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Protein engineering approaches to creating ferritin templates

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Abstract

Binding of one protein to another is involved in nearly all biological functions, yet the principles governing the interaction of proteins are not fully understood. The general aim of this review work is to study the rules that govern the control of folding and self assembly of ferritins. The scope of this review is to summarize all the available information regarding hot spots for a better atomic understanding of their structure which helps to use ferritin as a model to explain many protein structures. Ferritin templates could help to synthesize nanoparticles with novel size and shape. Finally, the review work will help to provide information on the development of nanoparticles to design different drug, synthetic proteins and re-engineering defective proteins including ferritins.

Keywords: Ferritin template, Ferritin nanocage, Hot spot, Nanoparticles, Self assembly of ferritin.

Introduction

Materials scientists have drawn an inspiration of how biological systems construct materials by self-assembled macromolecular templates in a modest synthetic condition. Cage likes protein architecture such as viral capsids and ferritins are examples of such biological template.[1] Ferritin is a self-assembled nanostructure intracellular protein which involves in the storage and release of iron in a fashionable way. This protein is found virtually in bacteria, algae, and higher plants, animals.[2]

Three distinguishable interfaces like the interior, the exterior and the interface between the subunits have been found in these protein cages that are synthetically used. These subunits can be modified by means of genetically or chemically so that the function of different surface of cages can be designed these protein cages shows multifuntionality of a single cage. Thus, the functional cages help to the development of new materials for biomedicine and electric field.[1]

From the study of native or reconstituted ferritin by X-ray diffraction and TEM, it is found that a single crystalline or poly crystalline structure in the protein shell build up the core of a ferritin.[3-5] Recently electron nanodiffraction experiments also exposed that the ferritin core posses a single phase of shapeless structure or partially crystalline.[3-5] The widely distribution of Ferritin manifest the role of attaching iron in the cell. Because of its wide importance it is studying extensively so that its high resolution structures may be determined, as we know different monomers folded into four- helix to design a ferritin theme (already the de Novo design community studied a fold ferritin

that acts as a model system for studying the protein engineering dimension).[6, 7]

The key process of self-assembly protein is initialized by protein-protein interaction between their subunits which ultimately combined and form structure in a nano-scale. Protein – protein interaction has become important by the discovery of "hot spot".[8] This protein-protein interaction helps in the designing of drugs and the diligence of development of antibodies, non-immunogenic antigens and the engineering of single cell organism.[9]

Biotemplates (a protein engineering approaches to make proteins self-assembly to turn into nanostructure and predict the protein properties) may be the hopeful way to synthesize nanoparticles as it keeps the constant shape and size of the protein. Ferritins have cavities in the center which could be used as a biotemplate for nanoparticles development. But the problem is that few metals like silver cannot bind to the inner surface of ferreting cavity, so biotemplate can't synthesize nanoparticles. But protein engineering can solve the problem by binding of ferritin peptide to the silver cations. Thus ferritin templates synthesize silver nanopartical.[10]

We are inspired by a group of researchers Nanyang Technological University directed by Brendan P. Orner in Singapore as they successfully disassemble a complex biological nanostructure. They discovered the rules of analyzing the interaction between 19 ferritin subunits and they succeeded over it. Their discovery facilitated us to conduct further engineering on ferritin in order to prepare ferritin templates to synthesize a diverse level of nanoparticles. Therefore, the aims of the present review work are to make a good understanding about the fundamentals of self assembly of ferritin and mechanisms of nanoparticles growth and what type of protein engineering approaches could be used further in order to synthesize ferritin template which finally would help to generate nanoparticles with novel sizes and shapes inside the ferritin cages.

Structure of Ferritin

Basically Ferritin consists of 24 polypeptide subunits and this subunit form a hollow spherical shape (800A in diameter and a thickness of 100 A) as they assemble directly (Figure 1). The total molecular weight of ferritin is 474,000 g/mol. However ferritin has two categories of channels. Fourfold channel (shown in the center of Figure 2) made by the overlap of four peptide subunits. Threefold channel (shown on the corner of Figure 2) formed by the overlap of three peptide subunits. As these channel posses' different chemical properties, the ferritin shows a variety of functional performances.[2]

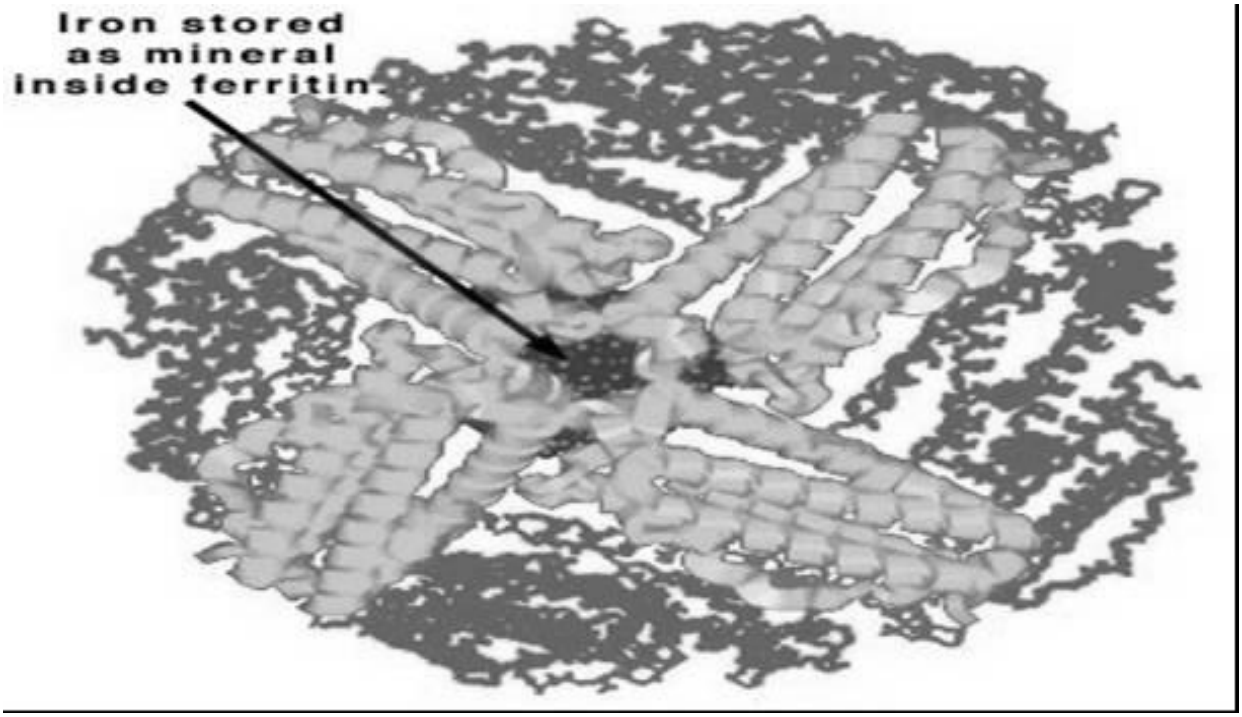

Figure 1: This figure demonstrates the three-dimensional representation of ferritin. Iron (brownish) is stored as a mineral inside the sphere of the protein. It is determined by x-ray crystallography

Crystal Structure of Ferritin

Literally two types of ferritin nanocage are available. These are mini-ferritin made by 12 subunits and maxi-ferritin build up with 24 subunits.[11] Each cage has the diameter 12/8 nm along with 8/5 nm interior cavity. The ferritin cages may come from minor multimeric species like two cages immers and three timers, and thus long chain of ferritin cages in crystalline form have been reported that are bound to bacterial nucleoids.[12, 13] As shown in (Figure 2b) ferritin cages possess 60% of total cage volume along with buffer and hydrated Fe (III) O mineral.[3]

Amino acid can mask the self-assembly code due to the variability of their sequence (vary up to 80%). Active sites of mini-ferritins are present in a small number of iron ligand between two subunits (three for Fe1 and one for Fe 2).[3, 14]. But in maxi-ferritin this active sites (di-Fe (II)) are absent and require an oxidant for binding to Fe (II) substrates.[15] The size, composition and crystallinity of ferritin cages vary respectably. However the mineral size is free of restraints enforced by the protein cage comparing with nanomaterials in ferritins which keep the bioavailability for mineralization of iron below the maximum capacity. The mean numbers of iron atoms/ ferritin cages are 1000–1500 normally, but it can vary zero to 2500.[3]

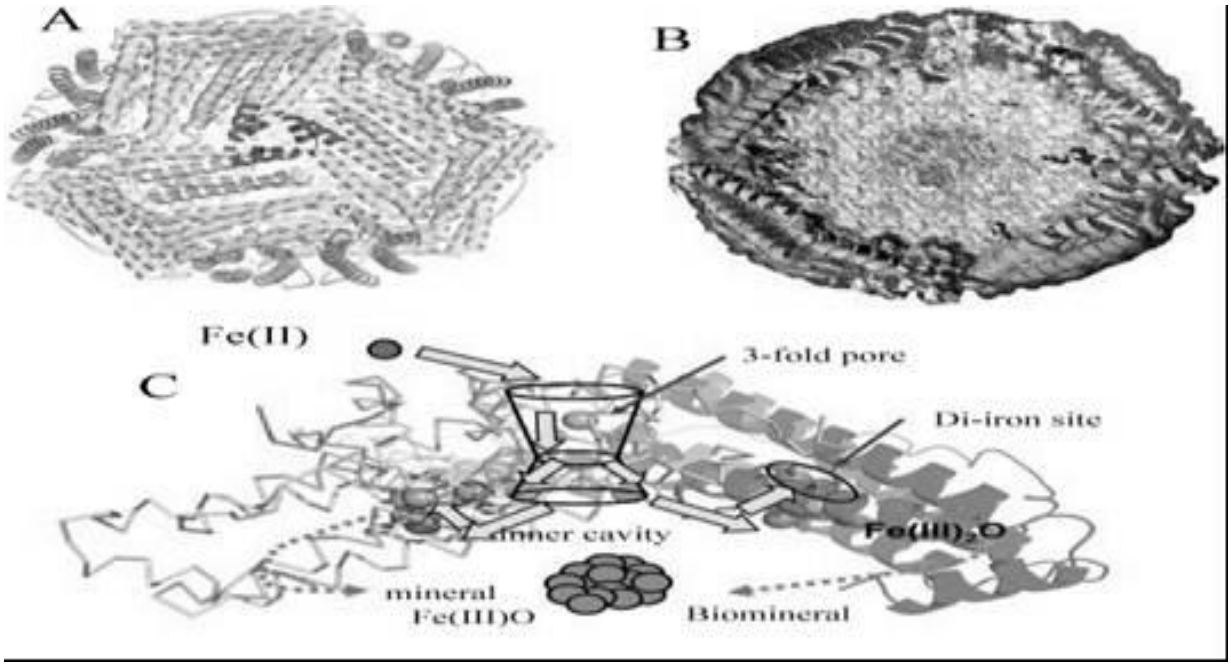

Figure 2: Protein cages of ferritin

Figure 2 is the protein cages of ferritin. The passing of the iron is facilitated by ion channels (16A°) into the cages of the 3-fold cage axes, where it is connected to the exterior and internal cage pores, and distributes Fe (II) substrate to each of three oxidoreductase sites. (A) An outside view where an ion channel is around the threefold axis (gold-helix bundles, red-unfolded sections of three subunits around the eight pores). (B) Cross section of a ferritin protein cage. (Where gold are assembled helix bundles, gray-cartoon represents the large mineral, found when iron is overload and red/white represents the ion channel region). (C) A sketch of iron passing through external pores of ferritin to ion channels of Fe (II) and oxidoreductase sites, after that it passes through Fe (III) O nucleation channels into the mineralization cavity. Spheres: Red-Fe (II), pink-Co (II), orange-Fe (III), green-Mg (II). The residues contributing to the ion channels are the subunit from helix like: (green-helix 1, pink-helix 2, and gray-helix 3. Arrows are paths of iron through the cage: blue-Fe (II) and pink-Fe (III).[3]

Iron Entry into Ferritin Cages and Protein catalysis

The Fe (II) substrate spans around the protein cage from the cytoplasmic surface multiple active sites. The ion channels that exist between the outer surface of the cage and pores on the inner surface of the cage are coordinate

by Cocrystallized metal ions (Figure.3c and 4a). The substrates are allocated throughout the eight channels of 24 active sites (Figure 3c, 4a). The mechanism of allocation is complicated by the cluster of three metal ions around the exits of the ion channels into the protein cage cavity so that each metal ion is oriented toward one of three catalytic sites where Aspartate residues contributed by each subunit that creates the channel (Figure 3a).[16] Positioning of ferritin ion pores and channels needs a functional feature to carry ferrous ion substrate to the multiple active sites in the cage of maxi-ferritins and mini-ferritins.[16, 17] When the entry of two Fe (II) ions into the cage, they bind to the active sites and then react with O2 to produce diferric oxo products in eukaryotes.[3]

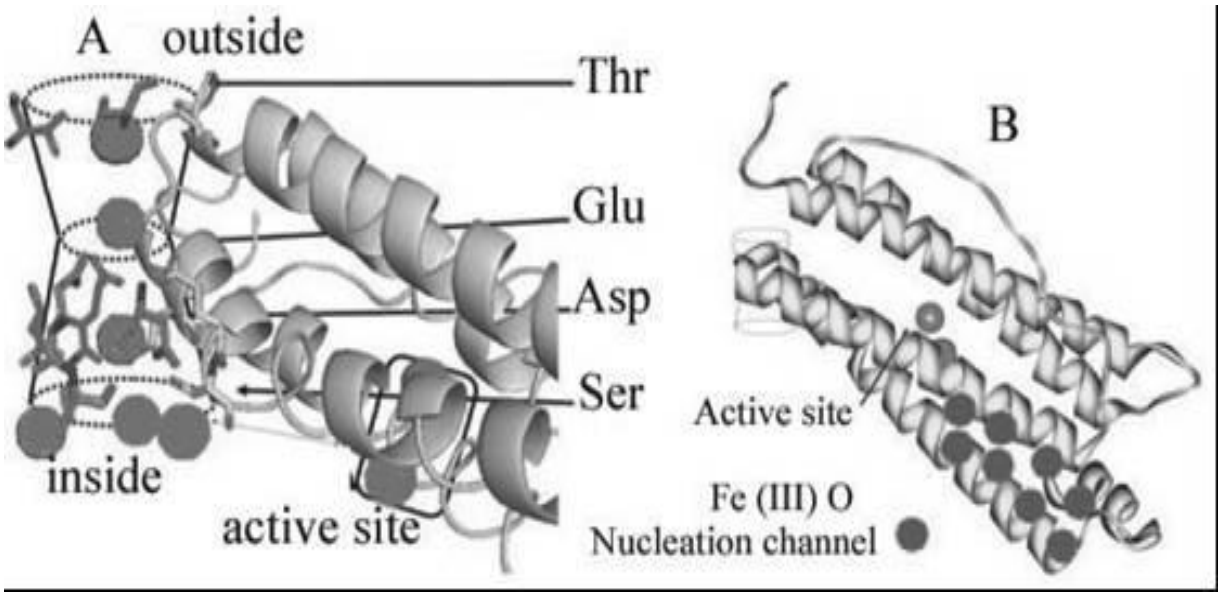

Figure 3: The mechanism of iron entry to the ferritin protein nanocages: Fe (II) ion channels, Fe (III) O nucleation channels and the ligands of active site. (A) An ion entry channel with nearby active site like (T Tosha, pdb file 3KA3). Green —Mg (II), stick figures aspartate 127 from gold — helix 1, blue —helix 2, pink— helix 3, gold ribbon — helix 1. (B) A nucleation channel called ferritin protein cage that receives the di-Fe (III) O reaction products and produces multiferric oxo mineral nuclei: gold — ribbon depiction of a four-helix bundle subunit. The color — represents the metal ions at a catalytic site and — represents catalytic products of the diferric oxo-bridged

Ferritin Iron Exit (Mineral Dissolution)

The exiting iron from ferritin mineral is the releasing of electrons, protons and water during mineralization; Fe–O–Fe bridges are attached when water is released.[16] Water is aligned to each Fe (II) at the diiron at the sites of oxidoreductase.[18] The actual mechanism protons and water entering and exiting the protein cage and releasing of product, nucleation of minerals and dissolution are unknown.[19] However the Fe(II) ions exiting the protein cages of animal ferritins is partially accomplished by the replacements of amino acid but not much known in case plant or bacterial ferritins although some plants accumulated ferritin during the development of nodule and leaf. Human absorb iron directly from legume seed ferritin in order to compensate their iron nutritious deficiency.[20]

The three fold of ion channel in ferritin cages control the Fe (II) entry and exit while unfolding remains around the pores (Figure 2a) and in the presence of reductant it takes part in rapid mineral dissolution. The stabilization of the pores is done by conserved hydrophobic (leu134–Leu110), ionic (Arg 72–Asp122) and hydrogen bond (N-terminus-Arg 72) interactions.

Mechanism of Nanoparticles Growth

Theoretically, the potential of the outer surface of ferritin is net positive and the inner surface is a net negative (Fig. 5).[21] The inner and outer surfaces are joined by channels. Six of them are found in positively charged in 4F-channel and eight are negative charge in 3F-channel (also called hydrophilic channel). 3F-channel paves the way of cation entry to the cavity.[22]

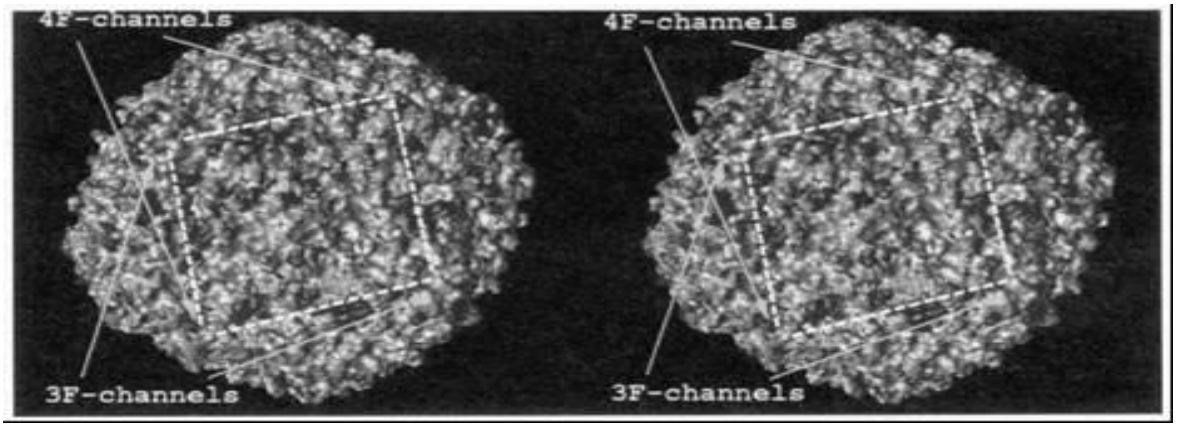

Figure 4: This figure demonstrated the Electrostatic potential of ferritin surfaces, where the Blue color indicates positive potentials and the red negative potentials. The dashed segment is the repetition of eight times that covers the whole surface

Another type of template for synthesizing nanoparticle is Ferritin-like-protein (FLP). This is made by the complexation of cobalt oxide/hydroxide and iron oxide. But the mechanism is similar to the ferritin. A great advantage of this mechanism is that viruses like CCMV can also used as biotemplates because it has a protein shell and an RNA cavity along with channels between outer and inner surfaces. RNA is possible to remove from the cavity and grow nanostructure by controlling pH. (Channels are open at high pH (>6.5) and blocked at low pH).[23]

Along with providing nanoparticles uniform size, biotemplates have some drawbacks. Some metals cannot synthesize bio templates as they can't bind to the inner surface of the protein. For this reason protein engineering is a wonderful solution to this problem. As we know ferritin cannot synthesize silver nanoparticles. So the introduction of peptide that can bind to silver cation along with ferreting can synthesize silver nano particle. Another example is CCMV hinders iron nanoparticles synthesis due to its positive inner surface.[24]

Hot Spots of Ferritin

A maxi-ferritin (nano-cage protein) named bacterioferritin from *Escherichia coli* was selected for the study of alanine-shaving mutagenesis aiming at the discovery of key amino acid residues at symmetry-related protein-protein interfaces for controlling protein stability and self-assembly. These interfaces and virtual alanine scanning helped nine mutants to design, express, purified, and characterize by the transmission of electron microscopy, size exclusion chromatography, dynamic light scattering, PAGE, and temperature dependent CD. Many of the selected amino acids are found to act as hot spot residues. Among them four are (Arg-30, found in the twofold axis, and Arg-61, Tyr-114, and Glu-128, at the threefold axis). But when they are mutated separately to alanine, they entirely shut down the detectable solution in 24-mere formation which favors the cooperative folding dimmers, suggesting oligomerization which we called "switch residues." Two more residues, Arg-30 and Arg-61, after changing to alanine form mutants become thermodynamically more stable than the original protein.[9]

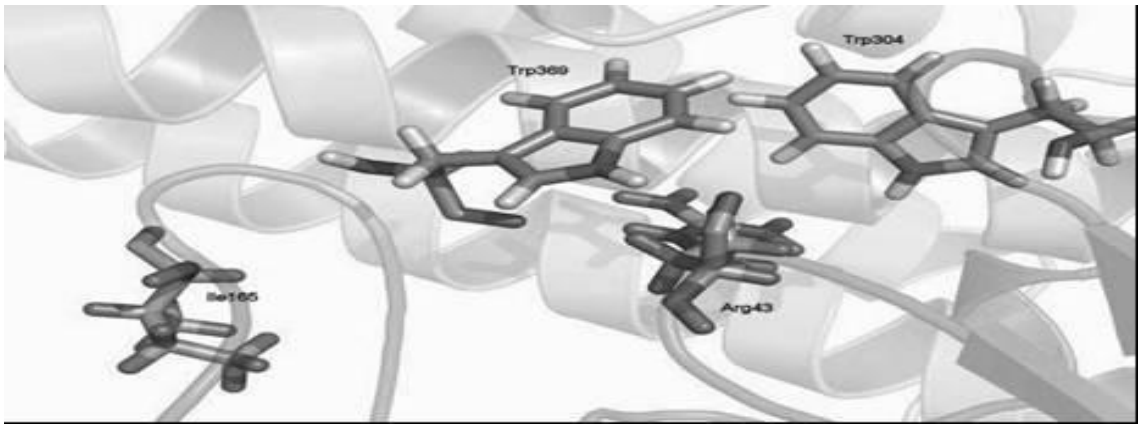

Figure 5: This is the figure of human growth hormone complex (cyan color) along with its receptor (blue color). The representation four hot spot residues are highlighted by a stick and the two tryptophan residues are demonstrated in pink color. The PDB ID is 1a22. The Pymol software52 was used to produce this figure

Engineered Protein Cages for Nanomaterial Synthesis

A silverbinding peptide, expressed by self-assembled particles of human L subunit ferritin (genetically engineered) was used as a nanoinstrumentration for silvernanoparticle synthesis. A dodecapeptide is exhibited by the inner cavity of the protein cage (a protein that is self-assembled), involved in reducing silver ions to metallic silver. When this protein cage is incubated in the presence of silver nitrate to show how silver nanocrystal grows in its cavity.[25]

In figure 6 it is shown how fusion contrast is designed. The L subunit of human ferritin (Genebank ID: M11147) cloned to the NdeI- BamHI sites of pET11b expression vector (Novagen, San Diego, CA) by PCR technology. The C-terminus of ferritin light chain gene was ligated to AG4 sequence (encoded by a short DNA oligomer by Integrated DNA Technologies, Coralville, IA) through glycine linker when matches restriction sites of pET11b-LCF. The pET11b-LCF-AG4 sequence ensures the coding sequence for the fusion of L subunit-AG4.[10]

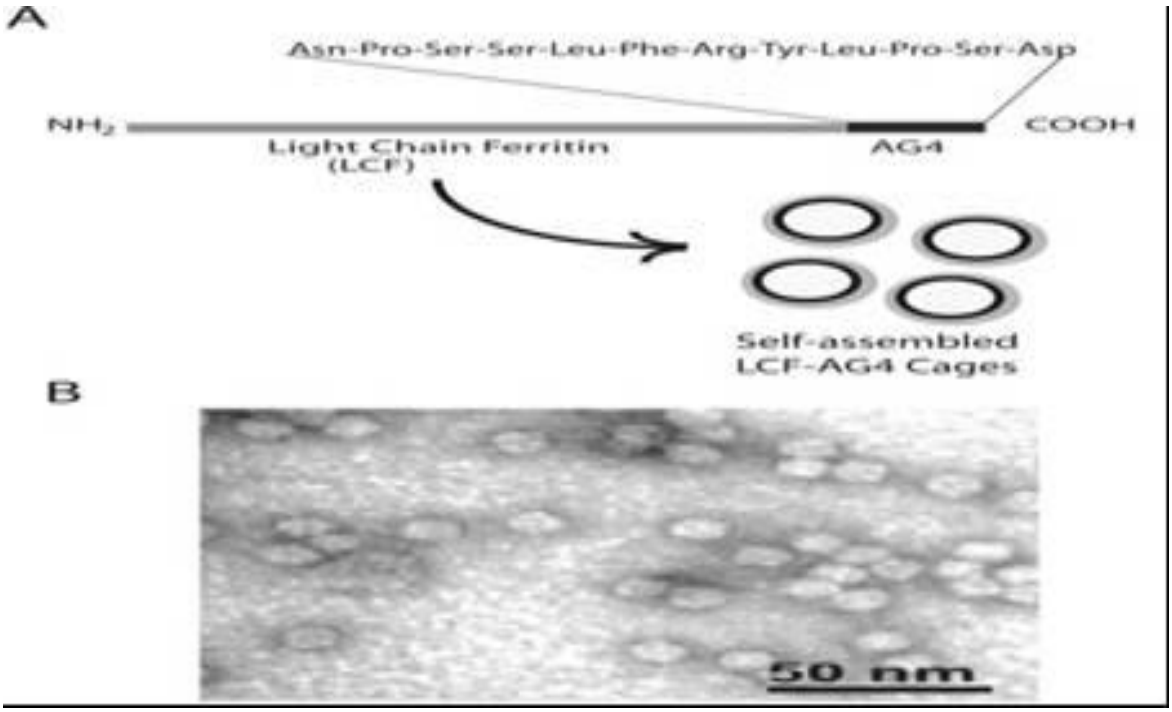

Figure 6: This figure shows how LCF-AG4 chimeric protein cages are created. (A) The fusion of the L subunit ferritin-AG4 (LCF-AG4) fusion and the selfassembled protein cage where the fusion of the AG4 peptide sequence and the C-terminal of LCF occur. (B) The presence of fully assembled protein cages is shown by staining negatively the TEM micrograph of LCF-AG4 protein cages negatively stained with uranyl acetate

Discussion

Although several methods are available to synthesize nanoparticles, but challenges still exist when synthesize under mild conditions and control of shape and size of that nanoparticles. The ferritin-like-protein (FLP), chaperonin and Cowpea Chlorotic Mottle Virus (CCMV), have cavities in the center. These protein cavities are used as template for nanoparticles growth maintaining the uniform size and shape.

Different specific peptides are already identified for the synthesis of inorganic nanoparticles from a phage peptide library display. These peptides have the ability of nucleation and the growth of nanoparticles can also be controlled in vitro system. As for example, the AG4 peptide along with amino acid sequence -Asn-Pro-Ser-Ser-Leu-Phe-Arg-Tyr-Leu-Pro-Ser-Asp-, are specifically involved in the nucleation and control silver nanoparticles growth. The human L-chain ferritin could be fused with these specific peptides at the carboxy-termini. In recombinant bacteria the product of gene fusion are made express and finally purified by the technique of ion-exchange chromatography and gel filtration chromatography. The gene fused protein is purified so that they could form a hollow ferritin cage.[10]

The developments of ferritin template for nanoparticles can posse's alternative method that is, if mutation occurs in the region of hot spot of ferritin during protein engineering which is responsible for protein engineering. Hot spot has found a nonrandom composition instead of typical amino

acid composition during systemic analysis, most commonly in tryptophan (21%), arginine (13.3%), and tyrosine (12.3%).[26] The significance of the tryptophan residue is clearly observable in Figure 5, where the complexation of the human growth hormone and the growth hormone binding protein are clearly illustrated. In figure 5 only four hot spots are observed out of 29 interfacial residues and among them two of its tryptophan. On the contrary, leucine (not isoleucine), serine, threonine, and valine residues are not found as hot spots. But their distinct protein structures are very important. Tryptophan seems to act as a unique function due to its large size and aromatic nature.[26, 27] It can also contribute to aromatic π-interactions, which acts as a hydrogen bonding donor, as it possess a large hydrophobic surface, and can protect slightly hydrogen bonds from water.[28] Ultimately the mutation of tryptophan to alanine creates a large cavity, this is possible due to size difference which ultimately results complex destabilization. Due to third highest preservation tendency Tyrosine also show a hydrophobic surface, aromatic π-interactions and the hydrogen bonding capability of its 4-hydroxyl group. For this reason (participation on hydrogen bonding) tyrosine has three times higher possibilities of a hotspot than phenylalanine.[23] These average percentages of aromatic residues being formed hot spots apparently established the importance of protein interactions which are encoded by their amino acid. Arginine also shows various types of interactions like hydrogen bonding arrangement. Along with its positive charge on guaninidinium theme, it produces five hydrogen bonds and a salt-bridge. Various investigations on these complexes have shown that aspartate and aspargine are preferred to glutamate and glutamine due to entropy difference of their side chain conformation.[26] Isoleucine has frequency of 9.62% as a hot spot, but leucine has only 0.83% (more than 10 times), although they are chemically isomer.

The scope of this review work is to use ferritin as a model to explain many protein structures. Ferritin templates could help to synthesize nanoparticles with novel size and shape. Finally, the development of nanoparticles could help to design different drug, synthetic proteins and re-engineering defective proteins including ferritins.

Conclusion

We know that Iron plays a significant role in molecule formation in our body as a trace element. Ferritin is the protein that involves in iron storage and release through its channel. But it is important for ferritin to be the tree-dimensional structure for functioning within the body. As ferritin is a self-replicating molecule the protein-protein interaction of this molecule is used in various significant functions like drug screening engineering of single cell etc. From the self-assembly of protein we can prepare nanostructure and study the properties of ferritin protein with constant shape and size using biotemplate.

The di-ferrous substrate used in synthesizing of ferric complex by oxo-bridge which initializes the mineralization in the large cavity of the proteins cages, and these protein cages involve in accumulation of thousand of iron and oxygen molecule. Whereas the other substrate (dioxygen in eukaryotes, or dioxygen or hydrogen peroxide in archaea and bacterial facultative anaerobes) involve in gene expression of ferritin which is iron or oxidant regulated and also used in (ARE-DNA) and (IRE-RNA) regulation.

Although researchers run lots of work with ferritin nanotemplate, Some questions are still to be unanswered ,they are about the exit of proton and water during mineralization, movement of Fe(III)o through protein helices, the consequence of ferritin pore dynamic conditions (when open and close), The possibility of viral protein cage to use same ferritin self-assembly code, The designing of nanomaterials when ferritin cages are used as nanomaterialtemplates in controlling nucleation to complementexiting models. And can these protein cages use in cancer therapy. Protein engineering approach using ferritin template has provided tremendous information by showing the structure and working procedure of ferritin template, nanocage that form nanopartical, nanostructure through ion channel and the iron mediated reaction that takes place in our body. And these can help us to control of iron in our body, also can provide the solution of related problems. More studies need to continue to realize the sophistication and complexity of ferritin cage for practical use. As past age, ferritin gave billions of evolutionaries to nawe need to improve the functional complexity of ferritin cage.

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