Journal of Visualized Experiments

Synthesis of cationized magnetoferritin for ultra-fast magnetization of cells
--Manuscript Draft--

Manuscript Number:	JoVE54785R2
Full Title:	Synthesis of cationized magnetoferritin for ultra-fast magnetization of cells
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	magnetoferritin; magnetic nanoparticles; protein cage; cationization; surface functionalization; magnetic labeling; magnetic cell separation; Stem Cells
Manuscript Classifications:	10.1.637.512.600.500: Metal Nanoparticles; 5.5.242.363: Cell Separation
Corresponding Author:	Sara Correia Carreira University of Bristol Bristol, Avon UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	S.Carreira@bristol.ac.uk
Corresponding Author's Institution:	University of Bristol
Corresponding Author's Secondary Institution:	
First Author:	Sara Correia Carreira
First Author Secondary Information:	
Other Authors:	James P.K. Armstrong
	Mitsuhiro Okuda
	Annela M Seddon
	Adam W Perriman
	Walther Schwarzacher
Order of Authors Secondary Information:	

Abstract:	Many important biomedical applications, such as cell imaging and remote manipulation, can be achieved by labeling cells with superparamagnetic iron oxide nanoparticles (SPIONs). Achieving sufficient cellular uptake of SPIONs is a challenge that has traditionally been met by exposing cells to elevated concentrations of SPIONs or by prolonging exposure times (up to 72 h). However, these strategies are likely to mediate toxicity. Here, we present the synthesis of the protein-based SPION magnetoferritin as well as a facile surface functionalization protocol that enables rapid cell magnetization using low exposure concentrations. The SPION core of magnetoferritin consists of cobalt-doped iron oxide with an average particle diameter of 8.2 nm mineralized inside the cavity of horse spleen apo-ferritin. Chemical cationization of magnetoferritin produced a novel, highly membrane-active SPION that magnetized human mesenchymal stem cells (hMSCs) using incubation times as short as one minute and iron concentrations as lows as 0.2 mM.

Author Comments:	
Additional Information:	
Question	Response
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

TITLE:

Synthesis of cationized magnetoferritin for ultra-fast magnetization of cells

AUTHORS:

Sara Correia Carreira
Bristol Centre for Functional Nanomaterials
University of Bristol
Bristol, UK
s.carreira@bristol.ac.uk

James P K Armstrong
Department of Materials
Imperial College London
London, UK
James.armstrong@imperial.ac.uk

Mitsuhiro Okuda
Self Assembly Group
CIC nanoGUNE
San Sebastian, Spain
m.okuda@nanogune.eu

Annela M Seddon
Bristol Centre for Functional Nanomaterials
University of Bristol
Bristol, UK
Annela.Seddon@bristol.ac.uk

Adam W Perriman
School of Cellular and Molecular Medicine
University of Bristol
Bristol, UK
chawp@bristol.ac.uk

Walther Schwarzacher
H.H. Wills Physics Laboratory
University of Bristol
Bristol, UK
W.Schwarzacher@bristol.ac.uk

CORRESPONDING AUTHOR:

Sara Correia Carreira
s.carreira@bristol.ac.uk

Walther Schwarzacher
W.Schwarzacher@bristol.ac.uk

KEYWORDS:
Magnetoferritin, magnetic nanoparticles, protein cage, cationization, surface functionalization, magnetic labeling, magnetic cell separation, stem cells

SHORT ABSTRACT:
A protocol for the synthesis and cationization of cobalt-doped magnetoferritin is presented, as well as a method to rapidly magnetize stem cells with cationized magnetoferritin.

LONG ABSTRACT:
Many important biomedical applications, such as cell imaging and remote manipulation, can be achieved by labeling cells with superparamagnetic iron oxide nanoparticles (SPIONs). Achieving sufficient cellular uptake of SPIONs is a challenge that has traditionally been met by exposing cells to elevated concentrations of SPIONs or by prolonging exposure times (up to 72 h). However, these strategies are likely to mediate toxicity. Here, we present the synthesis of the protein-based SPION magnetoferritin as well as a facile surface functionalization protocol that enables rapid cell magnetization using low exposure concentrations. The SPION core of magnetoferritin consists of cobalt-doped iron oxide with an average particle diameter of 8.2 nm mineralized inside the cavity of horse spleen apo-ferritin. Chemical cationization of magnetoferritin produced a novel, highly membrane-active SPION that magnetized human mesenchymal stem cells (hMSCs) using incubation times as short as one minute and iron concentrations as lows as 0.2 mM.

INTRODUCTION:
Surface binding or internalization of superparamagnetic iron oxide nanoparticles (SPIONs) has enabled magnetization of a variety of cell types for applications such as imaging and remote manipulation.[1] However, achieving sufficient cellular magnetization can be a challenge, particularly when the interaction between the SPION and the cell surface is weak.[2] In the past, prolonged exposure or high SPION concentrations have been employed as strategies to overcome this challenge.[3,4] Nevertheless, these strategies are problematic because they increase toxicity[5,6] and have very limited success in cell types with low internalization rates, such as lymphocytes.[7] To enhance cellular uptake of SPIONs, several surface functionalization approaches have been explored. For instance, antibodies have been used to promote receptor-mediated endocytosis,[8] while non-specific uptake can be achieved using transfection agents[9,10] or cell-penetrating species, such as HIV tat-peptide.[11,12] However, antibody and peptide functionalization approaches are limited by expensive reagents and complex synthetic preparation, while transfection agents can induce nanoparticle precipitation and adversely affect cell function.[13,14]

We recently reported the synthesis of chemically-cationized magnetoferritin, a novel SPION which was highly effective in magnetizing human mesenchymal stem cells (hMSCs) using incubation times as short as one minute.[15] Magnetoferritin is synthesized by reconstituting a

SPION inside the de-mineralized cavity of the iron storage protein ferritin. [16] This protein-based SPION combines many properties that make it well suited for cell magnetization, such as control over the magnetic properties of the magnetic core,[17-19] and biocompatibility and aqueous solubility conferred by the protein shell. Furthermore, surface functionalization is easily achieved due to addressable amino acids that can be chemically[20-22] or genetically modified.[23-25] We have shown that chemical cationization of the acidic amino acid residues of the protein shell generates a stable nanoparticle that readily interacted with anionic domains on the cell surface leading to rapid and persistent cell magnetization. This procedure eliminates the need for laborious functionalization and lengthy incubation protocols, and due the non-specific labelling mechanism this rapid magnetization strategy should find wide-spread application in other cell types. Here, we present an in depth report of the ultra-fast cell labeling method, including detailed protocols of the synthesis, purification and surface functionalization of cationized magnetoferritin.

PROTOCOL:

Human mesenchymal stem cells (hMSC) were harvested from the proximal femur bone marrow of osteoarthritic patients undergoing total hip replacement surgery, in full accordance with Bristol Southmead Hospital Research Ethics Committee guidelines (reference #078/01) and after patient consent was obtained.

1.   Magnetoferritin synthesis and purification
1.1.   General remarks
1.1.1.   Perform magnetoferritin synthesis in a sealable, double-jacketed reaction vessel at 65 °C under nitrogen atmosphere to restrict oxidation of the metal precursors. Inject metal salt solutions and hydrogen peroxide through access ports in the lid into the reaction vessel using a syringe pump.

1.1.2.   After magnetoferritin synthesis, purify the protein by anion-exchange chromatography (see section 1.4) to remove nanoparticles not enclosed in the protein cavity, followed by size-exclusion chromatography (see section 1.5) to isolate protein monomers. Determine protein concentration using a Bradford assay (see section 1.6).

1.2) **Preparations prior to synthesis**
1.2.1) Deoxygenate 500 mL of deionized water by placing a tube connected to a nitrogen gas cylinder in the water, sealing the vessel with cling film and bubbling through nitrogen gas for approximately 60 minutes.

1.2.2) Heat a water bath connected to the double jacketed reaction vessel to 65 °C. Add 75 mL of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.6) into the reaction vessel, seal the vessel, and deoxygenate by bubbling nitrogen gas through the buffer solution for approximately 20 minutes. At the same time, stir the buffer solution using a magnetic stirrer.

After deoxygenating the HEPES buffer solution, add apo-ferritin to achieve a final concentration of 3 mg/mL. Continue the magnetic stirring, but reduce the stirring speed if foaming occurs.

1.2.4) Prepare a 25 mM stock solution of cobalt sulphate heptahydrate by dissolving 0.19 g in 50 mL of deoxygenated deionized water.

1.2.5) Prepare a 25 mM solution of ammonium iron sulphate hexahydrate solution by dissolving 0.98 g in 100 mL of deoxygenated deionized water.

1.2.6) Remove 2.5 mL of the ammonium iron sulphate hexahydrate solution and replace with 2.5 mL of the cobalt sulphate heptahydrate.

1.2.7) Prepare an 8.33 mM solution of hydrogen peroxide in deoxygenated deionized water by first adding 965 µL of a hydrogen peroxide solution (30% w/w) to 9 mL of deoxygenated deionized water, and then adding 1 mL of this sub-stock to 99 mL of deoxygenated deionized water.

1.3) Magnetoferritin synthesis
1.3.1) Inject 10.1 mL of both the iron-cobalt precursor and the hydrogen peroxide simultaneously into the apo-ferritin solution (magnetically stirred) at a flow rate of 0.15 mL/min using two syringe pump (total volume injected: 20.2 mL).

1.3.2) During the injection period, deoxygenate another 500 mL of deionized water.

Note: The contents of the reaction vessel gradually adopt a brown color as the reaction proceeds.

1.3.3) Shortly before completion of the first injection, prepare another 100 mL of iron-cobalt precursor and hydrogen peroxide solution using the freshly deoxygenated deionized water.

1.3.4) Repeat the injection step described in 1.3.1 using freshly prepared solutions of iron-cobalt and hydrogen peroxide.

1.3.5) During the injection period, deoxygenate another 500 mL of deionized water, and proceed as described in 1.3.3 and 1.3.4.

1.3.6) After the third injection, leave the solution to mature for 15 minutes under stirring.

1.3.7) Add 1.5 mL of a 1 M sodium citrate solution to the reaction vessel to chelate free metal ions in solution.

1.3.8) Remove the solution from the reaction vessel into 50 mL centrifuge tubes, centrifuge for 30 minutes at 4350 x *g* and pass the supernatant through a 0.22 µm syringe filter. At this stage,

the filtered supernatant can be stored at 4 °C until purification.

1.4) **Ion exchange chromatography**
1.4.1) Load the sample onto a column (2.5 cm diameter, 20 cm long) containing a cationic matrix using a peristaltic pump at a flow rate of 10 mL/min.

1.4.2) Wash the column with approximately 100 mL of running buffer (50 mM Tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.0) using a gradient pump at a flow rate of 10 mL /min.

1.4.3) To elute the protein, wash the column at 10 mL/min with increasing concentrations of sodium chloride (NaCl) in Tris buffer: 150, 500 and 1000 mM final NaCl concentration, 150 mL of each concentration.

1.4.4) As the protein elutes at a NaCl concentration of 500 mM, collect it in 50 mL fractions using an automated fraction collector.

Note: For a detailed protocol of ion exchange chromatography consult previously published protocols.[26]

1.5) **Size exclusion chromatography**
1.5.1) Concentrate the 150 mL of magnetoferritin to a volume of approximately 2 mL using a 15 mL centrifugal filter unit followed by a 4 mL volume unit. Refer to the manufacturer's instructions of the centrifugal filter units (see material list) for a detailed protocol of this procedure.

1.5.2) Load the concentrated sample onto a gel filtration column using an injection loop.

1.5.3) Wash the column with running buffer (50 mM Tris buffer, pH 8.0, containing 150 mM NaCl) at a flow rate of 1.6 mL/min.

1.5.4) Collect 6 mL fractions using an automated fraction collector. Protein monomers elute last. At this point, purified magnetoferritin can be stored at 4 °C until cationization.

Note: For a detailed protocol of size exclusion chromatography using a gel filtration column consult previously published protocols.[27]

1.6) **Determining protein concentration**
1.6.1) Prepare ferritin standards of 0.06, 0.125, 0.25, 0.5 and 1 mg/mL in 50 mM Tris buffer using commercially available horse spleen ferritin.

Note: The protein concentration of commercially available ferritin is stated on the bottle. If not, contact the supplier for this information.

1.6.2) Dilute magnetoferritin samples of unknown concentration in 50 mM Tris buffer until the color of the solution approximately matches the color of the 0.5 mg/mL standard. Make a note of the dilution factor.

1.6.3) Add 10 μL of each standard and magnetoferritin sample in triplicate into wells of a 96 well plate.

1.6.4) Add 200 μL of ready-made Bradford reagent to each well (refer to materials list for Bradford assay reagent details).

1.6.5) Incubate at room temperature for 8 minutes.

1.6.6) Measure the absorbance at λ= 595 nm using a microplate reader.

1.6.7) Plot the mean absorbance of the ferritin standards as a function of protein concentration and use the slope and intercept of the linear fit to calculate the concentration of the magnetoferritin sample.

Note: Take into account the dilution factor that was used to adjust the magnetoferritin sample to the standard curve.

2. Magnetoferritin cationization
2.1) General remarks
Note: For magnetoferritin cationization, *N,N*-dimethyl-1,3-propanediamine (DMPA) was coupled to aspartic and glutamic acid residues on the MF surface using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC).

2.1.1) Carry out the reaction at room temperature under stirring. The protocol given below is for the cationization of **1 mg of magnetoferritin in a total volume of 0.5 mL** (protein concentration 2 mg/mL). However, scale up or down by keeping the protein:DMPA:EDC ratios consistent, as well as the volumes of the buffer and magnetoferritin solution.

2.1.2) Prior to the cationization reaction, dialyze the magnetoferritin samples into 50 mM phosphate buffer (pH 7) containing 50 mM NaCl. This is important to remove Tris elution buffer and avoid unwanted reactions between the Tris amine and the EDC-activated carboxylic acid residues. Determine the protein concentration after dialysis and adjust it to 4 mg/mL prior to cationization.

2.2) Cationization protocol
2.2.1) Weigh out 37.4 mg of DMPA and dissolve in 0.25 mL of 200 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer.

2.2.2) Adjust the solution pH to approximately 7 using concentrated (6 M) hydrochloric acid (HCl).

CAUTION: Perform this step in a fume hood, as addition of the acid to DMPA releases toxic fumes!

2.2.3) Add 0.25 mL of magnetoferritin solution at 4 mg/mL (final concentration of MES is 100 mM, final concentration of magnetoferritin is 2 mg/mL, total amount of protein is 1 mg).

2.2.4) Add a magnetic stirrer and stir for 2 h to equilibrate.

2.2.5) Carefully adjust the solution to pH 5.0 using 1 M HCl.

2.2.6) Add 14.1 mg of EDC powder to the DMPA/magnetoferritin solution.

2.2.7) Continue stirring for 3.5 h.

2.2.8) Filter the solution through a 0.22 μm syringe filter to remove any precipitates.

2.2.9) Add the solution to dialysis tubing with a 12–14 kDa molecular weight cut-off and dialyze against 4 L of 50 mM phosphate buffer (pH 7) containing 50 mM NaCl for 2 days at 4 °C, replacing the dialysis buffer at least three times during that period.

Note: At this point, cationized magnetoferritin can be stored at 4 °C until further use.

3.   Human mesenchymal stem cell labeling with cationized magnetoferritin
3.1) General remarks
3.1.1. Perform all cell culture using class II laminar flow cabinets and humidified incubators at 37 °C and 5% carbon dioxide atmosphere.

3.1.2. Culture cells as monolayers using 175 cm^2 flasks and 20 mL of culture medium, which was replenished every 3 - 4 days. Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM), containing 1000 mg/L glucose, 10% (v/v) foetal bovine serum, 1% (v/v) penicillin/streptomycin solution, 1% (v/v) L-alanyl-L-glutamine solution and 5 ng/mL freshly supplemented human fibroblast growth factor.

3.2) Magnetic labeling of hMSC with cationized magnetoferritin
3.2.1) Seed 150 000 hMSCs into 25 cm^2 flasks and leave to adhere overnight at 37 °C.

3.2.2) Filter sterilize cationized magnetoferritin through a 0.22 μm syringe filter and determine the protein concentration.

3.2.3) Keeping sterile conditions, prepare a 0.5 μM solution of cationized magnetoferritin in phosphate buffered saline (PBS). Keep capped in a sterile culture hood until use.

3.2.4) Wash the plated cells with 2 mL of room temperature PBS.

3.2.5) Add 1 mL of the sterilized cationized magnetoferritin solution to the plated cells and incubate for the desired time period (1 minute to 1 hour).

3.2.6) Wash cells with PBS, and then harvest cells by adding 0.5 mL of trypsin/ ethylenediaminetetraacetic acid (EDTA) and incubating at 37 °C for 5 min.

3.2.7) Add 1 mL of culture medium to inactivate the trypsin/EDTA, transfer the solution to a 15 mL centrifuge tube and centrifuge for 5 min at 524 x g.

3.2.8) Discard the supernatant and re-suspend the cell pellet in 0.5 mL magnetic separation buffer, consisting of 0.5% (w/v) fetal bovine serum and 2 mM EDTA in PBS.

3.3) Magnetic cell separation
3.3.1) Attach the magnet to the multi stand, and add a magnetic separation column to the magnet. Place a pre-separation filter on the column.

3.3.2) Place 0.5 mL of magnetic separation buffer in the pre-separation filter and let it run through both the filter and the column to wash and wet them.

3.3.3) Place a 15 mL centrifuge tube under the column.

3.3.4) Add 0.5 mL of the cell suspension in the filter reservoir of the magnetic separation column.

3.3.5) When the reservoir is empty, add 0.5 mL of magnetic separation buffer.

3.3.6) When the reservoir is empty, add further 0.5 mL of magnetic separation buffer.

3.3.7) Repeat one more time (the total volume of magnetic separation buffer used for washing should be 1.5 mL). This wash step elutes all non-magnetized cells from the column (non-magnetized cell fraction).

3.3.8) Remove the column from the magnet and place it in a fresh 15 mL centrifuge tube. Remove filter from the column reservoir.

3.3.9) Add 1 mL of magnetic separation buffer to the reservoir and immediately push through the column using the plunger supplied by the manufacturer. This elutes the magnetized cells from the column into the centrifuge tube (magnetized cell fraction).

3.3.10) Centrifuge both tubes for 5 min at 524 x g.

3.3.11) Discard the supernatant and re-suspend the cell pellets in 0.3 mL PBS.

3.3.12) Count the number of cells numbers in each fraction using a hemocytometer.

3.3.13) Determine the fraction of magnetized cells, *M(%)*, using the following equation:

$$M(\%) = 100 \cdot \frac{n(M)}{n(M+NM)}$$

where *n(M)* is the number of cells in the magnetized fraction and *n(M+NM)* is the sum of the number of cells in the magnetized and non-magnetized fractions.

3.4) Preparing hMSCs labeled with cationized magnetoferritin for iron quantification using inductively-coupled plasma optical emission spectroscopy (ICP-OES)
Note: The protocol may need to be adapted for other ICP-OES instruments. It is advisable to consult with the responsible technician.

3.4.1) Centrifuge a known number of cells for 5 min at 524 x *g*.

3.4.2) Remove the PBS supernatant and add 0.25 mL of 50% (v/v) nitric acid

3.4.3) Vortex mix the acidified cell suspension.

3.4.4) Incubate at room temperature overnight.

3.4.5) Add 4.75 mL of distilled water to each sample and vortex mix.

3.4.6) Analyze with ICP-OES.[28]

3.4.7) Normalize the measured iron content to the number of cells to determine a value for cellular iron content.

REPRESENTATIVE RESULTS:
TEM was used to confirm nanoparticle mineralization inside the apoferritin cavity and determine the average core size (Figures 1A and 1B). Image analysis of unstained magnetoferritin samples gave an average core diameter of 8.2 ± 0.7 nm, and aurothioglucose stain confirmed the presence of nanoparticles within the protein cage. Note that the images show a magnetoferritin sample that was further purified using magnetic separation to isolate uniform nanoparticle cores. Magnetoferritin samples that were not magnetically purified have a slightly broader core size distribution.[29] Analysis of the structure of the magnetoferritin core using selected-area electron diffraction indicated the possible presence of the inverse spinel structure based on magnetite (Fe_3O_4) and/or maghemite (γ-Fe_2O_3), as well as the spinel structure due to Co_3O_4. Furthermore, Raman spectra revealed peaks attributed to Fe_3O_4, small amounts of γ-Fe_2O_3, and a cobalt ferrite (Figure 1 C). ICP-OES analysis of magnetoferritin showed an average of 102 µg of iron and 0.9 µg of cobalt per milligram of magnetoferritin.

A schematic is included, illustrating the subsequent cationization step (Figure 2 A). The hydrodynamic diameter of magnetoferritin and cationized magnetoferritin was 11.8 ± 1.1 nm and 12.5 ± 1.4 nm, respectively, as determined by dynamic light scattering. The cationization efficiency of covalent DMPA-coupling to magnetoferritin was assessed using zeta potentiometry and matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry. The zeta potential changed from -10.5 mV for MF to + 8.3 mV for cationized magnetoferritin, confirming a change in surface potential from negative to positive (Table 1). Mass spectrometry experiments found a subunit molecular weight of 20.1 kDa for native apo-ferritin and 21.1 kDa for cationized apo-ferritin (Figure 2 B). This mass increase corresponds to approximately 12 coupled DMPA molecules per protein subunit, and the cationization of 288 residues on the entire 24-subunit protein.

Magnetic saturation and susceptibility were measured using SQUID magnetometry, and transverse and longitudinal relaxivity were measured using magnetic resonance imaging. Magnetic properties were similar for magnetoferritin and cationized magnetoferritin, indicating that cationization had negligible impact on the magnetic properties of the enclosed SPION (Table 1). Furthermore, these properties are similar to other iron oxide based nanoparticles,[19,30] demonstrating that cationized magnetoferritin would be as suitable as conventional SPION-based MRI contrast agents in enhancing imaging contrast.

After a 30 minute exposure, the cell surface was densely covered with cationized magnetoferritin (Figure 3 A). However, after one week, no nanoparticles were found on the cell surface (Figure 3 B). Cationized magnetoferritin was remarkably effective at magnetically labelling hMSCs. Notably, exposing the cells to cationized magnetoferritin for one minute resulted in the magnetization of 92% of the cell population and the delivery of 3.6 pg of iron per cell. Increasing the incubation time to 15 minutes resulted in the magnetization of the entire cell population (Figure 3 C).

Figure 1: Characterization of magnetoferritin cores doped with 5% cobalt.
TEM images of magnetoferritin stained with aurothioglucose (A) and unstained (B). Inset shows corresponding electron diffraction with magnetite indices. Scale bar: 20 nm. (C) Raman spectrum for magnetoferritin. The arrows indicate the main Raman vibration modes for cobalt ferrite (T_{2g}), magnetite and maghemite (both A_{1g}).[31,32] The laser wavelength used was 532 nm. (Image adapted from Okuda *et al.*[18]). Note that this magnetoferritin sample had been further purified using magnetic separation, which isolated uniformly loaded magnetoferritin particles.

Figure 2: Cationization of magnetoferritin.
A) Solvent accessible surface area representations showing the distribution of acidic (red) and basic (yellow) amino acid residues on the protein surface. Magnetoferritin (1) is modified to cationized magnetoferritin (2) by carbodiiide-mediated crosslinking of DMPA to acidic amino acid residues on the protein surface (3). B) Mass spectrometry analysis of apo-ferritin and cationized apo-ferritin subunits.
Mass-to-charge (m/z) spectrum of apoferritin (ApoF) and cationized apoferritin (cat-ApoF) generated by MALDI-TOF. A mass increase from 20.1 kDa to 21.1 kDa is observed after

cationization. (Image adapted from Correia Carreira et al.[15])

Figure 3: Magnetic labeling and cell separation of hMSCs incubated with cationized magnetoferritin.
A) TEM image of hMSCs after a 30 minute incubation with cationized magnetoferritin. The arrow indicates the presence of magnetoferritin cores densely packed on the cell surface. Scale bars: 200 nm. B) TEM image of hMSC one week after labeling. The cell surface is clear of cationized magnetoferritin. C) Investigating the rapidity of magnetic labeling: 92% of the cell population were magnetized after a one-minute exposure with 0.5 μM cationized magnetoferritin, and the entire cell population was magnetized within 15 minutes. Iron content per cell was determined using ICP-OES. Average and standard deviation from three biological replicates are shown. (Image adapted from Correia Carreira et al.[15])

Table 1: Physicochemical characterization of magnetoferritin (MF) and cationized magnetoferritin (cat-MF). (Table adapted from Correia Carreira et al.[15])

DISCUSSION:
TEM of magnetoferritin samples stained with aurothioglucose revealed the successful mineralization of nanoparticles inside the protein cage. Electron diffraction and Raman analysis of the nanoparticle core indicated the presence of a cobalt ferrite, indicating successful doping of the nanoparticle core with cobalt. This demonstrates that mixed-oxide nanoparticles can successfully be mineralized within the apo-ferritin cavity. Furthermore, we have shown previously that cobalt doping can be varied by altering the amount of cobalt precursor added to the reaction mixture, which enables tuning of the magnetic properties.[18]

Magnetoferritin synthesis can be performed in a variety of vessels, as long as they are tightly sealable and have access ports through which reactants can be introduced (e.g. a three-neck round bottom flask). The reaction temperature should be maintained at 65 °C either by placing the vessel in a water/oil bath or using a double-jacketed vessel. Here, we used a double-jacketed electrochemical cell setup to perform the synthesis. To guarantee successful synthesis, maintaining the correct pH and avoiding oxygen contamination of the aqueous solutions is crucial. Metal salt solutions should always be prepared freshly prior to use rather than in advance. Furthermore, commercial apoferritin solutions can vary in quality and affect synthesis outcome (e.g. size of nanoparticle core mineralized). It can help to dialyze the apoferritin solution into 50 mM HEPES buffer (pH 8.6) prior to synthesis to remove any residual reducing agent used by the manufacturer. It is useful to make a note of the batch number of the apo-ferritin solution used for synthesis, so it can be specifically requested from the manufacturer should additional material need to be purchased. Furthermore, the protein concentration of commercially-available apo-ferritin should be stated on the bottle, which can be used to calculate the volume of apo-ferritin solution needed for synthesis. If this is not the case, contact the supplier for this information.

The advantage of gradual addition of metal salts and hydrogen peroxide - as presented here and in previous reports- is that mineralization of the nanoparticle core can be controlled such

that different loading factors (*i.e.* nanoparticle sizes) can be achieved.[33] Furthermore, it is possible to purify magnetoferritin further using a magnetic separation column, *e.g.* a column packed with stainless steel powder secured inside an electromagnet.[34] Thus, highly monodisperse nanoparticle cores can be isolated from the bulk magnetoferritin sample. However, for magnetic cell labelling as presented here this is not required. A limitation of magnetoferritin synthesis is the relatively low synthesis yield of about 10%, and the relatively high cost of commercial apo-ferritin solutions. However, apo-ferritin may also be prepared from cheaply available horse spleen ferritin by following established de-mineralization protocols.[16]

Cationization of magnetoferritin was achieved by adding a molar ratio of 250 molecules of DMPA and 50 molecules of EDC per negatively charged residue (calculations based on the amino acid sequence of horse spleen ferritin). This excess of reagent over protein resulted in high cationization efficiencies, comparable also to previously reported results for the cationization of ferritin.[35] For MALDI-TOF analysis, apoferritin and cationized apoferritin were used because of the excessive molecular mass of the magnetoferritin core. To yield high cationization efficiencies, optimal pH is also crucial. EDC-mediated crosslinking is most effective under mildly acidic conditions, and we found that pH 5 yielded optimal cationization results for magnetoferritin. However, for other proteins cationization pH may need to be optimized. Cationization at or close to the isoelectric point of the protein should be avoided, because this may lead to severe precipitation.

Stem cell magnetization with cationized magnetoferritin was highly efficient and could be achieved using incubation times well below 30 minutes. Even a one-minute incubation resulted in a cellular iron content of 3.6 pg, which is within the reported range required to influence T2 and T2* contrast for MRI.[36,37] It is also remarkable that this efficient labeling is achieved with low extracellular iron concentrations. For example, previous studies using anionic nanoparticles report iron levels of 10 pg per cell after a 30 minute incubation period with 5 mM iron.[38] In comparison, incubation with a cationized magnetoferritin solution containing 0.5 μM protein corresponds to incubation with approximately 0.2 mM iron and also yields approximately 10 pg of iron per cell after 30 minutes. We were not able to clearly identify any endocytotic vesicles using TEM. However, previous studies using cationized ferritin found that internalization occurred within the first ten minutes of exposure.[39,40] Cationized ferritin could be localized in coated vesicles, indicating clathrin- or caveolin-dependent endocytosis. The same studies also reported that after 30 minutes of incubation, cationized ferritin was still present on the cell surface, as well as in multivesicular bodies, resembling lysosomes.

Further applications could include cationization of apo-ferritin cages loaded with other nanoparticles and/or functional molecules, such as anti-cancer drugs[41] or quantum dots[42]. Cationization of these ferritin constructs could result in faster and more efficient delivery of their cargo to cells.

**DISCLOSURES:**

The authors have nothing to disclose.

REFERENCES:

1   Gupta, A. K. & Gupta, M. Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. *Biomaterials* **26** (18), 3995-4021, doi:10.1016/j.biomaterials.2004.10.012 (2005).

2   Decuzzi, P. & Ferrari, M. The role of specific and non-specific interactions in receptor-mediated endocytosis of nanoparticles. *Biomaterials* **28** (18), 2915-2922, doi:10.1016/j.biomaterials.2007.02.013 (2007).

3   Cunningham, C. H., *et al.* Positive contrast magnetic resonance imaging of cells labeled with magnetic nanoparticles. *Magn. Reson. Med.* **53** (5), 999-1005, doi:10.1002/mrm.20477 (2005).

4   Song, H. T., *et al.* Surface modulation of magnetic nanocrystals in the development of highly efficient magnetic resonance probes for intracellular labeling. *J. Am. Chem. Soc.* **127** (28), 9992-9993, doi:10.1021/ja051833y (2005).

5   Daldrup-Link, H. E., *et al.* Targeting of hematopoietic progenitor cells with MR contrast agents. *Radiology* **228** (3), 760-767, doi:10.1148/radiol.2283020322 (2003).

6   Singh, N., Jenkins, G. J., Asadi, R. & Doak, S. H. Potential toxicity of superparamagnetic iron oxide nanoparticles (SPION). *Nano Rev.* **1**, doi:10.3402/nano.v1i0.5358 (2010).

7   Dodd, S. J., *et al.* Detection of single mammalian cells by high-resolution magnetic resonance imaging. *Biophys. J.* **76** (1), 103-109, doi:10.1016/S0006-3495(99)77182-1 (1999).

8   Ahrens, E. T., Feili-Hariri, M., Xu, H., Genove, G. & Morel, P. A. Receptor-mediated endocytosis of iron-oxide particles provides efficient labeling of dendritic cells for in vivo MR imaging. *Magn. Reson. Med.* **49** (6), 1006-1013, doi:10.1002/mrm.10465 (2003).

9   Arbab, A. S., *et al.* Efficient magnetic cell labeling with protamine sulfate complexed to ferumoxides for cellular MRI. *Blood* **104** (4), 1217-1223, doi:10.1182/blood-2004-02-0655 (2004).

10   Bulte, J. W. M., *et al.* Magnetodendrimers allow endosomal magnetic labeling and in vivo tracking of stem cells. *Nat. Biotechnol.* **19** (12), 1141-1147, doi:10.1038/nbt1201-1141 (2001).

11   Josephson, L., Tung, C. H., Moore, A. & Weissleder, R. High-efficiency intracellular magnetic labeling with novel superparamagnetic-tat peptide conjugates. *Bioconjugate Chem.* **10** (2), 186-191, doi:10.1021/bc980125h (1999).

12   Lewin, M., *et al.* Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. *Nat. Biotechnol.* **18** (4), 410-414, doi:10.1038/74464 (2000).

13   Hunter, A. C. Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. *Adv. Drug Delivery Rev.* **58** (14), 1523-1531, doi:10.1016/j.addr.2006.09.008 (2006).

14   Montet-Abou, K., Montet, X., Weissleder, R. & Josephson, L. Cell internalization of magnetic nanoparticles using transfection agents. *Mol. Imaging* **6** (1), 1-9, doi:10.2310/7290.2006.00028 (2007).

15   Correia Carreira, S., *et al.* Ultra-fast stem cell labelling using cationised magnetoferritin. *Nanoscale* **8** (14), 7474-7483, doi:10.1039/C5NR07144E (2016).

16   Meldrum, F. C., Heywood, B. R. & Mann, S. Magnetoferritin: in vitro synthesis of a novel magnetic protein. *Science* **257** (5069), 522-523, doi:10.1126/science.1636086 (1992).

17   Klem, M. T., *et al.* Synthetic control over magnetic moment and exchange bias in all-oxide materials encapsulated within a spherical protein cage. *J. Am. Chem. Soc.* **129** (1), 197-201, doi:10.1021/ja0667561 (2007).

18   Okuda, M., Eloi, J. C., Sarua, A., Jones, S. E. W. & Schwarzacher, W. Energy barrier distribution for dispersed mixed oxide magnetic nanoparticles. *J. Appl. Phys.* **111** (7), doi:10.1063/1.3676229 (2012).

19   Uchida, M., *et al.* A human ferritin iron oxide nano-composite magnetic resonance contrast agent. *Magn. Reson. Med.* **60** (5), 1073-1081, doi:10.1002/mrm.21761 (2008).

20   Danon, D., Skutelsk.E, Marikovs.Y & Goldstei.L. Use of Cationized Ferritin as a Label of Negative Charges on Cell Surfaces. *J. Ultrastruc. Res.* **38** (5-6), 500-&, doi:10.1016/0022-5320(72)90087-1 (1972).

21   Valero, E., *et al.* Magnetic Nanoparticles-Templated Assembly of Protein Subunits: A New Platform for Carbohydrate-Based MRI Nanoprobes. *J. Am. Chem. Soc.* **133** (13), 4889-4895, doi:10.1021/ja110014p (2011).

22   Zborowski, M., *et al.* Immunomagnetic isolation of magnetoferritin-labeled cells in a modified ferrograph. *Cytometry* **24** (3), 251-259, doi:10.1002/(SICI)1097-0320(19960701)24:3<251::AID-CYTO8>3.0.CO;2-K (1996).

23   Ikezoe, Y., *et al.* Growth of Giant Two-Dimensional Crystal of Protein Molecules from a Three-Phase Contact Line. *Langmuir* **24** (22), 12836-12841, doi:10.1021/la802104f (2008).

24   Uchida, M., *et al.* Targeting of cancer cells with ferrimagnetic ferritin cage nanoparticles. *J. Am. Chem. Soc.* **128** (51), 16626-16633, doi:10.1021/ja0655690 (2006).

25   Yamashita, K., *et al.* Selective nanoscale positioning of ferritin and nanoparticles by means of target-specific peptides. *Small* **2** (10), 1148-1152, doi:10.1002/smll.200600220 (2006).

26   Williams, A. & Frasca, V. Ion-Exchange Chromatography. In *Current Protocols in Protein Science* Coligan, J.E. *et al.* eds, Ch. 8.2, John Wiley & Sons, Inc., doi:10.1002/0471140864.ps0802s15 (2001).

27   Hagel, L. Gel-Filtration Chromatography. In *Current Protocols in Protein Science* Coligan, J.E. *et al.* eds, Ch. 8.3, John Wiley & Sons, Inc., doi:10.1002/0471142727.mb1009s44 (2001).

28   Hou, X. & Jones, B. T. Inductively coupled plasma-optical emission spectrometry. In *Encyclopedia of Analytical Chemistry* Meyers, R.A. ed, 1-25, John Wiley & Sons, Ltd., doi:10.1002/9780470027318.a5110.pub2 (2000).

29   Correia Carreira, S., *et al.* Ultra-fast stem cell labelling using cationised magnetoferritin. *Nanoscale*, doi:10.1039/C5NR07144E (2016).

30   Simon, G. H., *et al.* T1 and T2 relaxivity of intracellular and extracellular USPIO at 1.5 T and 3T clinical MR scanning. *Eur. J. Radiol.* **16** (3), 738-745, doi:10.1007/s00330-005-0031-2 (2006).

31   Jubb, A. M. & Allen, H. C. Vibrational Spectroscopic Characterization of Hematite, Maghemite, and Magnetite Thin Films Produced by Vapor Deposition. *ACS Appl. Mater.*

Interfaces **2** (10), 2804-2812, doi:10.1021/am1004943 (2010).

32 Wang, Z. W., *et al.* High-pressure x-ray diffraction and Raman spectroscopic studies of the tetragonal spinel CoFe2O4. *Phys. Rev. B* **68** (9), doi:10.1103/PhysRevB.68.094101 (2003).

33 Wong, K. K., Douglas, T., Gider, S., Awschalom, D. D. & Mann, S. Biomimetic synthesis and characterization of magnetic proteins (magnetoferritin). *Chem. Mater.* **10** (1), 279-285, doi:10.1021/cm970421o (1998).

34 Okuda, M., *et al.* Fe3O4 nanoparticles: protein-mediated crystalline magnetic superstructures. *Nanotechnology* **23** (41), 415601, doi:10.1088/0957-4484/23/41/415601 (2012).

35 Perriman, A. W., Cölfen, H., Hughes, R. W., Barrie, C. L. & Mann, S. Solvent-Free Protein Liquids and Liquid Crystals. *Angew. Chem. Int. Ed.* **48** (34), 6242-6246, doi:10.1002/anie.200903100 (2009).

36 Billotey, C., *et al.* T-cell homing to the pancreas in autoimmune mouse models of diabetes: in vivo MR imaging. *Radiology* **236** (2), 579-587, doi:10.1148/radiol.2362040603 (2005).

37 Smirnov, P., *et al.* In vivo cellular imaging of lymphocyte trafficking by MRI: a tumor model approach to cell-based anticancer therapy. *Magn. Reson. Med.* **56** (3), 498-508, doi:10.1002/mrm.20996 (2006).

38 Wilhelm, C., *et al.* Magnetic control of vascular network formation with magnetically labeled endothelial progenitor cells. *Biomaterials* **28** (26), 3797-3806, doi:10.1016/j.biomaterials.2007.04.047 (2007).

39 MacLean, I. & Sanders, E. Cationized ferritin and phosvitin uptake by coated vesicles of the early chick embryo. *Anat. Embryol.* **166** (3), 385-397, doi: 10.1007/BF00305925 (1983).

40 Van Deurs, B., Nilausen, K., Faergeman, O. & Meinertz, H. Coated pits and pinocytosis of cationized ferritin in human skin fibroblasts. *Eur. J. Cell Biol.* **27** (2), 270-278, PMID: 7117270 (1982).

41 Xing, R. M., *et al.* Characterization and cellular uptake of platinum anticancer drugs encapsulated in apoferritin. *J Inorg Biochem* **103** (7), 1039-1044, doi:10.1016/j.jinorgbio.2009.05.001 (2009).

42 Wong, K. K. & Mann, S. Biomimetic synthesis of cadmium sulfide-ferritin nanocomposites. *Adv. Mater.* **8** (11), 928-932, doi:10.1002/adma.19960081114 (1996).

Figure

A

(1)

(2)

(3)

12 nm

B
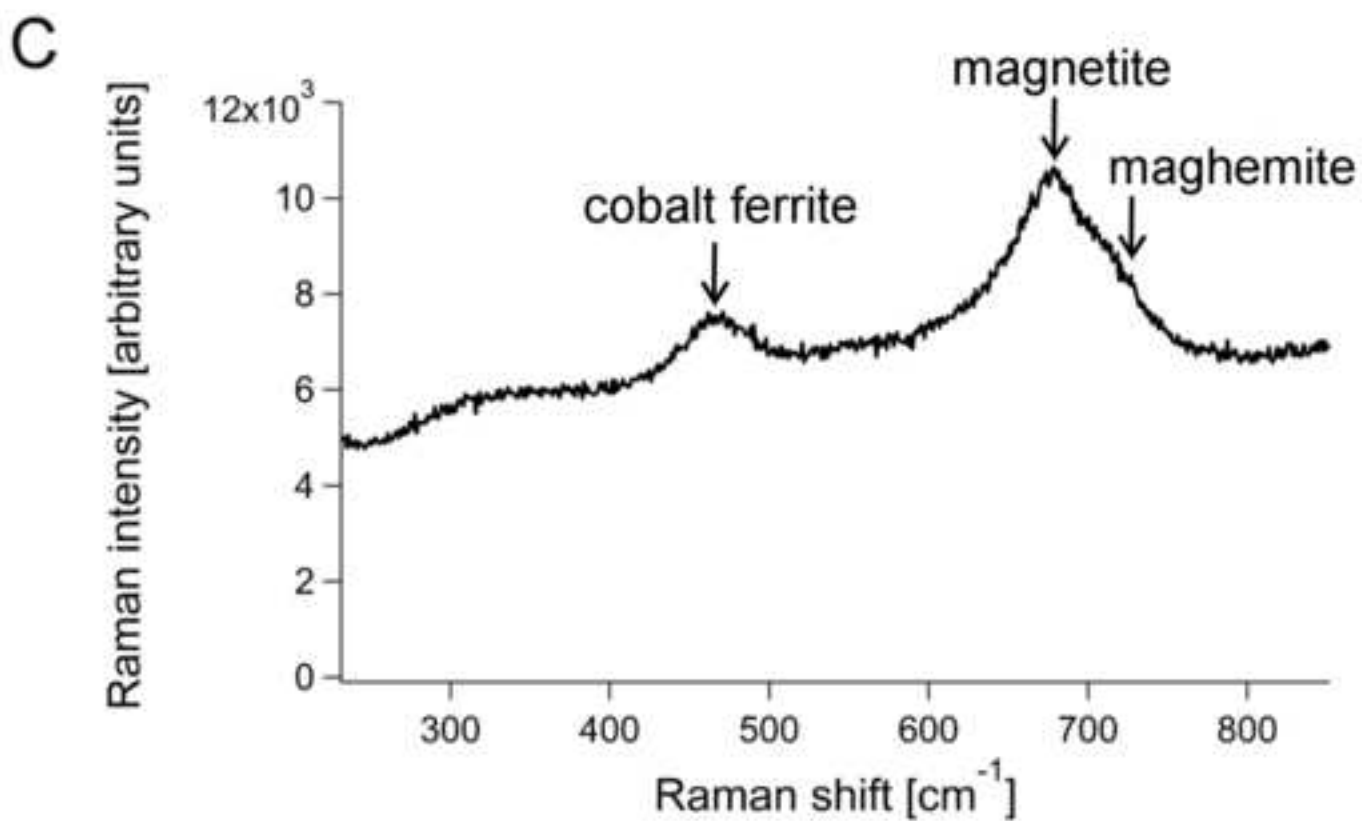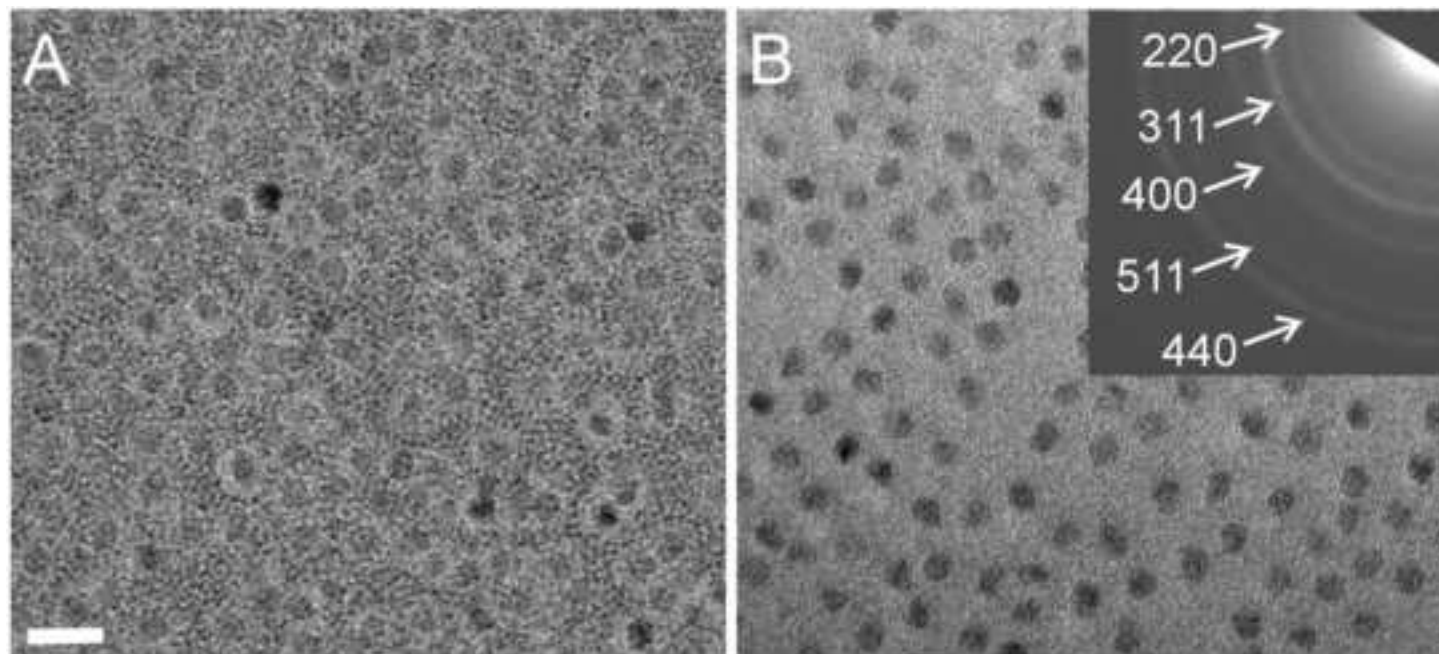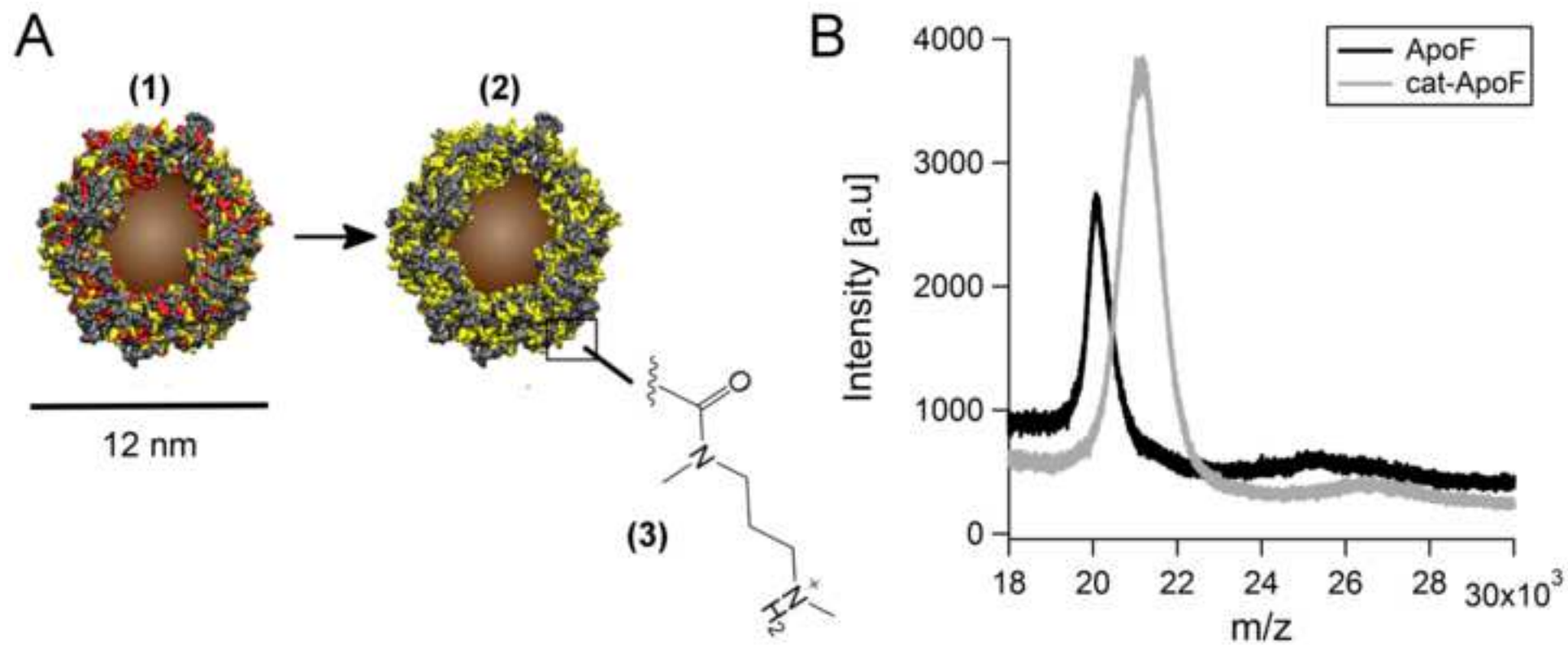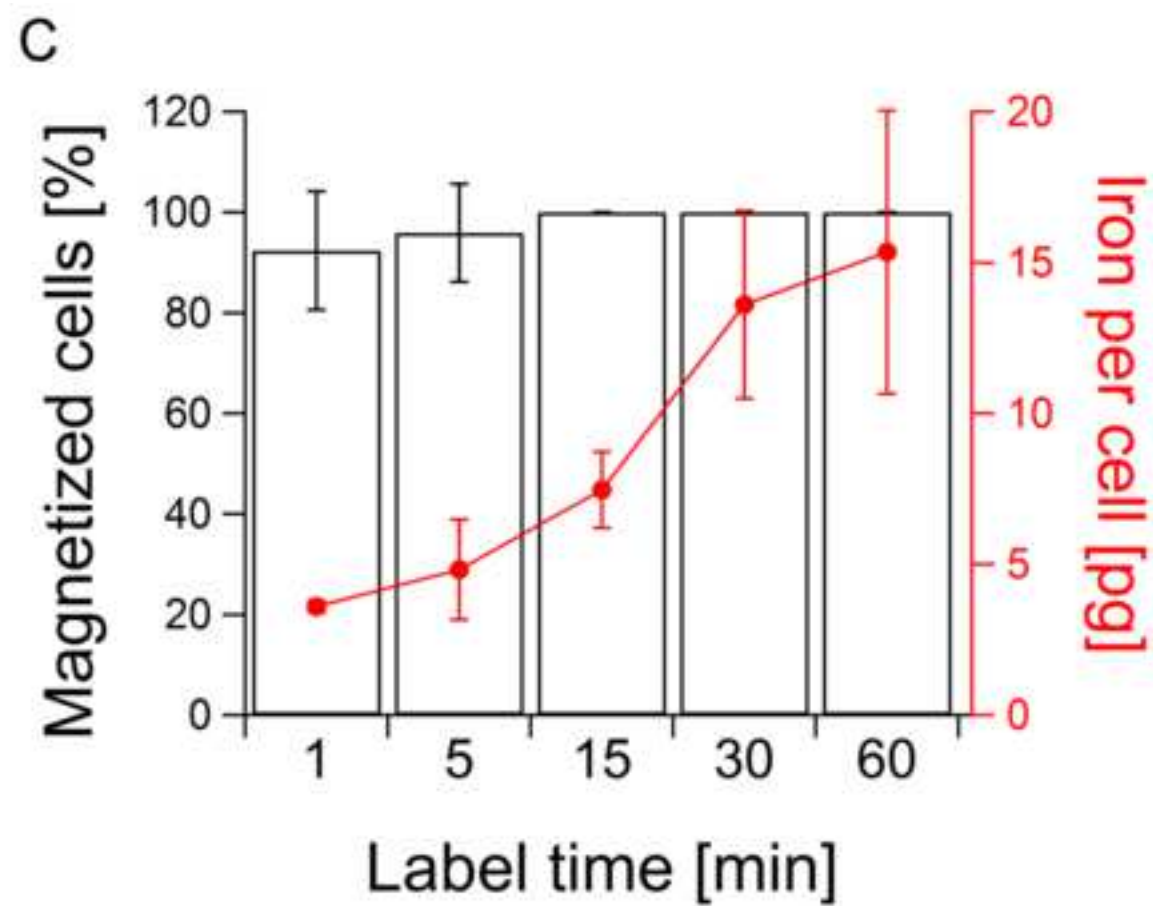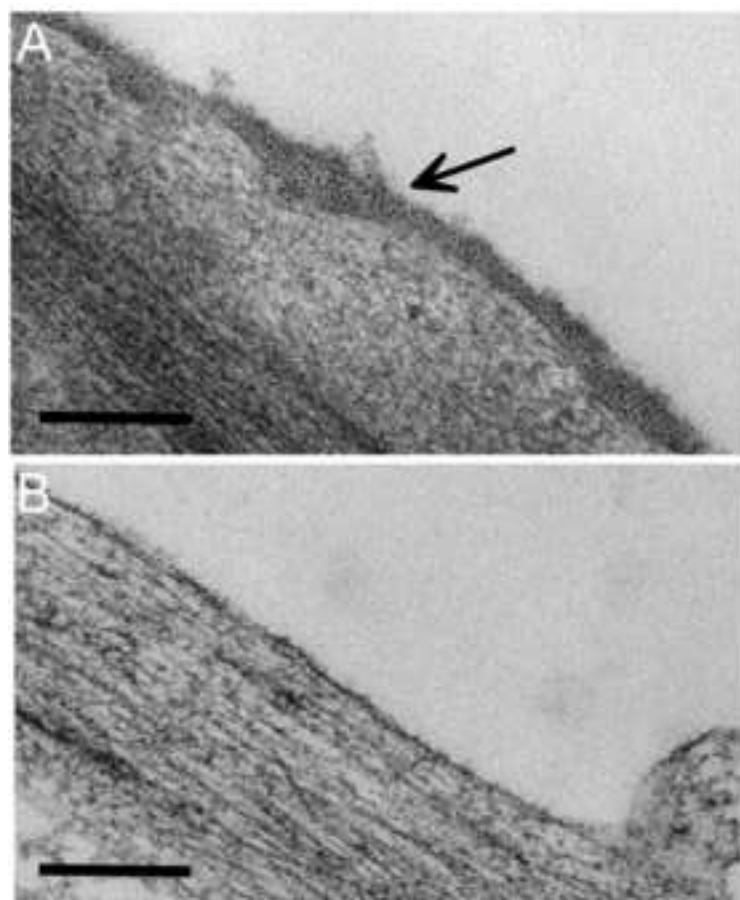
	MF	cat-MF
Hydrodynamic diameter [nm]	11.8 ± 1.1	12.5 ± 1.4
Zeta potential [mv]	(-)10.4 ± 0.2	8.3 ± 0.7
Magnetic saturation moment [Am^2 kg^-1]	54.9 ± 1.6	55.3 ± 1.4
Mass susceptibility [x 1E04 m^3 kg^-1]	1.75 ± 0.08	1.75± 0.07
Longitudinal relaxivity [mM^-1 s^-1]	2.6 ± 0.1	2.3 ± 0.1
Transverse relaxivity [mM^-1 s^-1]	44.6 ± 1.0	52.8 ± 0.8

Legend:
MF: magnetoferritin
cat-MF: cationized magnetoferritin

Excel Spreadsheet- Table of Materials/Equipment

Name of Reagent/ Equipment	Company	Catalog Number
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Fisher Scientific	BPE310-1
apoferritin from equine spleen	Sigma Aldrich	A3641
cobalt sulfate heptahydrate	Sigma Aldrich	C6768
ammonium iron sulphate hexahydrate	Sigma Aldrich	F1543
hydrogen peroxide solution (30%)	Sigma Aldrich	216763
sodium citrate	Sigma Aldrich	S1804
Millex GP filter unit, 0.22 micron	Merck Millipore	SLGP033RS
Trizma base	Sigma Aldrich	T1503
sodium chloride	Sigma Aldrich	31434
Centriprep centrifugal filter units	Merck Millipore	4310
Amicon Ultra-4 centrifugal filter untis	Merck Millipore	UFC801024
ANX Sepharose 4 Fast Flow	GE Healthcare	17-1287-04
HiPrep 26/60 Sephacryl S-300 HR column	GE Healthcare	17-1196-01
ÄKTA purifier system	GE Healthcare	28406264
sample pump P-960	GE Healthcare	18-6727-00
automated fraction collector Frac-950	GE Healthcare	18-6083-00
Bradford assay reagent	Sigma Aldrich	B6916
Ferritin, Type I: from horse spleen	Sigma Aldrich	F4503

N,N-dimethyl-1,3-propanediamine (DMPA)	Sigma Aldrich	308110
N-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC)	Sigma Aldrich	E6383
2-(N-morpholino)ethanesulfonic acid (MES)	AppliChem	A0689,0500
dialysis tubing cellulose membrane	Sigma Aldrich	D9652
Dulbecco's Modified Eagle's Medium (DMEM), 1000 mg/L glucose	Sigma Aldrich	D5546
fetal bovine serum	Sigma Aldrich	F7524
penicillin/streptomycin solution	Sigma Aldrich	P0781
glutamax solution	Gibco	35050-087
human fibroblast growth factor	PeproTech	100-18B
phosphate buffered saline	Sigma Aldrich	D8537
trypsin/EDTA solution	Sigma Aldrich	E5134
ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich	E5134
bovine serum albumin	Sigma Aldrich	A7030
MACS multi stand	Miltenyi Biotec	130-042-303
MACS MS columns	Miltenyi Biotec	130-042-201
MiniMACS separator magnet	Miltenyi Biotec	130-042-102
MACS column pre-separation filter	Miltenyi Biotec	130-041-407

| Nitric acid solution, 64-66% | Sigma Aldrich | 7006 |
| Titrando 907, syringe pump | Metrohm | 2.907.0020 |

Equipment used to characterize magnetoferritin and cationized magnetoferritin

SpectraMax M5	Molecular Devices
JEM 1200 EX	JEOL
InVia Raman spectrometer	Renishaw
Torus DPSS laser	Laser Quantum

| Bruker UltrafleXtreme | Applied Biosystems |

| ZetaSizer Nano-ZS | Malvern Instruments |

| Magnetic Property Measurement System | Quantum Design |

| Magnetom Skyra | Siemens |

| Tecnai 12 BioTwin Spirit | FEI |
| 710 ICP-OES | Agilent |

Comments/Description

powder; prepare a 1 M stock solution at pH 8.6 and dilute to 50 mM prior to use. Check the pH carefully prior to synthesis!

we used LOT# 081M7011V

prepare fresh solutions from the salt prior to synthesis

prepare fresh solutions from the salt prior to synthesis

prepare fresh solutions from the salt prior to synthesis

powder; a 1 M solution can be prepared and kept at room temperature for several months

syringe filter

powder; prepare a 1 M stock solution at pH 8.0 and dilute to 50 mM prior to use

poweder; add to buffers as required

Ultracel YM-50 membrane, 12 mL volume; use for initial concentration until the magnetoferritin solution has been concentrated from about 150 mL to 20 mL

Ultracel-10 membrane, 4 mL volume; use to concentrate magnetoferritin solution from about 20 mL to 2 mL

we packed this column ourselves

this column was bought ready packed

load sample at a flow rate of 10 mL/min

solution ready to use

prepare ferritin standards from this solution to determine magnetoferritin concentration

CAUTION: when adjusting the pH of a DMPA solution, perform this step in a fume hood

keep in freezer but bring to room temperature before opening the bottle

powder; prepare a 200 M stock solution at pH 5

soak 10 min in deionized water before use

warm in 37 °C water bath before use

add to stock DMEM bottle, 10 % (v/v) final concentration

add to stock DMEM bottle, 1 % (v/v) final concentration

add to stock DMEM bottle, 1 % (v/v) final concentration

add to DMEM freshly into cell culture flask with each media change; final concentration 5 ng/mL

sterile solution, for cell cultrue

keep in freezer and defrost in 37 °C water bath before use

powder; make a 2 mM solution in PBS

add 0.5 % (w/v) into 2 mM EDTA solution in PBS; carefully stir with magnetic stirrer, avoid foaming; filter sterilize through a 0.22 micron syringe filter

for attachment of MACS magnet

disposable; intended for single use, but if sterility is not required, they can re-used: wash with deionized water and 100 % ethanol, and placed in a drying oven; discard if you observe rusty patches

can be bought as a starter kit, together with columns and stands

30 µm filter

Used to measure absorbance in the Bradford assay
Used for TEM imaging of magnetoferritin
Used for Raman spectroscopy
Used for Raman spectroscopy
Used for MALDI-TOF analysis of apoferritin and cationized apoferritin
Used to measure hydrodynamic diameter and zeta potential of magnetoferritin and cationized magnetoferritin
Used to measure magnetic saturation moment and magnetic susceptibility
Used to determine longitudinal and transverse relaxivity
Used for TEM imaging of hMSC labeled with cationized magnetoferritin
Used to determine iron content in cells

jove
JOURNAL OF
VISUALIZED EXPERIMENTS

17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

Title of Article: *SYNTHESIS OF CATIONIZED MAGNETO FERRITIN FOR ULTRA-FAST MAGNETIZATION OF CELLS*

Author(s): *SARA CORREIA CARREIRA, JAMES P.K. ARMSTRONG, MITSUHIRO OKUDA, ANNELA M. SEDDON, ADAM W. PERRIMAN, WALTHER SCHWARZ-ALTER*

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☐ Standard Access ☑ Open Access

Item 2 (check one box):

☑ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. <u>Defined Terms</u>. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution 3.0 Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by/3.0/us/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;*

"**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. <u>Background</u>. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. <u>Grant of Rights in Article</u>. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and

(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article and Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. <u>Retention of Rights in Article</u>. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the

1

612542.6

jove
JOURNAL OF
VISUALIZED EXPERIMENTS

17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This Section 5 applies if the "Standard Access" box has been checked in Item 1 above or if no box has been checked in Item 1 above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to Section 7 below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This Section 6 applies only if the "Open Access" box has been checked in Item 1 above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to Section 7 below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in Item 2 above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict

612542.6

shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including,

ARTICLE AND VIDEO LICENSE AGREEMENT

without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the

Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or

damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of

JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

AUTHOR:

Name: SARA CORREIA CARREIRA

Department: H. H. WILLS PHYSICS LABORATORY

Institution: UNIVERSITY OF BRISTOL

Article Title: SYNTHESIS OF CATIONIZED MAGNETOFERRITIN FOR VLTRA-FAST MAGNETIZATION OF CELLS

Signature: *Carreira*   Date: 30.3.16

Please submit a signed and dated copy of this license by one of the following three methods:
1) Upload a scanned copy as a PDF to the *JoVE* submission site upon manuscript submission (preferred);
2) Fax the document to +1.866.381.2236; or
3) Mail the document to *JoVE* / Attn: *JoVE* Editorial / 17 Sellers St / Cambridge, MA 02139

For questions, please email editorial@jove.com or call +1.617.945.9051.

MS # (internal use): _____

3

612542.

Editorial comments:

1. Formatting: Please number steps sequentially. For instance, there are two steps 3.4.6.

We have corrected this.

2. Grammar:
-All sentences should begin with capital letters, including those in notes.
-3.2.1 – "over night"

We have corrected this.

3. Visualization: We suggest highlighting some of the cationization procedure for filming, such that total protocol highlighted for filming is >2.75 pg.

We have added selected steps from the cationization procedure. Highlighted areas now add up to a little more than 2 pages.

4. Additional detail is required:
-1.3.1 – Are these injected simultaneously using two syringes?
-3.4.6 (first one) – How is this performed? Please provide a citation.

We have added additional information and a citation for ICP-OES.

5. Branding should be removed from 3.1.2 – glutamax

We have removed the branding.

6. Results: Figure 3 legend – Please indicate how Iron per cell was determined.

We have added this information.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
N/A

Major Concerns:
N/A

Minor Concerns:
I miss in the paper the type of equipments which were used to samles characterization. (e.g. TEM images were used by JEOL JEM 1200 EX? The Raman spectra, Zeta potential, magnetic properties, etc.?)

We have added this information to the materials table

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
Accept.

Major Concerns:

N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
The paper is well structured and all sections are well explained. The manuscript has been revised well and could be published in the journal.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #4:
Manuscript Summary:
I think this is a valuable study and well-written. The major advantage of their SPIONs is they can quickly label cells. This is clinically useful for emergency procedures that may benefit from magnetic cell targeting, but they do not mention this. I think this is fine for publication and I don't have any major concerns. Some specific thoughts are included.

Major Concerns:
None

Minor Concerns:
* 1.3.1 is confusing: "Inject a total of 30.3 mL of both the iron-cobalt precursor and the hydrogen peroxide…" Make it clear to add 30.3 mL of iron-cobalt precursor and 30.3 mL of hydrogen peroxide (which is 60.6 mL total).

We have clarified this.

* 1.3.2 states the reaction will turn brown, but this is already stated in 1.3.1.

We have removed this.

* Would be helpful to see a representative TEM image of hMSC labeled with SPIONs. Are the SPIONs internalized and stored in endosomes in the cytoplasm?

We have included a TEM image of labeled cells in Figure 3 and added a comment about the presumed internalization mechanism in the discussion section.

We have added the
* A cartoon schematic of a SPION with chemical components, approximate dimensions, etc. would be helpful.

We have included this in Figure 2.

Additional Comments to Authors:
N/A

License for Figure 1

Click here to access/download
Supplemental File (as requested by JoVE)
Copyright Clearance Center for Figure 1.pdf