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Characterization of pH – Responsive Nanocage Based on the Ferritin Iron Storage Protein

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Characterization Of pH – Responsive Nanocage Based on The Ferritin Iron Storage Protein

By

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DEDICATION

It is with genuine gratefulness and warmest regard that I dedicate this work to my parents, Arun K Singh. and Ritaja Singh, my brother Shubham Singh, and sister-in-law Shubha Singh, who made incessant sacrifices supporting my highest hopes and praising my smallest successes. Secondly, I dedicate this work to my cousin sister and my mentor Pragya Singh, who guided me in my most difficult moments. Thirdly, I dedicate this work to my lab friends Dr. Angela-Nadia Albetel, Claire Fisher, David Eap, Alison Kimsey, Evan A Talib, Terrell Carter, Prajwal Patel, Leily Daneshian, and Anyway B. Kapingidza to make each moment spent in lab joyful when research was stressful. Finally, I dedicate this work to my best friends, Amit Bhandari, Rishabh Nautiyal and Kritika Sharma for keeping me sane throughout this arduous journey and being my companion through many long nights of writing!

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Last but not the least I place a deep sense of gratitude to my family members and my friends who have been constant source of inspiration during the preparation of this project work.

ABSTRACT

The iron-storage protein ferritin (*Ftn*) assembles into a protein cage structure with 24 subunits and octahedral (4-fold, 3-fold, 2-fold) symmetry. Each monomeric subunit contains a robust four-helix bundle fold. The fully assembled Ftn structure has a high degree of thermal stability (up to 100°C), a mono dispersed size (12 nm in diameter), and a large central cavity (7-8 nm in diameter). The central cavity stores ferric iron in phylogenetically diverse group of organisms, including humans. The central cavity has been used for encapsulation of cargoes such as other metals, contrast agents for imaging, small molecule drugs for therapy, as well as smaller nanoparticles. Ferritin has been widely used for several biomaterials, ranging from nanostructured composite materials to semiconductors to imaging reagents due to its attractive properties. Here we describe our initial efforts to design, generate, and test a series of pH-responsive derivatives of Ferritin (Ftn) protein nanocages that are competent for drug delivery through the endocytic pathway and may be helpful as responsive scaffolds for biomaterials construction. We plan to use a combination of analytical, biochemical, and biophysical techniques to characterize the Ftn derivates, thereby providing new insight into the principles for engineering bio-macromolecular assemblies for stimuli responsiveness.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Currently the commonly used drug delivery methods are oral consumption of pills and liquid medicines, intravenous injections, nasal and respiratory inhalation, and the topical or dermal application of ointments and creams. The basis of these methods is systemic release of drug all over the body which causes lower bioavailability at the target. To successfully circulate and release of drug at the target there have been surge in research and development of smart drug delivery systems (DDS).[1] Nanomaterial based DDS has been developed in past with great therapeutic efficacy.[2, 3] Despite great efficacy, these nanoparticles are accompanied with many disadvantages like they are not biocompatible and biodegradable that could cause cell toxicity.[4] Moreover, for internalization of these nanoparticles chemical derivatization is required.[5,6] Nanoparticles based on biological molecules can be perfect for DDS as they will be biocompatible and biodegradable.[7- 9]

For normal function of cells, constant flow of nutrients and waste is required. This process of flow, through specialized pathways known as membrane trafficking pathway, controls important cellular activities.[10] This pathway can be divided into two basic pathways called endocytosis and exocytosis. Endocytosis (Figure 1.1) is a mechanism to internalize and transport macromolecules using endosomal scaffold vesicles (endosomes). Lysosomes which are the last form endosomes have pH 5.5 – 6.0, is the most important feature which can be used for drug release at the target. In this project we are focusing on receptor mediated internalization using endocytic pathway of novel drug delivery system

that has the ability to use the change in pH property when internalized by an endosome in a cell.[11]

Ferritin, a ubiquitous protein used for intracellular iron storage and release, is an exciting candidate for use in drug delivery. Ferritin is an oligomer protein comprised of 24 subunits, which are a heterogeneous combination of H (heavy) and/or L (light) chains and octahedral (4- fold, 3-fold, 2-fold) symmetry. Each subunit consists of five α-helices (A-E) and an extended loop (BC loop). The fully assembled Ftn (Figure 1.2) structure has a high degree of thermal stability (up to 100°C), a small and mono dispersed size (12 nm in diameter), and a large central cavity (7-8 nm in diameter). The central cavity (Figure 1.3) has also been used for encapsulation of cargoes such as other metals, contrast agents for imaging, small molecule drugs for therapy, as well as smaller nanoparticles.[12-14] Human ferritin heavy (hFtnH) and light (hFtnL) chains are expressed in many cell types through the body as well as in the extracellular space and circulating plasma. Human Ferritin is, therefore, biocompatible, and likely to be nonimmunogenic (depending on the extent of modification). Unmodified hFtn nanocages can enter cells through receptor-mediated endocytosis using native cell surface receptors such as transferrin receptor 1 (Tf1) and the scavenger receptor Scara5.[15] Non-immunogenicity of ferritin provides one of the main reason to choose it for this project. On the other hand, surface derivatization of Ftn, either by chemical ligand-conjugation or genetic modification, can be used to target Ftn to non-native receptors or other cell targets selectively.[13]

There have been many previous literatures published on application of other caged protein which can be modulated by pH change.[16 - 20] Viral nanocages, Norwalk capsids or Cowpea Chlorotic Mottle Virus can assemble at low pH and disassemble at high pH. [16-18]

However, in case of ferritin this property is totally opposite. It remains assembled at very high ranged of pH [21] and disassembles in very high acidic pH solution.[20,22] Native human Light Chain Ferritin can disassemble in very harsh acidic conditions (pH ~2.0 - 3.0), which is not favorable for many drugs.[23] There have been no reports published that mentions any nanocage protein which has the ability to assemble and disassemble near physiological pH.

Many have hypothesized that interactions that causes assembly and disassembly of these nanocage protein can be controlled by modifying sequences responsible for interactions[24]. Based on this hypothesis, N-terminal modification was tested to alter viral capsids assembling ability, which caused decrease in volume of nanocage.[17,25] Similar modification was applied on dihydrolipoamide acetyltransferase by Wang and coworkers which resulted in successful disassembling at pH 5.[26,27] According to the published structures by Wang group of light chain ferritin, it lacks N-terminal embracing arm which was important for assembling of other cage proteins.[21] So this makes it impossible to apply the approach of deleting N-terminal arm (Figure 1. 4) to induce pH responsive switching in human light chain ferritin. However, similar approach was applied on human heavy chain ferritin in which 13 residues from N-terminal of E-helix was deleted. This approach failed in stopping reassembling at pH 5.[19] Other approaches like substituting amino acid residues in hydrophilic channel of four helix bundle were also reported but similar results were observed.[20] All these approaches mentioned in previous literatures confirm that deleting or substituting amino acid in A, B, C, and D helices will not cause ferritin to disassemble at pH 5. To increase pH responsive switching in ferritin, peptide sequences that have been developed for the application of drug delivery by pH variation will be used in this project.[28 - 30] Previous study concluded that 13 H-chain variants were less stable as

compared to L-chain variants in denaturing condition so, we decided to use L-chain ferritin homopolymer for this project.[20]

In this study, *E. coli* BL21 DE3 is used as model organism for its ability of high expression.[31] The significant time in this project was used to design a protein that can have a high yield and is adequately folded in 24-mer form when in buffered solution at physiological pH. To date, hFtnL-E4 has been purified using the process defined in appendix. Now further disassembling and assembling of the protein will be analyzed using size exclusion chromatography (SEC), Circular Dichroism (CD) spectra, and Isothermal Titration Calorimetry (ITC).[32 - 44] Later on, protein nanocage will be loaded with doxorubicin- Cu(II), and the structure of unloaded and loaded hFtnL-E4 will be analyzed using static light scattering (SLS), SAXS, TEM, and X-ray crystallography.

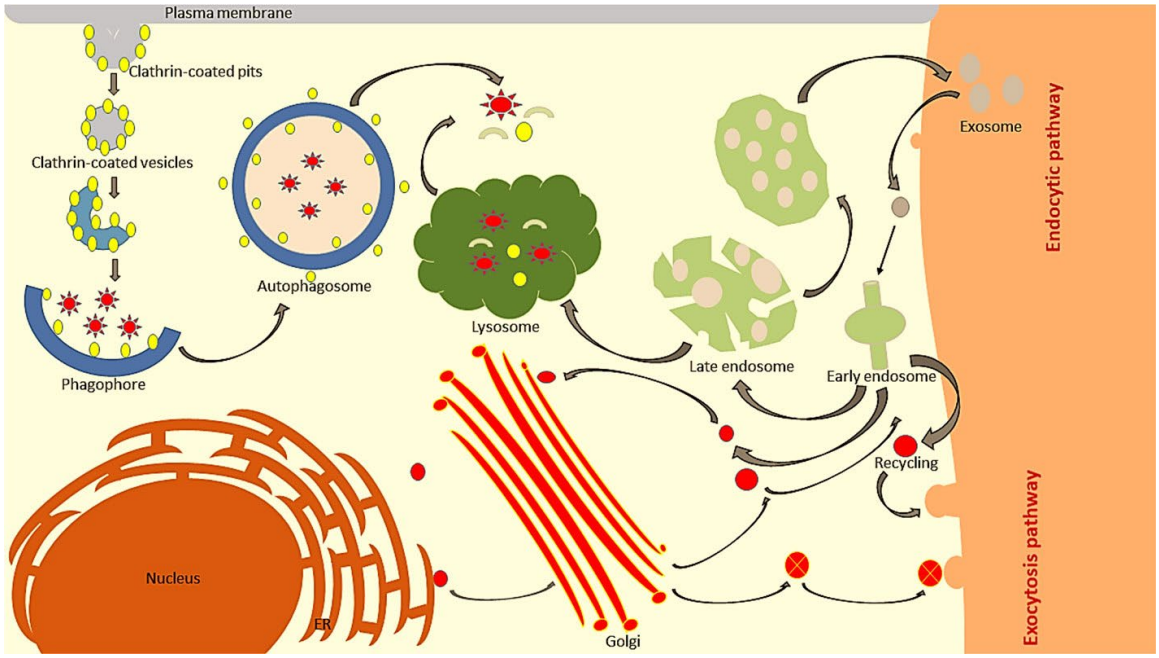


Figure 1.1 Pathway for molecule Internalization.
In eukaryotic cells, membrane trafficking is divided into two major pathways. In endocytosis, proteins are internalized from the outer cell surface into the early endosomes. The early endosome determines the trafficking route for the internalized material.

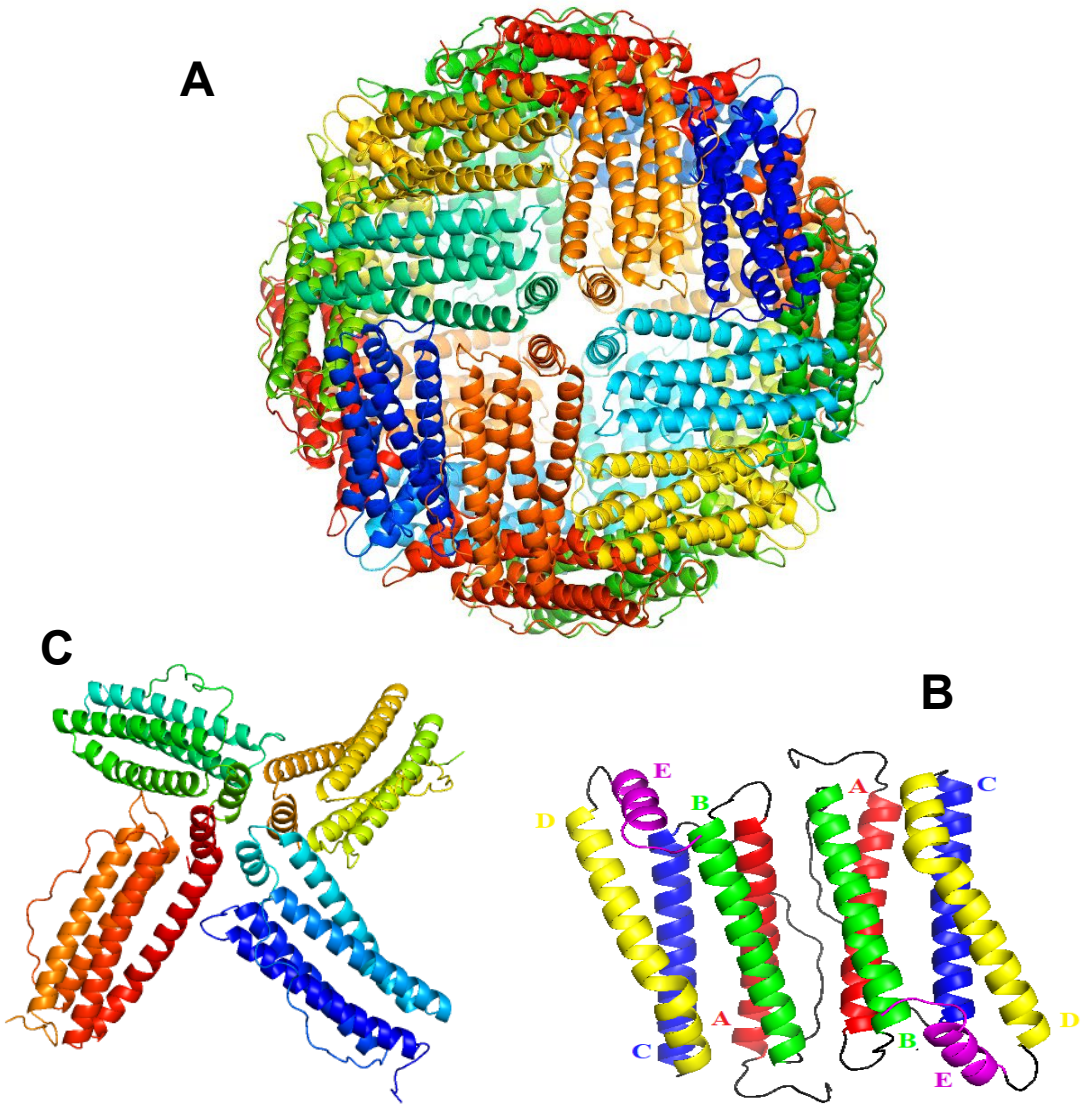


Figure 1.2 Human light chain Ferritin structure depicted using PyMol.
A) 24-mer form showing channel formed by E-helices at the center. B) Antiparallel alignment of protein monomers. C) 4-Fold axis alignment at the channel.

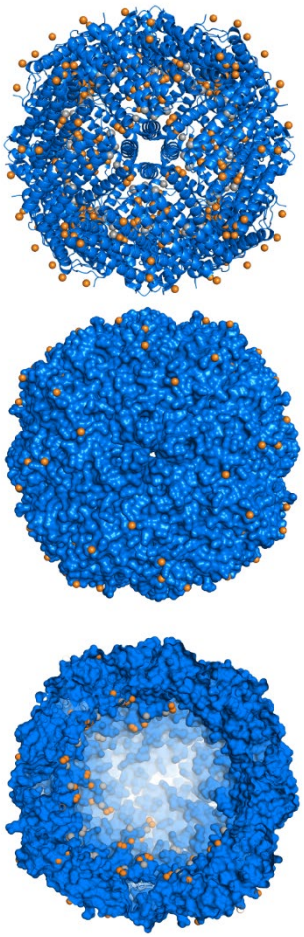


Figure 1.3 Horse spleen light chain ferritin.
Horse spleen light chain ferritin complexed with carboplatin (PDB code: 5mij) with 24 helical protein subunits shown in blue, Cd in orange, and Pt in silver. Top, ribbon view of structure. Middle, surface representation of structure. Bottom, partial cut-away of structure showing interior cavity.

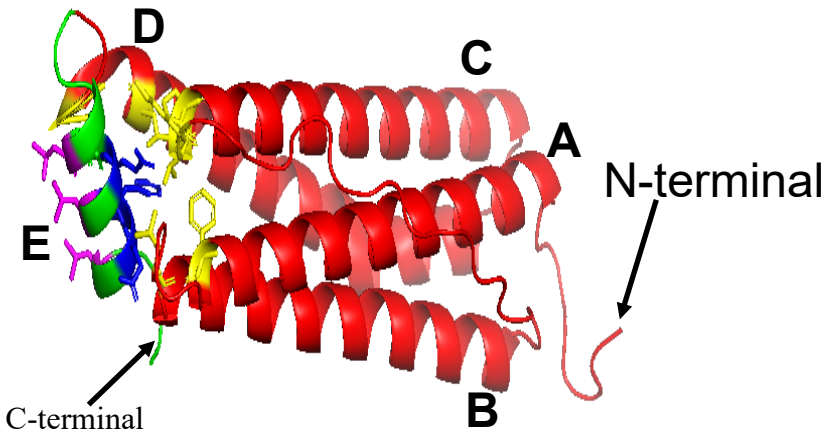


Figure 1.4 Monomer of Human Light chain Ferritin showing N-terminal and C-terminal arm.

CHAPTER 2: STRUCTURAL CHARACTERIZATION OF hFtnL-GALA6

2.1 BACKGROUND AND INTRODUCTION OF hFtnL-GALA6

The GALA peptide consists of the core amino acid sequence Glu-Ala-Leu-Ala repeated up to 7 times in series. Under neutral pH conditions (>pH=7.0), this sequence adopts a random coil structure, but when the pH is shifted to mildly acidic (pH=5.0), the amino acids assemble into an a-helix.[23, 21] It was previously shown that the native E-helix of hFtnL could be replaced with a peptide of repeating Glu-Ala-Glu-Ala ("GALA") units, without significantly altering the assembly of the Ftn 24-subunit nanocage at pH=7.0 (Figure 2.1).[28] Depending on the number of GALA repeats that are inserted, when the hFtnL-GALA nanocages are shifted to pH<6.0, the coil-helix (Figure 2.2) transition in the GALA peptide causes disassembly of the nanocage into smaller subunits, presumably by disrupting monomer-monomer interactions at the 4-fold symmetry axis within the nanocage (Figure 2.3). The greater pH-responsiveness of hFtnL-GALA make it more useful as a biomaterial, especially for disassembly and delivery of cargo in the pH range 4.5 - 5.0 of the lysosome within the endosomal pathway.[29, 30] Although the hFtnL-GALA was partially characterized in vitro, the assembly/disassembly kinetics and thermodynamics have not been carefully analyzed, nor has it been structurally characterized beyond examining the oligomeric state in solution.[28] There are no published

attempts to load this pH-responsive nanocage with cargo, nor has the hFtnL-GALA6 been directly tested for interactions with native cell receptors (such as Scara5).

Unexpectedly, the construct described in the literature did not express with high yields of soluble protein, and the sample that was purified had limited solubility in the absence of Imidazole and ultimately precipitated upon acidification below pH 7.[28] These complications limited the progress toward the goals using GALA6 repeats. However, two main features were concluded from the evaluation of GALA. (1) Glu is the main driving force for the random coil and α-helix formation. (2) The solubility issues originate from the high hydrophobic content of the GALA peptide.

2.2 EXPRESSION AND PURIFICATION OF hFtnL-GALA6

Over the years there have been multiple attempts to purify light chain of ferritin. The first time a modified pH-responsive hFtnL was expressed and purified was by Choi *et al*. In that published work, a polyhistidine tag was inserted at the N-terminus of light chain ferritin and the E-helix was replaced by six GALA repeats. *E. coli* BL21 (DE3) cells were used for overexpression of hFtnL-GALA6 (MW – 21593.28 Da) and it was purified by Immobilized Metal Affinity Chromatography (IMAC) using HisTrap FF 5 mL column followed by Size Exclusion Chromatography (SEC). Overall net yield reported in the previous paper was 1L in 2 mL protein solution.

In this research we first sought to reproduce the published pH-responsive ferritin as a standard to compare against other modified ferritins. The E-helix of human light chain ferritin was replaced with EALAEALAEALAEALAEALAEALAE (GALA6) and the chimeric gene was cloned into the pET28a plasmid to fuse it to a six-histidine tag at the N-terminus of the protein. *E. coli* BL21 (DE3) containing the pET28a-hFtnL-GALA6 plasmid

was grown in one-liter of Luria-bertani (LB) cultures were grown to an OD_{600} of 0.8 and induced with 200 μM isopropyl β-D-1-thiogalactopyranoside (IPTG), cooled to 20°C, and grown overnight. Harvested cell pellet was resuspended in ice-cold lysis buffer (50 mM Tris–HCl, pH 8.0, 100 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride) and homogenized with an ultrasonic processor. Cells were lysed by sonication and lysate (supernatant) was separated from lysed cell pellet by centrifuging at 20,000 ×g for 45 min. Lysate was loaded on a HisTrap FF (Cytiva) 5 mL column equilibrated with 50 mM Tris–HCl, pH 8.0, 100 mM sodium chloride, 20 mM imidazole. Bound proteins were eluted using 50 mM Tris–HCl pH 8.0, 100 mM sodium chloride, 500 mM imidazole with linear gradient method using 20 Column Volume (CV) solution. Extraction efficiency and fractions containing hFtnL-GALA6 was assessed using 15% SDS-PAGE gel. Chromatogram (Figure 2.4) from first purification showed multiple peaks however, analysis of lysed cell pellet (CP), Lysate, Flowthrough (FT), fractions (19, 26, 36) proved that all the hFtnL-GALA6 remained in the insoluble portion of the cell pellet. This could be due to two reasons (1) formation of inclusion bodies or (2) poor protein extraction by this method. Table 2.1 shows all the attempts to extract maximum protein from cells.

Lysis Buffer (50mM Tris pH 7.4, 300 mM NaCl, 1mM PMSF, 1mg/mL Lysozyme, 0.8% cholic acid) gave the best result for extraction of protein from the insoluble pellet (Figure 2.5). However, following the IMAC purification step, the protein started precipitating when total solution collected from fractions was decreased to 5 mL while preparing 2 mL concentrated sample for SEC purification. Similar results were observed after addition of 10 mM β-mercaptoethanol and 5% glycerol to the buffer. Sort of naively thought that histidine tag might be causing aggregation of protein so we thought of

decreasing pH near to Histidine to pKa value so it might function as buffer and not cause protein precipitation at higher concentration. Moreover, to facilitate this decrease in pH we chose Sodium Phosphate buffer which has pH range of 5.8 – 8.0. So, we decreased pH to 6.5 and Tris to Sodium phosphate buffer. At this point concentration of 2 mL double purified protein sample was 1mg/mL. this sample was used for Differential Scanning Fluorimetry.

Monitoring changes in the thermal stability of a protein under different buffer conditions can be used to identify buffer conditions that would favor higher solubility of pure protein in solution. Thermal stability of hFtnL-GALA6 was screened under different buffer ionic strength and pH conditions using differential scanning fluorimetry (DSF).[45] A Bio-Rad CFX96 RT-PCR instrument was used for DSF. A working concentration of 50 mM for the buffer was used for all the conditions with a pH range of 4.0−9.5 (in 0.5-unit increments) and a salt range (sodium chloride) 0.15−1.15 M (0.15, 0.275, 0.40, 0.65, 0.775, 0.9, 1.025 and 1.15 M). Samples for DSF were prepared according to previously published literature.[13] The melting temperature (T_m) of hFtnL-GALA6 was determined in each condition using Bio-Rad CFX Manager software. Average T_m was calculated by performing the experiment three times.[13, 28]

The highest melting temperature that was determined for hFtnL-GALA6 was 56°C. As shown in Table 2.2 (black box) it was found that buffers having pH 6.5-7.4 with NaCl concentration 0.15-0.65 M are favorable for protein thermal stability. Considering the results from DSF, we made 50 mM Sodium Phosphate buffers pH 7.4, 300 mM NaCl, 10 mM BME. We also tested addition of different concentrations of glycerol to the buffer. We also attempted to decrease the formation of inclusion bodies during hFtnL-GALA6

expression in vivo. The chaperone plasmid pGKJE8 from Takara Bio was introduced into the cells expressing hFtnL-GALA6. This plasmid expresses both the *dnaK-dnaJ-grpE* chaperone system (under control of the *araB* promoter induced using 0.5mg/mL L-Arabinose) and the *groES-groEL* chaperone system (under control of the *Pzt-1* promoter induced using 5 ng/mL Tetracyclin). Having two promoters in plasmid provided the ability to induce either or both protein folding chaperone systems. Even though amount of hFtnL-GALA6 protein extracted from cell was increased after induction of both the chaperone proteins from pGkJE8, which could be due to proper folding of hFtnL-GALA6, the hFtnL-GALA6 protein still precipitated at concentrations above 1 mg/mL, making it difficult to perform other downstream processes. So finally, cholic acid was maintained in all the solutions during purification of hFtnL-GALA6.[21]

Table 2.3 shows the attempts that were done after DSF to get highest concentration of protein solution. As for the final protocol, Lysis/Washing buffer (50 mM Sodium Phosphate pH 7.4, 300 mM NaCl, 0.8% cholic acid, 2% glycerol, 10 mM BME, 20 mM imidazole) was used for extraction and protein was eluted from Nickel column using Elution Buffer (50 mM Sodium Phosphate pH 7.4, 300 mM NaCl, 0.8% cholic acid, 2% glycerol, 10 mM BME, 500 mM imidazole). Eluted hFtnL-GALA6 was then concentrated to 3.2 mg/L of cells in 2.4 mL with less precipitation and loaded onto Superdex 200 Hiload 16/60 prep grade column to separate the contaminants using SEC buffer (50 mM Sodium Phosphate pH 7.4, 300 mM NaCl, 0.8% cholic acid, 2% glycerol, 10 mM BME). This also removed imidazole from the solution. hFtnL-GALA6 was observed in two different peaks (Figure 2.6 & 2.7) so, fractions from both peaks were collected and concentrated into one sample. Overall yeild of protein after this purification was 2.6 mg/L of cells.

2.3 MOLECULAR WEIGHT DETERMINATION USING SIZE EXCLUSION CHROMATOGRAPHY

For the first characterization step for hFtnL-GALA6 the apparent solution molecular weight was determined using an analytical grade superdex 200 10/300 GL column. Table 2.4 lists the standards, their molecular weights, and the concentrations that were used in determining molecular weight of protein sample. Gel Filtration Markers kit was ordered from Milipore Sigma for molecular weights 29,000 – 700,000 Da and Cytochrome C was also ordered separately. The hFtnL-GALA6 sample that was in SEC buffer. So, this buffer was used to make solutions of all the standards. Amount of standards were calculated for 100 µL sample and then all the standards were mixed separately first in 500 µL. These standard solutions were mixed together and then concentrated to 100 µL. 100 µL Blue dextran was prepared separately and then injected first in Superdex 10/300 gl column to obtain V_o. Then 100 µL standard solution prepared was injected and eluted. Then 100 µL of 2.6 mg/mL solution of hFtnL-GALA6 was injected on the column and then eluted. Elution volume V_e of standards was obtained from the chromatogram. Since there were multiple peaks observed in protein chromatogram so SDS-PAGE was used to detremine the peaks that had hFtnL-GALA6. For standard curve, ratio of V_e/ V_o was plotted in Microsoft Excel. Using the standard curve equation, the unknown molecular weight of hFtnL-GALA6 was obtained. Since there were two peaks it can be infered from the molecular weights that there are two forms of hFtnL-GALA6, a 24-mer and dimer. Figure 2.8 shows the overlapped chromatograms of standard and protein. Figure 2.9 and Table 2.5 shows the molecular weight calculation.

Ferritin has been proved to be a good nano-molecule that has the ability to store molecules and is used for transportation. However, in previous attempts to load ferritin buffer pH must be decreased to 2. This change in pH causes disassembling of protein. Most of the drugs are unstable or degrade in acidic pH. To increase structure responsiveness near neutral pH, the E-helix of human light chain Ferritin was replaced with six repeats of GALA. This replacement was proved to increase the pH responsiveness in range pH 6.0 – 7.4 which was good for loading the nanocage. However, using leucine (L) in the GALA sequence increased hydrophobicity of the protein and this may have caused overall yield to be very low. It is possible to do sevral structural studies on this much low yield however, using this protein industrially will not be very cost effective. Moreover, multiple peaks of protein on analytical Superdex 200 10/300 GL column suggested that it was not properly folded or have multiple forms even after adding so many additives. Whether the protein is properly folded, or it is in dimer, trimer or tetramer form can only be confirmed using Circular Dichroism Spectroscopy (CD spectra). However, these results from hFtnL-GALA6 protein purification made it unsuitable to work any further.

Table 2.1 Attempts to solubilize hFtnL-GALA6.

<table>
<tr><th colspan="4">Condition</th><th rowspan="2">Method</th><th rowspan="2">Observations</th></tr>
<tr><th>Buffer (50mM)</th><th>pH</th><th>NaCl (mM)</th><th>Additives</th></tr>
<tr><td rowspan="5">Tris-HCl</td><td>8</td><td rowspan="2">100</td><td rowspan="2">None</td><td>Sonication</td><td>• No extraction</td></tr>
<tr><td rowspan="3">7.5</td><td rowspan="4">Lysozyme, Cholate, PMSF, Sonication</td><td>• Extraction possible but too much contamination</td></tr>
<tr><td rowspan="3">300</td><td rowspan="3">10mM BME, 5% glycerol</td><td>• Extraction possible but less contamination</td></tr>
<tr><td>7.4</td><td>• Better extraction,
• zero contamination, unable to concentrate</td></tr>
<tr><td rowspan="2">Sodium Phosphate</td><td rowspan="2">6.5</td><td>• Very less extraction,
• more contamination,
• protein lost in Flowthrough</td></tr>
<tr><td>Dnase 1, MgCl2, Lysozyme, Cholate, PMSF, Sonication</td><td>• Very less extraction, more contaminated, protein lost in Flowthrough during Nickel column,
• Concentration possible but still aggregation,
• Multiple peaks due to contamination in SEC purification,
• Pure protein obtained after SEC,
• Concentrated to 1mg/ml</td></tr>
</table>

Table 2.2 Differential Scanning Fluorimetry
This table shows the average temperature recorded after two experiments. Red color shows very high temperature and blue color shows low temperature. T higher the temperature, the more the protein is stable in that condition.

pH →	**4**	**4.5**	**5**	**5.5**	**6**	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**
NaCl (mM) ↓ 0.15	56	49	54	50	53	**56**	**55**	38	30	30	30	30
0.275	48	51	60	52	51	**55**	**56**	53	43	35	30	51
0.4	48	59	54	53	54	**55**	**55**	54	54	54	35	35
0.65	48	51	54	53	54	**56**	**54**	43	54	30	31	31
0.775	48	51	55	53	55	42	55	42	47	30	35	38
0.9	50	51	49	48	54	43	42	54	31	31	36	32
1.025	39	42	41	50	38	35	30	31	32	32	32	32
1.15	39	46	49	46	46	46	46	39	49	46	46	32

Table 2.3 Attempts to solubilize the hFtnL-GALA6

Plasmid	Condition				Method	Observations
	Buffer (50mM)	pH	NaCl (mM)	Additives		
pET 28a-hFtnL-GALA6	Sodium Phosphate	7.4	300	10mM BME, 2%Glycerol	DNase 1, $MgCl_2$, Lysozyme, Cholate, PMSF, Sonication	• No contamination, • still inclusion bodies are formed, • concentrated till 1 mg/ml
pET 28a-hFtnL-GALA6 + pG-KJE8(Protein Folding Chaperone)				10mM BME, 2%Glycerol		• Better extraction, • No contamination, • Concentrated till 1 mg/ml
				10mM BME, 10%Glycerol		• Extraction hindered due to high glycerol concetration
				10mM BME, 2%Glycerol, 0.8% Cholic Acid		• Best extraction, • no contamination, • concentrated till 6 mg/ml

Table 2.4 Gel Filtration standards for molecular weight determination.

Standards	Molecular weight (kDa)	Concentration (mg/mL)
Thyroglobulin	660	5
Apo ferritin	440	0.4
Alcohol Dehydrogenase	150	5
BSA	67	8
Ovalbumin	43	4
Cytochrome C	12	5

Table 2.5 Calculated molecular weight.

Standards	**Molecular Weight (kDa)**	**Ve (mL)**	**Ve/Vo**
Thyroglobulin	669	8.8	1.13
Ferritin	474	10.99	1.42
Alcohol Dehydrogenase	150	13.06	1.68
BSA	66	13.92	1.79
Oval albumin	45	14.8	1.91
Cytochrome C	12.3	17.33	2.23
Sample 1	540.2	9.57	1.23
Sample 2	72.9	15.07	1.94

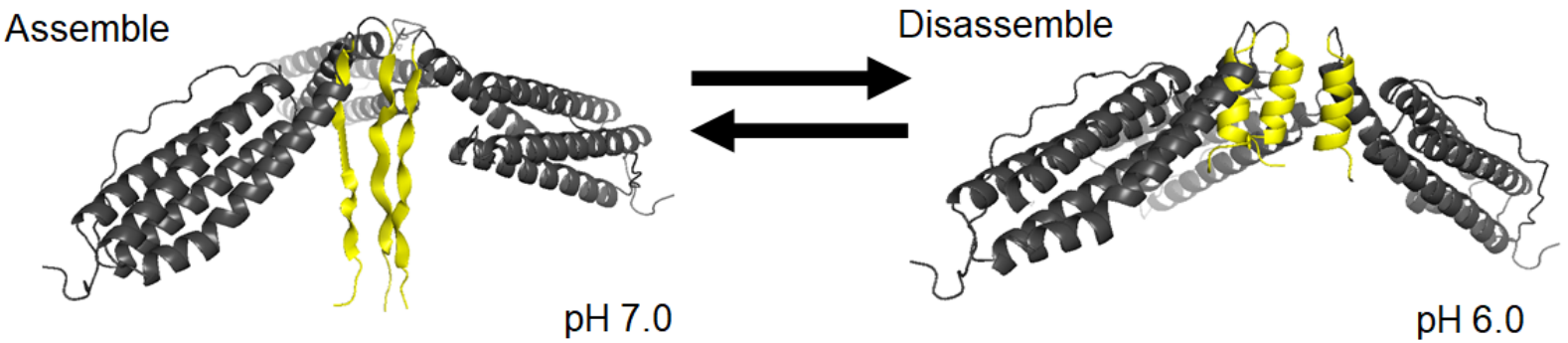


Figure 2.1 Coil to Helix transition of E-helix by changing pH.

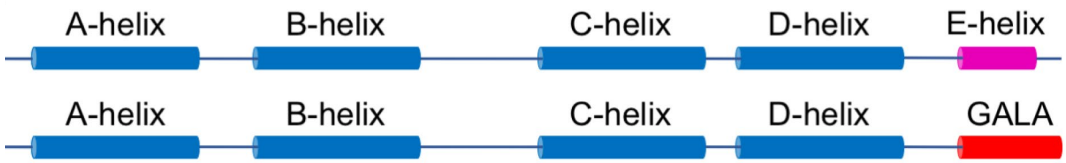


Figure 2.2 Monomer of Human light chain Ferritin and hFtnL-GALA.
α-helical secondary structure (cylinders) in hFtnL (top) and hFtnL-GALA (bottom). Helix labels are per hFtnL nomencla

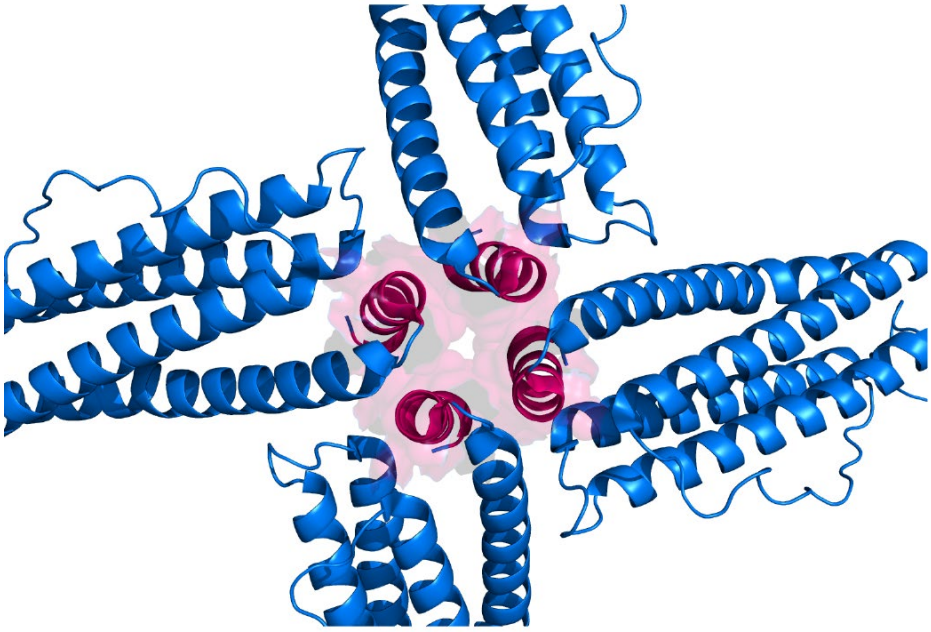


Figure 2.3 Location where E-helix will cause disassembly.
Same 4 monomers magnified with the E-helix of each monomer shown in magenta as helix and transparent surface representation. Other monomers not shown.

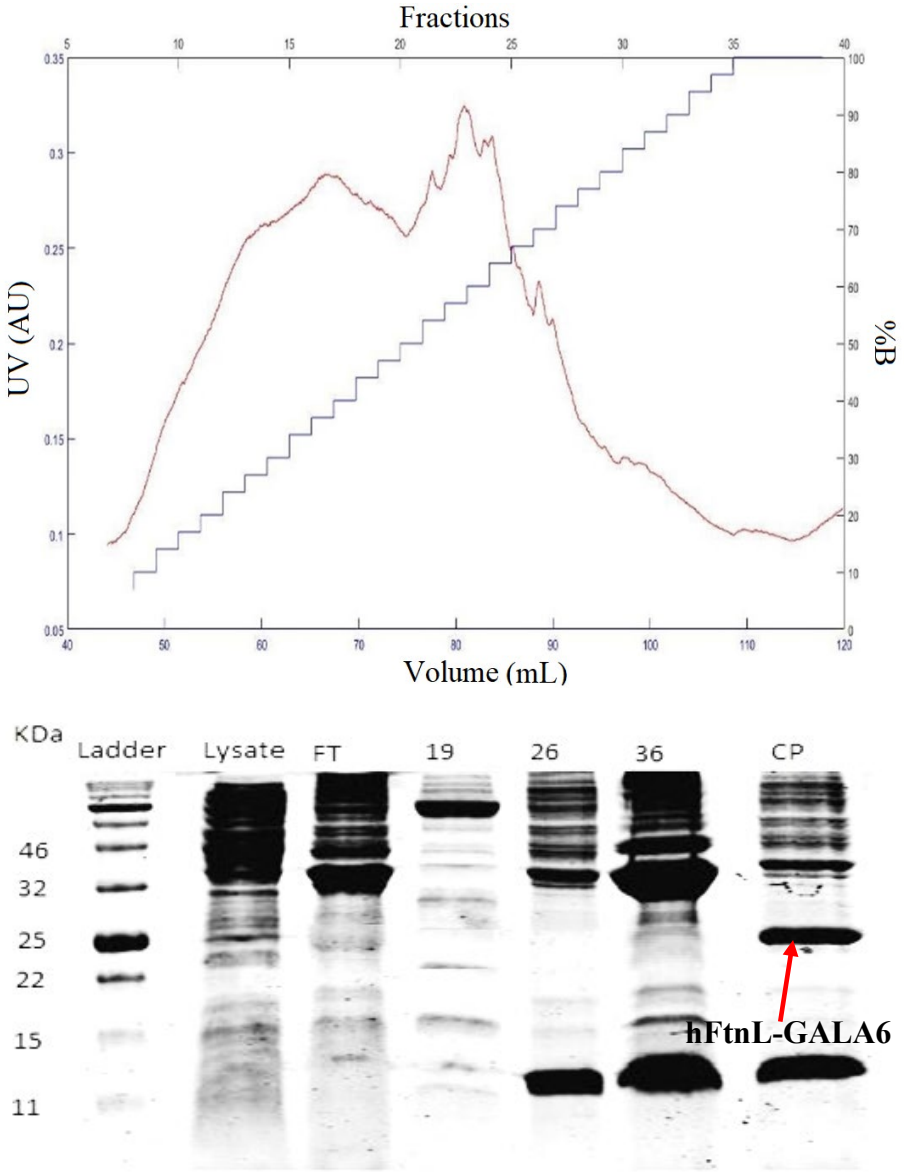


Figure 2.4 First attempt of purification. MW - 21593.28 Da
Protein extraction using Lysozyme Cholate method. Buffer Conditions - : **Lysis Buffer** – 50mM Tris-HCL, 100mM NaCl pH 7.5. **Wash Buffer** – 50mM Tris-HCl, 100mM NaCl, 20mM Imidazole pH 7.5. **Elution Buffer** – 50mM Tris-HCl, 100mM NaCl, 500mM Imidazole pH 7.5. **[A]** Chromatograph obtained after using HisTrap column. **[B]** Samples observed on 15% SDS-PAGE.

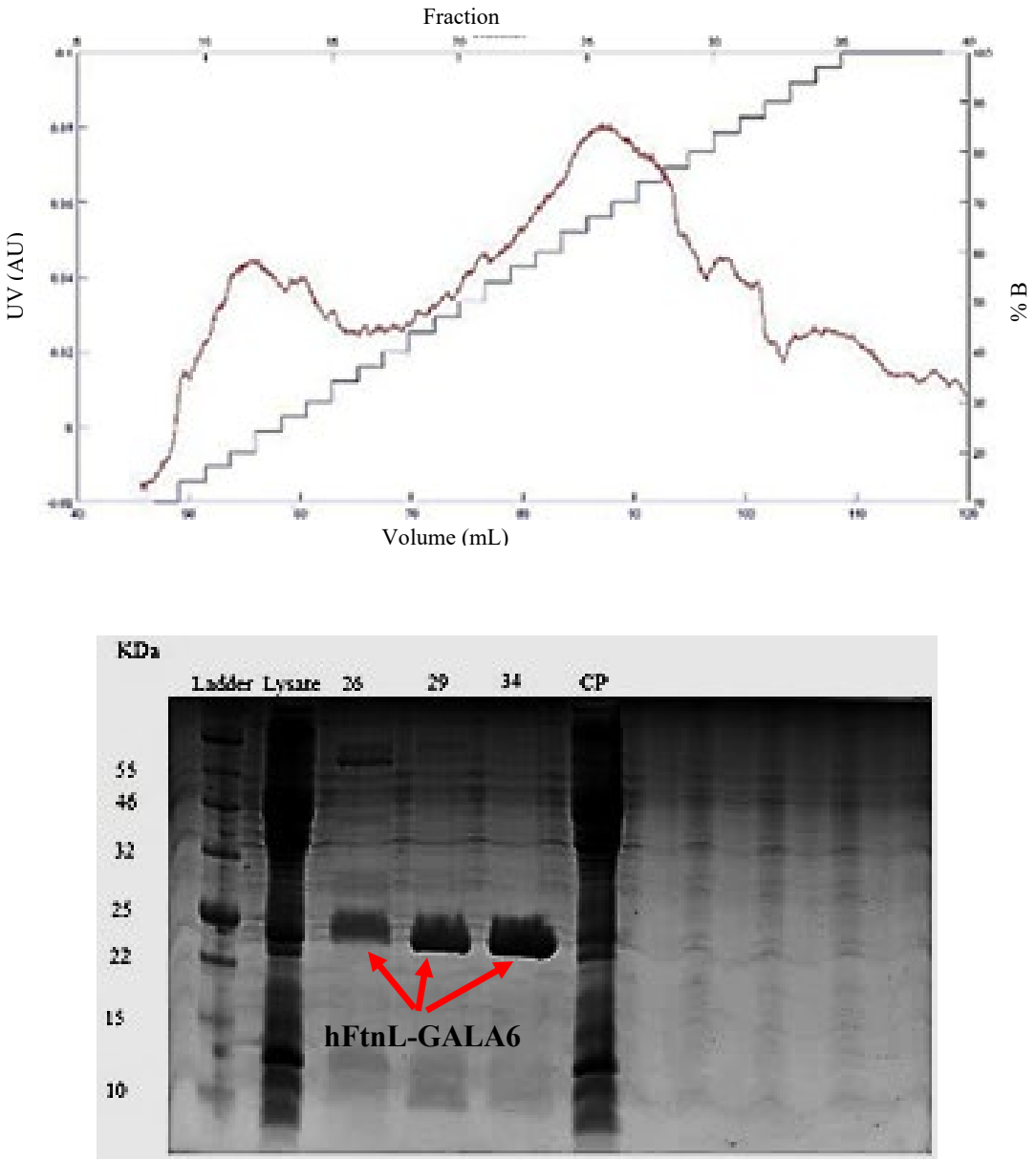


Figure 2.5 Purification using HisTrap column showing increased extraction. Protein extraction with pH 7.4. Buffer conditions -: **Lysis/Washing Buffer –** 50mM Tris-HCl, 300mM NaCl, 20mM Imidazole, 10mM BME, 5% Glycerol. **Elution Buffer** - 50mM Tris-HCl, 300mM NaCl, 500mM Imidazole, 10mM BME, 5% Glycerol. **[A]** Chromatograph obtained after using His Trap column. **[B]** SDS- PAGE gel showing fractions, lysate, and lysed cell (CP) after protein extraction.

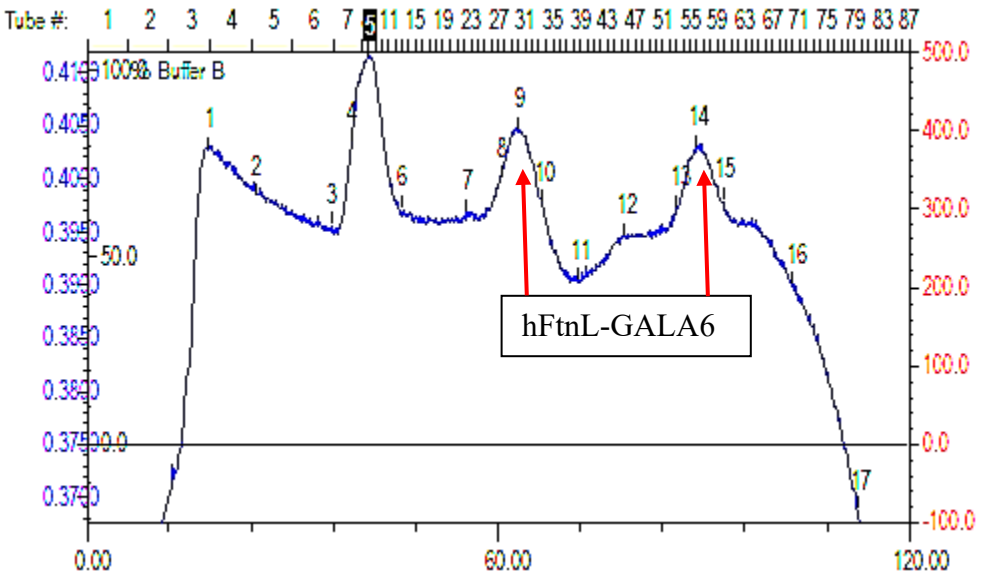


Figure 2.6 SEC Chromatogram of final purification of hFtnL-GALA6

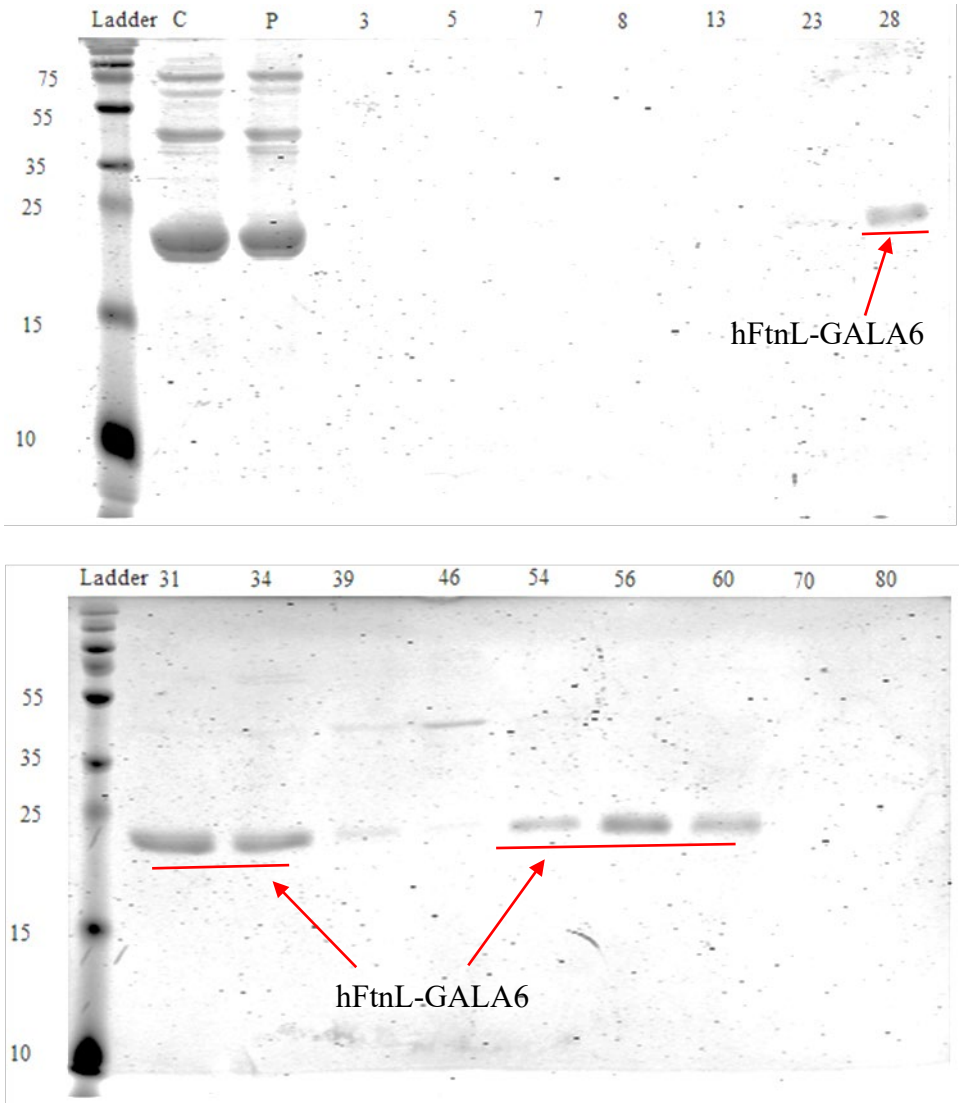


Figure 2.7 15%SDS-PAGE gel showing hFtnL-GALA6 bands in fractions from SEC purification.

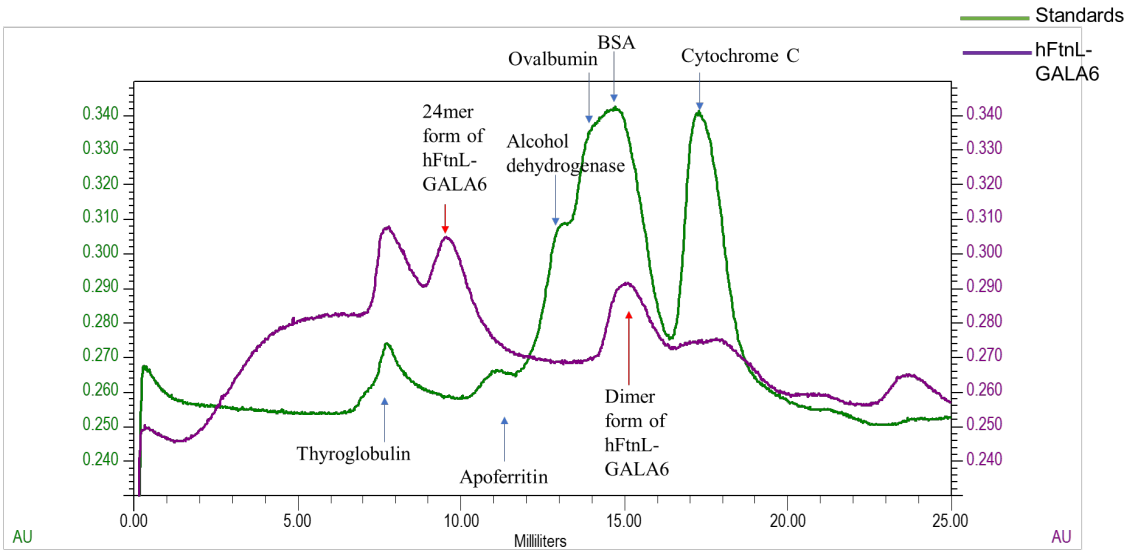


Figure 2.8 Molecular weight determination chromatogram
Overlapped chromatogram of standards and hFtnL-GALA6 protein solution. Concentration of hFtnL-GALA6 is 0.4 mg/mL

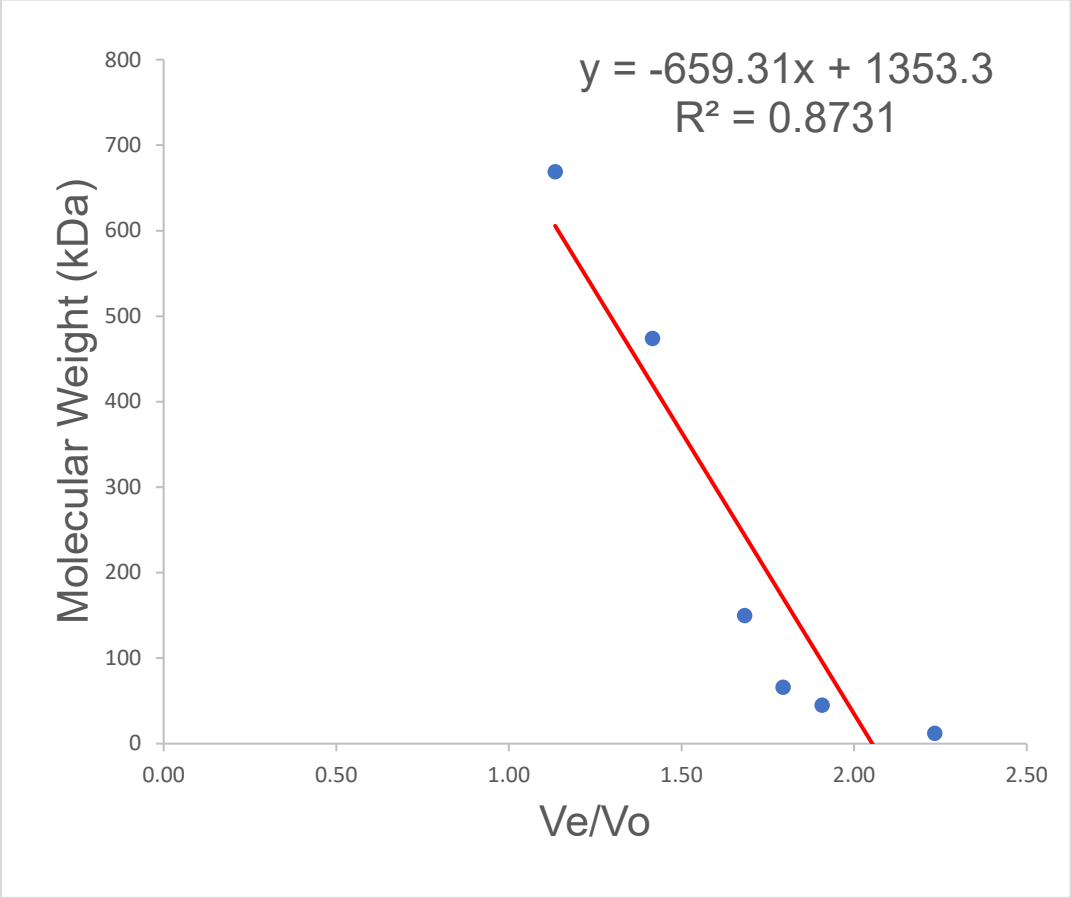


Figure 2.9 Standard curve for molecular weight determination.

CHAPTER 3: STRUCTURAL CHARACTERIZATION OF hFtnL-E4

3.1 BACKGROUND

Working on hFtnL-GALA6 allowed us to understand the working of the EALA sequence. In this peptide, Glutamate (E) was the primary driving force that would change the E-helix structure to control disassembling and assembling of the protein. So moving forward, we found another peptide sequence, EAQA, designed using Ensemble Allosteric Model (EAM), and its efficiency to cause structural changes were analyzed by modifying allosteric protein c4, which fuses maltose-binding protein (MBP) and TEM1 β-lactamase (BLA).[30] Initially using PyMol [46], PHYRE2 [47], and FATCAT [48] we tried to estimate the structure of EAQA modified hFtnL by completely replacing E-helix. Results of this modification suggested the formation of the helix. However, analysis of WT-hFtnL revealed that some of the amino acids of the E-chain were near other chains (A, B, C, D) to form different types of interactions. These kinds of synergy suggested that the WT-hFtnL might be more stable and soluble to be produced in higher concentrations than GALA modification.[32] Comparing these data, we decided to mutate every fourth amino acid with Glutamate in the E-helix of WT- hFtnL as well as to add the EAQA repeat on C-terminus to form an extended E-helix with four glutamate amino acid residues spaced appropriately to form a pH-responsive helix similar in nature to GALA, but less hydrophobic. Analysis of secondary structure using Pymol of the newly modified hFtnL,

produced using PHYRE2 to model the mutant structure, showed that E-chain amino acid residues that might be interacting with other helices were not disturbed. Table 3.1 summarizes all the sequences that were tested. WT-hFtnL, hFtnL-E4, and hFtnL-E6 were the proteins that were ordered after comparing GRAVY scores. Grand Average of Hydropathicity index (GRAVY) indicates hydrophobicity of protein. GRAVY score below 0 are more hydrophilic protein. We hypothesized that the lower the GRAVY score, the better would be the solubility. Due to the lack of any data available for new modifications of Human Light Chain Ferritin, we decided to start from a small scale and increase it to laboratory level large-scale production.

3.2 EXPRESSION AND PURIFICATION OF hFtnL-E4

The parent plasmid chosen was pET28a+. To form hFtnL-E4, pET28a+ was digested using Ndel at 5' end and Xhol at 3' end and then the hFtnL-E4 sequence was cloned into the plasmid. N-terminal of the protein is tagged with 6x Histidine for Nickel affinity purification. E4 modification is present at the C-terminal. Similar method is applied for hFtnL-E6. WT-hFtnL has no modification so it is simply cloned in pET28a+ using above mentioned restricition enzyme with 6x Histidine tag at N-terminal.

Analyzing various sequences for hydrophobicity in comparison to hFtnL-GALA6, hFtnL-E4 was found to be least hydrophobic. In this sequence instead of totally replacing E-helix, every fourth amino acid of the native E-helix sequence was replaced with glutamic acid (E). hFtnL – PEAG**E**GEY**E**FER**E**TLK**E**AQAE (hFtnL-E4) was the sequence that was analyzed first. There is no data available for this sequence so small-scale overexpression analysis was performed. All three plasmids that were ordered, were overexpressed at 37°C for 4h and 6h with IPTG of concentrations 0.1 mM, 0.25 mM, 0.5

mM, 1 mM. B-per (thermo fisher) was used to lyse the cells. All the samples were sonicated using a water bath sonicator for 15 min. Non-induced, induced, soluble protein solution and lysed cell pellet were collected and observed on 15 % SDS-PAGE gel. Results (Figure 3.1 A & B) showed overexpression of proteins (hFtnL-WT, hFtnL-E4 and hFtnL-E6) but there was no extraction as no band was observed near 25 kDa ladder. As all the modified proteins were new except WT-hFtnL, we decided to troubleshoot expression of WT-hFtnL so we could use that information to improve purification of other proteins. WT-hFtnL was expressed in *E. coli* BL21 DE3 cells using pET28+ plasmid. This plasmid adds six Histidine tag at the N-terminal of the protein. According to the literature on Histidine-tagged WT-hFtnL, we decided to use 20°C to grow cells overnight.[32] Protein overexpression was induced using 1mM IPTG. For the proper folding of the protein and decreasing formation of inclusion bodies, we added the pGKJE8 chaperone plasmid.[32] Even though we were able to highly overexpress protein, during initial extraction using a 1x PBS buffer, the protein was degraded to form a thick dark band near 15 kDa. This can be inferred from Figure 3.2 in which Induced non – lysed cells did not have any band at 15 kDa but lysed cells and soluble protein showed the band at 15 kDa as these samples were subjected to sonication or lysis. Although this can only be confirmed by Mass Spectrometry. However, since we still did not have any protein band near 25 kDa so, we decided to analyze different parameters for the buffer and lysis method. While analyzing suitable buffer conditions to keep the protein most stable and decrease degradation of protein, we used to collect only 1 mL of cells, and we decided to vortex cells for 1 min as the sample amount was very low for sonication or any other lysis instrument. At the end of the analysis best lysis buffer decided was 50 mM Sodium phosphate buffer with 150 mM

NaCl, 20% Glycerol, 1 mM EDTA at pH 7.4. Modulating different sonication parameters and comparing them with the French press method for lysis of 2 g *E. coli* BL21 DE3 pET28a-WT- hFtnL pGKJE8 cells mixed in 30 mL lysis buffer, it was clear that using sonicator at 50% amplitude for 50 sec with 10 sec O/N and 30 sec OFF will give us the best result. Table 3.2 summarizes all the variations that were attempted to overexpress the protein and make the best protocol for extraction of protein. Figure 3.3 clearly shows the effect of all the conditions that were decided at the end of small-scale overexpression.

For characterization of pH-sensitivity in hFtnL-E4 structure, it is vital to produce the protein at a large scale. Our main aim in this part was to have hFtnL-E4 in 24mer form at a very high concentration. Conditions defined during small-scale overexpression study was used at a large scale. Although Human L-chain Ferritin does not have a ferroxidase center, one of the analyses suggested mutation in this protein can disrupt Ferritin function leading to neurodegenerative disease.[33] So we decided to add Ferric Citrate while overexpression, Although Human L-chain Ferritin does not have a ferroxidase center, one of the analyses suggested mutation in this protein can disrupt Ferritin function of iron mineralization, leading to neurodegenerative disease.[33] So we decided to add Ferric Citrate during overexpression and protein extraction, to increase protein folding with significantly less degradation (15kDa band). FtnL-E4 was overexpressed in *E.coli* BL21 DE3 pGKJE8 cells using defined condition from small scale analysis with 3 mg/L Ferric citrate, except 0.5mM IPTG was used to induce the protein production. After harvesting, cells were mixed in Lysis buffer + 1 mg/mL lysozyme. After heating the cell solution at 70°C and sonicating them, lysate was separated from cells using centrifugation at 16000 RPM for 45 min. hFtnL-E4 was purified using Affinity chromatography through a nickel column. Two peaks

were observed during Nickel column purification containing hFtnL-E4 (Figure 3.3 Top). Chaperone proteins that were used for proper folding were also observed on SDS-PAGE in the same fractions in which Ferritin was present (Figure 3.3 Botom), as there were bands observed at 70, 40 and 15 kDa ladder,[49] 18 mg/mL protein concentration in 2.4 mL solution was achieved without any precipitation which was further purified using a SEC column. There was single peak observed corresponding to 24-mer of hFtnL-E4 during SEC purification. However, chaperone proteins were still present with hFtnL-E4. Moreover, precipitation was observed while concentrating the protein below 3 mL. Precipitated protein pellet was separated by centrifugation and then again solubilized in Nickel column elution buffer, which contained 500mM imidazole. Single peak was observed in SEC purification of resuspended precipitated protein. Figure 3.5 shows the comparison between chromatograms of soluble protein, resuspended protein, and Horse spleen apoferritin (obtained from Sigma). All three peaks have almost similar retention time which meant that precipitated protein was successfully resuspended in elution buffer, and it is also in 24-mer form. As Ferric Citrate was used during cell growth and overexpression, so there is a chance that native *E. coli* Ferritin might be getting overproduced due to which there were two distinct peaks observed in Nickel column. Two peaks could mean that there could be one protomer, a mix of hFtnL-E4 and native *E. coli* Ferritin subunit, and another protomer that could have only hFTnL-E4 subunit. This was the hypothesis so; next purification was performed without using Ferric Citrate at any point.

Figure 3.4 Top shows the single peak shifted towards higher Imidazole concentration which was observed after not using of Ferric Citrate during overexpression. This confirms that E. coli native Ferritin was getting overexpressed. Shifting of the peak

towards higher Imidazole concentration was the result of more tight binding of hFtnL-E4 suggesting that there is only His-tagged protein is present. However, during purification of protein using SEC column two peaks were observed (Figure 3.4 Bottom). Both the peaks contained hFtnL – E4 which meant that there are two forms of hFtnL-E4 are present. This was observed for the first time which could be result of not using Ferric Citrate during extraction. This confirmed that Ferric Citrate helped in proper folding of hFtnL-E4 in 24mer form after cell lysis.

Analyzing many SDS-PAGE gel during all the troubleshooting we observed that there were three bands that were always present in the same fractions and same thing was observed when pGkJE8 was co-expressed with hFtnL-GALA6. These observations and previous literature confirmed that chaperone proteins remain attached to protein which could be result of incomplete protein folding mechanism. Morales et al. showed that chaperone proteins could be removed by using Mg-ATP during Nickel column purification, but this resulted in precipitation of protein while concentrating it[14]. Since ATP is required for the removal of so may be adding extra source energy during overexpression in growth media could help in completion of folding mechanism by chaperones. So instead of adding glucose to LB media, Terrific Broth (TB) media was used as it contained glycerol which could also work as ATP source. At last TB media used for the growth of overexpressed cells and Ferric Citrate was used during extraction. As hFtnL-E4 was always eluted after 47% of Elution buffer in linear gradient method. For more detailed purification protocol refer appendix.

In Final protocol pGKJE8 + pET28a hFtnL-E4 transformed cells were overexpressed in 1L Tb media with 0.5 mg/mL L-Arabinose, 5 ng/mL Tetracyclin, 50

µg/mL Kanamycin, 20 µg/mL Chloramphenicol and 1mM IPTG. Cells were harvested at 6000 RPM for 10 min. Cell pellet was resuspended in Lysis buffer (50 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA, 20% Glycerol, 20 mM Imidazole, 1mg/mL Lysozyme, pH 7.4) and then frozen in -80°C. Frozen cell pellet was liquified at 37°C and then again diluted to 120 mL using Lysis buffer. 200 µL Ferric citrate was added to neutralize EDTA and for proper folding of protein. Then cell solution was heated at 70°C for 15 min. 1mM PMSF was added, and sonication was performed to lyse cells. Lysate was separated at 16000 RPM for 45 min. HisTrap HP 5 mL column was equilibrated using washing buffer (20 mM Sodium Phosphate, 500 mM NaCl, 20 mM Imidazole, pH 7.4) and then Lysate was loaded on the column. Eluted protein obtained after using gradient method on Nickel column was concentrated to 2.4 mL and 5% glycerol was added before loading 2 mL sample on Superdex 200 Hiload 16/60 column. Samples were observed on 15% SDS-PAGE and then solution was concentrated to 2.5 mL. 2 mM hFtnL-E4 was confirmed using Bradford protein estimation assay. Single peak in chromatograms obtained from both the columns confirmed formation of properly folded protein (24mer) which can be seen in Figure 3.6

Table 3.1 List of sequences designed and there GRAVY score comparison.

Name	**E-helix sequence**	**No. of amino acids**	**GRAVY score**
WT-hFtnL	PEAGLGEYLFERLTLKH	13	-0.594
hFtnL-EALA6	PEAG**LAEALAEALAEALAEALAEALAE**	23	-0.436
hFtnL-EAQA4	PEAG**QAEAQAEAQAEAQAE**	15	-0.655
hFtnL_EAQA6	PEAG**QAEAQAEAQAEAQAEAQAEAQAE**	23	-0.663
hFtnL-E6	PEAG**E**GEY**E**FER**E**TLK**E**AQA**E**AQA**E**	21	-0.721
hFtnL-E4	PEAG**E**GEY**E**FER**E**TLK**E**AQAE	17	-0.718

Table 3.2 Troubleshooting performed during Small-scale overexpression to determine best conditions to extract protein.

Plasmid	Overexpression condition	Lysis buffer condition		Lysis method	Best condition inferred
		Constant	Variable		
pEt 28a+ -hFtnL E4 with pGKJE8 (GroEL-GroES and dnaK-dnaJ-grpE).	50µg /mL Kanamycin, 20µg /mL Chloramphenicol, 0.5mg/mL L-Arabinose, 20ng/mL Tetracyclin, 20°C, overnight, and 1mM IPTG.	100mM NaCl, 5mM EDTA, pH 7.4	20- and 50-mM Sodium Phosphate	1 mg/mL Lysozyme and 1 min Vortexing	50 mM Sodium Phosphate. Degradation of protein observed
		50 mM Sodium Phosphate, 5mM EDTA, pH 7.4	100, 150, 500 mM, and 1M Sodium Phosphate		150, 500 mM and 1 M had same result. Degradation observed. 150 mM NaCl was used for further analysis.
		50 mM Sodium Phosphate, 5mM EDTA, pH 7.4, 150mM NaCl	10%, 20%, 50% Glycerol		20% Glycerol was best. Still degradation was observed.

		50 mM Sodium Phosphate, 5mM EDTA, pH 7.4, 150mM NaCl, 20% Glycerol		1mg/mL Lysozyme	Sonication, French press, Freeze thaw.	Except sonication none of the technique was able to Lyse cells. So sonication was further analyzed
					Multiple conditions of Sonication	Sonication – 50sec, 50% amplitude, 10 sec ON, 30 sec OFF resulted least degradation and better extraction.
		50 mM Sodium Phosphate, pH 7.4, 150mM NaCl, 20% Glycerol	1 mM and 5 mM EDTA	1mg/mL Lysozyme and Sonication – 50sec, 50% amplitude, 10 sec ON, 30 sec OFF		1 mM and 5 mM EDTA sonication had same result so, 1 mM EDTA was used as final condition.

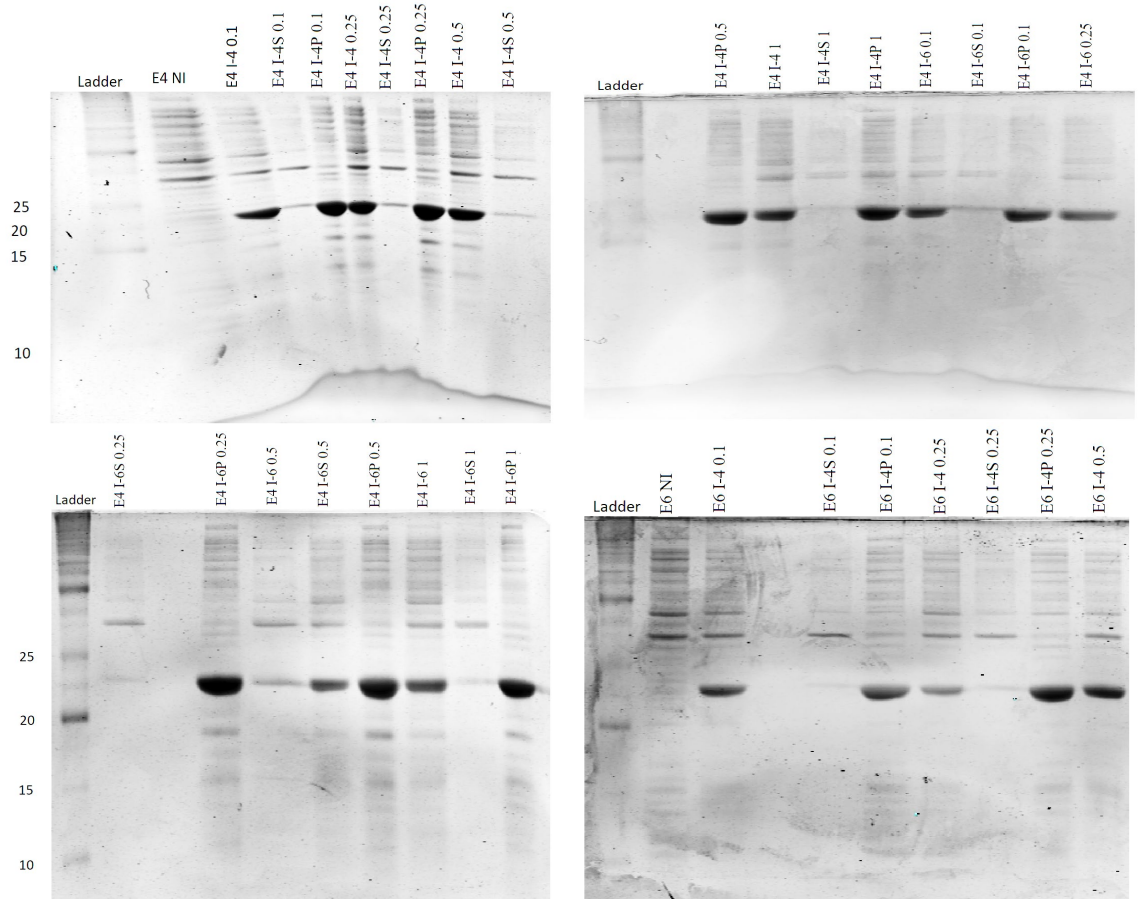


Figure 3.1 A WT-hFtnL, hFtnL-E4, hFtnL-E6 overexpressed in various conditions. Overexpression Temperature - 37°C, induced using IPTG – 0.1, 0.25,0.5, 1 mM, Induction time – 4 hours and 6 hours.
WT I-4 0.1- WT-hFtnL induced cells for 4 hours using 0.1 mM IPTG, **WT I-4P 0.1 -** Lysed WT-hFtnL pellet collected after protein extraction, induced for 4 hours using 0.1 mM IPTG, **WT I-4S 0.1 –** Solublized protein obtained after lysis**.**

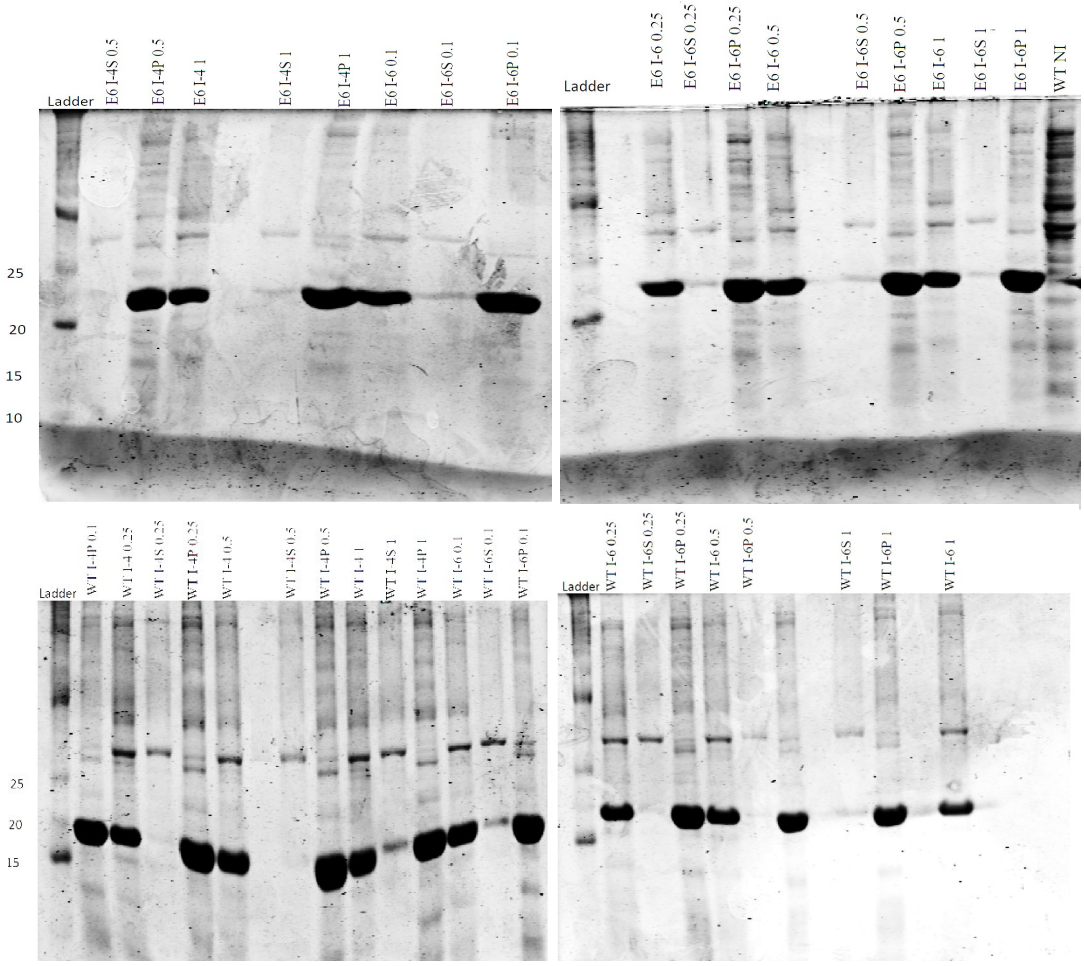


Figure 3.1 B WT-hFtnL, hFtnL-E4, hFtnL-E6 overexpressed in various conditions. Overexpression Temperature - 37°C, induced using IPTG – 0.1, 0.25,0.5, 1 mM, Induction time – 4 hours and 6 hours.
WT I-4 0.1- WT-hFtnL induced cells for 4 hours using 0.1 mM IPTG, **WT I-4P 0.1 -** Lysed WT-hFtnL pellet collected after protein extraction, induced for 4 hours using 0.1 mM IPTG, **WT I-4S 0.1 –** Solublized protein obtained after lysis**.**

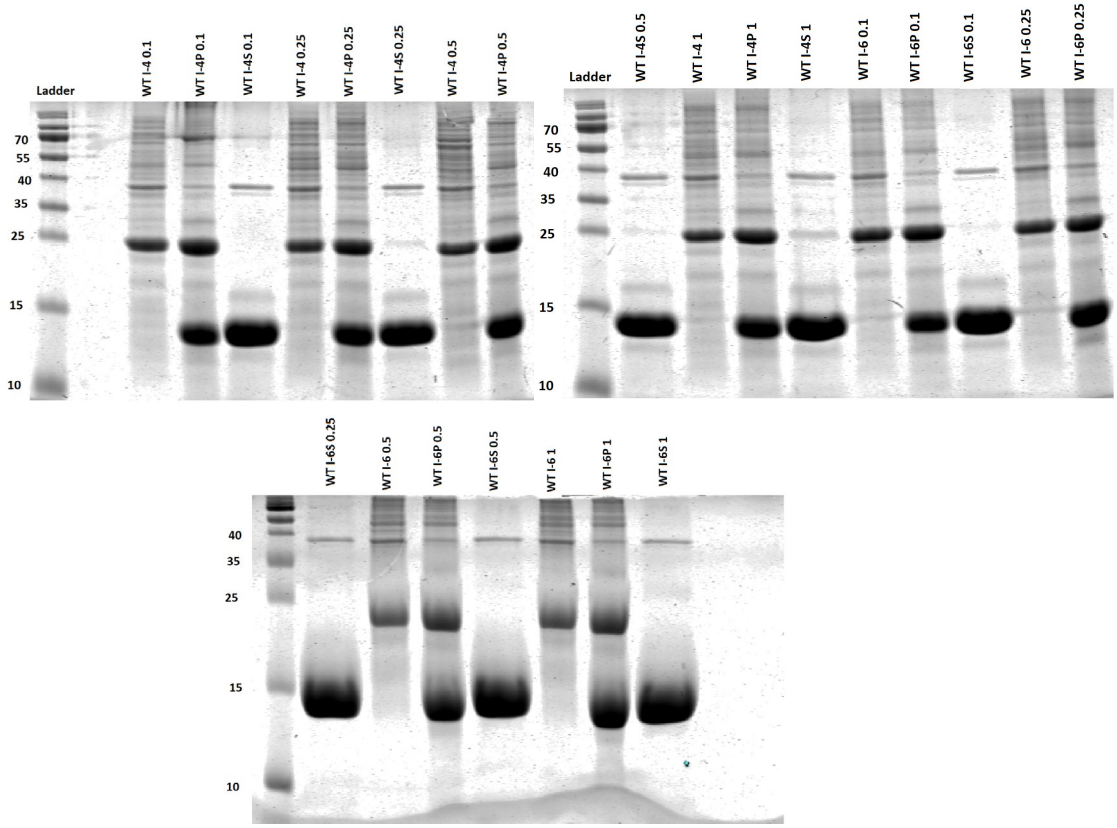


Figure 3.2 WT-hFtnL overexpressed in various conditions. Overexpression Temperature - 20°C, induced using IPTG – 0.1, 0.25,0.5, 1 mM, Induction time – 4 hours and 6 hours.
WT I-4 0.1- WT-hFtnL induced cells for 4 hours using 0.1 mM IPTG, **WT I-4P 0.1 -** Lysed WT-hFtnL pellet, induced for 4 hours using 0.1 mM IPTG, **WT I-4S 0.1 –** Solublized protein obtained after lysis**.**

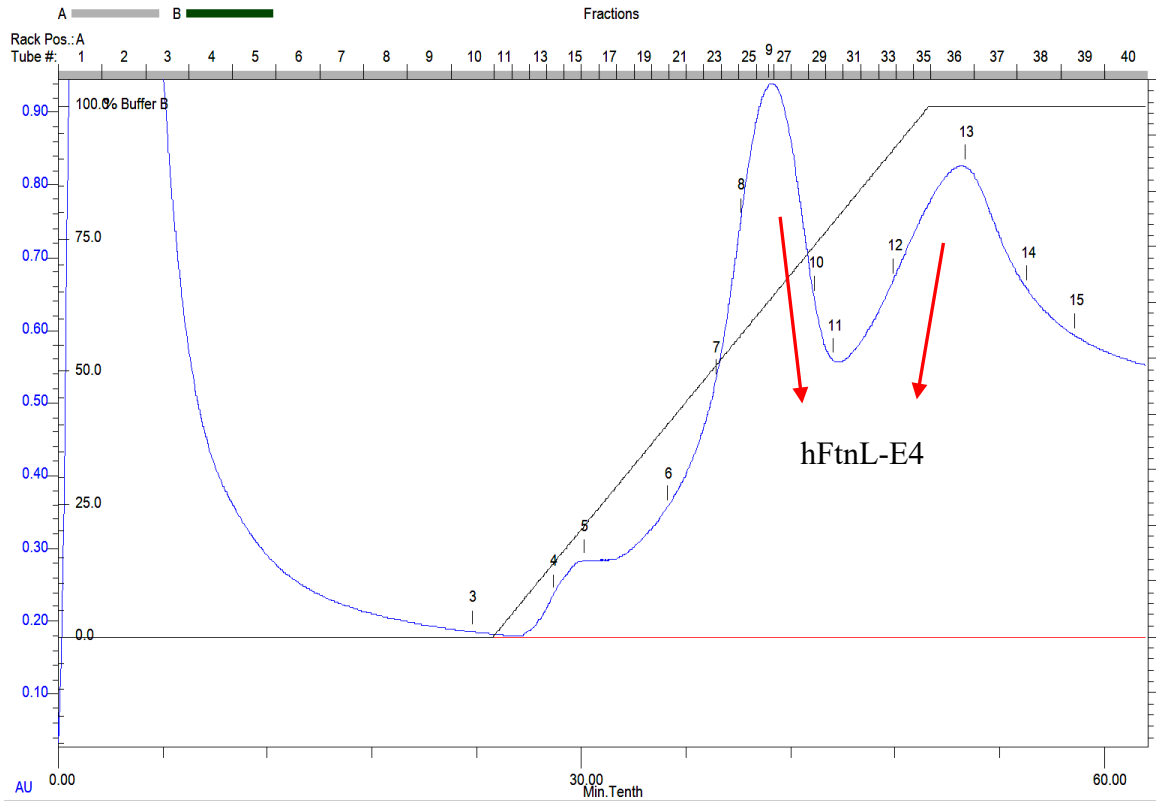


Figure 3.3 Chromatogram for Purification of hFtnL-E4 with Ferric Citrate using 5 mL HisTrap column.
3 mg/L Ferric Citrate is used during protein overexpression. 4 mg/mL Ferric Citrate is used during Protein extraction.

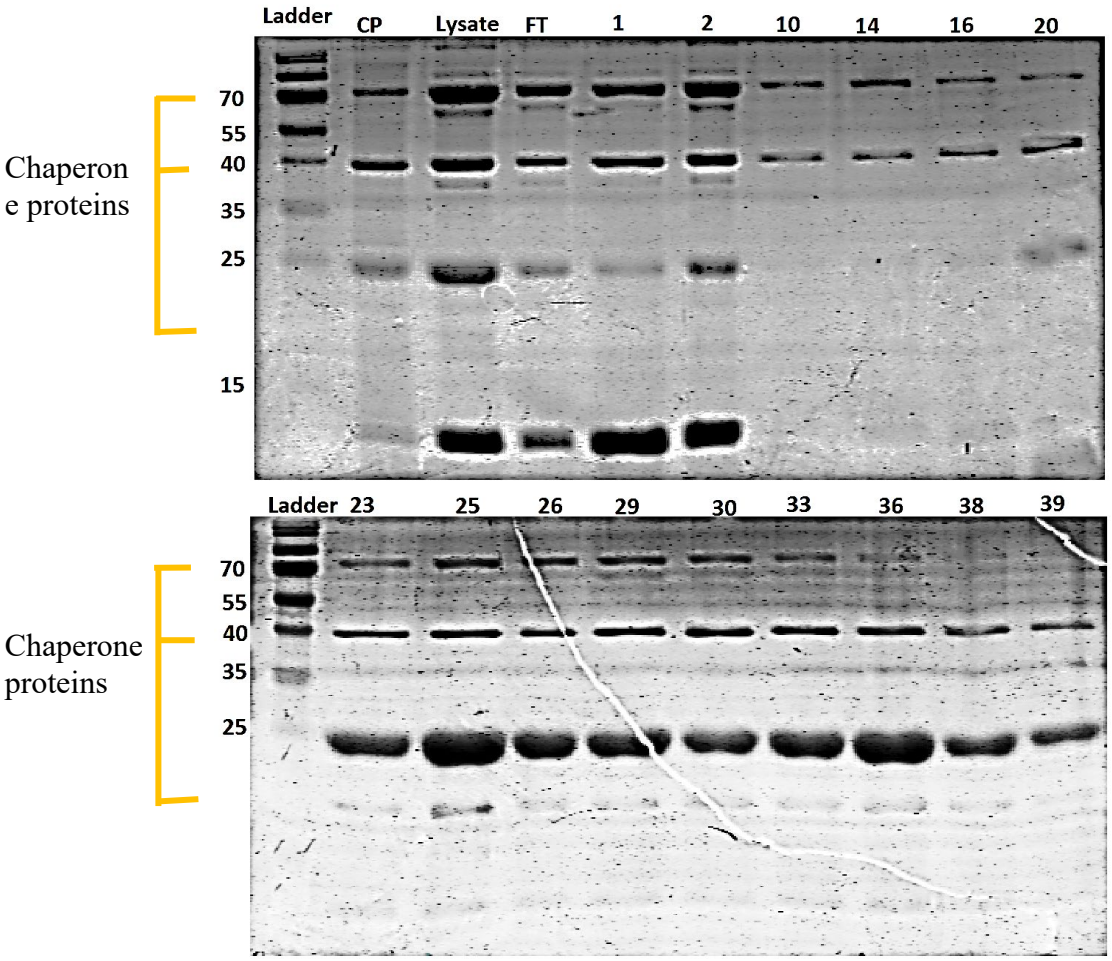
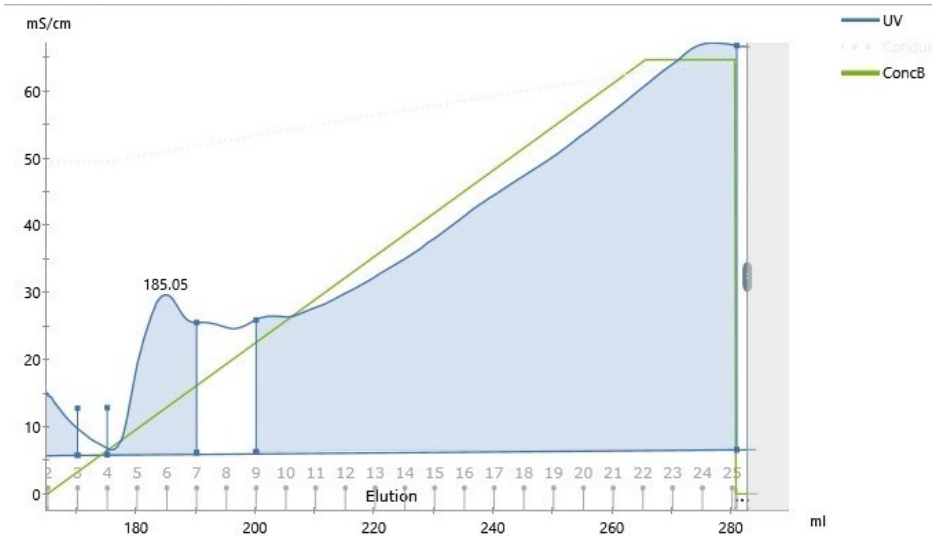


Figure 3.4 15% SDS – PAGE of hFtnL-E4 with Ferric Citrate purified using 5 mL HisTrap column.
3 mg/L Ferric Citrate is used during protein overexpression. 4 mg/mL Ferric Citrate is used during Protein extraction.

A



B

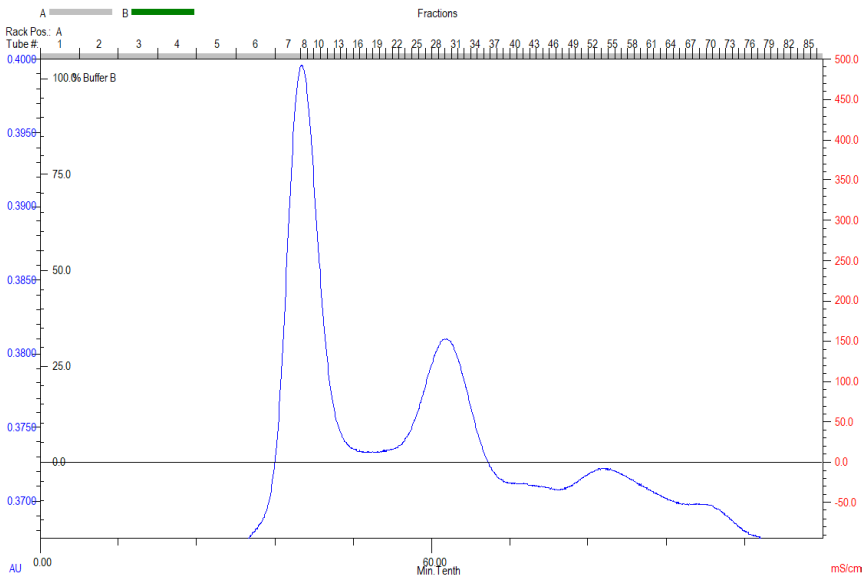


Figure 3.5 Purification of hFtnL-E4 without adding Ferric citrate.

A) No peak observed in HisTrap protein purification indicating only single type of protein being overexpressed.

B) Concentrated protein solution showed 2 peaks of hFtnL-E4 concluding that addition of Ferric citrate during extraction helps in proper folding of protein.

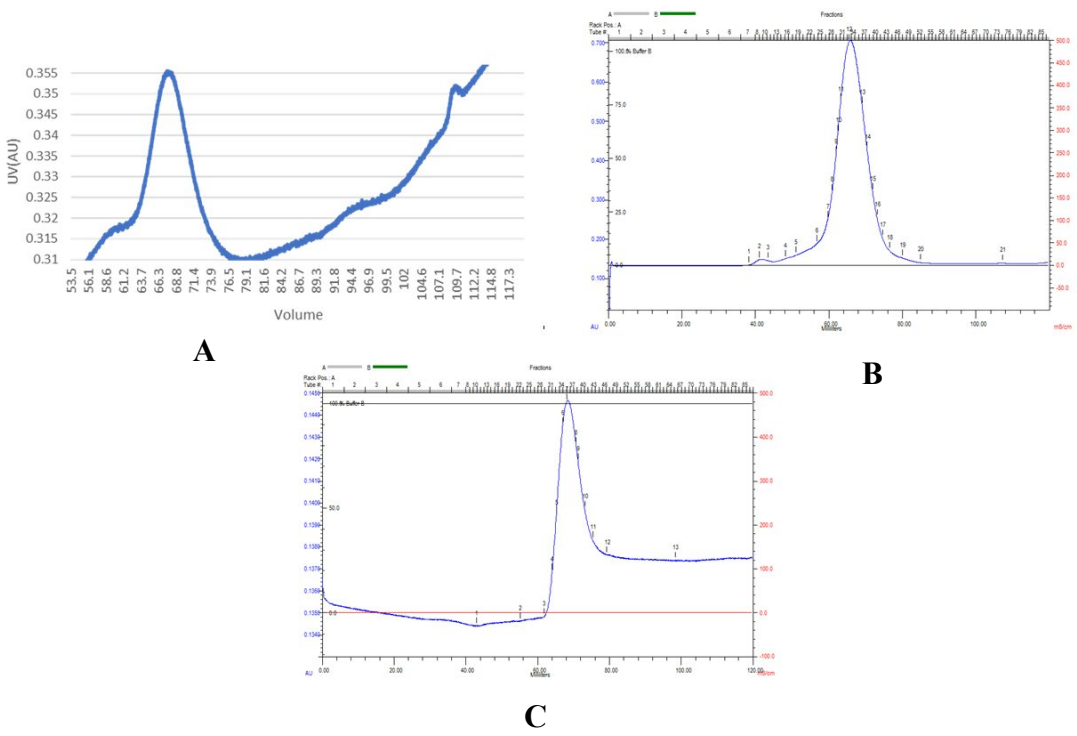


Figure 3.6 Comparison between **(A)** Horse spleen Apoferritin, **(B)**Soluble hFtnL-E4, and **(C)** resuspended hFtnL-E4.

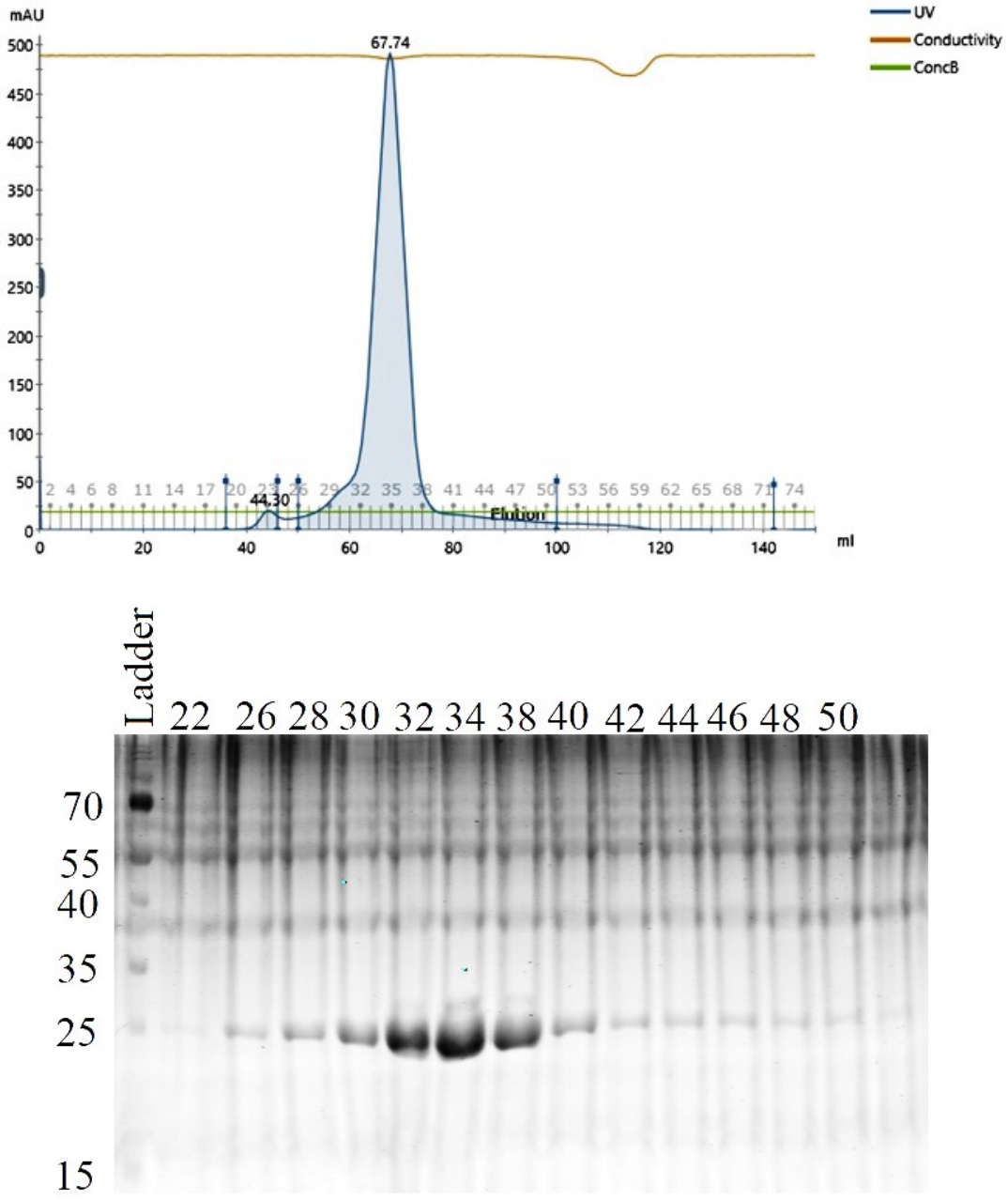


Figure 3.7 Final Superdex 200 Hiload 16/60 purification of hFtnL-E4 using elution buffer.
Elution Buffer – 20 mM Sodium Phosphate buffer, 500 mM NaCl, 500 mM Imidazole pH 7.4

CHAPTER 4: FUTURE DIRECTIONS

4.1 EXPRESSION AND PURIFICATION OF OTHER MODIFICATIONS OF FERRITIN

After many troubleshooting a protocol to extract and purify Ferritin based protein was formed. WT-hFtnL, hFtnL-E6 is another modification of human light chain ferritin that was ordered with hFtnL-E4. Moreover, hFtnL-EALN6 is another modification that was formed by our collaborator Dr. N. Grossoehme. These proteins are embedded in pET28a plasmid. All these plasmids should be extracted and purified using the finalized protocol. If results are similar for all the proteins as compared to hFtnL-E4 then we can generalize this protocol for any human light chain ferritin-based protein.

4.2 IDENTIFICATION OF BEST MODIFICATION

After purified hFtnL-E6, hFtnL-EALN6 is obtained then we need to study the characteristics of all the modifications. At the end of purification protocol proteins remain in high concentration of Imidazole (500 mM). There are two contradictory research papers that showed that Imidazole is good for solubilizing a protein and another shows that it has inhibitory effect on protein activity.[50,51] Keeping both the papers in mind protein when need to be stored for very long time then buffer having high concentration of Imidazole should be used. However, disassembling and assembling protein is protein-protein interaction so to have effective interactions Imidazole should be removed from the solution. Now the later step in which Imidazole will be removed from the solution might cause

protein precipitation. At this step if any of the above-mentioned modification do not precipitate can be considered very soluble is good for further studies.

The buffer exchange step is good step to identify soluble protein but should be considered secondary method to identify a good modification. The ferritin modification which shows maximum disassembling and assembling of protein at very short range of pH change should be the first iterative method to identify the best modification. According to previous data available on hFtnL-GALA6 disassembling of protein occurs at pH 6 so, if any ferritin modification that disassembles in pH range 7 – 6 should be considered a better modification. According to our main aim we are modifying the ferritin to load various drugs in the nanocage by simply making it pH responsive. Even native ferritin is pH responsive, it requires very acidic condition to dissociate which most of the therapeutic drugs cannot withstand. So, any modification that requires smaller change in pH to cause disassembling and assembling will be best as it will cause less side effect on drug that is being loaded.

4.3 CHARACTERIZATION OF pH RESPONSIVENESS

hFtnl-E4, hFtnL-E6 and hFtnL-EALN6 extraction and purification completes the first part of Aim 1. To complete second part of Aim 1, pH responsiveness needs to be characterized. Table 4.1 shows the molecular weight of 24mer (assembled), monomer, and dimer (disassembled) form of all three modifications. The techniques that will be used for characterization are Size Exclusion Chromatography, Circular Dichroic Spectra, and Dynamic Light Scattering. In case of Size Exclusion chromatography peaks referring to oligomer state and monomeric or dimeric state should be observed. To initiate characterization, whatever analytical SEC column will be used first standards needs to be

run. Each standard that will be used should be run separately instead of mixing in one solution since some standards tend to coelute which will cause changes in V_e value and ultimately give wrong R^2 value. Additionally, we do not need to run all the standards as we just have to analyze assembled and disassembled state so only Thyroglobulin, Apoferritin, Alcohol dehydrogenase, BSA and Cytochrome C. This step with standards needs to be repeated till we get $R^2 = 0.99$. To prepare sample, buffer of the protein solution should be exchanged with Analysis buffer (20 mM Sodium Phosphate buffer pH 7.4, 100 mM NaF). This step is important as the same sample must be used for all the techniques that will be used for characterization of pH responsiveness. Additionally, Imidazole and NaCl has very high absorption value which will cause problem in analyzing the protein using CD and DLS. Moreover, the concentration required for all three techniques is very less so there might not be requirement to troubleshoot the buffer condition to get high concentration of protein in solution. Sample and standard volume for SEC should be decided according to the manual provided with the column. Concentration of hFtnL-E4, hFtnL-E6 and hFtnL-EALN6 should be equal to the concentration of Apo ferritin. Most probably single peak should be observed which should have retention time between Thyroglobulin and Apoferritin. Using this data molecular weight of assembled state can be easily determined. Circular dichroism measures the secondary structure present in protein structure. According to the hypotheses at pH above 7, E-helix is supposed to form irregular structure and at pH below 6 E-helix should form α-helix. However, the change in helical structures for other peptides (A, B, C, D) of protein subunit, while changing pH are not recorded previously for native ferritin. Previous studies on hFtnL-GALA6 show decrease in overall α-helical structure when pH was decreased from 7 to 6 and the same sample was used for

SEC column and it showed peak corresponding to monomeric state of protein.[38] So, in our experimentation with E4, E6, and EALN6 modifications higher irregular structure should correspond to disassembled state of the protein. CD analysis should be done in Far-UV range (180 – 280 nm). Jasco CD model J-815 will be used with Spectrocell Inc. 1mm quartz cuvette. It is recommended to not exceed 0.1 mg/mL for 1mm path length.[52] For all the analysis, conditions should not change. Blank buffer solution should be analyzed first and then sample should be analyzed after subtracting blank. Scanning speed should be 10 nm/min at 25°C.[38] There three most important points to keep in mind firstly, the concentration of protein should be accurately measured as it will be used in analyzing the sample on different software. Secondly, consistency very important which include cuvette size, shape, path length and company. Also, the side of cuvette facing detector and lamp should be consistent. Third is clarity of sample. Since there is a chance that protein might get absorbed by 0.22 μm filter the best way to clear the protein solution of any precipitates is by centrifuging at 13000 RPM for 2 min. After successful analysis, data obtained can be analyzed using CAPITO software which available online.[53]

Non-invasiveness of Dynamic Light scattering (DLS) makes it widely accepted protein characterization technique. DLS in this project will be used to determine molecular weight and radius of gyration of protein molecule. For the initial experimentation sample prepared for CD should be used. However, if the good result is not obtained then either the concentration of protein sample or buffer solution conditions should be troubleshooter.

To analyze disassembled state of protein, protein precipitation needs to be analyzed. If protein precipitates at pH 6 then pH titration must be done. pH titration is important to monitor the pH at which protein starts precipitating out. The main reason behind this step

is to keep the protein in the solution while it is disassembled. Once the lowest pH at which the protein remains soluble is determined then that pH can be used to make a new buffer. Then perform buffer exchange of protein sample and again perform all the steps that were done at pH 7.4.

4.4 STRUCTURAL STUDY

To fully understand the structure X-ray crystallography and Small Angel X-ray Scattering (SAXS) must be performed. Crystallizing protein will "freeze" the structure which can be analyzed using X-ray. PDB deposit 2FFX shows the crystal structure of wild type human light chain ferritin has been published.[54] Wang et al. analyzed multiple crystals in different buffer conditions out of which the conditions out of which 2FFX was published can be used to analyze new protein modifications. If the published conditions to crystallize structure do not work efficiently then the course of action will be to give sample to a lab which has expertise in this technique. Although X-ray crystallography will help in analyzing structure but analyzing structure in solution is very important. Protein structure in solution might behave differently which can be analyzed by SAXS. However, SAXS will be very new technique for our lab so many conditions will need to be troubleshooter to get best structural data.

4.5 LOADING OF NANOCAGE

Second aim of this project includes loading the Nano cage with doxorubicin. As our main aim of this project is to develop pH responsive human light chain Ferritin nanocage which will be able to deliver drug through endocytic pathway we need to load the new modifications of ferritin with doxorubicin. Loading of doxorubicin on Apo ferritin (horse spleen) has been tested in previously literature. In this literature they used Rose

Bengal (RB) which can stimulate disassembling of the protein using low pH and laser irradiation. The techniques and method published in this paper can be used for loading doxorubicin on nanocage of novel proteins and can be analyzed *in vitro, in vivo* in cancer cells and mouse models.

Table 4.1 List of proteins and the molecular weight of monomer, dimer, and 24 – mer.

Protein Name	Monomer	Dimer	24 - mer
hFtnL-E4	21397.89	42,795.78	513,549.36
hFtnL-E6	21797.29	43,594.58	523,134.96
WT-hFtnL	20958.64	41,917.28	503,007.36

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APPENDIX A: OVEREXPRESSION

Protocol

1. Made sure whether we have *E. coli* BL21 DE3 cells with pGKJE8 and pET28a - hFtnL-E4 in -80°C or transformation is required.
2. After confirmation leave them back in -80°C till the time starter culture.
3. Two 1L TB media in 4L flask was made. Composition of Large-scale TB media-
    a. TB is a highly enriched medium used for the cultivation of bacteria.
    b. 900 mL TB medium contain tryptone 1.2%, yeast extract 2.4%, glycerol 0.5% and 100 mL of 10X TB salt phosphate buffer (89 mM)
    c. TB medium:
        i. 1.2% tryptone = 12 g/L
        ii. 2.4 % yeast extract = 24 g/L
        iii. 0.5 % glycerol = 5 g/L
        iv. Dissolved tryptone, yeast extract and glycerol in water to a final volume of 900 ml and autoclave for 15 min at 121°C. Let it cool down to room temperature before adding the 10X TB salt solution.
        v. Made only 250 mL of 10X TB salt solution since only 200 mL will be used. Here is composition for 1 L 10X TB salt solution -
            1. 0.17 M KH_2PO_4 = 23.1 g/L

2. 0.72 M K_2HPO_4 = 125.4 g/L

vi. Dissolved 5.8 g KH2PO4 and 31.35 g K2HPO4 in water to a final volume of 250 mL and autoclaved for 15 min at 121°C.

d. Kept the autoclaved 10X TB salt solution at room temperature to cool down. Salts tend degrade the media at high temperature. So, waited till next day to make sure that all the solutions have cooled down to room temperature.

4. Terrific Broth media will be used for the cell growth. For the starter culture stock TB media was used. 20mL (1:50 of volume of large culture) starter culture was added to 50 mL falcon tube.

5. Made two starter culture for 2 Large-scale culture. Composition of starter culture

   a. 20 mL TB media from stock bottle in 50 mL falcon tube.

   b. Kanamycin – 50 µg/mL from 50 mg/mL stock solution in 20 mL starter media.

   c. Chloramphenicol - 20 µg/mL from 20 mg/mL stock solution in 20 mL starter media.

6. After making above solution take out the frozen cells described in first step and used 200 µL tip to scratch the top of frozen cells and mixed the cells in starter culture by shaking the tip in the solution. This step was performed very quickly before defrosting of the stock cells and as then the cells were stored back in -80°C quickly.

7. Incubated the starter culture in 37°C water bath shaker for overnight.

APPENDIX B: PROTEIN OVEREXPRESSION

1. Mixed 100 mL of 10x TB salt in TB medium. Then kept the 4L flasks in floor shaker and set the temperature to 37°C. Do not press start button otherwise it will start shaking the flask. At this point you just need to maintain temperature of the flask no shaking is required.
2. Now make the solution of antibiotics and chaperone inducers. Composition for 1L media -:
    a. Kanamycin – 50 µg/mL from 50 mg/mL stock solution i.e., 1 mL of stock solution. If there is not enough stock solution, then make 50 mg/mL stock solution in ddH2O.
    b. Chloramphenicol - 20 µg/mL from 20mg/mL stock solution i.e., 1 mL of stock solution. If there is not enough stock solution, then make 20 mg/mL stock solution in 100% Ethanol.
    c. L-Arabinose - you need 0.5 mg/mL in 1L media from 200 mg/mL stock solution. So, make 2.5 mL stock solution.
    d. Tetracycline - 5 ng/mL in 1 L media. concentration of 10 µg/mL. Tetracycline stock solution is already in -20°C.
    e. Added all the above solutions in 2.5 mL L-arabinose stock solution.
3. Poured above solution and 20 mL starter culture to Large-scale cultures and started shaking the cultures at 200 RPM.

4. Checked OD600 after 2 hours. Used TB media as blank. If the OD600 is around 0.5 then lower the temperature to 20°C. After 30 mins add 1mM IPTG and then leave it overnight.

APPENDIX C: CELL HARVESTING

1. Transfer the media to 1 L centrifuge bottles. Equalize the weight and then centrifuge the bottles at 6000 RPM for 10 min at 4°C.
2. Remove the supernatant and transfer the pellet to 50 mL Falcon tube.
3. Add 15 mL Lysis solution.
4. Make 1 mg/mL Lysozyme solution using lysis buffer according to the total volume of cell solution. For example, if the total cell solution is 30 mL then measure 31 mg Lysozyme. Mix it with 0.8 mL Lysis solution. Make up the volume to 1 mL and then transfer this solution to cell solution. Do not mix Lysozyme in water always use Lysis solution.
5. Vortex the cell solution to make sure that all the cells have dissolved in the solution and there is no lumps present.
6. Freeze the cells at -80°C.
7. Make sure you have all the buffers available for extraction purification.
    a. Lysis Buffer - 50 mM Sodium Phosphate, 150 mM NaCl, 1mM EDTA, 20% Glycerol, 20 mM Imidazole, pH 7.4
    b. Binding/Washing Buffer - 20 mM Sodium Phosphate, 500 mM NaCl, 20 mM Imidazole, pH 7.4
    c. Elution Buffer and gel filtration buffer - 20 mM Sodium Phosphate, 500 mM NaCl, 500 mM Imidazole, pH 7.4

APPENDIX D: EXTRACTION AND PURIFICATION

1. Take out the cells keep them in water bath shaker to defrost. Keep it shaking for faster defrosting. Also switch on 70°C water bath heater so that it is hot up by the time you need it.
2. Once solution liquifies add some more lysis solution to make the volume to a level so that you can divide whole solution in 4 equal volumes. So let's say if the volume is 30 mL then I will increase it to 40 mL by adding Lysis buffer. Then divide the solution in 4 equal parts.
3. At this point you must have four 50 mL falcon tubes with 10 mL cell solution in it. These solutions are still very concentrated, so you need to dilute the solution. I would increase the total volume to 30 mL in each falcon.
4. Adding Lysis solution will decrease the lysozyme concentration too. So, you need to add 1 mg/mL Lysozyme again in each falcon tube. Follow exactly same procedure as described in DAY 3 step 4.
5. Keep the tubes in 37°C water bath shaker for 30 min.
6. Add 200 µL Ferric citrate solution in each falcon. Then keep them in 70°C water bath heater for 10 min.
7. Make 1 mL of 200 mM PMSF solution in Isopropanol. Then add PMSF to each falcon tube to final concentration of 1 mM.
8. Switch on the centrifuge. Fix the small rotor and then leave it to cool down to 4°C.

9. Transfer all the solution in one beaker for sonication. Sonication Method - 50% amplitude, 50 sec sonication, 10 sec ON and 30 sec OFF.
10. After sonication centrifuge the solution using centrifuge tube at 16000 RPM for 45 min.
11. Attach the Nickel column and start the purification procedure. Refer Table D1 for the purification method.
12. Transfer the supernatant to a beaker carefully without disturbing the pellet.
13. Drop the sample tube in the Lysate beaker. At this point procedure must have stopped on its own after elution is complete. Open the sample port to remove air from the tube using syringe. Do not throw the lysate solution while removing air. Add it back to the solution in beaker. Click on the continue to start the purification.
14. Collect 20 µL sample from the fractions and run SDS-PAGE to confirm the fractions containing hFTnL-EALN6. Do not forget to run Lysed cells, Lysate and Flowthrough.
15. Once you confirmed the fractions that contain hFtnL-EALA6 collect those fractions in one Falcon tube.
16. Start concentrating the protein to 2.4 mL. You need to centrifuge concentrating tubes at 4000 RPM for 20 min. This has to be repeated as many times required to bring down the solution collected from fraction to 2.4 mL.
17. Now do the Bradford assay:
    a. For standards, mix solutions according to the Table D2.
    b. Make these solutions in 1.5 mL microcentrifuge tubes.

c. Now make your samples. For sample I will first check the color by mixing 2 µL in 798 µL and then add 200 µL Bradford. If the solution turns very dark blue compared to standard no. 6, then you need to do more dilution.
    i. To prepare more diluted sample mix 2 µL of protein sample in 1000 µL ddH2O this solution has dilution factor = 500. Now take 10 µL and mix it in 790 µL ddH2O and add 200 µL Bradford. So, your final solution for Bradford will have DF = 50,000. Calculation -
        1. DF = (1000/2) × (1000/10) = 50,000
d. Check the OD of standards first and then unknown sample. R^2 should be near 0.99.

18. 5% of glycerol should be in the final concentrated protein. (If the sample is 2.4 mL then 5% of that should be glycerol which means adding 120 uL of 100% glycerol)
19. Attach a Superdex 200 16/60 column and start the purification procedure.
    a. Buffer A will be the elution buffer that was use in the Nickel column. Buffer B will be ddH20 filtered and autoclaved. Buffer C is 20% ethanol filtered.
    b. Before loading the concentrated protein, wash the sample (injection) loop with ddH20 twice, then elution buffer, and finally insert air into it until you see the air bubble.
    c. Loading the concentrated protein, watch out that the air bubble has been fully removed and you can see a tiny part of the sample. It should not be too out as that is lost of protein.
    d. Protocol for purification using Superdex 200 Hiload 16/60 is mentioned in Table D3.

Table D.1 HisTrap FF 5mL Column Purification. Buffer A – Binding/Washing Buffer, Buffer B – Elution Buffer

Method Step	Flowrate	Volume	Solution
Column Wash	5 mL/min	10 CV	Flitered dH_2O
Equilibration		5 CV	Buffer A
			Buffer B
			Buffer A
Washing		10 CV	Buffer A
Elution (Gradient)		20 CV	Buffer A 100 % à 0 % Buffer B 0% à 100%
Column Wash		5 CV	Buffer A
		10 CV	Flitered dH_2O
Storage	2 mL/min	5 CV	Filtered 20% Ethanol

Table D.2 Standard preparation for Bradford Assay.

S. no.	ddH_2O (µL)	BSA (µL)	Bradford (µL)	Concentration (mg/mL)
Blank	800	0	200	0.000
1	800	0	200	0.000
2	798	2	200	0.002
3	796	4	200	0.004
4	794	6	200	0.006
5	792	8	200	0.008
6	790	10	200	0.010

Table D.3 Superdex 200 Hiload 16/60 purification method

Method	**Flowrate**	**Volume (Column Volume = CV)**	**Solution**
Column Wash	1 mL/min	2 CV	Filtered dH_2O
Equilibration			Elution Buffer
Elution		1.5 CV	Elution Buffer
Column Wash		1 CV	Filtered dH_2O
Storage			20% Ethanol