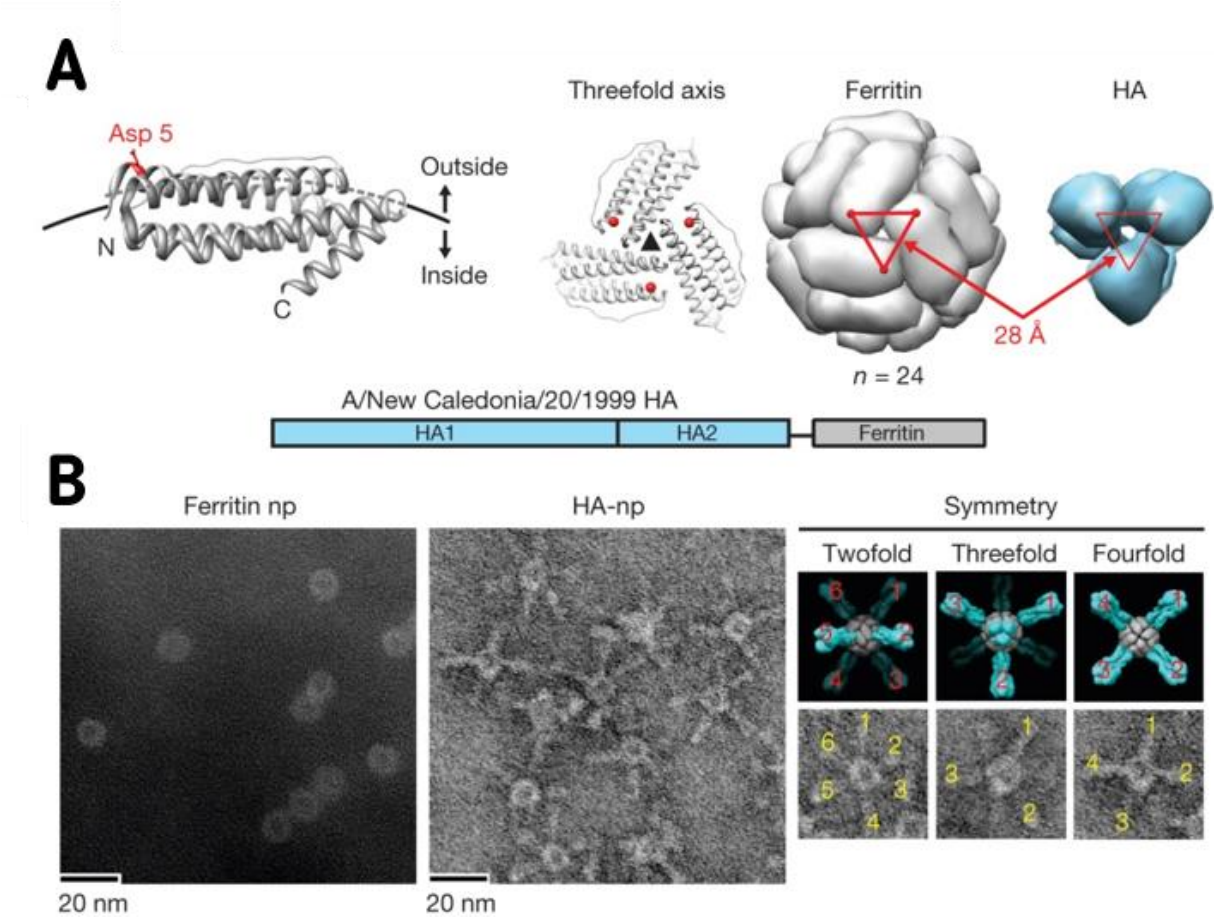


Figure 14. The first ferritin-based vaccine against influenza. **A** Graphical description of chimeric protein complex HA-ferritin. **B** The HA-ferritin nanoparticles. Reprinted from the paper[47].

Ferritin as a base for fused recombinant vaccines allows a bivalent antigen representation[240]. A possibility to make a bivalent virus protein exposition on one nanoparticle is based on the fact that some ferritins have two types of monomers: heavy and light chains. After deletion of a few amino acids on N-termini, this method allows a bivalent representation of trimeric antigen.

The first clinical study of ferritin vaccines was started in 2017[241] and was finished two years later (Figure 16). This was a phase I study directed to explore dose, safety, tolerability, and immunogenicity. No severe side effects of vaccination were observed. Now some other phase I clinical studies of vaccines, based on this technology, are in progress[241–245].

The nCov-2019 pandemic gave a significant boost in this area. A series of papers[246–248] with new investigations against COVID were published since the start of the virus spreading around the globe. S-proteins of the coronavirus SARS-CoV-2 form trimers (as well as influenza hemagglutinins) and could assemble in similar nanoparticles. In such nanoparticles, there is no overlapping of the S-protein trimers

with each other. Vaccines based on the ferritin from *H. pylori* and the trimer of S-protein of the SARS-CoV-2[249] or its RBD domain[250] were developed. At this moment, one of these vaccines[248] was tested on non-human primates[251]. After two doses plus adjuvant, a strong B- and T-cell response was observed.

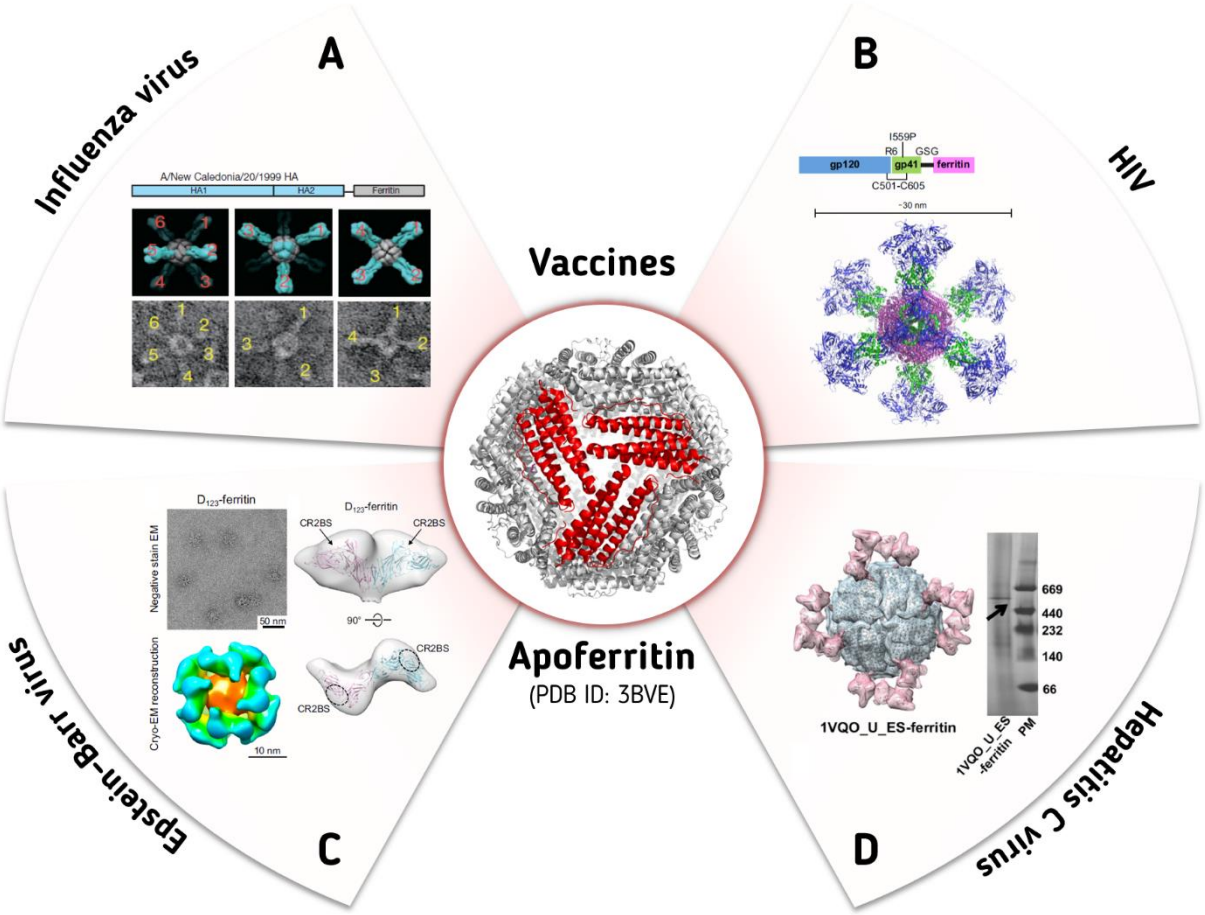


Figure 15. Ferritin-based recombinant vaccines. **A** Influenza vaccine[47], **B** Vaccine against HIV[234], **C** Vaccine against Epstein-Barr virus[235], **D** Vaccine against HCV[236]. Adapted from corresponding refs.

This technology could also be a good platform for development of antitumor vaccines[232,239,252]. Tumor antigens exposed to human ferritin heavy chain target lymph nodes and cause CD8+ cell response stronger than the same antigens shown on other protein nanoparticles[232].

Future tasks in this direction include the following: second phase clinical trials, investigations of new variants of multimeric form in one nanoparticle, development of a

simple method for post-translational modifications of viral antigens, and deep understanding of the self-assembly process and biomineralization mechanism.

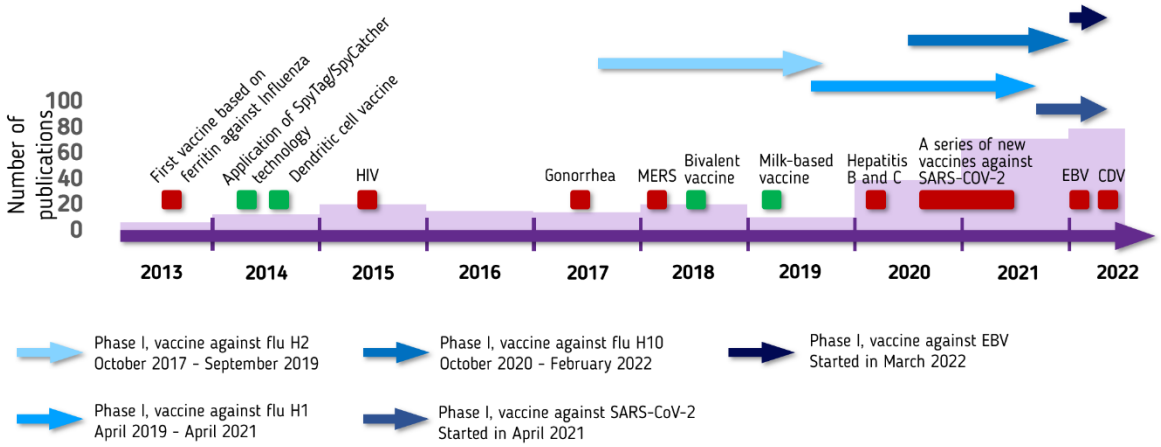


Figure 16. Development of ferritin-based vaccines. A timeline of successful cases of ferritin-based vaccines is shown: blue arrows indicate phase I clinical trials, red squares represent the publications about new vaccines, green squares – applications of new techniques into vaccine development. Purple histogram in the background show publication activity (Pubmed search "ferritin vaccine"). There are following cases: the first ferritin-based vaccine[47], application of SpyTag/SpyCatcher[238], dendritic cell vaccine[252], HIV vaccine[234], Gonorrhea[281], MERS[282], bivalent vaccine[240], milk-based vaccine[283], hepatitis B[284] and C[285], a series of vaccines against SARS-COV-2[246–249,286,287], EVB[288], and CDV[289]. Clinical trials: against flu H2[242], against flu H1[290], against flu H10[243], against SARS-CoV-2[244], and against EBV[245].

4. Self-assembly

A unique property of ferritins is spontaneous formation of a globular structure from individual subunits through protein-protein interactions. Individual ferritin subunits are not found in cells; rather, they are combined into a globular structure of 24 subunits (12 in case of DPS). Although the self-assembly intermediates may differ in different organisms, the result of self-assembly is always the same: a 24-meric complex is formed.

The 24-meric protein globule is very stable and undergoes disassembly only under harsh conditions[253]. For example, a point-mutated ferritin from *Crassostrea gigas* (oyster) is stable at 90°C[254] and ferritin from *Listeria innocua* is stable below pH 2[255] (see section "(Apo)Ferritin as a model for structural studies"). Recently, it was shown that the self-assembly process depends on pH and ionic strength of the solution[256]. Self-assembly is accelerated with an increase of ionic strength and with a decrease of pH (from 7.5 to 5.5). pH-dependent disassembly of the protein shell is widely used in different practical applications (see section "Applications")[229].

In general, self-assembly is an important feature, which plays a crucial role in protein functionality. Despite their importance, the molecular mechanisms of self-

assembly are still unclear. Nevertheless, there is some information about which regions are responsible for the formation of interparticle interaction interfaces, as well as about the effect of mutations on self-assembly and the sequence of intermediates during self-assembly.

4.1. Various self-assembly pathways

Most studies observe a dimer of ferritin subunits as an essential state towards protein assembly[21,257]. Dimers were also mentioned as stable intermediates by Gerl et al.[258]. The formation of dimeric complexes is quite stable for some ferritins[259,260] (for example, they are even found as an separate state in a protein solution of *E. coli* BFR[261,262]), supporting the consideration that the dimer is a necessary intermediate in self-assembly process. Carmona et al. in 2017 studied the formation of human ferritin dimers by FRET[136]. They found that formation of heterodimer (H-L) is prevalent over the formation of homopolymer (H-H)[136]. Despite these facts, the role of dimers as a starting state in ferritin self-assembly is still questioned, considering the possibility of monomers participation in different stages of this process[256,258]. Despite the stability of dimers, some studies[256,258] show that folded monomers could be observed as a separate state: the rate of dimer appearance is not fast enough to prevent the presence of free monomers in solution. This supports the idea that self-assembly is possible through odd-numbered intermediates (e.g., trimers).

Different researchers noted contradictory statements about the kinetics of the process (Table 2, Figure 17). The first (and more or less conventional) scheme was proposed by Gerl and Jaenicke in 1987[258] using horse spleen ferritin. The authors carried out reversible ferritin dissociation by 2,3-dimethylmaleic anhydride (DMMA) and studied the self-assembly process by cross-linking and circular dichroism experiments. The authors suggested the formation of 24-subunit ferritin complexes via intermediates composed from 1 - 2 - 3 - 6 - 12 - 24 subunits (Figure 17, arrow I). The authors underlined that “folded monomers” could be observed separately, demonstrating that folded monomers were very rapidly combined into stable dimers. Later, the same group proposed structurally-based representation of the assembly mechanism of apoferritin[263]. It takes into account already obtained structural data[21]. It should be noted that there are two possible types of hexamers, not only one that was proposed in the paper[263] (Figure 17, arrows I and IV, M_6’ and M_6’’, respectively).

Table 2. Comparison of ferritin self-assembly studies. The column "Self-assembly pathway" shows the number of ferritin subunits comprising intermediate oligomers and the path of reassembly of ferritin globule. For every scheme the corresponding arrow in Figure 17 is shown.

Research	Method for studying of self-assembly dynamics	Starting material	Host	Disassembly	Time scale evaluated?	Self-assembly pathway
Gerl et al. 1987, 1988[258,263]	Cross-linking, SDS-page	Folded monomers	*Equus caballus* (Horse)	Addition of DMMA in 60:1 molar ratio to protein	N	1 - 2 - 3 - 6 - 12 – 24, (Figure 17, arrow I)
Stefanini et al. 1987[257]	Cross-linking, analytical ultracentrifugation	Apoferritin subunits (probably partially unfolded monomers/dimers)	*Equus caballus* (Horse)	Lowering pH to 1.8 by dialysis in glycine-HCl buffer	N	1 - 2 - 4 - 8 - ?, (Figure 17, arrow III)
Sato et al. 2015, 2016[264,265]	Time-resolved small-angle X-ray scattering	Folded dimers	*Escherichia coli*	Lowering pH to 2.5 by phosphoric acid	Y	1 - 2 - 4 - 6 - 12 - 24, (Figure 17, arrow II)
Mohanty et al., 2021[256]	Dynamic light scattering	Partially unfolded monomers/dimers	*Lithobates catesbeianus* (Bullfrog)	Lowering pH by incubation in HCl (pH 1.5)	N	1 - ? - 24, all possible variants, including shown on Figure 17

At the same time with Gerl and Jaenicke, Stefanini et al. investigated the self-assembly of horse spleen ferritin by sedimentation velocity and near-UV circular dichroism[257] (Table 2). The authors obtained results contradictory to the other studies, and showed that tetramers and octamers are possible intermediates of the process (Figure 17, arrow IV). However, formation of octamers is a dead end of self-assembling, because it is topologically unable to assemble a 24-meric ferritin core from three identical octamers (Figure 17, arrow VI). In particular, two 4-folded octamers require four dimers

to complete the formation of 24-mer (Figure 17, arrow V). Zhang Yu also noted that octamers might appear as a result of violation of the self-assembly process[130]. For example, they were likely observed in the case of chimerical fusion of ferritin N-terminus with a huge viral antigen[196,197], which may lead to steric hindrance near the 3-fold channel. Thus, an octameric oligomer might exist as a partially folded complex with broken 3-fold contacts.

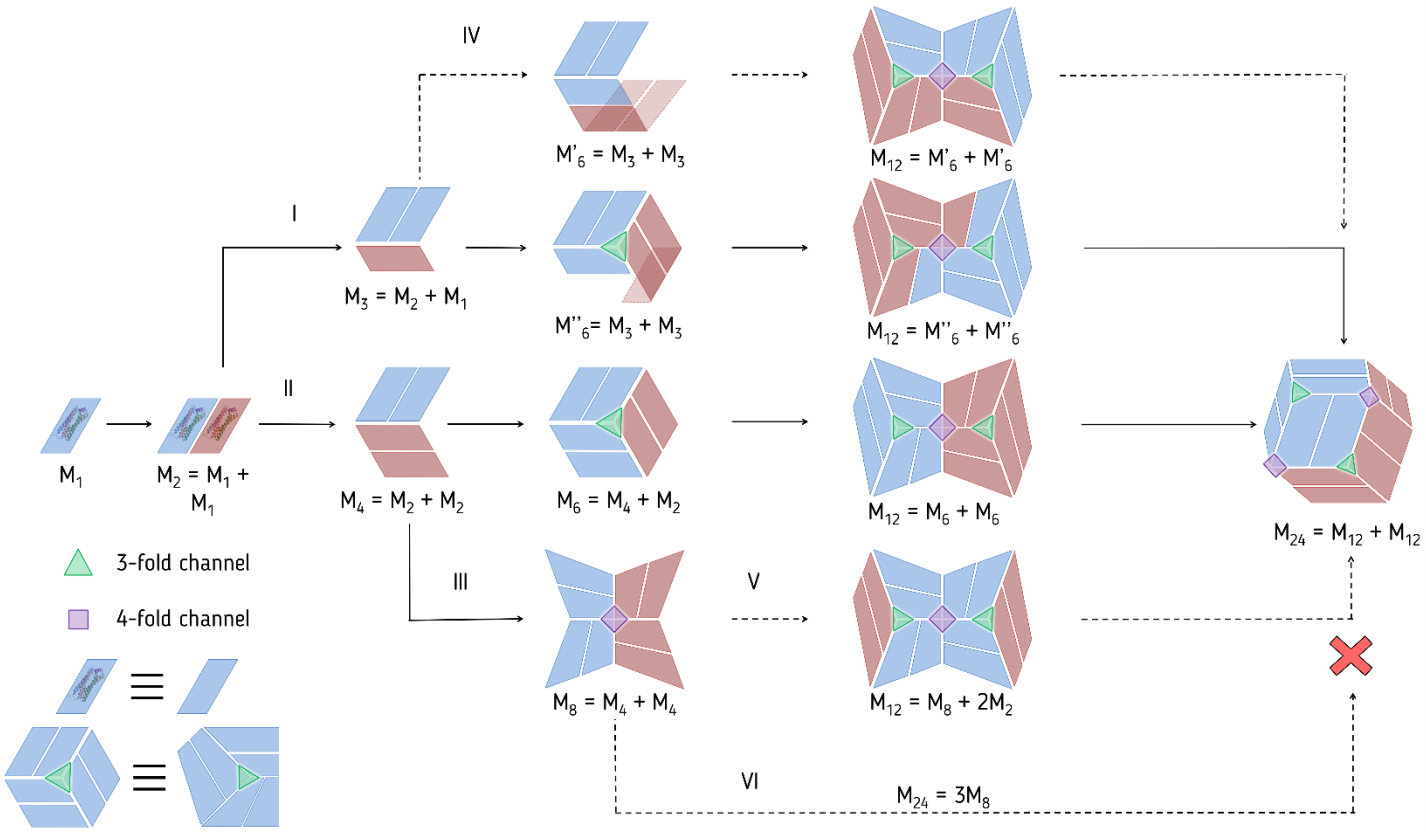
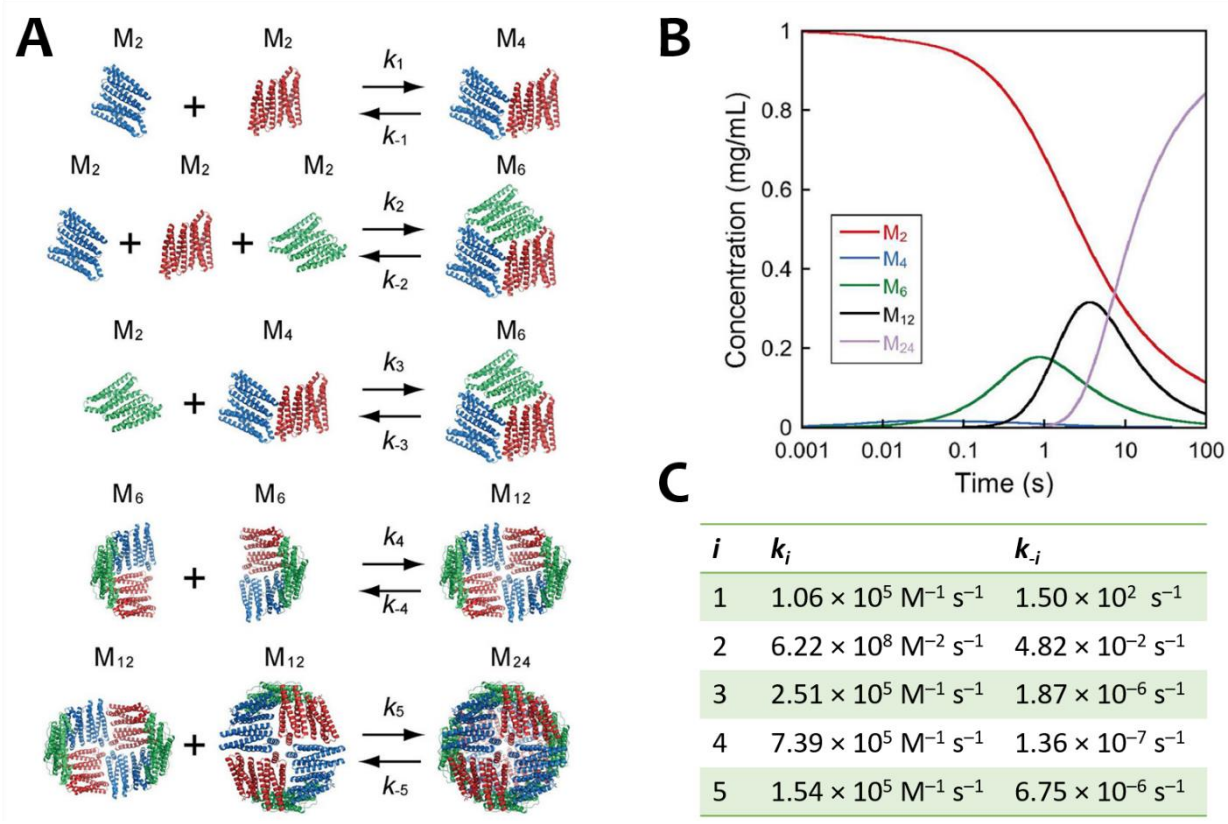


Figure 17. Different mechanisms of ferritin self-assembly. The paths described in literature are shown with solid arrows: (**I**) – Gerl et al.[263], (**II**) - Sato et al.[265], (**III**) - Stefanini et al.[257]. The paths that we also consider as potentially possible are shown with dashed arrows: (**IV**) - alternative M_6 hexamer formation from two M_3 trimers, (**V**) - the only possible way for the formation of M_{12} followed by 24-meric cages from M_8 octamers, (**VI**) - a dead-end path associated with the impossibility of forming 24-mers and three octamers. M_N denotes the N-numbered oligomeric intermediate. Blue colored subunits correspond to previous step of self-assembly, while red colored correspond to added subunits at a current step.

Recent studies also consider the self-assembly mechanism (Table 2). In 2015 Sato et al. used time-resolved SAXS to clarify the mechanism of self-assembly for *Escherichia coli* ferritin A (*Ec*FtnA)[265]. The authors built a model of the self-assembly process (Figure 17, arrow II). Sato et al. carried out reversible ferritin dissociation of *Ec*FtnA by lowering pH to 2.5 (Table 2). The *Ec*FtnA reassembly was initiated by alkali addition for a step adjustment of pH up to 8.0 (a standard reassembly condition). SAXS

curves were measured during the reassembly process at fixed time points. The data were fitted using structural models of assumed intermediate oligomers (Figure 18A). The authors numerically solved kinetic equations for concentration of self-assembly intermediates at time points corresponding to SAXS measurements. Thus, the authors obtained kinetic constants (Figure 18C) of subsequent steps of self-assembly process (Figure 17, arrow II).

Interestingly, assumed structural models of hexameric and 12-meric intermediates have C3 and C2 symmetries, respectively, following the overall structural arrangement of the whole globule. In particular, hexameric state was shown as a 3-fold channel (it contains three dimers), and the dodecameric state was shown as a result of a formation of a 4-fold channel (Figure 18A). Channel contacts between subunits may indeed play an important role in self-assembly and were additionally investigated by the same group[264].



i	k_i	k_{-i}
1	1.06×10^5 M^{-1} s^{-1}	1.50×10^2 s^{-1}
2	6.22×10^8 M^{-2} s^{-1}	4.82×10^{-2} s^{-1}
3	2.51×10^5 M^{-1} s^{-1}	1.87×10^{-6} s^{-1}
4	7.39×10^5 M^{-1} s^{-1}	1.36×10^{-7} s^{-1}
5	1.54×10^5 M^{-1} s^{-1}	6.75×10^{-6} s^{-1}

Figure 18. A Ferritin self-assembly mechanism according to ref.[265]; k_i denotes the rate constants of corresponding reactions. **B** Time-dependent plot of concentrations of self-assembly intermediate oligomers at total protein concentration of 1 mg/ml. The plot was calculated using the rate constants shown in panel **C**. The panels **A** and **B** are reprinted from[265], Panel **C** refers to the data from[265].

Despite a number of advantages of the work[265], the method of *Ec*FtnA dissociation by increasing the acidity makes it possible to obtain only dimers of ferritin subunits as a starting material (Table 2). This approach limits the range of possible intermediates by

excluding those comprising an odd number of subunits. In a living system, monomers are initial. Therefore, the self-assembly pathway obtained in the paper[265] can fully correspond to the natural mechanism only if the dimerization constants for ferritin subunits significantly exceed all other interactions, but it seems to not be a general case[256,258].

In addition, Sato et al. noted that the calculated dynamic scattering patterns are in good agreement with the experimental curves only at initial concentrations of 3–4 mg/mL, but not so well for data at 1 or 5 mg/mL. This may be the result of simplifying the model to the exclusion of all other possible intermediates that are likely to exist in real samples during the self-assembly process. In particular, the octameric state, corresponding to a 4-fold channel formation (Figure 17, arrow IV), was not considered. This intermediate is believed to almost not be present, since it is a deadlock state, and because the formation of 4-fold channels is slower than 3-fold ones in this case, which is confirmed by the rate constants of 6-meric and 12-meric oligomers formation (Figure 18C, k_2 and k_4). The rate of a 3-fold channel formation should be higher than that of a 4-fold one in the beginning of self-assembly, because these rates are proportional to dimer concentration to the power of three and four, respectively. Still, the formation of 3-fold channel may be delayed under certain conditions due to violation of local intersubunit contacts (e.g., in case of protein denaturation or mutation). Thus, consideration of the parallel pathways $2\times M_4 \rightarrow M_8$ and $M_8 + 2\times M_2 \rightarrow M_{12}$ (Figure 17, arrows IV and V) may improve fitting and provide additional information about self-assembly kinetics.

In the study by Mohanty et al.[256], the authors investigated kinetics of self-assembly of the bullfrog M ferritin, *Bf*MF (structural and functional analogue of human H ferritin) by laser light scattering. In contrast to Sato et al.[265], the authors suggest the possible presence of intermediates comprising odd number of subunits (e.g., M_1 and M_3 in Figure 17) along with intermediates comprising even number of subunits, which is in line with the findings of the work[258]. Gerl et al.[258] noted an importance of the fact that the starting material for reassembly experiments was presented by folded monomers. However, in contrast to the study[258], the method of *Bf*MF disassembly via incubating the protein in 40 mM HCl, pH 1.5 (Table 2)[256] allowed obtaining only partially unfolded monomers as a starting material. Therefore, the results of the work[256] cannot be considered as direct experimental evidence for the possibility of ferritin self-assembly pathway via intermediates comprising an odd number of subunits.

Some recent papers contradict the scheme[266], depending on the method of the research. For example, in 2011 Kim et al. observed pH-dependent disassembly of a ferritin by SAXS, showing structural models of the protein in different solutions[266]. They obtained 20-meric, dodecameric, trimeric, and dimeric asymmetrical oligomers. These models were obtained from averaged scattering profiles and shouldn't be considered as

intermediate states of self-assembly. Thus, an unambiguous scheme of ferritin self-assembly still has not been reached.

4.2. Effect of various protein modifications on self-assembly

The molecular mechanism of self-assembly is not clear. There are a number of mutation studies of different ferritins, but their results cannot be generalized, as they are unique for every single protein. There are a number of known ferritins whose stability can be violated by a point mutation[259,267–269].

Interestingly, a self-assembly can depend on the presence of a cofactor, as for salt-dependent ferritins[259]. Many studies observed the stability of human H-chain ferritin. It turns out that the C-terminus region is not important for H-ferritin: elimination of twenty-two C-terminal amino acids (including the whole E-helix) does not lead to destabilization of protein globule and impairment of function[268]. A similar truncation of the N-terminus (thirteen amino acids) does not influence protein functionality, either[268]. At the same time, removal of even a single residue from the BC loop (L82 or I85) leads to protein disassembly[270]. This may occur due to destabilization of dimeric interaction.

Similar studies were also performed for *E. coli* ferritins (BFR, FtnA), but their results do not follow the results of corresponding studies on human protein. For example, depletion of E helix of *E. coli* BFR results in formation of dimeric oligomers instead of complete globular complexes[262]. For more information about studied mutations and their effect on protein stability, see the following review[130]. A wide range of studied mutations with quite an ambiguous effect on protein self-assembly underlies the complexity of ferritin self-assembly. Mutations have unpredictable effects on different proteins, showing evolutionary and structural diversity of ferritin self-assembly processes.

4.3. Possible view on molecular mechanisms of self-assembling through interface context

Intersubunit contacts play a key role in understanding mechanisms of self-assembly. These contacts create a unique interface context determining the stability of intermediate oligomers and the whole ferritin globule. The detailed analysis of these interfaces should be the focus of further studies towards controlled self-assembly of protein complexes.

An approach for analyzing the interface context of ferritin subunits was introduced in the paper[130] (Figure 19). An accurate analysis of interaction interface requires taking several factors into account: hydrogen bonds, surface charges, hydrophobicity, ionic strength, and pH of local environment and steric hindrance. Such local conditions were more or less mentioned above for existing studies in the field (Table 2). The usage of existing high-resolution structures is not very suitable in this case due to an incorrect local environment. For example, the presence of hydrogen bonds in high-resolution models of proteins strongly depends on sample preparation, a method of obtaining the structure, accuracy of model refinement, etc.

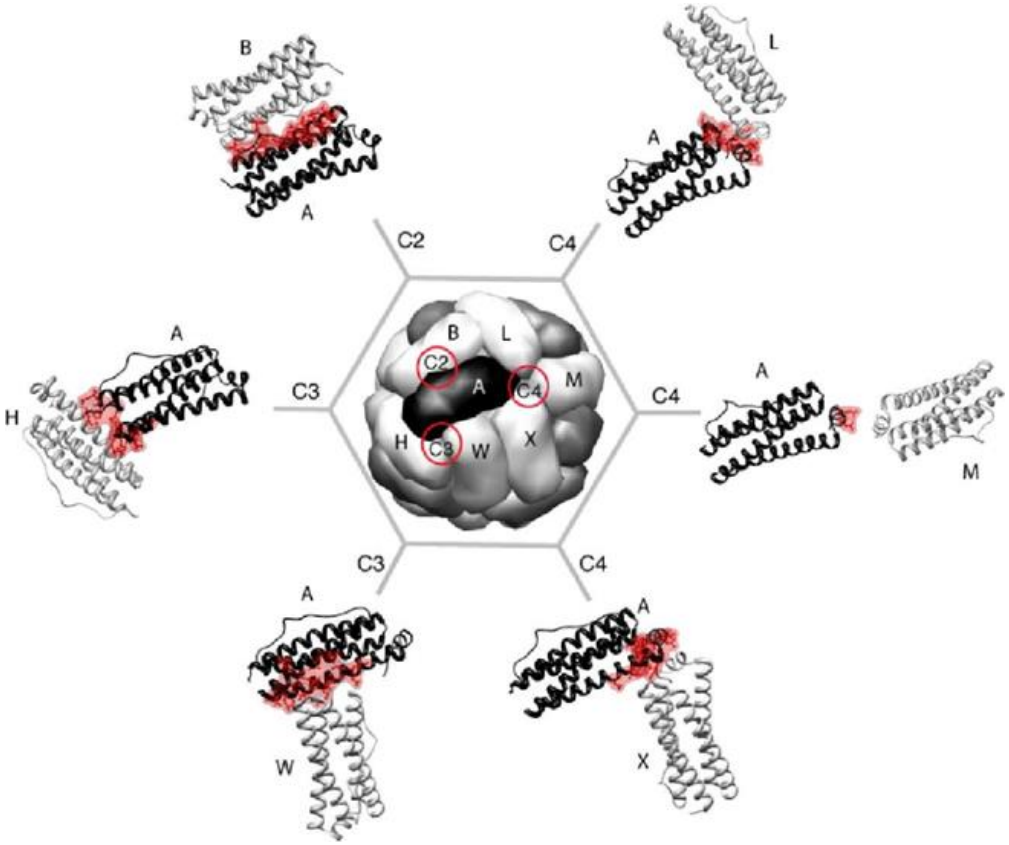


Figure 19. An approach for analyzing the interface context of ferritin subunits. Each monomer in ferritin (PDB ID: 1BFR - *E. coli* bacterioferritin) is involved in six unique inter-subunit interactions at the respective symmetry related interfaces (C2, C3, C4 are highlighted with red circles). The subunits (B, H, W, X, M, and L) which interact with subunit A are indicated. The residues in subunit A that are involved in the inter-subunit interactions are shown in red. Reprinted from the paper[130].

The time-scale of intersubunit interface formation is even more important for understanding of self-assembly than intersubunit contacts themselves. There is likely a kind of competition between different self-assembly pathways, and its result depends on local interface context. In the work by Sato et al.[265] time-dependent concentration curves for different self-assembly intermediates were calculated. Total protein

concentration at the starting point in one of the cases was 1 mg/ml (Figure 18B). In this case, the peak concentration of hexamers was observed approximately at 0.83 sec after the beginning of assembly reaction. The peak concentration of 12-meric oligomers, which formation is associated with the formation of 4-fold channels (according to author's scheme, Figure 17, arrow II), was observed approximately at 3.55 sec. Thus, the formation of 4-fold channel is about 4 times slower than the 3-fold one.

The ratio between 4-fold and 3-fold channels formation rates is a sufficient condition for complete assembly of the globule[258,265] (Figure 17, arrows I, II, IV), although its exact values might slightly vary in some cases. In the case of predominance of a 4-fold channel formation rate over 3-fold one, the deadlock octameric state of self-assembly occurs[196,197,257] (Figure 17, arrows III, V). If the rate of 4-fold channel formation is approximately the same as the 3-fold one, a mixture of octamers and the whole globules might be observed. Such condition seems to be realized in the work by Stefanini et al.[257], where both octamers and 24-mers were observed.

It is important to note that the intermediate oligomers (Figure 17) may be artificial due to non-native conditions which influence the interface context of ferritin subunits. For example, electrostatic potentials mapped onto molecular surfaces of a single ferritin subunit differ at neutral and acidic pH (Figure 20). The ionic composition of buffers is another factor that can affect the interaction of macromolecules[271] and, consequently, the self-assembly process. However, the influence of different ions on the self-assembly of ferritin requires further study.

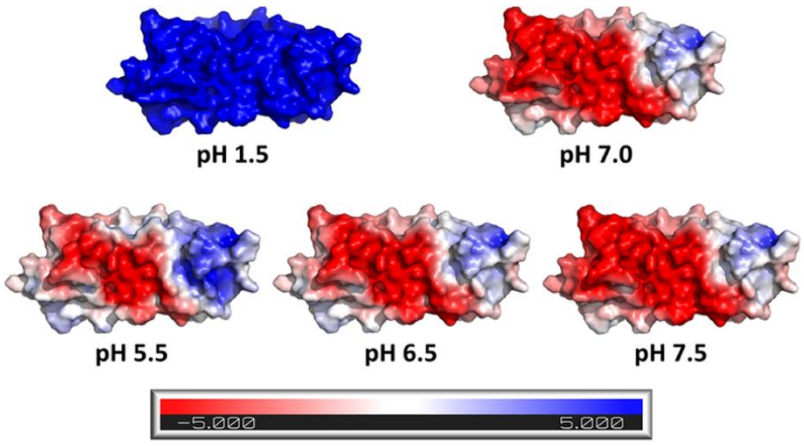


Figure 20. Effect of pH on the surface electrostatic profile of ferritin subunits (figure adapted from paper[256]). At pH = 1.5 the 3D-structure might be different due to protein unfolding; therefore, this representation might not reflect a real structure.

Therefore, decreasing pH[256,257,265] to obtain the starting material impacts the interface context. It may change a self-assembling pathway regardless of folded or

semi-folded starting material. This remark also applies to works[258,263], since their disassembly method is also based on changing the surface electrostatic profile (Table 2). Therefore, we are convinced that studying self-assembly under native conditions (including *in vivo*) allows one to obtain unambiguous experimental information. The hydrogen bonds, surface charges, hydrophobicity and steric hindrance, and other factors should also be concerned for an accurate model to predict self-assembly pathways. Obtaining high-resolution structures can also help understand the self-assembly processes.

Although it is challenging to investigate self-assembly processes *in vivo*, one can study the influence of different mutations on the interface context of ferritin subunits, which is crucial for self-assembly. For example, one can try to change the rate of formation of different intermediate oligomers and control the equilibrium of the formation of a deadlock octameric state. Using the ratio between the rates of formation of different intermediates may allow a self-assembly control. In addition, monitoring of the self-assembly might be implemented via studying the final step of the reaction, for example, by measuring of the ratio between octameric and 24-meric ferritin fractions.

5. Outlook

Ferritin is a well-studied protein, which is actively used in different fields of science and industry. The range of its applications is from fundamental biology, biophysics, calibration of instruments for structural biology, and synthesis of nanoparticles, towards engineering of protein complexes, diagnostics, anti-cancer drug development, drug-delivery systems, and recombinant vaccines against a wide spectrum of diseases.

However, its active application and understanding of its biological role is limited by the lack of information about molecular mechanisms of self-assembly processes. This is a bottleneck in a number of tasks, especially for biomedical applications. Unexpectedly, the process of self-assembly is poorly studied in native conditions, which may be crucial for understanding its molecular mechanisms, significantly affecting the final state of the protein complex. It appears that deadlock states of intermediate oligomeric assemblies may prevent assembly of the whole ferritin or ferritin-based chimeric recombinant protein complexes, which is a significant challenge for vaccine development, drug-delivery, and drug-design. Therefore, deeper understanding of these mechanisms is strongly recommended for further research.

In this review, we illustrate some of possible kinetic schemes of ferritin self-assembly (Figure 17). In general, they follow previously observed mechanisms[258,265]. There is also some evidence about the existence of an octameric state, which can be

observed under specific conditions[196,197,257]. It seems to be a deadlock state preventing normal self-assembly. For example, modification of N-terminus (especially addition of a large chimeric domain) might lead to a steric hindrance and destabilization of the 3-fold channel, with consequent formation of such an octameric complex (which is stabilized by 4-fold channel contacts)[196,197]. Further formation of the whole globule in this case is unlikely due to the high stability of the deadlock state intermediates.

The mechanisms of self-assembly of large recombinant chimeric protein complexes based on ferritin carriers are feasible, since they follow internal protein symmetry and general rules of chemical reactions. Internal symmetry of the protein is important in this process, because more symmetrical mutants of ferritin can show more thermal stability than the native one[269]. We suppose that appropriate modifications of the protein surface or some structural regions should lead to the formation of different oligomeric states (hexamers, octamers, etc.). For example, chimeric attachment of some folded domain to the C-terminus may lead to the formation of deadlock hexamer intermediates, via interaction of 3-fold channel contacts. Thus, it might be possible to stop self-assembly in different states, observing corresponding oligomers in the solution. Such oligomers (even deadlock ones) may be useful for research of ferritin self-assembly and other molecular mechanisms (for example, iron uptake).

Furthermore, they may be improved for further use as a biotechnological tool. Such a “molecular constructor” can help obtain different geometrical shapes, which might have a wide range of applications, even beyond a single protein globule. For example, this approach was already used to obtain ferritin-based octameric nanotubes[272] and arrays of ferritin globules[273,274]. This approach was partially applied in the recent work on controlled assembly of ferritin nanoparticles[275].

In addition, the geometry of three-folded channels allowed attaching viral proteins to its subunits (influenza HA protein, SARS-CoV-2 S-protein, etc.). Such chimers were shown to be high-efficient vaccines against a wide range of diseases due to multiple antigen presentation and adjuvant effect of ferritin.

Self-assembly is a distinctive feature of all ferritins. Despite being universal among ferritins, it might have specific properties in every single case. Its sensitivity to modifications of interface context makes self-assembly a useful tool for different practical applications. Information about molecular mechanisms of self-assembly processes is a keystone of the rapid development in the number of biotechnological fields. Ferritin has already become a tool for immunology, oncology, material science, etc., and it opens new perspectives for future technologies based on self-assembly matter.

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Individual Contribution

VVS designed, conceived and wrote the manuscript.

SMD strongly contributed to the section “Applications”, contributed to all sections, edited the text of the manuscript.

SB strongly contributed to the section “Biological role of ferritin”, contributed to all sections, edited the text of the manuscript.

SVB strongly contributed to sections “Regulation of ferritin expression” and “Structure and function”, edited the text of the manuscript.

YLR strongly contributed to the section “Self-assembly”, edited the text of the manuscript.

VNU strongly contributed to the section “Functional intrinsic disorder in ferritins”, edited the text of the manuscript.

NAB strongly contributed to the section “Vaccines”, edited the text of the manuscript.

SDO contributed to all sections and edited the text of the manuscript.

AEM contributed to the section “Short historical retrospective”, edited the text of the manuscript.

DDK contributed to all sections and edited the text of the manuscript.

TNM contributed to all sections and edited the text of the manuscript.

IVM strongly contributed to the section “Diversity of ferritins and ferritin-like proteins” and edited the text of the manuscript.

AVR organized funding acquisition, edited the text of the manuscript.

VIG contributed to all sections and edited the text of the manuscript.

IYG contributed to all sections and edited the text of the manuscript.

AIK contributed to the section “(Apo)Ferritin as a model for structural studies”, edited the text of the manuscript.

AVV supervised the project, strongly contributed to all sections and edited the text of the manuscript.

All authors have read and agreed to the published version of the manuscript.

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