SRI VENKATESWARA COLLEGE INTERNSHIP PROGRAM IN RESEARCH & ACADEMICS (SRI-VIPRA 2024)

SRI-VIPRA

Project Report of

2024 SVP-2407

"Protein folding studies by biophysical techniques"

IQAC
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SRI-VIPRA 2024

Title: Protein folding studies by biophysical techniques.

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(Dr. T. Vasantha)

Certificate of Originality

This is to certify that the aforementioned students from Sri Venkateswara College have participated in the summer project SVP-2407 titled **"Protein folding studies by Biophysical Techniques"**.The participants carried out the research project under my guidance and supervision from 1st July 2024 to 30th September 2024. The work is original and carried out in hybrid mode (offline and online).

Signature of Mentor
(Dr. T. Vasantha)

Acknowledgement

We express our deepest gratitude to **Sri Venkateswara College** for offering us this invaluable opportunity. Our sincere thanks extend to the **Department of Chemistry, Sri Venkateswara College,** for their unwavering support throughout the project.

We are profoundly grateful to our mentor, **Dr. T. Vasantha** for her patient guidance, enthusiastic encouragement, and insightful critiques. Her invaluable contributions and dedication, particularly in providing constructive suggestions during the planning and execution of this research, have been instrumental in shaping the success of this project.

We also extend our deepest gratitude to **Prof. P. Venkatesu, Department of Chemistry, University of Delhi,** and his scholar **Ms. Pooja** for their cooperation, resources and her diligent efforts, unwavering patience, and providing lab facility guidance throughout this journey.

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List of Abbreviations

Abbreviation	Full Form
XRD	X-ray Diffraction
SAXS	Small-Angle X-ray Scattering
DSC	Differential Scanning Calorimetry
TEM	Transmission Electron Microscopy
XPS	X-ray Photoelectron Spectroscopy
DFT	Density Functional Theory
SEC	Size Exclusion Chromatography
DLS	Dynamic Light Scattering
CD	Circular Dichroism
ITC	Isothermal Titration Calorimetry
NMR	Nuclear Magnetic Resonance
Cryo-TEM	Cryogenic Transmission Electron Microscopy
IR	Infrared Spectroscopy
SEM	Scanning Electron Microscopy
ET	Electron Tomography
PXRD	Powder X-ray Diffraction
FTIR	Fourier-Transform Infrared Spectroscopy
ESR	Electron Spin Resonance
ICP	Inductively Coupled Plasma

Introduction

Proteins are biological macromolecules made up of amino acid residues linked by amide bonds, which then fold into 3-D shapes unique to each protein. A protein adapts to the most energetically favorable shape and performs various biological functions. However, proteins are prone to denaturation when subjected to various stresses like temperature, change in pH, and exposure to organic solvents, to name a few. Among many proteins, one such protein is ferritin which is the oldest known protein involved in iron metabolism. It was first described in 1894 by the German pharmacologist Oswald Schmiedeberg who noted an iron-rich component in horse livers [1]. However, it was not until 1937 that ferritin was purified from horse spleen by the Czech biologist Vilém Laufberger who proposed that it "must be a substance which serves as a depot for iron in the organism" [1,2]. The early isolation of ferritin was facilitated by several distinct biochemical characteristics: its stability at high temperatures (N80 °C), relative insolubility in ammonium sulfate solutions, and its crystallization with cadmium salts. Ferritin is a 450 kDa hollow nano-cage (outside diameter 12–13 nm; inside diameter 8 nm) capable of incorporating up to 4500 iron atoms in a non-toxic but bioavailable form [4,5]. In mammals, each ferritin complex is composed of 24 subunits that form a spherical symmetrical protein shell. Each ferritin subunit folds into a 4-helix bundle with a fifth short helix in close proximity to the C-terminus [6,7]. In its assembled form, the ferritin complex has eight hydrophilic channels which have been proposed to serve as entry ports for ferrous iron [8,9]. Iron is stored within the ferritin cavity as mineralized ferrihydrite ($Fe_2O_3 \cdot 9H_2O$) with traces of phosphorus and nitrogen [10]. Two functionally and genetically distinct ferritin subunits exist: L-ferritin and H-ferritin (also known as light-chain and heavy-chain ferritin). In humans, but not all species, their molecular masses are 19 and 21 kDa respectively [11,12]. Although the two subunits share approximately 55% of their sequence as well as their multi-helical three-dimensional structures, they are functionally distinct [12,13]. The H subunit possesses enzymatic activity and can oxidize ferrous iron into ferric iron. The ferroxidase center in H-ferritin is composed of several residues (mainly glutamic acid) which are buried within the H-ferritin helical bundle and serve as metal ligands [6,14]. The ferroxidase activity of H-ferritin is not dependent on the assembly of the full-ferritin complex and can be detected in the monomeric form [15]. The presence of a ferroxidase center within the ferritin subunit is essential and sufficient for rapid iron uptake [13,14,16]. A mutant of H-ferritin generated by mutating two residues within the ferroxidase center (Glu62 and His65) was capable of forming stable ferritin complexes but lacked detectable ferroxidase activity [17]. Furthermore, the introduction of several glutamic acid residues necessary for the ferroxidase center into L-ferritin was sufficient to increase its iron

incorporation capacity to similar levels as H-ferritin [18]. L-ferritin lacks enzymatic activity and thus does not contribute to iron oxidation and uptake. However, it has a higher number of carboxy groups lining the ferritin cavity which serve as iron nucleation sites [16,19]. In vitro experiments with recombinant L-ferritin homopolymers showed that it is capable of mineralizing iron faster than H-ferritin homopolymers [19]. Moreover, the L-ferritin monomer contains a salt bridge within its helical fold which confers greater stability on the ferritin complex in acidic and reducing conditions [20].

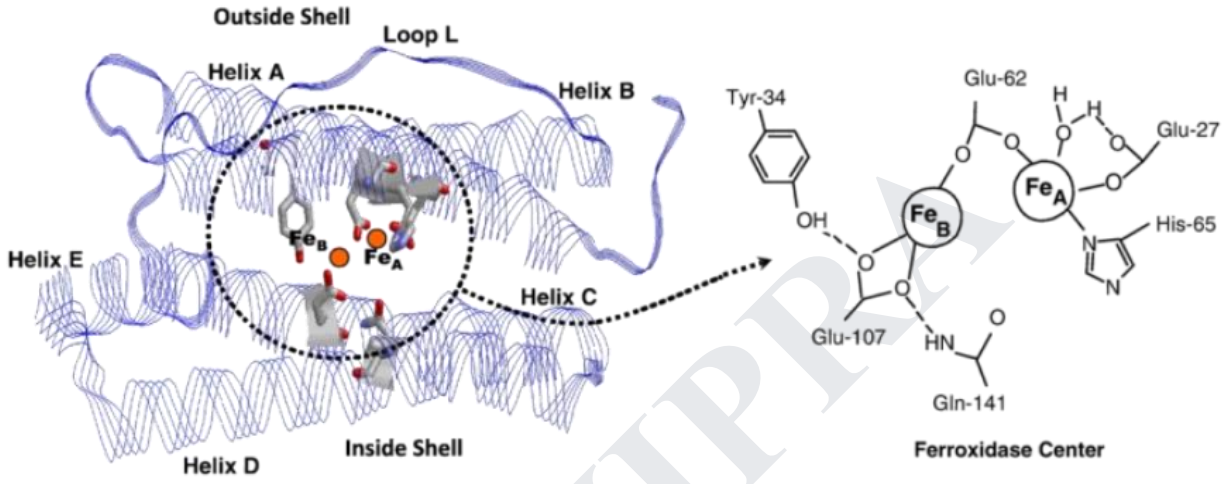

Fig. (a) Mechanism of iron oxidation in the active center of ferritin subunit. A general overview of an active site. On the right side, a diagram of the dinuclear ferroxidase center is shown.

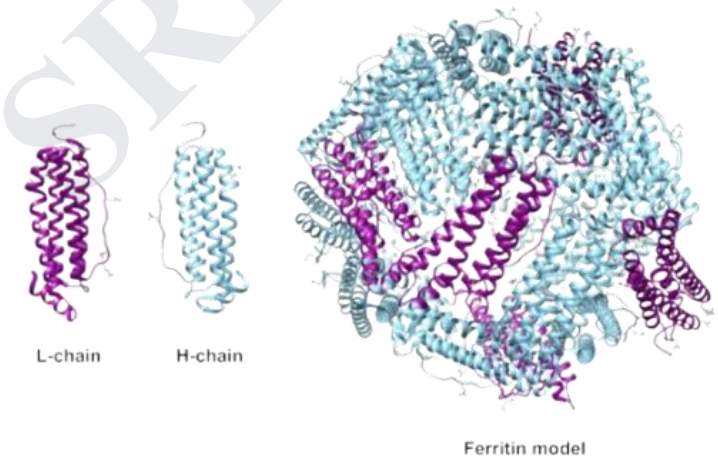

Fig. (b) Light (L) (PDB 2FFX) and heavy (H) (PDB 2FHA) human ferritin subunit chains combine to form the human ferritin shell in specific ratios. The H:L ratio is dependent on the tissue where the protein is synthesized. The model seen here was created from the crystal structures of individual subunits, not a biologically obtained structure.

Ferritin has vast biomedical and biophysical applications. Also, it serves as a good model object for SAS and electron microscopy.

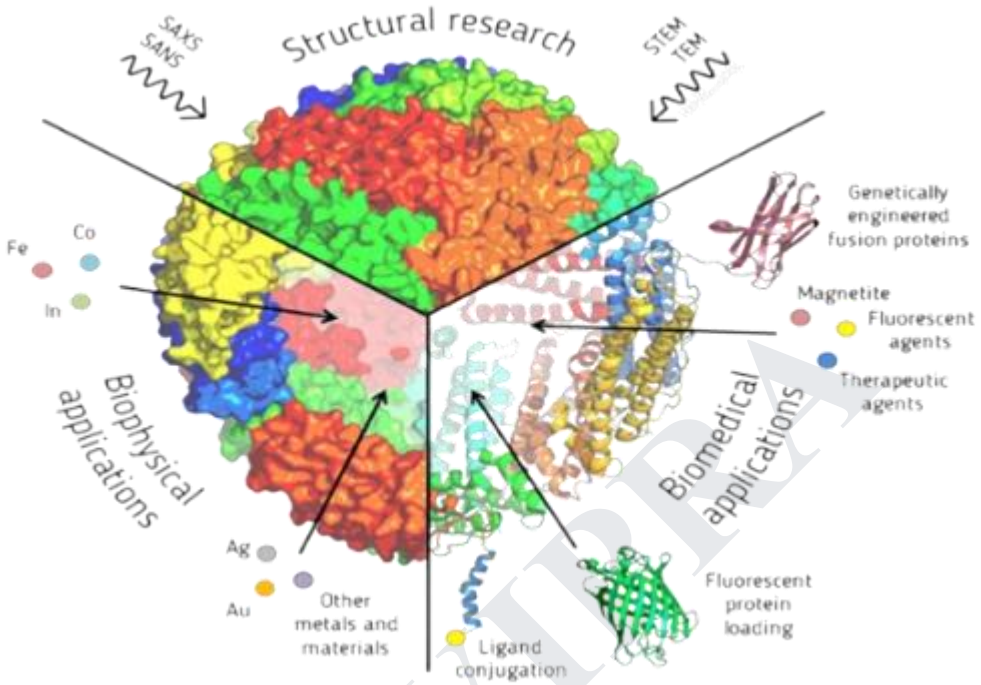

Fig. (c) An overview of applications of ferritin. Biophysical applications: ferritin is used in the synthesis of nanoparticles from different metals and materials for magnetic resonance imaging, electron cryotomography, etc.

1. **Ferritin as a model for structural studies:** Since 1991, many structural studies of ferritins have been carried out, and today the PDB database contains >1500 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 [21]. In recent years, the number of structures obtained by electron microscopy has grown.
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 [22]. Based on this property, it is possible to purify water from the phosphates using ferritin. In nature, ferritin forms a core of ferrihydrite. 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. The synthesis of nanoparticles from divalent cations does not need manipulations with the protein[23]

There are several methods to eliminate a protein shell after nanoparticle synthesis, for example, by heating and UV treatment [24]. The obtained nanoparticles can be employed as memory gates, contrast agents for electron cryotomography, and magnetic resonance imaging (MRI) [23].

3. **Biomedical applications:** Ferritin is used as a drug carrier, a contrasting agent both in vitro and in vivo, and for vaccine production. Nanoparticles from metal compounds are widely used in biotechnology, e.g. in diagnostics and treatment of diseases including cancer [25] Ferritin-like proteins are excellent platforms for multi-modal nanoparticle development [26]. They combine properties from different materials and perform several functions simultaneously. For instance, ferritin can be loaded by magnetite or fluorescent dyes [27] and be detected by magnetic resonance or fluorescence imaging. Loading drugs into ferritin is carried out in three ways, mainly passive loading, pH-dependent disassembly-reassembly, and disassembly-reassembly using chaotropic agents [28]. Another important biotechnological application of ferritin is vaccine production. Due to the oligomerization of this protein, it is possible to provide multimeric antigen presentation. The first example of a ferritin-based vaccine was designed in 2013 against influenza viruses [29]. Vaccine against HIV [30], Vaccine against Epstein-Barr virus, Vaccine against HCV are also produced.

Despite having vast applications, conditions such as changes in pH, temperature, and exposure to chemical denaturants can disrupt its secondary and tertiary structure by weakening the interactions like hydrogen bonding, electrostatic interactions, and hydrophobic interactions. The denatured protein changes its physical state while maintaining its chemical composition. Thermal denaturation is the most common stress and causes loss of protein globular structure at elevated temperatures. During thermal denaturation, the unfolded protein molecules associate rapidly to form aggregates [31]. Various chemical factors might also be responsible for ferritin's instability, mainly including hydrolysis, oxidation, and deamidation. Oxidation of proteins is due to the presence of side chains of amino acids such as methionine, cysteine, histidine, tryptophan, and tyrosine. Methionine and cysteine residues may undergo oxidation even by atmospheric oxygen and normal storage conditions [32]. Protein may undergo auto-oxidation due to the presence of trace amounts of metal ions, peroxides, and free-radical initiators in the protein formulations

and oxidations can be caused by atmospheric oxygen and normal storage conditions as in the case of methionine and cysteine [32]. Factors like temperature and pH might cause the hydrolysis of the Asp-Pro peptide bond and Asp-Gly, resulting in protein fragmentation [33,34]. Deamidation is another type of hydrolytic reaction responsible for the instability of proteins. The amide groups of asparaginyl or glutaminyl residues are hydrolyzed to a free carboxylic acid because of susceptibility to extreme pH. Deamidation of protein therapeutics may affect the bioactivity, half-life, aggregation, immunogenicity, and conformation. Alam et al [35] investigated the deamidation of IgG1 and IgG4 at a pH range of 3.8-7.4 and results revealed that at pH value (3.8), the protein started aggregating. Even during the lyophilization process, protein therapeutics may encounter a variety of stresses such as crystallization, pH changes, dehydration stress, ionic strength change, interfacial stress (ice liquid), and ice crystal formation. The stress generated during the lyophilization process may result in the denaturation/unfolding of the protein, eventually leading to loss of biological activity. When ferritin denatures, it loses its ability to maintain its three-dimensional structure, affecting its ability to bind and release iron.

MOFs can be proven as effective materials for maintaining the protein's stability. MOFs are porous 3-D frameworks with high thermal and chemical stability. They form by coordinating metal nodes and linkers. There is scope to significantly increase ferritin's stability by conjugating it with metal-organic frameworks or incorporating it into them. Ferritin-based MOFs are a new and expanding field of study that combines the special qualities of ferritin with the adaptability of metal-organic frameworks.

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Table 1: Comparison between different Ferratin@MOF composites and their applications.

Se. No.	MOF-Protein Composite	Stress Conditions	Result	Biophysical Technique Used
1	p-bdh-Ni-Ferritin MOF	Heating up to 75°C	Maintained stability; thermally stable at higher temperatures compared to Zn-MOFs	X-ray Crystallography, SAXS, DSC
2	m-bdh-Ni-Ferritin MOF	Heating between 25°C and 75°C	Thermally stable but no phase transition observed	X-ray Crystallography, SAXS
3	fdh-Ni-Ferritin MOF	Reversible first-order phase transition at 33°C with cooling and heating	Reversible 4% volumetric contraction at 33°C, with a hysteresis window of ~10°C; high stability	SAXS, DSC
4	p-bdh-Zn-Ferritin MOF	Heating up to 60°C	Lost structural integrity; thermally unstable compared to Ni-based MOFs	X-ray Crystallography, SAXS, DSC
5	m-bdh-Zn-Ferritin MOF	Temperature range 25°C to 60°C	Displayed pseudo-tetragonal distortion but no phase transition was observed	X-ray Crystallography, SAXS
6	fdh-Zn-Ferritin MOF	Heating and cooling up to 60°C	Thermally unstable; no significant phase transition or contraction observed	X-ray Crystallography, SAXS, DSC
7	Ferritin encapsulated in ZIF-8 and Eu-BDC	Variations in ligand-to-metal ratio, protein concentration, and denaturing agents	Protein localization and aggregation within MOF crystals; 3D spatial distribution visualized	SEM, TEM, ET, PXRD, FTIR

Result and Discussion

Several studies were conducted to gain insights into the design and structure of Ferritin-Based MOF**.** For instance, Jake B. Bailey et al. (2020) investigated how ferritin-MOF conjugates display unexpected thermomechanical behaviors. In this study, ferritin served as the protein node, and the frameworks were assembled using metal ions and ditopic hydroxamate-based linkers. Six different ferritin-MOF frameworks were constructed and studied by varying the metal composition (Ni or Zn) and the linker molecules. The study showed that Ni-ferritin MOFs were more thermally stable than their Zn counterparts. The Ni-based MOFs retained their crystalline structure up to 75°C, while Zn MOFs lost their order around 60°C. The most significant finding was the discovery of a reversible, first-order phase transition in the fdh-Ni-ferritin MOF. This transformation occurred near room temperature, around 33°C, and was marked by a 4% volumetric change within a narrow temperature range (less than 1°C). This type of phase transition, where a material switches between two crystal structures without losing its solid form, is very rare in biological materials. The transition showed hysteresis (a delay in the response to temperature change). Despite the apparent flexibility of the linkers, the six Protein-MOFs were found to be quite rigid. Only p-bdh-Zn-ferritin MOFs displayed a slight structural transformation at low concentrations of additives, hinting at the influence of lattice packing on the rigidity of the material. The structural dynamics during the phase transition were investigated using X-ray diffraction techniques. It shows how the modular MOF design allows it to determine the properties of new materials that are difficult to predict.

To gain insights into protein behavior within these frameworks, R. Dhaoui et al. (2023) presented a report on the influence of synthetic parameters on ferritin behavior within ZIF-8 and Eu-BDC using a combination of SEM, TEM, and electron tomography. The characteristic iron oxide core from the horse spleen (Fn) was utilized as a contrast agent for imaging protein localization and arrangement in the MOF. ZIF-8 was chosen because of its known properties as a protein carrier with its Zn (II) ion and 2-methylimidazole linkage, and Eu-BDC was chosen because of its high atomic number, which allows easier visualization using electrons. Results showed that manipulating factors such as protein concentration and ligand-to-metal ratio can tune the spatial arrangement and aggregation of proteins in MOF crystals. Low ligand-to-metal ratios in ZIF-8 promoted surface-bound proteins, while higher ratios resulted in more uniform protein encapsulation, providing important insights into the design of more efficient protein-based bio-composites.

Conclusion

Metal-organic frameworks (MOFs) have emerged as highly effective materials for various applications in protein folding, denaturation, and stability. Their unique structure, characterized by a high surface area, tunable porosity, and diverse functionality, makes them an ideal composite for these processes. Ferritin-based metal-organic frameworks represent a promising frontier in the field of nanomaterials, merging the unique biological functions of ferritin with the versatile structural and functional properties of MOFs. In the context of protein folding, MOFs have demonstrated the ability to stabilize proteins through selective interactions between the metal nodes and organic linkers, which helps guide the protein to its native conformation.

However, despite the progress made, several challenges remain in optimizing their synthesis, improving their stability, and fully understanding their interactions at the molecular level. Advances in characterization techniques, computational modeling, and the exploration of novel metal ions and linkers will be key to overcoming these obstacles. Continued interdisciplinary research will be essential in harnessing the full potential of these bioinspired materials and advancing them from laboratory research to real-world applications.

Emerging Trends & Future Prospects

The field of ferritin-based MOFs is rapidly evolving, with numerous studies exploring new applications in drug delivery, catalysis, and cancer therapy. The ability to design MOFs with tunable properties by incorporating ferritin nanocages opens new avenues for developing multifunctional materials with precise control over their structural and functional properties.

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