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Preparation and labeling of surface-modified magnetoferritin protein cages with rhenium (I) carbonyl complex for magnetic targeted radiotherapy

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Abstract

New rhenium radiolabeled compounds are of general interest due to their nuclear characteristics which allow radiotherapy and in situ monitoring of tumor uptake. Besides biocompatible magnetic nanoparticles capable of transporting radionuclides, providing MRI contrast agent properties for imaging and therapeutic effect in the target tissue simultaneously are highly desirable. Herein we describe the preparation of magnetoferritin samples, and their labeling with rhenium in the form of low oxidation state rhenium (I)-tri carbonyl complex, $[Re(CO)_3(H_2O)_3]^+$. A non-radioactive rhenium isotope (^{187}Re) was used in all studies. Rhenium complex was conjugated covalently to the surface lysine groups of protein cage via glutaraldehyde crosslinker and histidine modification. The analyses of conjugates were performed by inductively coupled plasma mass spectroscopy (ICP-MS) and size exclusion chromatography (SEC). Labeling efficiency was calculated as 22 ± 2 rhenium per protein cage. The in vitro stability of the rhenium carbonyl label was evaluated at room temperature and in human serum medium. It was found that 91.1 ± 1.8 % rhenium was retained on the surface of the magnetoferritin cage following 72 h of dialysis. Prussian blue staining revealed the uptake of rhenium labeled nanocages preferentially into the human breast metastatic adenocarcinoma, MDA-MB-231 cells lines. The cytotoxicity assay carried out with the same cell lines exhibited that there is no significant cytotoxic effect up to 72 hours of incubation with 1 mg labeled nanocages /mL (IC_{50} value).

Keywords: Rhenium Tricarbonyl Complex, Labeling, Magnetoferritin, Histidine, ICP-MS.

Introduction

Multifunctional nanoparticles capable of delivering radionuclides and concurrently providing imaging capability and therapeutic effect in the target tissue are highly desirable. Biocompatible magnetic nanoparticles were envisioned to improve the delivery of the radionuclides to the tumor site as well as to keep them in the target tissues under the force of an external magnetic field. Hence, magnetic nanoparticles, mineralized in a human ferritin protein cage, can provide an attractive platform to realize smart agents for cancer diagnosis and treatment [1-4].

Ferritin is composed of 24 subunits of two types, heavy-chain ferritin and light-chain ferritin with the outer diameter of 12 nm and inner cavity diameter of 8 nm, with storing capacity of up to 4500 iron atoms in the form of an iron oxide-hydroxide mineral [5]. Iron atoms can be removed from the cage by reductive dissolution through hydrophilic and hydrophobic channels penetrating the cage. Then, the empty cage, apoferritin, can be used in the synthesis of monodispersed inorganic oxides and sulfides of controlled size where the cavity acts as a template and the protein cage around the core provides surface functionalization for preventing agglomeration [6,7]. Ferritin is an excellent choice for biological studies, because it is already present in the living systems, thus it is not recognized as a threat by the immune system compared to the metal nanoparticles having the similar or higher concentrations. Its smaller size improve tissular diffusion and extend the sedimentation times [7,8]. Moreover, heavy-chain ferritin has intrinsic tumor targeting properties as it binds to human cells via interacting with the transferrin receptor 1 (TfR1) which is highly expressed on human cancer cells [9]. So, ferritin can function as carriers and storage devices for metal ions or targeting moieties for biomedical applications [10-12]. Magnetic iron oxide nanoparticles (Fe_3O_4, ɣ-Fe_2O_3) synthesized in apoferritin, form a biocompatible ferrofluid, namely magnetoferritin [1]. Magnetic properties of magnetoferritin, based on inducible magnetization of iron oxides, allow them to be used as negative contrast enhancing agents for MRI with high transverse relaxivity [13]. Based on the in vivo studies reported [14], long-term MRI properties of magnetoferritin are comparable or even better than those of standard superparamagnetic iron oxide particles widely used in biomedical applications. The possible reasons for the enhancement can be due to the narrow range of particle size distribution synthesized in the cavity [6].

For cancer threatment, chemotherapy and radiotherapy are the commonly used ways [15,16]. However, during radiotheraphy and chemotheraphy, healthy cells are destroyed as well as the tumor ones which may cause side effects. To overcome these problems an advanced treatment is needed where radionuclides are irreversibly attached to the magnetic nanoparticles and tumor is magnetically targeted with the radiolabeled nanoparticles causing an effective localized radiation theraphy [17-19]. Re-188 (Radio rhenium) with 2.1 MeV maximum beta energy and 16.9 hours half-life is the most routinely employed radionuclide in targeted therapy. Besides, 155 keV gamma emissions of ^{188}Re also allow in situ monitoring of tumor uptake as well as dosimetric calculations. ^{188}Re, that can be readily obtained by daily elution from ^{188}W/^{188}Re-generator is very suitable for routine clinical use [20,21]. Consequently, the production of the new compounds with this radiolabel is of general interest.

For radiotherapy, biomolecules such as intact antibodies, antibody fragments, peptides, DNAs and other oligomers have all been labeled with radiorhenium (^{188}Re) [22]. On the other hand, radiolabeling of magnetoferritin with ^{188}Re, to the best of our knowledge, has not been reported. There are several chelators that are used in the indirect radiolabeling of biomolecules with radiorhenium. Among them, ^{188}Re tricarbonyl core , $Re(CO)_3^+$, provides a new approach for labeling biomolecules with radiorhenium where rhenium is in +1 oxidation state [22]. It has been stated that low oxidation state complexes show high thermodynamic stability and kinetic inertness at neutral pH and they can also easily be attached to the biomolecules [23,24]. Jiaoyun Xia and at al has proposed an easy way for the preparation of an organometallic $[^{188}Re(H_2O)_3(CO)_3]^+$ carbonyl complex [25]. The single-step procedure consists of carboxylation and reduction of aqueous $^{188}ReO_4^-$ with carbon monoxide gas and sodium borohydride respectively at ambient pressure. Carbonyl complexes of metals preferentially react with ‘‘soft’’ sp^2 nitrogen of aromatic amines instead of aliphatic amines or thioethers [26]. It has been shown that the weakly bound water molecules are replaced with an incoming mono or multidentate ligands such as histidine, histamine, imidazole attached to a derivatized biomolecule [27]. The natural existence of amine and carboxylic acids groups on the surface of the ferritin protein provides functionalization of the surface for the attachment of biologically important agents in the use of imaging and cancer therapy. Compared with other macromolecules such as polymers, there are a limited number of chemical groups on the protein surface that can be used for conjugation. It is therefore challenging to integrate $[^{188}Re(H_2O)_3(CO)_3]^+$ on the surface of magnetoferritin, without disturbing the intact nature of the protein.

Herein, we report the synthesis of magnetoferritin and labeling of it with organometallic rhenium. The preparation of the tri carbonyl complex with nonradioactive rhenium, $[Re(H_2O)_3(CO)_3]^+$, have been investigated. Histidine groups linked to ferritin cage through glutaraldehyde crosslinking were used for the conjugation of rhenium carbonyl complex to magnetoferritin. The stability of this protein-rhenium complex has been evaluated utilizing ICP-MS technique. Bioassays of the resulting labeled nanoparticles were done to demonstrate that the protein retained intact nature and preferentially taken into the human breast metastatic adenocarcinoma, MDA-MB-231 cells lines.

Experimental section

Preparation of Apoferritin

Horse spleen ferritin (HoSF) used in this study was obtained from Sigma-Aldrich (Sigma F4503). The Fe (III) oxo-hydroxide core of HoSF was removed by a reductive dissolution process to form apoferritin according to established procedure [28]. Ferritin (10 mg, 0.2 mL) was placed into a dialysis bag (MWCO of 12-14 Da) and diluted (x5) with sodium acetate buffer (pH: 4.5). The bag was floated into 1L of sodium acetate buffer (pH: 4.5) and purged with N_2 for 15-30 min. Thioglycolic (TGA) acid (Sigma-Aldrich) (final concentration of 0.02 M) was added as a reducing agent into the buffer solution. After 2 hours, fresh TGA (final concentration of 0.01M) was added and dialysis continues for additional 1 h. The buffer was refreshed, purged with N_2 for 15-30 min and these cycles were repeated until the solution becomes colorless. Protein was exhaustively dialyzed against 0.1 M NaCl solution and then, subjected to size exclusion chromatography (SEC) using PD-10 desalting columns (GE Healthcare) prepacked with G-25 Sephadex column (1.5 cm x 5.5 cm) equilibrated with 0.1 M NaCI solution. 1 mL of fractions was collected for UV-Vis (PG Instruments, T80+ UV-VIS Spectrophotometer) masurements at 280 nm and then, the protein-containing fractions were isolated. Protein concentration was measured by the absorbance value at 595 nm in the UV-Vis spectrum according to Bradford Assay method (Coomassie Brilliant Blue G-250, Sigma).

Mineralization of iron oxide nanoparticles in apoferritin protein cage

In the mineralization procedure, ferrous ammonium sulfate ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$) (Sigma-Aldrich) and trimethylamine N-oxide (Me_3NO) (Sigma-Aldrich) were used as Fe(II) source and oxidant, respectively . The reaction was carried out at pH: 8.6, using 3-[(1,1-Dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid (AMPSO) (Sigma-Aldrich) buffer. All

the solutions were deaerated with N_2 before used. Apoferritin was added to buffer solution and reaction vessel was placed in a preheated (65 ^oC) water bath under N_2 atmosphere. The $(NH_4)_2Fe(SO_4)_2.6H_2O$ (12.5 mM) and and stoichiometric equivalents of Me_3NO solutions (3Fe(II) : $2Me_3NO$) were injected simultaneously into the reaction vessel at a constant rate of 100 Fe/(cage.min). Samples with theoretical loading of 2000 Fe atoms/protein molecule were prepared. After the mineralization procedure, 200 μL of 300 mM sodium citrate (Aldrich) was added to chelate any remaining free Fe [10,28]. The mineralization reaction in ferritin is shown in Equation 1.

$$3Fe(II) + 2Me_3NO + 4H_2O \rightarrow Fe_3O_4 + 2Me_3N + 6H^+ + H_2O_2 \qquad (1)$$

In order to get rid of the aggregation products from the mineralized apoferritin (magnetoferritin), protein was purified and analyzed by SEC using G-25 Sephadex column equilibrated with 0.1 M NaCI solution by following the absorbances at 280 nm and 410 nm for protein and mineral elutions, respectively.

Magnetoferritin sample was dissolved in 70 % nitric acid (1:1 v/v) overnight and 10 fold dilutions was done for Atomic Absorption Spectrometry (Varian Spectra AA140 with Varian GTA 120 atomizer) analysis to determine the iron concentration and the protein concentration were determined by Bradford Assay to find the actual number of iron atoms per protein cage.

Magnetoferritin sample was negatively stained with 1 % uranyl acetate solution and air-dried on a carbon coated copper grid. The core size of iron oxide nanoparticles were investigated by transmission electron microscopy (TEM, JEOL 2100 F) 100kV and the average particle size was determined by measuring the diameters of more than 100 particles in the TEM image. The T2 relaxivity values of magnetoferritin samples synthesized by similar method in Prof. Dr. Douglas's laboratory (Montana State University, USA) were measured at the same University utilizing Anasazi FT-NMR 90 MHz (2.1 T) and Bruker DPX 300 MHz (7.0 T) spectrometers. The magnetoferritin sample was dialyzed overnight against 0.1 M phosphate buffer (0.1 M NaCl, pH 7.0) at 4 ^oC for surface modification experiments.

Histidine immobilization to magnetoferritin protein surface via glutaraldehyde crosslinker

1 mg/mL of magnetoferritin sample in 0.1 M phosphate buffer (0.1 M NaCl, pH 7.0) and 10 µL 2.5 % glutaraldehyde solutions (Sigma-Aldrich) were mixed at room temperature. The reaction time for protein and glutaraldehyde interaction was varied as 15 min and 60 min and agglomeration behaviors of protein samples were characterized by SEC using G-25 Sephadex column. A brief centrifugation, 5 min, at 17000 g was applied to the mixture before introducing to the SEC column for removing the excess reagents and aggregated proteins. Then 1 mL of (5 mg/mL) freshly prepared L-Histidine (Sigma-Aldrich) solution in 0.1 M PBS buffer (0.1 M NaCl, pH 7.0) was added to the protein and mixed for 1 hour at room temperature and incubated overnight at 4 ^{0}C. This procedure is called as two step procedure where histidine was reacted with the protein already modified with glutaraldehyde. In one step crosslinking procedure, on the other hand, protein, glutaraldehyde and histidine were mixed altogether for 1h; the rest of the procedure was exactly the same as stated in two step crosslinking procedure.

Following histidine conjugation reactions, to reduce the resultant Schiff bases and any excess aldehydes, 50 µL of $NaBH_4$ (Merck) (final concentration of 1 mg/mL) was added to the protein solution and equilibrated for 30 min at 4 ^{0}C. Then protein was centrifuged at 17000 g for 5 min and dialyzed against 0.1 M phosphate buffer (0.1 M NaCl, pH 7.0) using a dialysis membrane with MWCO of 12-14 Da. The procedure was modified according to the literature [26]. The histidine conjugated magnetoferritin (his-Magnetoferritin) samples were then concentrated by using Amicon concentrator (100 K) for subsequent experiments.

Preparation and charazterization of $[Re(H_2O)_3(CO)_3]^+$

$[Re(CO)_3(H_2O)_3]^+$ complex was synthesized from ^{187}Re-perrhenate ($NaReO_4$ with the nonradioactive rhenium isotope, Sigma-Aldrich) through reductive carboxylation reaction using gaseous carbon monoxide as a source of carbonyl and amine borane ($BH_3.NH_3$) as reducing agent, according to a literature procedure with some modifications [23,29].

Briefly, 10 mg of $BH_3.NH_3$ (Aldrich) was added into a 10 mL glass vial. The vial was covered with a cap and then CO gas (OKSAN, Turkey) was passed from the vial over 10 min. The mixture of 2 mL of 4.4 x 10^{-5} M ReO_4^- and 60 µL H_3PO_4 (85 %) (Baker) was added to the vial and incubated in a water bath at 70–80 ^{o}C for 15 min. H_2 gas is formed during the reaction between water and amino borane; this should be taken into consideration in order to

keep the balance of H_2 gas with the help of 10 mL syringe. In order to compensate the possible changes in the yield of the rhenium carbonyl complex, which would directly affect the labeling efficiency measurements; products from several trials were collected and passed through an anion exchange resin containing a quaternary ammonium ion with the dimensions of 2 cm x 30 cm.

Re labeling of histidine immobilized magnetoferritin

The pH of rhenium complex solution was adjusted to pH: 6 with 0.01 M NaOH (Sigma-Aldrich). Then 500 µL of his-magnetoferritin sample and 100, 500, 1000 µL of rhenium complex solution were mixed in separate experiments with same initial concentrations of protein and placed into a shaker thermostated at 55 - 60 °C for 40 minutes [26].

Labeled magnetoferritin was purified by G-25 Sephadex column and eluted with 0.1 M PBS buffer (0.1 M NaCl, pH 7.0). The eluate was collected in 1 mL fractions, which were examined for their protein and rhenium content using UV-Vis and ICP-MS (Thermo Scientific X series instrument with a concentric nebulizer and Peltier effect cooled spray chamber) techniques.

Stability test in human serum

To test the stability of the label in human serum medium, 500 µL rhenium labeled magnetoferritin particles were mixed with 500 µL of both human serum (H4522 Sigma, from human male AB plasma, sterile-filtered, USA origin,) and in 0.1 M PBS buffer (0.1 M NaCl, pH 7.0) separately and each was dialysed against 0.1 M PBS buffer (0.1 M NaCl, pH 7.0) for 72 h. Aliquots of samples were removed after 12 h, 24 h, 48 h and 72 h from both of the dialysis bags and subjected to an ICP-MS analysis for Re determination.

The integrity of the protein samples (native ferritin, histidine immobilized magnetoferritin and Re labeled magnetoferritin) was also analyzed by a polyacrylamide gel electrophoresis (PAGE) experiment under native (nondenaturing) conditions on a Bio-Rad electrophoresis system (Mini-PROTEAN 3 Cell) using 4 % stacking gel and 6 % resolving gel. The samples were loaded with a loading buffer (50 % glycerol and 0.01 % bromophenol blue, pH 7.4). After electrophoresis, the gel was stained overnight with 0.25% Coomassie Brilliant Blue R-250, followed by destaining with an aqueous solution containing 7 % (v/v) acetic acid and 5 % (v/v) methanol.

In vitro cytotoxicity assay

The potential cytotoxicity of Re labeled magnetoferritin nanoparticles in human breast metastatic adenocarcinoma, MDA-MB-231 cells were investigated with the Cell Proliferation XTT Kit (Biological Industries, Israel) according to manufacture instructions. Briefly, the cells (10^4 cells/well) were incubated for 24, 48, and 72 hours in the presence of varying final concentrations (0.5, 1.0, 1.5 mg/mL) of nanoparticles. Then, XTT reagent was added and the absorbance of the dye was measured at a wavelength of 415 nm with Bio-tek ELISA reader linked to a PC supplied with KC Junior program at 37 ^{0}C. 3 independent experiments were performed and each one had triplicate wells and the inhibitory concentration (IC_{50}) values were determined by Graph Pad Prism Version5 according to dose-response curve drawn according to percent viability. The results were expressed in terms of percentage cellular viability with respect to concentrations.

Prussian blue staining

In order to visualize the uptake of the Re labeled magnetoferritin nanoparticles, human breast metastatic adenocarcinoma, MDA-MB-231 cells were incubated with the nanoparticles (1.0 mg/mL) at 37 °C in 5 % CO_2 atmosphere for 24 h. After incubation, cells were washed two times with DPBS (Lonza, Belgium) to remove the nanoparticles excluded from the cells and collected using 0.25 % trypsin (Lonza, Belgium). The cells were fixed with 4% of formaldehyde then washed with distilled water three times. Working solution for staining was prepared by mixing 5 % potassium ferrocyanide (Sigma-Aldrich) (stored in dark bottle) and 5 % hydrochloric acid solutions (Sigma-Aldrich) at a ratio of 1:1 just before use for staining. Fixed cells were incubated with the working solution for 30 min at room temperature, washed by distilled water three times, and counterstained with Nuclear Fast Red (Sigma-Aldrich, USA) for 5 min.

Results and discussion

In this study, we present data on the ferrimagnetic iron oxide nanoparticle synthesis in apoferritin cage and the incorporation of a radioactive label on the exterior surface of the protein cage. In addition, the efficacy of protein labeling and stability of the rhenium carbonyl label in human serum medium were demonstrated using ICP-MS measurements.

Mineralization of iron oxide nanoparticles in apoferritin

HoSF protein cages were demineralized by a reductive dialysis method and iron oxide nanoparticles were synthesized in the 8 nm sized cavity of the protein under elevated pH and temperature (pH 8.5, 65 °C) to direct the ferrimagnetic phase Fe_3O_4. The synthesized ferrimagnetic phase comes out with brown color.

Protein samples were purified and analyzed before and after mineralization by SEC. Elutions from the column were collected and monitored at both 280 nm and 410 nm for protein and iron oxide nanoparticles, respectively (Fig. 1).

Analysis of both apoferritin and magnetoferritin by SEC exhibited identical elution times which mean that the mineralization reaction did not disrupt the protein cage (Fig. 1). For the apoferritin (Fig. 1-a), iron oxide absorption peak was almost at the a back ground level whereas after mineralization (Fig. 1-b) the coelution of the protein cage (280 nm) and iron oxide formed inside the core (410 nm) were observed.

Aggregates were eliminated and only mono-dispersed mineralized proteins were used for subsequent experiments. From the AAS and Bradford analysis for the protein sample, iron loading factor was found to be 1855 iron atoms per protein cage (Fe/cage). Mineralized sample was negatively stained with 1 % uranyl acetate solution and investigated by TEM to determine the size of the iron oxide nanoparticles and to show protein shells around each particle. The average size of the particles was measured as 4.52 ± 1.2 nm and the image shows the expected spherical cage like structure (Fig. 2).

Size distribution histograms of the particles analyzed from the TEM image obtained by measuring the diameters of more than 100 particles. The value shown on the histograms are mean ± standard deviation.

MR relaxivity determination of the synthesized materials was performed in order to investigate the potential of the magnetoferritin as an MRI contrast agent. The most important characteristics are the longitudinal and transverse relaxivities R_1 and R_2 of the contrast agent. It is known that the transverse relaxivity of superparamagnetic contrast agents is far greater than their longitudinal relaxivity. Therefore they are used mainly as negative agents for T2-weighted imaging [30]. T2 relaxivity measurements of the magnetoferritin samples with the

loading factors of 1500 - 2000 Fe/protein (experimentally found iron loading factors) were done at room temperature and at a frequency of 300 MHz. The R_2 values were found around 70 $mM^{-1}.s^{-1}$ which were comparable with the R_2 values of the other commercially used iron oxide contrast reagents [13]. The result indicated that the magnetoferritin samples prepared can be used as T2 contrast agents in magnetic resonance imaging.

Surface modification of protein exterior surface

Our laboratories are not appropriate to work with radioactive elements, hence a non-radioactive rhenium isotope, $[Re(CO)_3(H_2O)_3]^+$, was used for the preparation of rhenium carbonyl complex. Optimization studies regarding complex formation yield were carried out previously with ICP-MS [29]. The Re carbonyl complex formation efficiency was found as 90.4 %

For the modification of lysine of protein before loading histidine and rhenium complex, the glutaraldehyde crosslinker, one of the mostly used one for the protein conjugation, was used. The N-terminal amino groups of cysteine and lysine of the peptides and proteins can be activated by the aldehyde groups of glutaraldehyde that can bind to the amino groups of the protein. Glutaraldehyde was found to react readily with the α-amino groups of amino acids to form mainly intermolecular cross-linkages and some other reactions may occur with tyrosine, histidine [31,32]. For the modification of lysine groups of protein, the amount of glutaraldehyde used is of importance, since this affects the conjugation procedure. The number of lysine groups on the exterior surface exposed to the solution was assumed as 60-70 [33] and 10 fold excess amounts of glutaraldehyde and histidine compared to the total number of surface amine groups were used for the surface modification experiments. When the glutaraldehyde concentration was increased further, the protein sample becomes more insoluble, resulting in the formation of protein precipitates [34,35].

Glutaraldehyde has tendency to form high molecular weight polymers due to its homobifunctional nature. However there should be a difference between the rate of reaction of glutaraldehyde with an amine group on the protein surface and the rate of crosslinking reaction of this fixed glutaraldehyde group with another protein. In principle, the former modification should be very rapid. Whereas, the crosslinking reaction which requires the correct alignment of two groups located on two different proteins should be relatively slow [32]. Therefore the duration of the protein glutaraldehyde interaction was varied as 15 and 60

minutes. Large aggregates were eliminated through centrifugation. The SEC profiles of glutaraldehyde conjugated magnetoferritin samples obtained at these two different time spans are presented in Fig. 3.

When the reaction was performed at short reaction time, protein elution was completed in the first eight fractions of the SEC profile (Fig. 3-a). At longer reaction time on the other hand, as seen in Fig. 3-b, protein absorption peak starts to appear at the fifth fraction, its absorbance value is decreased compared to the absorbance of the protein modified in 15 min reaction time and a small signal appears at higher fractions. These observations can be correlated to the homobifunctional nature of glutaraldehyde. At longer reaction times, protein loss was mostly seen as precipitates due to the formation of heavy protein-protein conjugates which were eliminated through centrifugation before passing the column. Moreover, at long time span, small polymeric structures may also form due to the self-reaction of glutaraldehyde molecules. These glutaraldehyde polymers absorb at the same wavelength as protein [36,37] and eluted at higher fractions in the SEC profile. Therefore 15 min reaction time was preferred in the modification of the magnetoferritin structures with glutaraldehyde.

Surface modification of protein with glutaraldehyde groups were tried to be followed by UV-Vis absorption measurements. As stated in the literature [34], the reactions of colorless proteins with glutaraldehyde can be monitored spectrophotometrically. The color of the protein solution turns into yellow following the glutaraldehyde addition and a new peak appears at 265 nm due to the reaction of lysine and glutaraldehyde [34]. Unfortunately the dark brown color of magnetoferritin solution did not allow any observable change during surface modification of the protein with glutaraldehyde.

For the histidine immobilization, mixing of the reagents simultaneously or in a sequence which are named as one-step and two-step procedures were tried. Following histidine immobilization protein samples were again characterized by SEC following the centrifugation step.

According to experimental observations, in the case of two-step process protein loss in the centrifugation step was less and the full width at half maximum in the SEC profile of was narrower compared to that of one step process (Fig. 4). Therefore it was thought that mixing all reagents simultaneously grounds the formation of both the large molecular weight

insoluble protein oligomers and small protein fractions together with intact ferritin nanoparticles. Therefore two-step procedure which mostly overcomes these problems and produces homogeneous particle size distribution was preferred.

Effect of histidine immobilization on the binding efficiency of Re carbonyl complex

100, 500 and 1000 μL of rhenium complex solution was coupled to both histidine modified and unmodified magnetoferritin proteins (1855 Fe/cage)) and protein samples were subjected to size separation by means of G-25 Sephadex column and 1 mL fractions were collected for UV-Vis and ICP-MS measurements to determine the protein and Re concentrations respectively. The distribution of rhenium and protein amounts in the collected fractions are shown in Fig. 5-a and Fig. 5-b.

In the SEC analysis, due to its small size compared to that of protein, rhenium carbonyl complex is expected to appear at longer retention times. It appears early only if it were appended to the protein surface. As can be seen in Fig. 5-a, in the absence of exogenous histidine groups on magnetoferritin cage, a small absorption peak for rhenium complex was observed corresponding to the protein containing fractions. This was probably due to the rhenium complex attached to protein either through naturally existed histidine or lysine groups on the surface or unspecific adsorption. The number of Re carbonyl label per protein cage was calculated as 4. In case of histidine modified magnetoferritin samples, on the other hand, the absorbance value of the rhenium complex was increased, and as the SEC elution profile displayed, rhenium carbonyl and magnetoferritin were eluted concurrently, Fig. 5-b. The other factor influencing the labeling efficiency was the amount of the rhenium carbonyl complex introduced to the reaction medium. The number of Re carbonyl label per protein cage was found as 15 and 22 when 100 μL and 500 μL of rhenium complex solution were used for labeling respectively. A 5 fold increase in the volume of the rhenium carbonyl complex was brought about 47 % increases in the labeling efficiency. However, when larger volumes of rhenium complex were used proteins were started to precipitate. Therefore, it was concluded that 500 μL of rhenium complex solution was optimum for the labeling and the introduction of histidine groups to the surface of the protein were enhanced the labeling efficiency almost 6 fold compared to that of natural magnetoferritin surface, Table 1.

Stability of rhenium carbonyl complex on magnetoferritin

The stability of the rhenium carbonyl complex on magnetoferritin is very important for its biological applications. Thus, it was tested in human serum medium and PBS buffer (0.1 M NaCl, pH 7.0) by exhaustive dialysis of the Re labeled magnetoferritin samples for 72 h. The equilibrated dialysate is replaced with fresh one at every 12 h. The amount of rhenium released through dialysis was followed by ICP-MS in 12 h periods. The percentages of rhenium retained on the surface of the protein at various time intervals are given in Fig. 6.

After 24 h dialysis at room temperature, 92.8 ± 1.7 % rhenium in human serum medium and 94.2 ± 2.0 % of the rhenium in PBS environment were retained on the surface of the protein. At the end of three days, these values were changed to 91.1 ± 1.8 % and 87.6 ± 1.9 % rhenium in human serum medium and PBS environment respectively.

Stability test was also applied to the rhenium labeled magnetoferritin samples stored in PBS buffer for 72 hours. Three days after the labeling process, proteins were purified by SEC using G-25 Sephadex column to remove the free Re carbonyl complex that might have broken away from the protein. As stated before, protein and Re concentrations were measured in the collected fractions and SEC profiles were obtained. It was found that 91.8 ± 0.09 % of rhenium carbonyl complex was still attached on the protein surface and the protein cage remains intact during this time interval, Table 2.

The percentage of the label retained on the protein after 72 hours of the labeling process in PBS environment, measured following SEC and dialysis separations are coinciding with each other. The small change may be due to the exchange of dialysate in 12 hours where diffusion may further force the release of rhenium carbonyl complex in the sample.

Native gel electrophoresis

The native ferritin cage, magnetoferritin cages surface modified with histidine and rhenium carbonyl complex were electrophoresed on polyacrylamide gel under native (non-denaturing) conditions (Fig. 7).

The gel chromatogram showed no significant differences in the elution profiles for native ferritin, histidine immobilized magnetoferritin and Re labeled magnetoferritin samples (Fig. 7). The co-migration of them indicated that the surface modified magnetoferritin cage

remained intact and did not change by the synthesis. We can also assume that the overall charge of the protein and the size of mineralized protein samples have not been changed significantly.

In vitro cytotoxicity study

Human breast metastatic adenocarcinoma, MDA-MB-231 cell line was chosen to evaluate in vitro cytotoxicity of Re labeled magnetoferritin nanoparticles. Since the radioactivity oriented toxicology is the wanted property of radiopharmaceuticals used in the therapy, cytotoxicity studies were designed to examine the chemical toxicity of the prepared particles containing stable isotope, ^{187}Re only [38].

The cells not treated with nanoparticles were used as control (untreated) and a concentration/time dependent cytotoxicity profile was obtained as seen in Fig. 8. The assay revealed an IC_{50} value of 1 mg/mL for the prepared nanoparticles. No significant cytotoxic effect was observed up to 72 hours of incubation at IC_{50} value. This result could also be explained by the presence of apoferritin shell that camouflaged the nanoparticles and the stability of rhenium complex on the protein surface that was above 90 % for 72 h since rhenium carbonyl complex affixed to the magnetoferritin does not come off easily before apoferritin was degraded.

Prussian blue staining

Prussian blue staining was utilized for qualitative detection of iron remained within the cells. Intra cellular uptake experiments of prepared ^{187}Re carbonyl labeled magnetoferritin nanoparticles were tested using MDA-MB-231 cell lines. The physiochemical properties of atoms, such as diffusion, depend on mass. In case of ^{187}Re and ^{188}Re isotopes, there is only one neutron difference. This mass difference may be important between the isotopes of the lightest elements, which is not the case in this study [39]. As a control experiment, MDA-MB-231 cells without incubation of nanoparticles were also stained (Fig. 9-a). Prussian blue staining results showed that labeled magnetoferritin cages (as blue dots) were taken up by cancerous cell lines and accumulated in cytoplasm (Fig. 9-b).

Conclusion

We have reported the first case of preparation of Re labeled magnetoferritin nanocages. Mineralization of magnetic materials (Fe3O4 / γ-Fe2O3) was successfully done in the ferritin cages with suitable characteristics for use as an MR contrast agent. Tricarbonyl complex of Re (I) was easily prepared from perrhenate and CO gas at atmospheric pressure in aqueous solution. Imidazole rings of histidine groups introduced to the surface were used to bind Re (I) strongly. It was calculated that 22 out of approximately 60 lysine groups on the protein surface were occupied by rhenium carbonyl complex. The stability of the label was evaluated by exhaustive dialysis of labeled magnetoferritin samples for 24 and 72 hours in human serum medium. The recoveries were above 90 percent for both cases which confirmed that rhenium carbonyl complex affixed to the the magnetoferritin does not come off easily. High labeling yield, in vitro stability and low toxicity of labeled magnetoferritin nanoparticles create opportunities for its use as a diagnostic and therapy agent.

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Conflict of ınterest: The authors declare that they have no conflict of interest.

References

1. F. C. Meldrum, B. R. Heywood, S. Mann, Science, 1992, 257, 522-523.

2. T. Douglas, D. P. Dickson, S. Betteridge, J. Charnock, Science, 1995, 269, 54.

3. F. C. Meldrum, T. Douglas, S. Levi, P. Arosio, S. Mann, Journal of Inorganic Biochemistry, 1995, 58, 59-68.

4. K. K. Wong, S. Mann, Advanced Materials, 1996, 80, 928-932.

5. D. Finazzi, P. Arosio, Archives of Toxicology, 2014, 88, 1787-1802.

6. H. Yoshimura, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2006, 282, 464-470.

7. M. Uchida, M. T. Klem, M. Allen, P. Suci, M. Flenniken, E. Gillitzer, Z. Varpness, L. O. Liepold, M. Young, T. Douglas, Advanced Materials, 2007, 19, 1025-1042.

8. P. Tartaj, M. Morales, T. Gonzalez-Carreno, S. Veintemillas-Verdaguer, C. Serna, Journal of Magnetism and Magnetic Materials, 2005, 290, 28-34.

9. K. Fan, C. Cao, Y. Pan, D. Lu, D. Yang, J. Feng, L. Song, M. Liang, X. Yan, Nature Nanotechnology, 2012, 7, 459-464.

10. M. Uchida, M. L. Flenniken, M. Allen, D. A. Willits, B. E. Crowley, S. Brumfield, A. F. Willis, L. Jackiw, M. Jutila, M. J. Young, Journal of the American Chemical Society, 2006, 128, 16626-16633.

11. N. Gálvez, B. Fernández, P. Sánchez, J. Morales-Sanfrutos, F. Santoyo-González, R. Cuesta, R. Bermejo, M. Clemente-León, E. Coronado, A. Soriano-Portillo, Solid State Sciences, 2009, 11, 754-759.

12. E. Valero, S. Tambalo, P. Marzola, M. Ortega-Munoz, F. J. López-Jaramillo, F. Santoyo-González, J. de Dios López, J. J. Delgado, J. J. Calvino, R. Cuesta R, Journal of the American Chemical Society, 2011, 133, 4889-4895.

13. M. Uchida, M. Terashima, C. H. Cunningham, Y. Suzuki, D. A. Willits, A. F. Willis, P. C. Yang, P. S. Tsao, M. V. McConnell, M. J. Young, Magnetic Resonance in Medicine, 2008, 60, 1073-1081.

14. M. Uchida, S. Kang, C. Reichhardt, K. Harlen, T. Douglas, Biochimica et Biophysica Acta (BBA)-General Subjects, 2010, 1800, 834-845.

15. C. A. Boswell, M. W. BrechbieL, Nuclear Medicine and Biology, 2007, 34, 757-778.

16. M. Hamoudeh, M. A. Kamleh, R. Diab, H. Fessi, Advanced Drug Delivery Reviews, 2008, 60, 1329-1346.

17. M. Hamoudeh, H. Salim, D. Barbos, C. Paunoiu, H. Fessi, European Journal of Pharmaceutics and Biopharmaceutics, 2007, 67, 597-611.

18. L. Ottobrini, P. Ciana, A. Biserni, G. Lucignani, A. Maggi, Molecular and cellular endocrinology, 2006, 246, 69-75.

19. H. Hong, Y. Zhang, J. Sun, W. Cai, Nano today, 2009, 4, 399-413.

20. U. Häfeli, G. Pauer, S. Failing, G. Tapolsky, Journal of Magnetism and Magnetic Materials, 2001, 225, 73-78.

21. B. C. Lee, B. S. Moon, J. S. Kim, J. H. Jung, H. S. Park, J. A. Katzenellenbogen, S. E. Kim, RSC Advances, 2013, 3, 782-792.

22. G. Liu, D. J. Hnatowich, Anti-cancer Agents in Medicinal Chemistry, 2007, 7, 367.

23. R. Alberto, R. Schibli, R. Waibel, U. Abram, A. P. Schubiger, Coordination Chemistry Reviews, 1999, 190, 901-919.

24. R. Schibli, A. P. Schubiger, European Journal of Nuclear Medicine and Molecular Imaging, 2002, 29, 1529-1542.

25. J. Xia, Y. Wang, J. Yu, J. Cao, C. Zhang, D. Yin, Journal of Radioanalytical and Nuclear Chemistry, 2005, 266, 313-316.

26. J. Cao, Y. Wang, J. Yu, J. Xia, C. Zhang, D. Yin, U. O. Häfeli, Journal of Magnetism and Magnetic Materials, 2004, 277, 165-174.

27. H. J. Pietzsch, A. Gupta, M. Reisgys, A. Drews, S. Seifert, R. Syhre, H. Spies, R. Alberto, U. Abram, P. Schubiger, Bioconjugate Chemistry, 2000, 11, 414-424.

28. K. K. Wong, T. Douglas, S. Gider, D. D. Awschalom, S. Mann, Chemistry of Materials, 1998, 10, 279-285.

29. Ü. Zengin, Preperation and characterization of silica coated magnetite nanoparticles and labeling with nonradioactive re as a surrogate of tc-99m for magneticly targeted imaging. M.Sc. thesis, 2010, Middle East Technical University, Ankara, Turkey.

30. H. Shokrollahi, Materials Science and Engineering: C, 2013, 33, 4485-4497.

31. A. F. S. A. Habeeb, R. Hıramoto, Archives of Biochemistry and Biophysics, 1968, 126, 16-26.

32. O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. C. Rodrigues, R. Fernandez-Lafuente, RSC Advances, 2014, 4, 1583-1600.

33. M. Li, S. Mann, Journal of Materials Chemistry, 2004, 14, 2260-2263.

34. D. Hopwood, C.R. Allen, M. Mccabe, Histochemical Journal, 1970, 2, 137-150.

35. D. T. Cheung, M. E. Nimni, Connective Tissue Research, 1982, 10, 201-216.

36. J. Chilková, V. Říha, V. Chvátalova, Chem. Papers, 1996, 50, 345-348.

37. V. Herzog, H. Fahimi, The Journal of Cell Biology, 1974, 60, 303-311.

38. European Medicine Agency, Radiopharmaceuticals, Directives 65/65/EEC, 75/318/EEC as amended, Directive 89/343/EEC, **http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003653.pdf** Accessed; November 15, 2015.

39. G. Friedlander, J.W Kennedy, J. M. Miller, Nuclear and Radiochemistry, 1964, 2 nd add, John Wiley&Sons, Inc., New York.

FIGURES LEGENDS

Fig. 1 Size exclusion chromatography (SEC) absorbance profiles of horse spleen ferritin (HoSF). (a) before mineralization reaction, (b) after mineralization with 2000 Fe/cage. Elution was monitored at both 280 nm (protein) and 410 nm (iron oxide mineral). Coelution of protein and mineral in profile (b) indicate the composite nature of the mineralized protein cage.

Fig. 2 (a) Transmission electron microscope (TEM) image of iron oxide cores inside apoferritin cages negatively stained with uranyl acetate.; 100 nm scale bar, (b) Size distribution diagram of nanoparticles within apoferritin cages.

Fig. 3 Size exclusion chromatography (SEC) absorbance profiles of glutaraldehyde conjugated magnetoferritin protein samples obtained at time spans of (a) 15 min (blue), (b) 60 min (red) reaction times.

Fig. 4 Size exclusion chromatography (SEC) absorbance profiles of histidine immobilized magnetoferritin samples following (a) one step (red), (b) two step (blue) modification procedures.

Fig. 5 Size exclusion chromatography (SEC) absorbance profiles of rhenium labeled protein samples using (a) magnetoferritin with naturally existed lysine groups (b) histidine immobilized magnetoferritin.

Fig. 6 Effect of time on stability of rhenium retained on the surface of the magnetoferritin at various time intervals in human serum and PBS buffer.

Fig. 7 Native polyacrylamide gel stained electrophoresis (Coomassie Brilliant Blue R250). Lane 1 is Re labeled magnetoferritin sample, lane 2 is histidine immobilized magnetoferritin sample and lane 3 is native ferritin sample.

Fig. 8 Cell viability of MDA-MB-231 cells after treatment with varing concentrations (0.5–1.5 mg/mL) of ^{187}Re labeled magnetoferritin nanoparticles over different exposure times.

Fig. 9 Light microscopy images of MDA-MB-231 cells that are stained with Prussian blue and following counterstained with nuclear fast red (a) control: MDA-MB-231 cells that are not treated with Re labeled magnetoferritin nanoparticles (1.0 mg/mL), (b) MDA-MB-231 cells that are treated with Re labeled magnetoferritin nanoparticles. The scale bar in the images is 200 μm.

Table 1 The average numbers of rhenium labeled to the magnetoferritin and histidine immobilized magnetoferritin samples that were calcutated over three experiments (N= 3) from size exclusion chromatography (SEC) results using protein and rhenium amounts in the collected fractions that are shown with straight line on the chromatograms.

Samples	Average number of Re / protein ± Std Deviation
his-Magnetoferritin	21.70 ± 1.81
Magnetoferritin	4.17 ± 0.70

Table 2 The average number of rhenium labeled to histidine immobilized magnetoferritin sample calcutated over three experiments (N= 3) from size exclusion chromatography (SEC) results that was obtained 72 h after the labeling process using protein and rhenium amounts in the collected fractions from size exclusion column.

Sample	Average number of Re / protein ± Std Deviation
his-Magnetoferritin (after 72 h experiment)	19.93 ± 1.39

Fig. 1

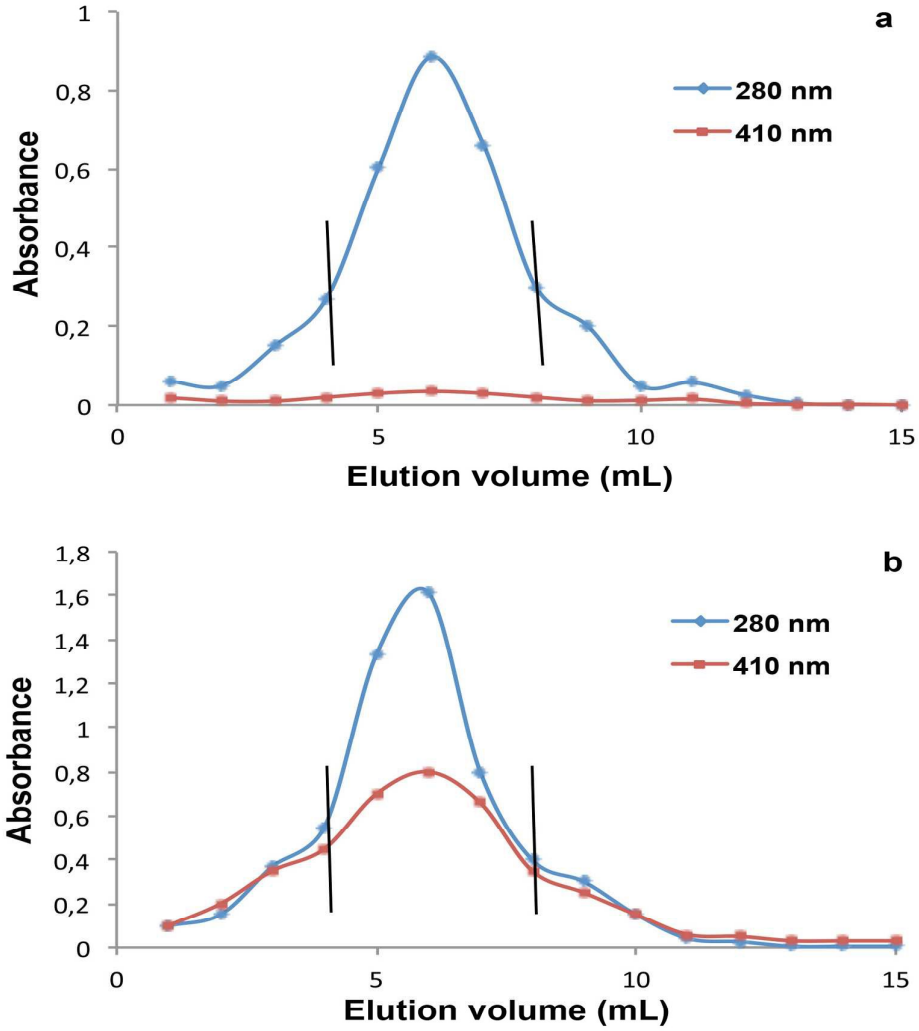


Fig. 2

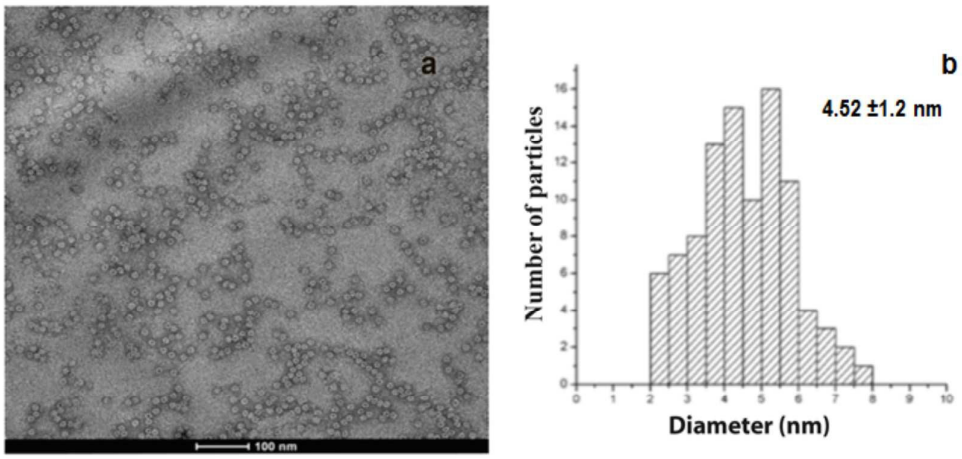


Fig. 3

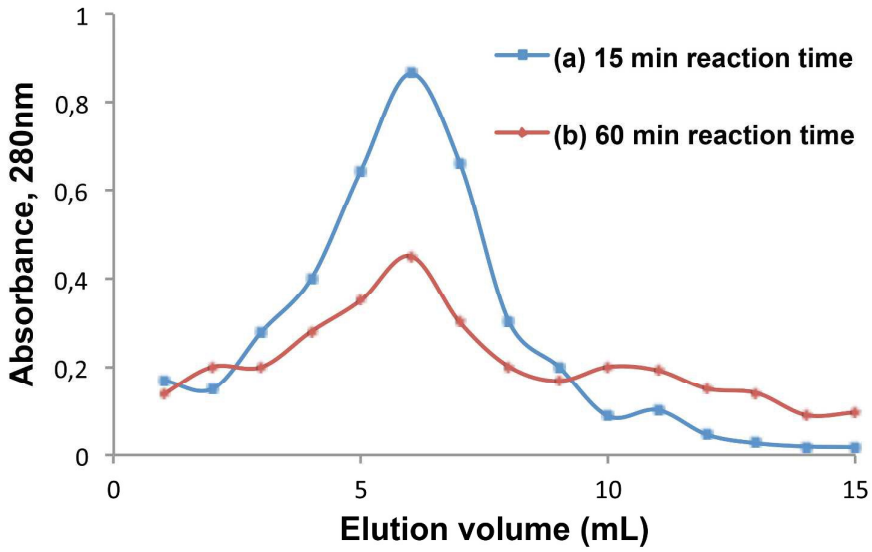


Fig. 4

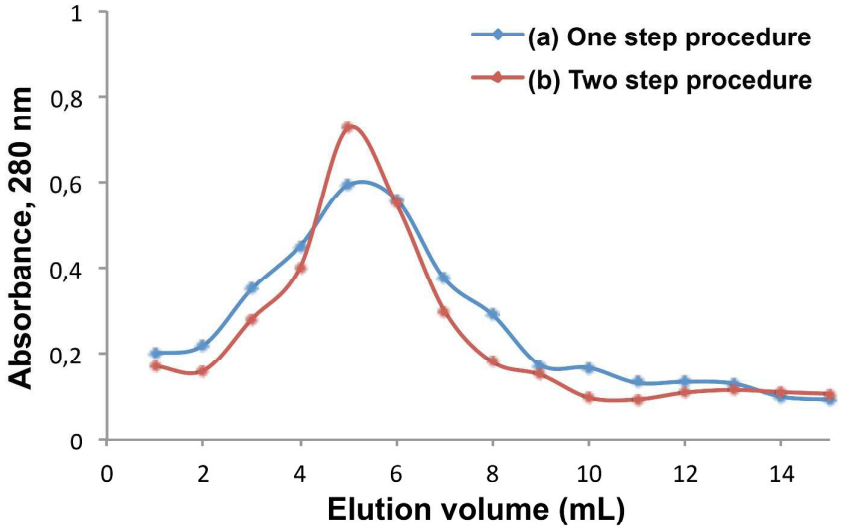
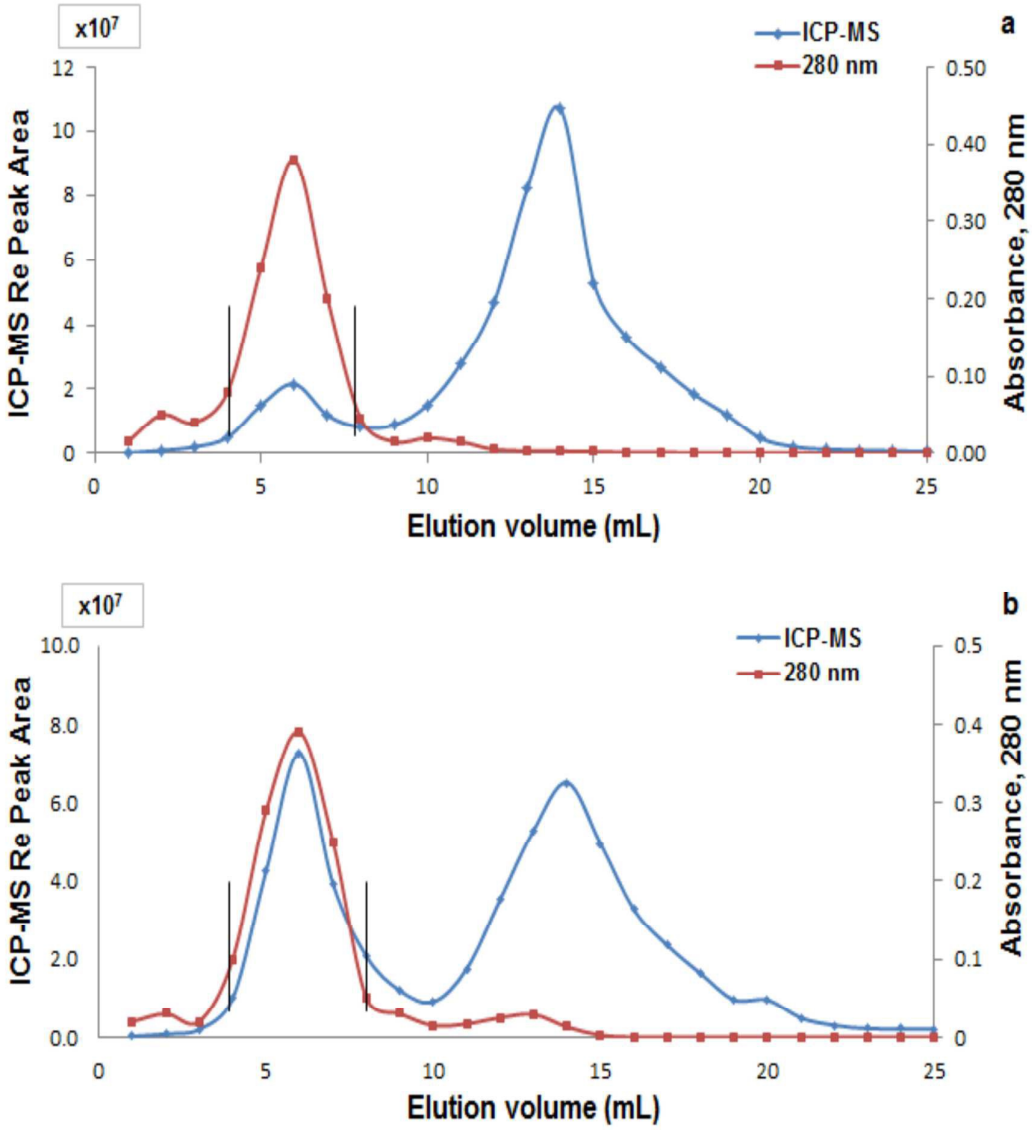


Fig. 5



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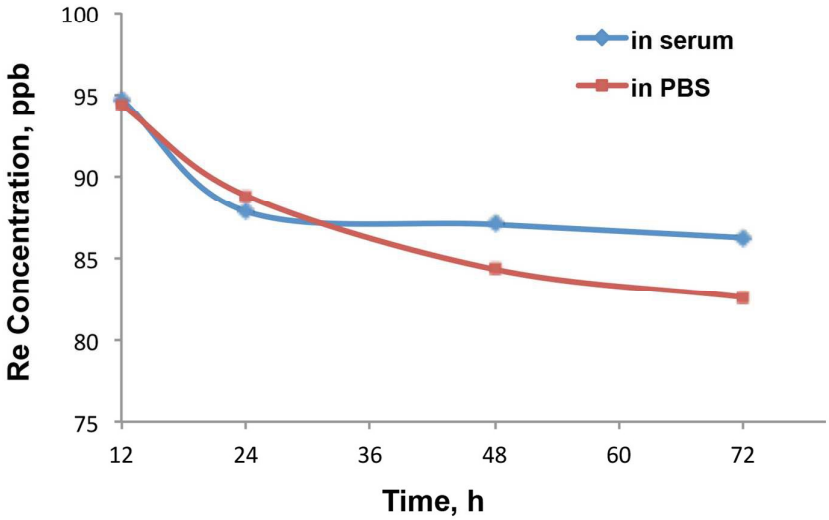
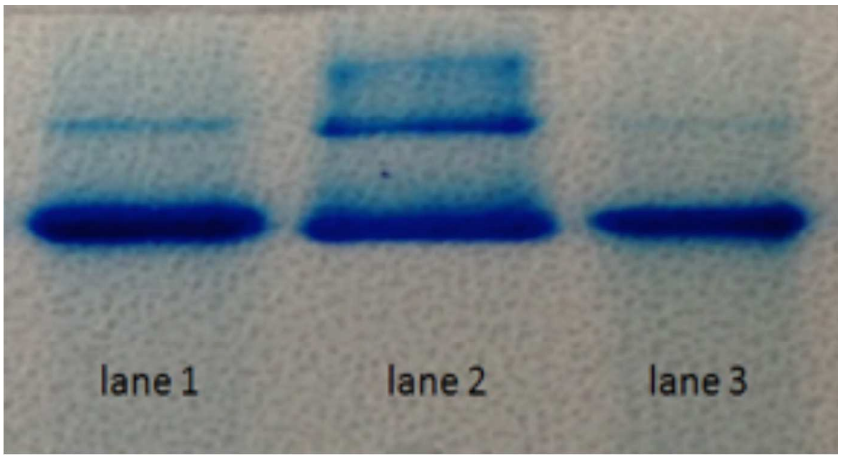


Fig. 7



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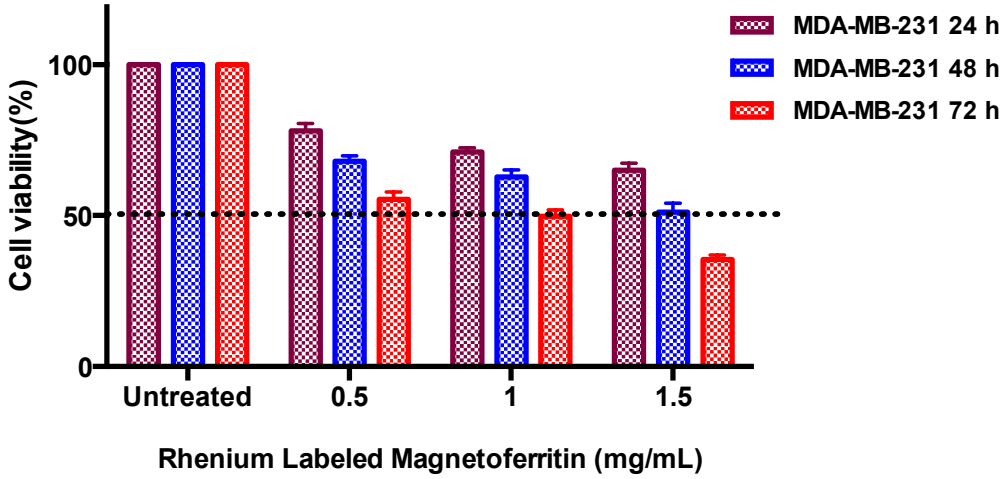

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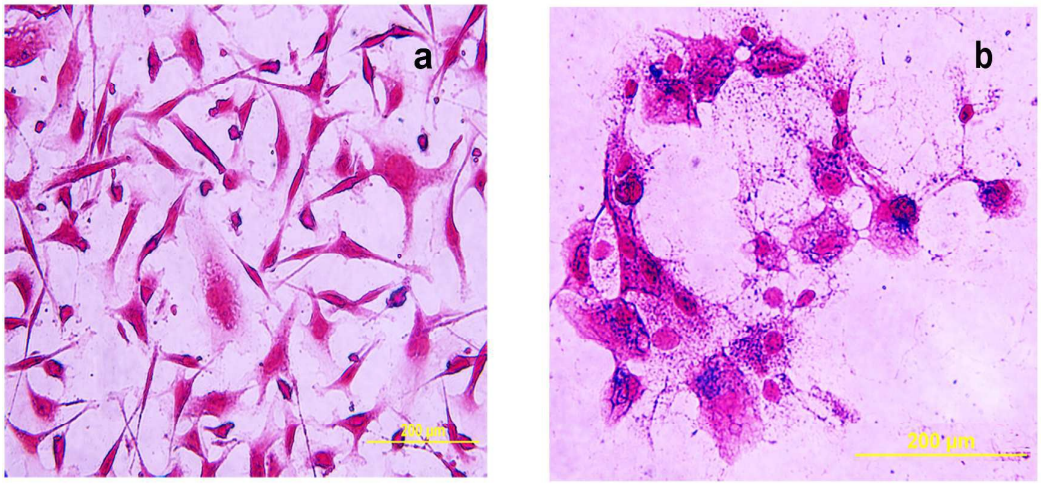
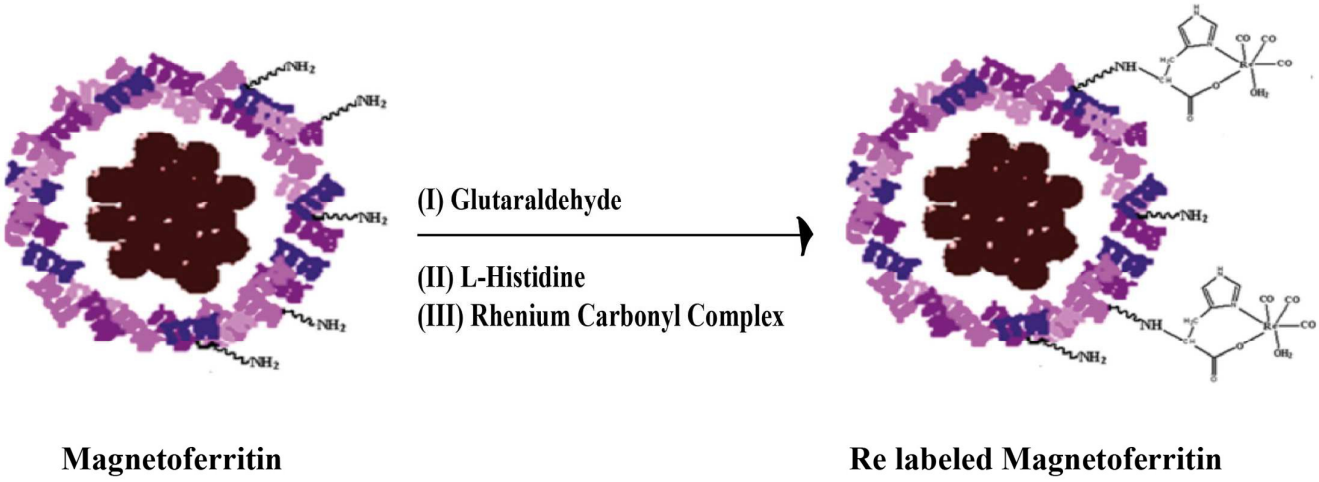


Graphical Abstract



Magnetoferritin **Re labeled Magnetoferritin**