Chapter

The Use of Ferritin as a Carrier of Peptides and Its Application for Hepcidin

Mohamed Boumaiza, Samia Rourou, Paolo Arosio and Mohamed Nejib Marzouki

Abstract

Hepcidin a 25-amino-acid and highly disulfide bonded hormone, is the central regulator of iron homeostasis. In this chapter we propose ferritin as a peptide carrier to promote the association of the hybrid hepcidin/ferritin nanoparticle with a particular cell or tissue for therapeutic or diagnostic use. Indeed, human ferritin H-chain fused directly (on its 5'end) with camel mature hepcidin was cloned into the pASK-43 plus vector and expressed using BL21 (DE3) pLys *E. coli* strain. The transformed *E.coli* produced efficiently hepcidin-ferritin construct (hepcH), consisting of 213 amino acids with a molecular weight of 24 KDa. The recovered product is a ferritin exposing hepcidin on outer surface. The hepcH monomer was characterized by immunoblotting using a monoclonal antibody specific for human ferritin and a polyclonal antibody specific for hepcidin-25. The results were also confirmed by MALDI-TOF mass spectrometry. The recombinant native human ferritin and the commercial human hepcidin-25 were used as controls in this experiment. The assembly of hepcH, as an heteropolymer molecule, was performed in presence of denatured human ferritin-H and -L chains. After cysteine oxidation of the recombinant nanoparticles, cellular binding assays were performed on mammalian cells such as mouse monocyte–macrophage cell line J774, HepG2 and COS7.

Keywords: camel hepcidin, chimeric nanoparticle, *engineered recombinant E. coli*, human ferritin, *protein folding*

1. Introduction

The combination of chemistry, biology, and nanotechnology is expected to make significant contributions to the field of medical diagnosis and therapeutics. In this framework, the use of nanoparticles in vaccine formulations allows not only improved antigen stability and immunogenicity, but also targeted delivery and slow release. Protein cage architectures such as virus capsids and ferritins are versatile nanoscale platforms willing to both genetic and chemical modifications. The incorporation of multiple functionalities within these nanometer-sized protein architectures reveals their potential to serve as functional nanomaterials with applications in medical imaging and therapy. For example, RGD-4C, a cell specific targeting peptide, which binds αvβ3 integrins upregulated on tumor vasculature, was genetically incorporated on the exterior surface of a human H-chain ferritin

nanoparticle [1]. Interestingly, this modified protein cage binds specifically cancer cells in vitro. Thus, the use of ferritin cage architecture is an exciting and promising strategy to serve as a multifunctional platform for the biomimetic synthesis of magnetic nanoparticles. It can be engineered for cell-specific targeting.

Ferritin is probably the most used protein in bio-nanotechnology. This is due to its well-known structural features, high stability, capability to mineralize metals in its cavity, self-assembly and possibility to redesign its interior and exterior by protein engineering. It has been used to encapsulate molecules, for the synthesis of inorganic cores, for functional nanostructured composite material, for magnetic nanoparticles for MRI applications and for carrying various epitopes. Most published studies used the human H or L ferritin chains, which are able to self-assemble in different proportions to produce a variety of heteropolymers. This allows the possibility to adorn ferritin surface with multiple functionalities through genetic and chemical modifications to achieve desired properties for therapeutic and/or diagnostic purposes. In particular, it can be used as a peptide carrier to target specific receptors. Unfortunately, there are no data published concerning functional biological peptides genetically fused to ferritin are missing.

In this chapter, we plan to exploit this approach by fusing hepcidin to the ferritin molecule. In fact, ferritin and hepcidin are central molecules implicated in the regulation of iron homeostasis and the fusion of the two can carry several advantages. For example, the injection of iron-loaded ferritin in a ten days wild mouse induces the expression of *BMP6* and *Hamp1* at dose-dependent manner. Such result cannot be obtained through an injection of FAC (ferric ammonium citrate) or holo-transferrin. However, the injection of uncharged ferritin has no effect on the expression of *BMP6* or *Hamp1*.

We also proposed to use ferritin to carry hepcidin, another key protein of iron metabolism. It is a small hormone peptide that control systemic iron homeostasis (ferritin is a major controller of cellular iron homeostasis). The production of chimeric ferritin complexes that expose on the surface of limited number of functional hepcidin is of interest. It is a tool that allows deep studies in relation to the mechanism of interaction between hepcidin and ferroportin and how the complex is degraded. It might indicate alternative approaches to control hepcidin activity and systemic iron homeostasis. Ferritin is composed of 24 subunits. Once defined the conditions to insert a novel function and to co-assemble different subunits in a highly stable molecule that carries are defined, it is possible to produce molecules with many more functions and it can be applied to other peptides and hormones.

The aim of this chapter is to describe an efficient strategy to fuse the full-length hepcidin to the N-terminus of ferritin-H chain (which in the assembled protein is exposed on the surface). The produced chimeric protein will be tested:

1. As iron regulatory hormone (by studying the hepcidin-ferroportin interaction) which can be useful for patients with iron disorders.

2. As drug-delivery agent.

2. Methods

2.1 Expression and solubilization of HepcH monomer

Human ferritin H-chain fused directly, on its 5'end, with camel mature hepcidin was cloned into the pASK-IBA 43 plus vector (**Table 1**) and expressed using *E. coli* BL21 (DE3) pLysS. Growth of the transformed *E. coli* was done in 1 L LB medium (10 g

Primers	Sequence 5′ to 3′	Using
NheI hFTH F	CAAATGGCTAGCACGACCGCGTCCA	Construction of pASK-IBA43 plus hFTH vector
BamHI hFTH R	TCGAGGGATCCCCGGGTTAGCTTTCATT	Construction of pASK-IBA43 plus hFTH vector
NheI H25 F	ATAGACGCTAGCATGGACACCCACTTCCCCATCTGC	Construction of pASK-IBA43 plus HepcD-hFTH vector
NheI H25 R	ATAGACGCTAGCGGTCTTGCAGCACATCCCAC	Construction of pASK-IBA43 plus HepcD-hFTH vector
pASK F	GAGTTATTTTACCACTCCCT	Sequencing
pASK R	CGCAGTAGCGGTAAACG	Sequencing

Table 1.
Sequences of the primes used in this study.

Tryptone, 5 g Yeast extract, 5 g NaCl), with100μg/ml AMP, at 37°C, for 1-2 h until OD = 0,5. Expression was induced by the addition of 200 μl anhydroteracycline (1 mg/ml), final concentration 200 μg/L, for 4 h. Cells were harvested by centrifugation at 7000 RPM for 10 min. The pallet was washed twice in Tris–HCl 20 mM pH 7,4 and sonicated, for cytoplasmic protein extraction, at the following conditions: 1 min: 30 sec, cycle5, power 72%. The sonicated pallet was centrifuged at 12000 RPM and washed twice in Tris 20 mM, 2 M Urea, 0,1% TritonX100, pH 7,4. The insoluble HepcH, precipitating with the inclusion bodies from the *E.coli* paste, was solubilized with a weight to volume ratio of 1:1 in 6 M Guanidine hydrochloride (GdnHCl) pH 4,7. The suspension was sonicated at the same conditions to homogenate the solution and incubated with stirring, for 18 h at 4°C.

2.2 Assembly and cysteine oxidation of HepcH-FTH heteropolymers

To enhance the assembling of HepcH as an heteropolymer molecule, it was mixed, with the molar ratio hepcidin/ferritin of 1:5, in presence of denatured FTH in 6 M GdnHCl pH 7. The mixture was then diluted at least 10-fold into 0,1 M sodium phosphate pH 7,4, in the presence of 5 mM beta-mercaptoethanol (3 mM DTT and 1 mM EDTA), and incubated with stirring for 18 h at 4°C, for the renaturation of the heteropolymer. The diluted solution was then clarified by centrifugation, at 4000RPM for 15 min, at least twice to separate the soluble fraction from the insoluble cellular debris. A 10-fold concentration was performed with a 100-KDa molecular weight cutoff membrane. The renatured HepcH-FTH heteropolymer was then purified using a gel filtration on a Sepharose 6B column and analyzed on native gel 8%. A slightly modified protocol of HepcH-FTH renaturation, using different molar ratios of hepcidin/ferritin, was described by Boumaiza et al. [2]. Both protocols showed to be efficient for the production of correctly assembled and functional HepcH-FTH heteropolymer. Cysteine oxidation for the final refolded HepcH-FTH renatured in the proportion hepcidin/ferritin = 1:5, was carried out using the glutathione redox system (GSH/GSSG) as described by Jordan et al. [3].

2.3 Cell culture

Mouse monocyte–macrophage cell line J774 (Lombardy and Emilia Romagna Experimental Zootechnic Institute) was cultured as previously described by

Delaby et al. [4]. Briefly, cells were grown in DMEM (PAA Laboratories GmbH), 10% endotoxin-free fetal bovine serum (Euroclone), 0.04 mg/mL gentamicin (Euroclone), 2 mM l-glutamine (PAA Laboratories GmbH), and maintained at 37°C in 5% CO2. They (200,000 cells/well) were seeded onto 12- well plates, and after 24 h were grown for 12 h in presence of 100 μM ferric ammonium citrate (FAC) to induce ferroportin expression. The day after, cells were incubated with HepcH-FTH at final concentrations of 0.5 μM and 0.2 μM. Controls were cells without ferric ammonium citrate (FAC) treatment and cells incubated with native FTH homopolymer. Experiments were performed at 37°C for 30 min and 2 h. After this time, the supernatant was discarded and the cells washed with cold PBS and lysed using cold buffer (200 mM Tris–HCl at pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol, 1 mM sodium fluoride, 1 mM sodium orthovanadate, complete protease inhibitor cocktail; Roche). Protein content was determined by colorimetric BCA assay (bicinchoninic acid assay, Pierce), 30 μg of total proteins were separated by native and denatured polyacrylamide gel electrophoresis and Western blotting, for the detection of proteins bound and internalized by the J774 cells, as well as the mass spectroscopy analysis were performed as described previously [5].

3. Results and discussion

Ferritin is probably the most used protein in bionanotechnology. This is due to its well-known structural features, high stability, capability to mineralize metals in its cavity, self-assembly and possibility to redesign its interior and exterior by protein engineering [6, 7]. Hepcidin, a 25 amino acid peptide belonging to the β-defensins family, was isolated for the first time from plasma and human urine respectively by Krause *et al*. [8] and Park *et al*. [9]. It is a cysteine-rich cationic peptide, engaged with four disulfide bridges, which plays a major role in innate immunity and iron homeostasis [10]. It is induced by iron and inflammation and is suppressed by iron deficiency and hypoxia. It binds and inhibits ferroportin1, the only cellular iron exporter, thus, high hepcidin reduces while low hepcidin increases systemic iron availability. This hormone acts via its N-terminal part and more precisely the 7–9 amino acids including a single thiol cysteine, comprised the minimal structure that retained hepcidin activity [11] and can be refolded *in-vitro*. The 3D structure of human hepcidin is known [3]. Recombinant human and mouse hepcidins were expressed in *E. coli* in fusion with a Thioredoxin and treated by two sequential proteolytic enzymes to obtain functional hepcidin, in low yield <10% [12]. We used this procedure to clone and express camel hepcidin, in collaboration with Prof. Marie-Agnès Sari in the laboratory of Chemistry and Pharmacological and Toxicological Biochemistry (UMR 8601) at the University Paris Descartes. We showed that this hepcidin is functionally equivalent to human one in binding and inhibiting ferroportin using mouse monocyte–macrophage cell line J774 treated with Fe-nitrilotriacetate. Camel hepcidin differs from human hepcidin in 2 residues, which make it more stable [13]. Thus, we planned to use camel hepcidin to make hybrid molecules with ferritin. For this purpose, we have approached the laboratory of Prof. Paolo Arosio in Brescia that has a long experience in the production of recombinant proteins and is working on ferritin and hepcidin. The aim of this work was to fuse the full length hepcidin to the N-terminus of ferritin-H chain, which is exposed on the surface in the assembled protein. The chimeric subunit was assembled in the 24-mer shells together with various proportion of H or L chains to produce hybrid heteropolymers. These latter that can carry 1 to 24 hepcidin moieties and other functions that can be engineered and can include the electron dense iron core as tracer.

We cloned the gene encoding camel hepcidin in fusion with the 5′ of the cDNA for heavy chain of human ferritin into the pASK-IBA 43plus vector (**Figure 1A, B**) for high expression in *E. coli*. The clone was verified and expressed the chimeric peptide in high amount but it was insoluble and could not be easily refolded in a soluble and active 24-mer shell. Thus, we planned to carry out this project with different strategies:

The construct was expressed in the prokaryotic system *E.coli* BL21 (DE3) pLysS, and the peptides were initially characterized on polyacrylamide gel in denaturing conditions (**Figure 2A**), western blotting (**Figure 2B**) and MALDI-TOF mass spectrometry. Hepcidin carries 8 cysteines that form 4 disulfide bridges in the folded molecule, while FTH has three cysteines, which do not form bonds. Thus, the presence of free -SH groups was also quantified throughout all the purification and refolding processes. We planned to produce bicistronic constructs that express the chimeric hepcidin-FTH and the FTH-WT, with an approach that has been found successful for the production of insoluble ferritin mutants [14]. We obtained ferritin shells with low proportion of chimeric hepcidin (**Figure 1C, D**). In parallel, we have performed co-renaturation together with ferritin-H or L chains. We obtained molecules with various amounts of hepcidin. To this aim the *in-vitro* refolding of the fusion protein hepcH was done with different molar ratio of H and L chains, in order to optimize the assembly of the hybrid nanoparticles [2]. Purified fusion hepcidin–ferritin H subunit (**Figure 3D**) assembled together with H- or L-chains at a ratio of 1:5, produced a stable and functional 24-mer ferritin exposing about 4 hepcidin per shell (**Figure 1C, D**; **Figure 3C**). HepcH-FTH heteropolymer was purified and characterized by analysis on polyacrylamide gel in non-denaturing condition and by western blotting (**Figure 3E, F, G, H**). MALDI-TOF spectra of the final oxidized HepcH-FTH heteropolymer exhibited, average mass peaks at m/z 21135.97 and at 243775.51 (**Figure 4A**) which corresponds to the theoretical average mass [M + H] + of 21225.64 of FTH (183 amino acids), and of 24410.50 to HepcH (213 amino acids).

These procedures have produced ferritin shells that expose on the surface the hepcidin moiety. In order to control the correct folding, the cysteines of the

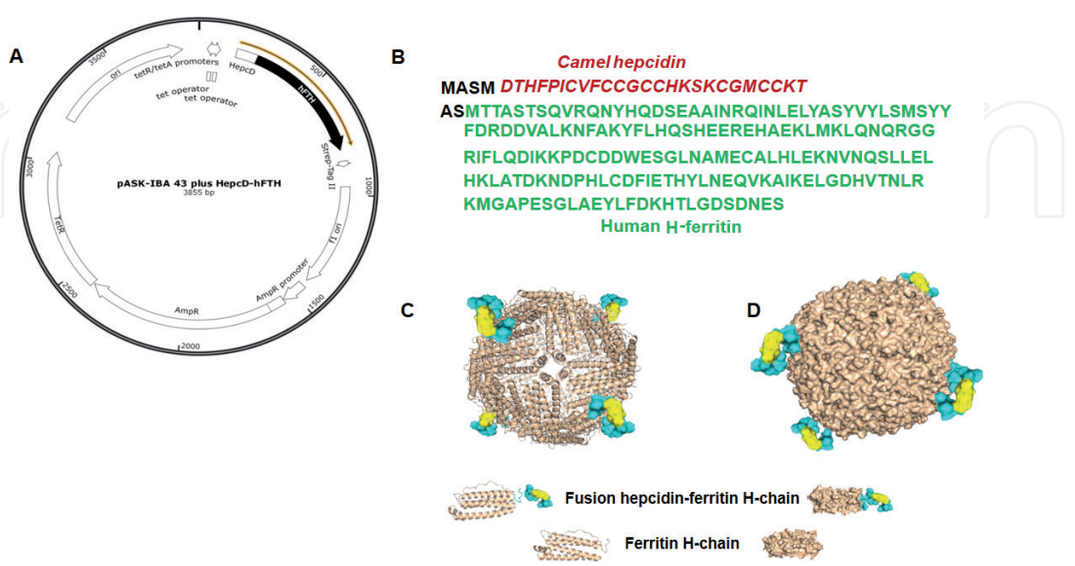

Figure 1.
(A) Construction of the recombinant HepcH pASK-IBA 43 plus vector. (B) Amino acid sequence of the fusion HepcH protein. In this recombinant protein, the coding sequence of camel hepcidin, in red and italic, was fused directly to the 5′ end of human H-ferritin, in green, and cloned into pASK-IBA43 plus vector. (C) Schematic representation (left) and space-filling diagram (right) of the assembled HepcH-FTH heteropolymer (with molar ratio hepcidin/ferritin = 1:5).

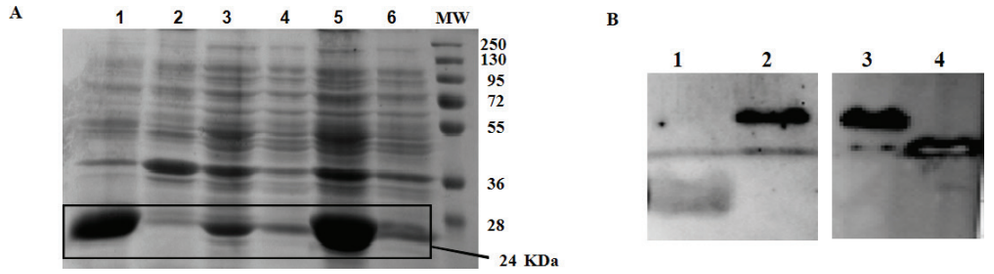

Figure 2.
(A) SDS-PAGE analysis of the induced and uninduced E.coli transformed by the recombinant HepcH-FTH pASK-IBA 43 plus vector. Lane 1 and 2, induced and uninduced pellet respectively (insoluble fraction). Lane 3 and 4, induced and uninduced supernatant (soluble fraction). Lane 5 and 6, induced and uninduced total sonicate (total protein). (B) Western blot analysis of the recombinant HepcH monomer (in denaturing conditions). Lane 1, rabbit hepcidin antibodies recognized the human hepcidin-25 (control). Lane2, rabbit hepcidin antibodies recognized the recombinant HepcH. Lane3, rH02 antibodies recognized the recombinant HepcH. Lane4, rH02 antibodies recognized the recombinant human H-ferritin (control).

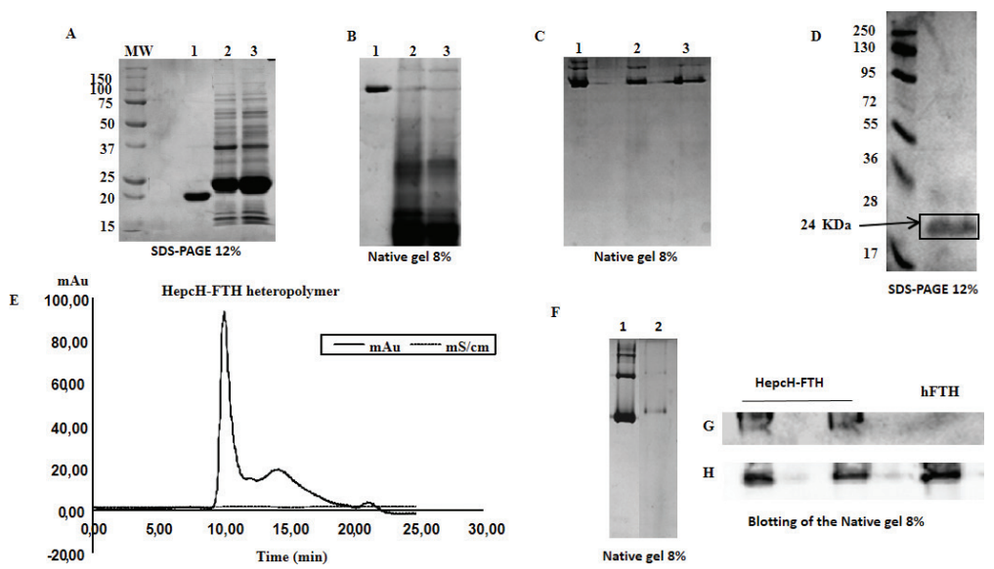

Figure 3.
Renaturation of the insoluble HepcH monomer in absence and in presence of FTH. (A) Purified FTH (line1) and solubilized HepcH monomer (line 2 and 3) analyzed on denaturing conditions. (B) FTH homopolymer (line1) and renatured HepcH, in absence of FTH, analyzed on non-denaturing conditions (line 2 and 3). (C) FTH homopolymer (line1) and renatured HepcH in presence of FTH with molar ratio of hepcidin/ ferritin = 1:5 (lane 2 and 3) analyzed on non-denaturing conditions. (D) SDS-PAGE analysis of the purified HepcH, from the insoluble fraction (IS), by gel filtration on a Sepharose 6B column (GE Healthcare, life sciences). (E) Fast protein liquid chromatography chromatogram showing the purification of HepcH-FTH heteropolymer using a gel filtration on a Sepharose 6B column and analyzer on non-denaturing conditions (F). (G) Western blot analysis of the purified HepcH-FTH heteropolymer using the anti-rabbit hepcidine-25 and the human ferritin-H antibodies, rH02, as control (H).

molecule was reduced and then slowly oxidized under controlled conditions (to allow the formation of the correct four disulfide bridges constituting the hormone). In case the cysteine of the ferritin interferes with the process or its monitoring by spectroscopic techniques, they could be removed by site-directed mutagenesis.

The functionality of the assembled heteropolymer was analyzed by its capacity to bind with high affinity the ferroportin, which is the natural hepcidin receptor. To this goal, we used the macrophagic cell line J774 that expose evident ferroportin after treatment with iron. The cells were incubated with the hybrid molecules and the binding analyzed directly with traced anti-ferritin antibodies. Binding specificity was analyzed by adding ferritin and synthetic hepcidin. This process was repeated with the various heteropolymer types. Next, we studied the biological activity of the chimera,

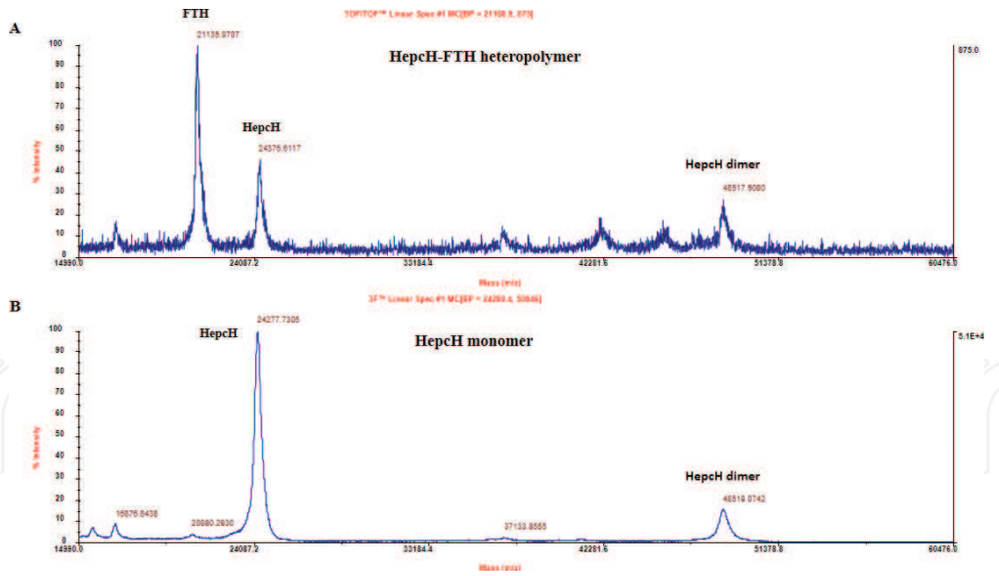

Figure 4.
(A) High-resolution MALDI-TOF/TOF mass analysis of the recombinant assembled HepcH-FTH heteropolymer and the recombinant human ferritin FTH homopolymer (B).

in particularly if it is taken up and degraded together with ferroportin, as it occurs with the hepcidin. Hybrid and native ferritin binding to murine J774 cells were monitored using monoclonal anti-human FTH antibody rH02 that does not cross-react with the mouse ferritins [15]. This was confirmed by treating J774 cells with mouse ferritin alone or together with HepcH-FTH. The obtained results (**Figure 5A**) showed that these antibodies are specific only to human H-ferritin. J774 cells treated with 100 μM FAC showed to be able to internalize HepcH-FTH heteropolymers after 30 min to 2 h of incubation at 37°C (**Figure 5B**). Hepcidin exerts it function by binding and then inducing ferroportin degradation, and in fact we observed that after 2 h incubation, with a final concentration of 0.2 μM, more efficiently than human hepcidin-25 used as control

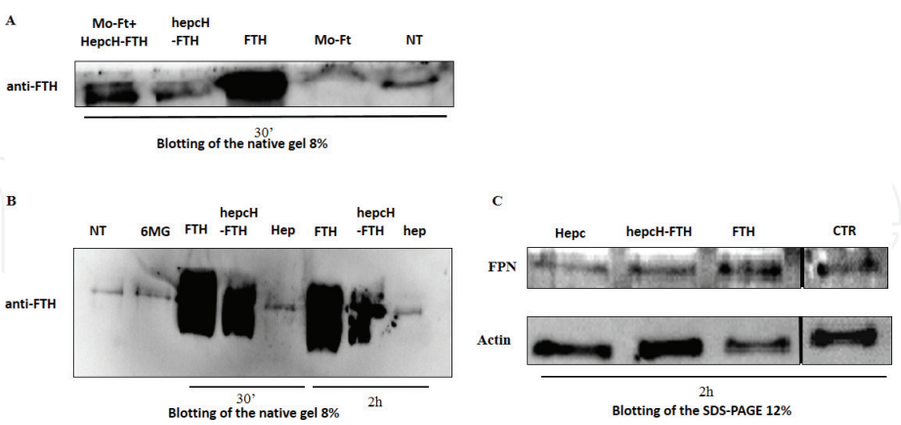

Figure 5.
(A) Western blot analysis of the J774 cells treated with mouse ferritin (Mo-Ft), FTH, human hepcidin-25 and HepcH-FTH heteropolymer using anti-FTH antibodies (rH02). Lane 1, cells treatment with 0.5 μM Mo-Ft and HepcH-FTH; lane 2, cells treatment with 0.5 μM FTH; lane 3, cells treatment with 0.5 μM Mo-Ft; lane 4, untreated cells. (B) Western blot analysis of the J774 cells treated with 0.5 μM HepcH-FTH heteropolymer and FTH, using anti-FTH antibodies (rH02). Lane 1, non-treated cells; lane2, cells treated with 6 M guanidine; lane 3, cells treated with 0.5 μM FTH for 30′; lane 4, cells treated with 0.5 μM HepcH-FTH for 30′; lane 5, cells treated with human hepcidin for 30 min; lane 6, cells treated with FTH for 2 h; lane 7, cells treated with HepcH-FTH for 2 h; lane 8, cells treated with human hepcidin for 2 h. (C) Western blotting analysis of the SDS-PAGE of cell lysates using polyclonal anti-rabbit ferroportin antibody. CTR: Untreated cells. FTH: Cells treatment with 0.5 μM native FTH for 2 h. HepcH-FTH: Cells treatment with 0.2 μM HepcH-FTH (with molar ration of 1:5) for 2 h. Hepc: Cells treatment with 0.5 μM synthetic human hepcidin-25 for 2 h. non-adjacent bands, from the same blot, were denoted by vertical black lines.

(**Figure 5C**). Indeed, the level of ferroportin in the J774 cells decreased, as it occurred in the cells treated with the synthetic hepcidin, while the incubation with FTH had no evident effect. This indicates that heteropolymer is biologically functional. Consequently, folded camel hepcidin activity against FPN1 could be enhanced thanks to its exposition, through its N-terminal part, at the H-ferritin surface nanocage [16]. This will probably offer a tool for a detailed study of the events after ferroportin binding the hepcidin. The heavy ferritin iron core and the fate of iron will facilitate the monitoring of the process.

As perspectives, the examination of the chronic effect of the purified heteropolymer injections on liver iron accumulation in a mouse model of hereditary hemochromatosis could be monitored. Indeed, hepcidin-1 knockout mice ($hepc1^{-/-}$) can be used, as a model, to eliminate any possibility of endogenous hepcidin contributing to the regulation of iron loading. This strategy can also be developed by producing ferritin subunits carrying other functionalities or epitopes, in order to have multifunctional complexes. Moreover, involving the approach can be applied for other peptides/hormones for the design of 'smart' molecular systems programmed to allow the transport in the body of potent anticancer agents in an innocuous manner toward safe tissues. Thus, these hybrid molecules will be useful for future therapeutic applications to improve health and life quality of a great number of patients with iron disorders or cancer diseases.

4. Conclusion

Ferritin is a well-known molecule with enormous potentiality in biotechnology. It has been already used to encapsulate molecules, as contrast in MRI and to carry epitopes. This chapter offers an original strategy to design a new bifunctional hybrid protein, which can be proposed as a stable iron regulatory molecule or a drug-delivery agent. The results could be exploited by scientists to produce ferritin subunits carrying other functionalities or epitopes, in order to have multifunctional complexes. It can be a new platform with utmost importance in the field of cell and gene therapies.

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Conflict of interest

Authors declare that they have no conflict of interest.

Abbreviations

HepcH	fusion camel hepcidin-human ferritin H-chain subunit
FTH	human ferritin H
HepcH-FTH	24-mer heteropolymer comprising camel hepcidinhuman ferritin H assembled with FTH

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