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TITLE:

Using Magnetometry to Monitor Cellular Incorporation and Subsequent Biodegradation of Chemically Synthetized Iron Oxide Nanoparticles

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KEYWORDS:

Iron oxide nanoparticle; non-aqueous sol gel nanoparticle synthesis; magnetism; stem cell; vibrating sample magnetometry; biodegradation

SUMMARY:

Iron oxide nanoparticles are synthesized via a nonaqueous sol gel procedure and coated with anionic short molecules or polymer. The use of magnetometry for monitoring the incorporation and biotransformations of magnetic nanoparticles inside human stem cells is demonstrated using a vibrating sample magnetometer (VSM).

ABSTRACT:

Magnetic nanoparticles, made of iron oxide, present a peculiar interest for a wide range of biomedical applications for which they are often internalized in cells and then left within. One challenge is to assess their fate in the intracellular environment with reliable and precise methodologies. Herein, we introduce the use of the vibrating sample magnetometer (VSM) to precisely quantify the integrity of magnetic nanoparticles within cells by measuring their magnetic moment. Stem cells are first labeled with two types of magnetic nanoparticles; the nanoparticles have the same core produced via a fast and efficient microwave-based nonaqueous sol gel synthesis and differ in their coating: the commonly used citric acid molecule is compared to polyacrylic acid. The formation of 3D cell-spheroids is then achieved via centrifugation and the magnetic moment of these spheroids is measured at different times with the VSM. The obtained moment is a direct fingerprint of the nanoparticles' integrity, with decreasing values indicative of a nanoparticle degradation. For both nanoparticles, the magnetic moment decreases over culture time revealing their biodegradation. A protective effect of the polyacrylic acid coating is

also shown, when compared to citric acid.

INTRODUCTION:

There is increased interest in the magnetic features of iron oxide nanoparticles for a wide range of biomedical applications. Their response to magnetic resonance makes them reliable contrast agents for magnetic resonance imaging (MRI), an advantage in regenerative medicine where cells labeled with magnetic nanoparticles can be tracked in vivo following implantation[1]. Using magnetic fields, cells can also be guided at a distance; this way, cellular spheroids[2,3], rings[4], or sheets[5] can be engineered magnetically and also remotely stimulated[6], an asset in the development of scaffold-free tissues. The range of possibilities for these nanoparticles also includes drug delivery systems[7,8] and magnetic and photoinduced hyperthermal treatment to kill cancerous cells[9–11]. For all these applications, the nanoparticles are integrated in the biological environment either by intravenous injection or via direct internalization in cells and are then left within, which brings into question their intracellular fate.

In vivo analyses conveyed a general understanding of the nanoparticles' fate in the organism: upon injection in the blood stream, they are first captured mostly by the macrophages of the liver (Kupffer cells), spleen and bone marrow, are progressively degraded, and join the iron pool of the organism[12–19]. Qualitative observations are only possible due to the circulation of the nanoparticles throughout the organism. Typically, transmission electronic microscopy (TEM) can be used to directly observe the nanoparticles and the presence of iron in the organs can be determined via dosage. More recently, their fate has been assessed directly on a pool of cells, meaning in close circuit with no iron escape, allowing a quantitative measurement of their biotransformations at the cell-level[20–22]. Such measurements are possible via the analysis of the magnetic properties of the nanoparticles that are tightly linked to their structural integrity. Vibrating sample magnetometry (VSM) is a technique where the sample is vibrated periodically so that the coil-measurement of the flux induced provides the magnetic moment of the sample at the applied magnetic field. Such synchronous detection allows for a rapid measurement, which is an asset for determining the magnetic moments of a large number of samples[20–23]. The macroscopic magnetic signature retrieved by VSM then gives a quantitative overview of the entire biological sample directly correlated to the nanoparticles' size and structure. In particular, it provides the magnetic moment at saturation (expressed in emu) of the samples, which is a direct quantification of the number of magnetic nanoparticles present in the sample, respective to their specific magnetic properties.

It has been shown that the intracellular processing of magnetic nanoparticles is tightly linked to their structural features[20]. These features can be controlled via optimal synthesis protocols. Each protocol presents advantages and limitations. Iron oxide nanoparticles are commonly synthesized in aqueous solutions via coprecipitation of iron ions[24]. To overcome the limitations of nanoparticles size polydispersity, other synthesis methods such as polyol-mediated sol-gel methods have been developed[25]. Nonaqueous approaches by thermal decomposition leads to the production of very well-calibrated iron oxide nanoparticles[26]. However, the use of massive amounts of surfactants like oleylamine or oleic acid complicates their functionalization and water transfer for biomedical applications. For this reason, we synthesize such magnetic nanoparticles

through a nonaqueous sol gel route leading to high crystallinity, purity and reproducibility[27]. This protocol produces well-controlled size nanoparticles that can be tuned through temperature variation[28]. Nevertheless, the microwave-assisted non-aqueous sol-gel route has an upper size limit of the obtained nanoparticles of around 12 nm. This procedure would not be adapted for applications using ferromagnetic particles at room temperature. In addition to the core synthesis, another main feature to be considered is the coating. Lying at the surface of the nanoparticle, the coating act as an anchoring molecule, helping the targeted internalization of the nanoparticles, or it can protect the nanoparticle from degradation. Since benzyl alcohol acts as an oxygen source and a ligand at the same time, bare nanoparticles are produced without the need for additional surfactants or ligands. The nanoparticles are then easily surface functionalized after synthesis without a surfactant exchange process.

Herein, two types of nanoparticles are assessed that possess the same core and differ in the coating. The core is synthesized using a fast and highly efficient microwave based technique. The two coatings compared consist of citric acid, one of the most used as coating agent in biomedical applications[29,30], and polyacrylic acid (PAA), a polymeric coating with a high number of chelating functions. VSM magnetometry measurements are then used first to quantify the nanoparticle uptake by the cells, and then as a direct assessment of the nanoparticle structural integrity upon internalization in stem cells. Results demonstrate that the incubation concentration impacts nanoparticle uptake and that the coating influences their degradation, with the large number of anchoring molecules of PAA protecting the core from degradation.

PROTOCOL:

1. Synthesis of magnetic nanoparticles

1.1. Core synthesis – microwave-assisted

1.1.1. Dissolve 400 mg of iron(III) acetylacetonate (>99.9%) in 10 mL of benzyl alcohol (BA, 99.8%) within a 30 mL monowave glass vial.

1.1.2. Increase the temperature of the suspension from 25 to 250 °C in 20 min (at a rate of 11.25 °C/min) and maintain it at 250 °C for 30 min using a microwave reactor.

1.1.3. Transfer the resulting nanoparticles suspended in benzyl alcohol to a glass vial and separate the nanoparticles using a neodymium magnet for 30 min.

1.1.4. Wash the precipitate in the previous 100 mL glass vial with 10 mL of dichloromethane, 1 M sodium hydroxide, ethanol, pH 7 water (three times) using a neodymium magnet for 5 min each step to pelletize nanoparticles and remove the supernatant.

1.1.5. Adjust the nanoparticle suspension in water to pH 2 using 1 M hydrochloric acid, and then centrifuge in 100 kDa ultracentrifugal filters at 2,200 x *g* for 10 min.

1.1.6. Remove the filtrate, add 12 mL of 10^{-2} M hydrochloric acid to the nanoparticles solution and centrifuge in 100 kDa ultracentrifugal filters at 2,200 x *g* for 10 min (three times). Then dilute the nanoparticles solution within 10 mL of 10^{-2} M hydrochloric acid before coating.

1.2. Coating

1.2.1. Dilute 175 mg of citric acid and polyacrylic acid in water at pH 2 in a 100 mL glass vial and adjusted the pH to 2 with a 1 M hydrochloric acid solution.

1.2.2. Add the 10 mL nanoparticles aqueous dispersion to the coating molecule solution, corresponding to a mass ratio of 5 between the coating molecule and the nanoparticles. Agitate for 2 h under magnetic stirring at room temperature.

1.2.3. After the reaction, adjust the pH to 7 with 1 M sodium hydroxide.

1.2.4. Centrifuge the nanoparticle solution 3 times with deionized (DI) water in 100 kDa ultracentrifugal filters at 2,200 x *g* for 10 min; remove the filtrate and dilute the nanoparticles solution in 12 mL of DI water.

2. Culture and magnetic labeling of stem cells

2.1. Culture human mesenchymal stem cells (MSC) in complete Mesenchymal Stem Cell Growth Medium (MSCGM) at 37 °C and 5% CO_2. When the cells are at 90% confluence, add 10 mL of trypsin-EDTA pre-warmed at 37°C per 150 cm^2 flask and leave for 2-3 minutes to detach the cells.

2.1.1. To know when the cells are detached, observe them with a bright-field microscope. Resuspend the detached cells in MSCGM and divide them in four 150 cm^2 flasks. Amplify the cells this way until passage 4 to 5.

2.2. Prepare the solution of magnetic nanoparticles for cell labeling: disperse the chosen concentration of iron oxide nanoparticles in serum free Roswell Park Memorial Institute medium (RMPI-1640) without glutamine. RPMI is used for nanoparticle incubation (and the related rinsing steps) as it has a lower ionic strength than DMEM and better prevents nanoparticle aggregation events.

2.3. When the cells are at passage 5 and 90% confluent, remove the MSCGM medium, rinse the cells with serum free RPMI (without glutamine) and add 10 mL of iron oxide nanoparticles solution per 150 cm^2 culture flask, which is the minimum volume required to cover all the cells.

2.4. Incubate at 37 °C and 5% CO_2 for 30 min and then discard the nanoparticle solution. Wash once with serum free RPMI-1640 (no glutamine) and incubate with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin (25 mL per 150 cm^2 flask) overnight at 37 °C and 5% CO_2 to allow a complete internalization of the nanoparticles.

3. Formation of stem cell-spheroids

3.1. Freshly prepare cell-spheroid culture medium composed of DMEM high glucose with L-glutamine supplemented with 50 µM L-ascorbic acid 2-phosphate, 0.1 µM dexamethasone, 1 mM sodium pyruvate, 0.35 mM L-proline, and 1% universal culture supplement containing Insulin, human Transferrin and Selenous acid (ITS-Premix).

3.2. Detach the magnetic MSCs by adding 10 mL of 0.05% trypsin-EDTA pre-warmed at 37 °C per 150 cm^2 flask for 2-3 minutes. When the cells are detached, immediately inactivate the trypsin by adding 1/3 of the volume of DMEM supplemented with 10% FBS and 1% penicillin-streptomycin pre-warmed at 37 °C.

3.3. Centrifuge the dissociated cells at 260 x *g* for 5 min, aspirate the media and re-suspend the cells in a small volume (less than 1 mL per 150 cm^2 flask) of cell-spheroid culture medium such as having 200,000 cells in about 50 µL of medium. Count the cell number using a hemocytometer and adjust the volume if needed.

3.4. Add 1 mL of freshly prepared cell-spheroid culture medium in a 15 mL sterile conical centrifuge tube and add the volume of resuspended solution corresponding to 200,000 cells.

3.5. Centrifuge these magnetically labeled cells at 180 x *g* for 3 min such as forming a cell pellet at the bottom of the tube and keep the supernatant, which is the cell culture medium.

3.6. Slightly open the tubes to allow air exchange and incubate at 37 °C with 5% CO_2 for up to 21 days, culture time along which the cells form a cohesive 3D structure that results in a fully formed spheroid. Change the medium twice a week.

3.7. On given days, wash the spheroids twice with cacodylate buffer made of 0.2 M cacodylate diluted in demineralized water and fix them using 2% glutaraldehyde in 0.1 M cacodylate buffer diluted in distilled water for 30 min at room temperature. Store the fixed spheroids in PBS until used for VSM measurement or TEM imaging. This fixation step stops all biological processes and allows long-term conservation.

4. Quantification of magnetic nanoparticles in solution and in cellulo using a vibrating sample magnetometer (VSM)

4.1. Place either a given volume of magnetic nanoparticle solution (maximum 10 µL) or a single cell-spheroid into the sample holder specifically designed to fit in the VSM.

4.2. Insert the sample in the VSM and scan for sample offset. Place the sample at the position corresponding to the magnetization maximum.

4.3 Perform the first measurement at a low magnetic field (between -1500 Oe and +1500 Oe)

with a 20 Oe/s rate.

4.4 Perform a second measurement at a high magnetic field (between -30 000 Oe and +30 000 Oe) with a 200 Oe/s rate.

5. Transmission Electron Microscopy (TEM) analysis

5.1. For the nanoparticle solutions, deposit 10 µL of the aqueous solution onto a carbon-coated copper grid and let it dry at room temperature.

5.2. For the nanoparticles internalized in cells, contrast the fixed cell-spheroids with Oolong Tea Extract (OTE) at 0.5% diluted in 0.1 M cacodylate buffer, post fix with 1% osmium tetroxide containing 1.5% potassium cyanoferrate and then dehydrate in graded ethanol baths, included in Epon. Slice ultrasections of 70 nm and deposit them onto cooper grids.

5.3. Take images using an electron microscope at 80 kV with a magnification from 1k for the observation of entire cells to 40k for the observation of endosomal compartments.

REPRESENTATIVE RESULTS:

Using the microwave-assisted synthesis, magnetic nanoparticles with a monodisperse 8.8 ± 2.5 nm core size are produced and coated with either citrate or PAA (**Figure 1A**). Stem cells are then incubated with these nanoparticles dispersed in culture medium at a given concentration for 30 minutes, resulting in their endocytosis and confinement within the cellular endosomes (**Figure 1B**). The magnetic stem cells are then suspended in medium, centrifuged, and the cell pellet formed is cultured for up to 21 days (**Figure 1C**). The spheroids obtained are fixed, such as stopping biological processes, and kept in PBS until being measured via VSM.

First, the magnetic moment of the nanoparticle solution is measured using the VSM: 10 µL of the nanoparticle aqueous dispersion containing 7 µg of iron is measured, and the obtained curve is displayed in **Figure 2A**. Due to the presence of water in this solution, and to the sample holder, a diamagnetic signal is captured in addition to the superparamagnetic signal of the nanoparticles. A diamagnetic constant (M_{dia}) corresponding to the slope of the second part of the curve can be measured, as shown in **Figure 2A**, and this constant can be subtracted such as obtaining the magnetic moment of the nanoparticles only ($M_{sample} = M - M_{dia}$). The saturation magnetization of the nanoparticles (M_s) can then be extracted; it corresponds to 518 µemu for the aqueous dispersion, meaning that the solution is at 74 emu/g of iron (Fe) corresponding to 52 emu/g of nanoparticles (Fe_3O_4).

The magnetic moment of cell spheroids can similarly be obtained. In this case, a cell-spheroid (made of 200,000 cells) is inserted in the sample holder, placed in the VSM, and measured (**Figure 2B**). The magnetic moment values obtained are here much lower than the ones of the initial nanoparticle solution; however, they remain within the detection range. The saturation magnetization of this particular sample is of 69 µemu. Besides, this spheroid contains 1.3 µg of nanoparticles (6.7 pg of nanoparticles per cell), consistent with the saturation value at 52

emu/g_{Fe3O4}. This value can thus be used to determine the amount of nanoparticles in cellular samples. In **Figure 2C**, spheroids corresponding to cells labelled with three concentrations of citrate-coated nanoparticles are measured one day after labelling. Results clearly show that the uptake of the nanoparticles depends on the incubation concentration, with concentrations of 0.125, 0.25 and 0.5 mM leading to uptakes of 0.3, 0.7 and 1.3 µg of iron per spheroid, meaning 1.3, 3,3 and 6.7 pg of iron per cell, respectively.

Attention has been brought on the importance of the surface coating, which directly interacts with the biological environment[20]. Two coatings have here been produced: a citrate coating, commonly used for biomedical applications, and a PAA coating, with a higher number of chelating functions. Stem cells are labeled with these two types of nanoparticles and centrifuged such as forming a cell pellet that then becomes a cohesive cell-spheroid (**Figure 3A**). Magnetism of these cell-spheroids is measured via VSM at day 1 and day 21 (**Figure 3B** and **Figure 3C**). Results demonstrate a decrease in magnetism upon the 21 days of culture, indicating the biodegradation of the nanoparticles, this degradation being more important for the citrate-coated nanoparticles (**Figure 3B**) than the PAA-coated ones (**Figure 3C**). TEM images confirm the degradation of the nanoparticles and show the appearance of smaller (6 nm in size) light grey dots, typical of ferritin loaded with iron. Some nanoparticles remaining intact can also be observed, particularly with the PAA coating.

Figure 1: Microwave-assisted synthesis of iron oxide (Fe_3O_4) nanoparticles, their internalization in stem cells and the subsequent culture of the cells as spheroids. (**A**) Schematic of the various steps of the nanoparticle synthesis. First, the core is synthesized via a non-aqueous sol gel procedure. The coating molecule, either Citrate (Cit) or polyacrylic acid (PAA), is then grafted at the surface of the iron oxide core. A representative TEM image shows the synthesized nanoparticles with a citrate coating. (**B**) Schematic of the nanoparticle internalization within stem cell, showing the nanoparticles confined in the endosomes upon internalization. Representative TEM images also show the citrate and PAA coated nanoparticles inside the cells, confined in the endosomes. (**C**) Schematic of stem cell-spheroids formation.

Figure 2: **Measurement of the samples magnetic moment via VSM.** (**A**) 10 µL of nanoparticles dispersed in an aqueous solution are measured via the VSM. The signal obtained represents the magnetic moment of this nanoparticle solution in function of the magnetic field (B). The diamagnetism coming from the presence of water and the sample holder can then be measured as it corresponds to the slope of the second part of the curve and subtracted such as obtaining the magnetic moment of the nanoparticles only. The magnetic moment at saturation (M_s) can then be determined. (**B**) The magnetic moment of cell spheroids can similarly be obtained; In this case a single cell-spheroid is measured at a given time period. (**C**) Curves of spheroids corresponding to cells labeled with citrate-coated nanoparticles at three independent concentrations of 0.125 mM (orange), 0.25 mM (grey) and 0.5 mM (blue).

Figure 3: **Quantification of magnetic nanoparticle degradation in cellulo via VSM.** (**A**) Upon cell labeling with magnetic nanoparticles, a cell pellet is formed by centrifugation (day 0). The cells then form a cohesive structure resulting in an easy to handle cell-spheroid that can be kept in

culture without cell loss for extended time periods (months). (**B, C**) Herein, two types of magnetic nanoparticles, coated with citrate (**B**) or PAA (**C**), are internalized in stem cells and the cells are cultured as spheroids for up to 21 days. Magnetism of spheroids cultured for 1 day (orange curves) and 21 days (grey curves) are measured with the VSM, with a decrease in magnetism indicating a degradation of the nanoparticles. Representative TEM images taken at day 21 show light grey dots about 6 nm is size within the endosomes and the cytoplasm of the cells, typical size and shape of ferritin, the iron storage protein (black arrows). Some intact nanoparticles can also be observed, mostly for the PAA-coated nanoparticles (brown arrow).

DISCUSSION:

Using a fast and efficient microwave-based synthesis, magnetic nanoparticles can easily be synthesized, with controlled size, and further coated with given molecules. A critical step is to stock the iron salt and the benzyl alcohol under vacuum to keep a small dispersion in size. The benzyl alcohol acts as both as solvent and ligand at the same time allowing to directly obtain calibrated bare iron oxide without the need of additional ligands. After nanoparticles transfer in water the bare magnetic nanoparticles can be easily coated with a large variety of ligands. This coating confers inter-nanoparticle and nanoparticle-cell interacting properties that can influence their cellular internalization, a parameter that needs to be tightly controlled as a minimum internalization is required for biomedical applications while a too high internalization could be damaging to the cells and potentially originate cytotoxic events[31]. Magnetometry is a powerful tool to assess this internalization as well as the fate of magnetic nanoparticles in cellulo. Upon the incorporation of magnetic nanoparticles into stem cells, spheroids can be formed via a simple centrifugation followed by culture in a medium that stops the proliferation of the cells and drives extracellular matrix production. The cells become highly cohesive and start creating a tissue. The spheroids are then very easy to handle and can be cultured for extended time periods (months) that allows for long-term tracking of magnetic nanoparticles' fate in a biological environment. By stopping cell division, there is no dilution of the nanoparticles from mother to daughter cell; moreover, it has been verified that, with this cell-spheroid model, there is no iron escape over a month of culture[21–23]. As a consequence, a decrease in magnetism values can only correspond to a degradation of the nanoparticles and not to iron being exported out of the cells.

Magnetism of the cell spheroids is here measured via VSM that provides a fast and precise quantification. Other methods have also been explored to measure cellular magnetism, such as using a Superconducting Interface Device (SQUID), a machine typically more precise than the VSM with a sensitivity around 10^{-7} emu compared to 10^{-6} emu for the VSM, but operational cost is higher[32]. The sensitivity of the VSM allowing measurements of magnetic iron dose inferior to 2 pg/cell in the experimental setup is herein sufficiently precise as iron doses inferior to 2 pg/cell would be too low for the aimed applications, such as cell attraction toward a magnetic for cell guidance and tissue engineering purposes. Both VSM and SQUID magnetometry, in addition to allowing a quantification of cell magnetism, can give additional details on the nanoparticles' features. By analysis of the signal obtained, the size of the nanoparticles can be deducted[22,23] and also general notion of its magnetism characteristics can be obtained, hysteresis and coercivity of nanomaterials can for example be revealed. Alternative magnetometry approaches also exist, such as magnetophoresis that consists in measuring the velocity of individual cells when attracted

toward a magnet and the magnetism of single cells is extrapolated[33,34]. The magnetism at the single cell level can this way be obtained; however, no additional nanoparticle features can be determined. Additionally, a small magnetic sensor that provides a signal proportional to the sample magnetism has recently been used with a similar cell spheroid model. This magnetic sensor allowed the tracking of magnetic nanoparticles' biodegradation in real-time, continuously, over 7 days[35]. However, the signal of this magnetic sensor cannot be directly translated into a magnetic moment as it depends on the size of the nanoparticles. For this reason, a calibration curve needs to be performed for each nanoparticle design, curve that can be realized using the VSM.

Measurements performed herein with the VSM demonstrate a progressive degradation of the magnetic nanoparticles in the cellular environment, indicated by a decrease in magnetism. They also evidence that a same core coated with different molecules is degraded at varying rates depending on the coating. When comparing a PAA and a citrate coating, PAA leads to higher core protection to degradation, most probably due to its strong anchoring to the magnetic core[20]. The fate of magnetic nanoparticles in the intracellular environment is assessed on cell spheroids; however, the method is not limited to it. Indeed, it can be extrapolated to cell suspensions, which has already been used to assess the effect of stem cell differentiation on the fate of the nanoparticles[22]. It revealed that, depending on the differentiation pathway, the magnetic nanoparticles are processed differently by the cells, with, in some cases, the neo-crystallization of iron upon its dissolution. VSM magnetometry is thus a useful tool to further explore the influence of both nanoparticle and cell features on the intracellular processing of iron oxide nano-objects.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

Click here to access/download;Figure;Figure1.psd

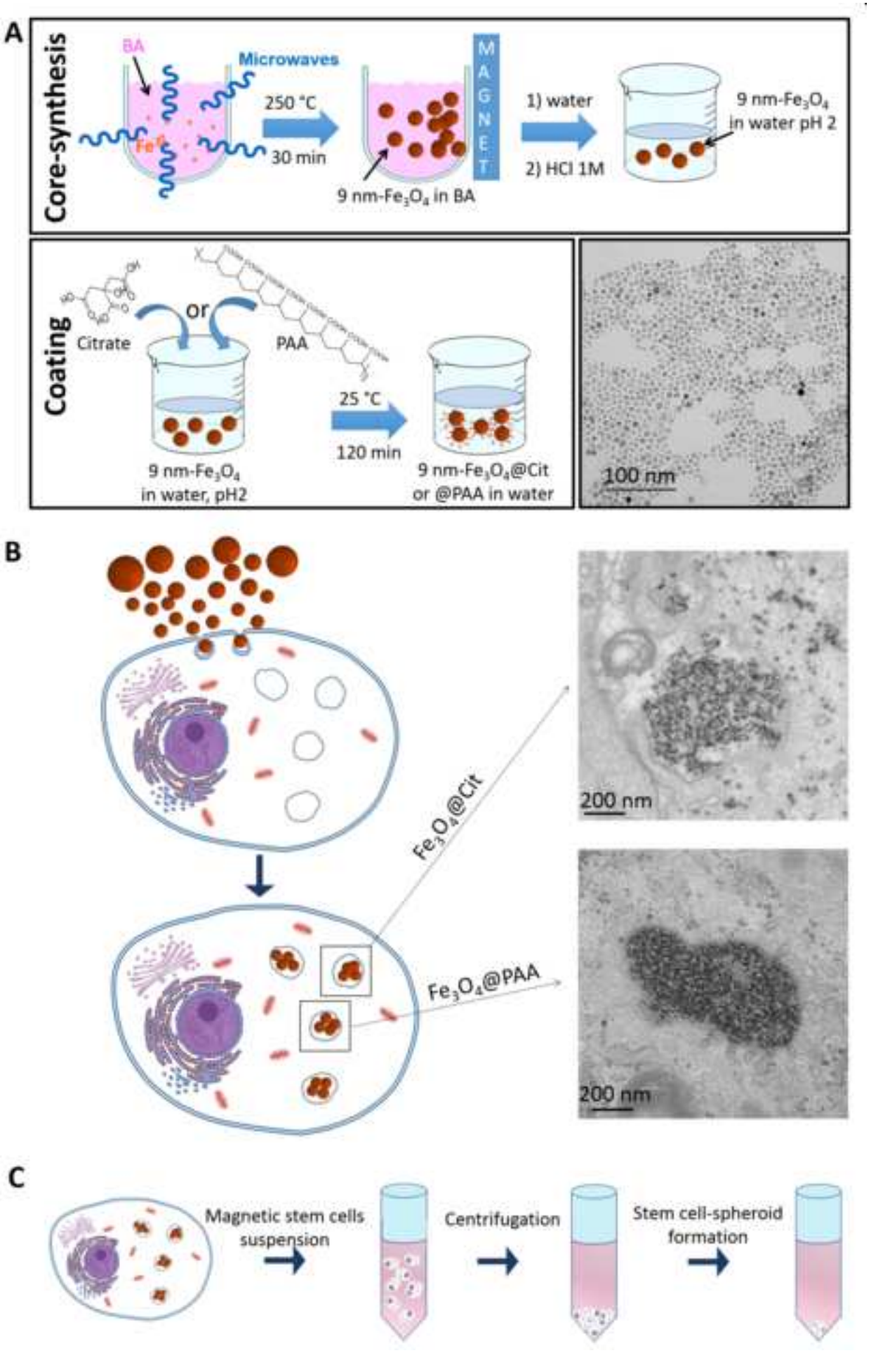


Figure2

Click here to access/download;Figure;Figure2.psd

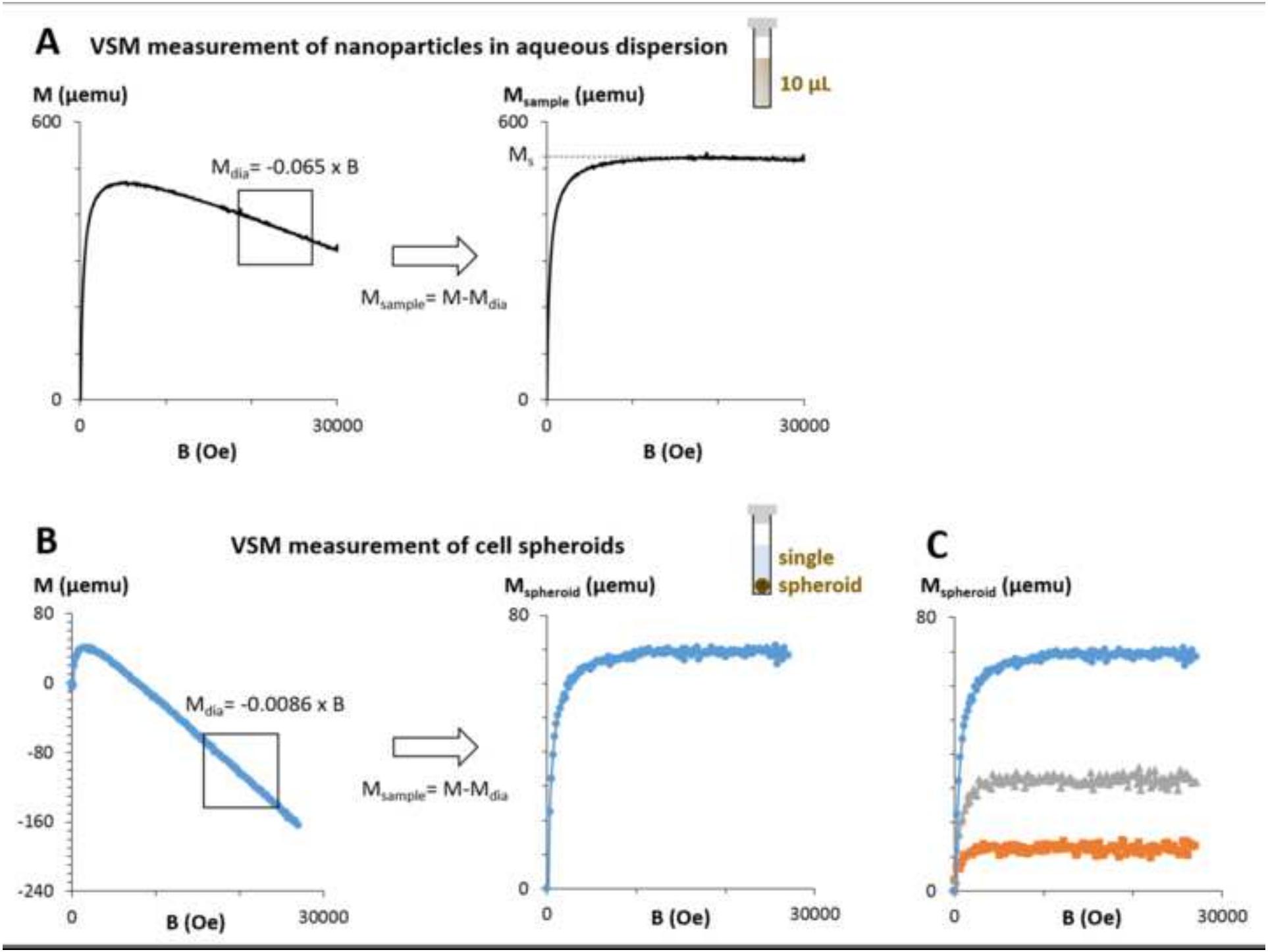
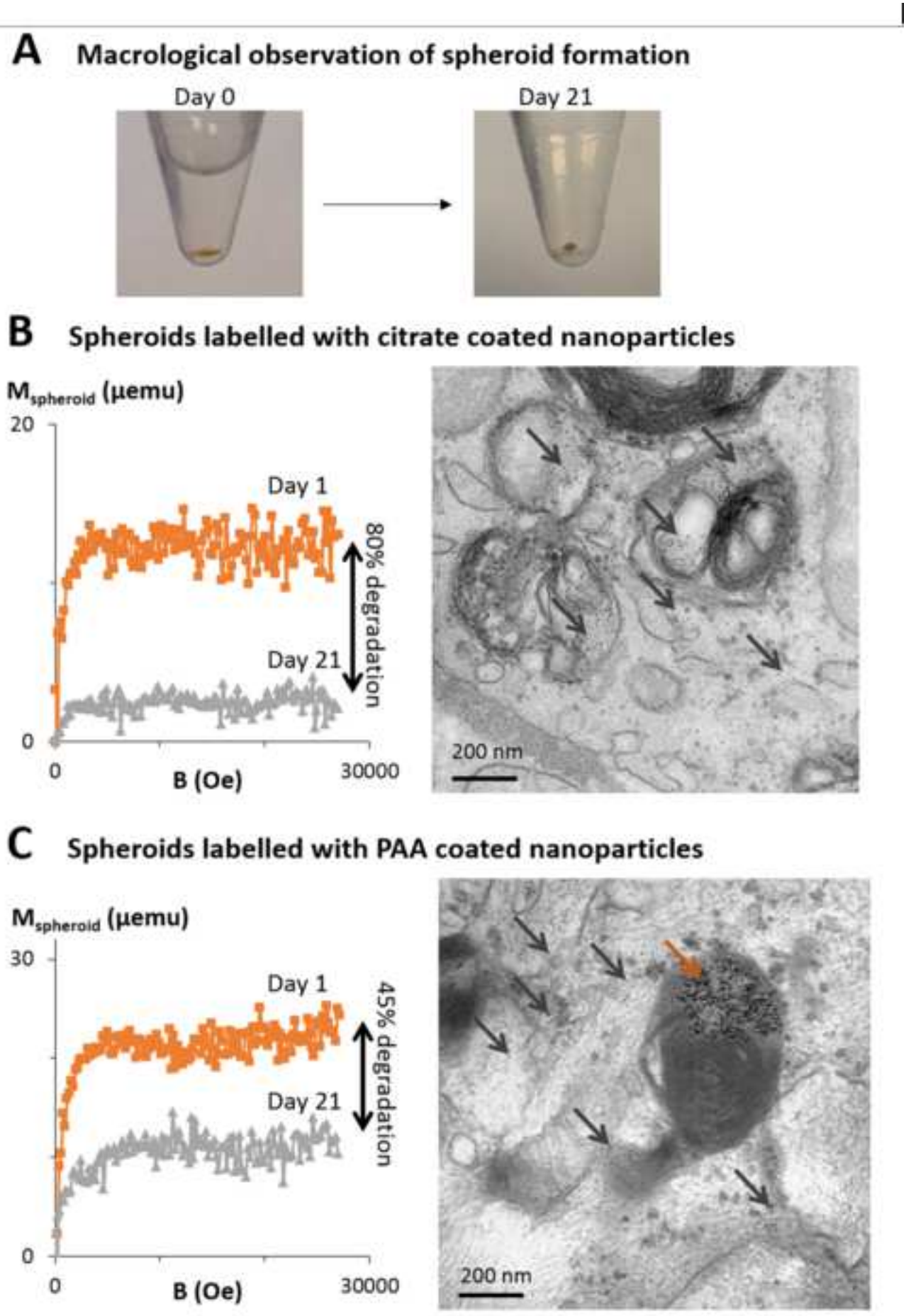


Figure3 Click here to access/download;Figure;Figure3.psd



Name of Material/Equipment	Company	Catalog Number	Comments/Description
0.05% Trypsin-EDTA (1x)	Life Technologies	25300-054	
Benzyl alcohol for synthesis	Sigma Aldrich	8.22259	
Dexamethasone	Sigma	D4902	Prepare a 1 mM stock solution diluted in Ethanol 100% and store at -20°C
Dichloromethane ≥99% stabilised, (	VWR Chemicals	23367	
DMEM with Glutamax I	Life Technologies	31966-021	No sodium pyruvate, no HEPES
Ethanol absolute	VWR	20821.310	
Fetal Bovine Serum	Life Technologies	10270-106	
Formalin solution 10% neutral buffered	Sigma	HT5012	
Hydrochloric acid, 1.0N Standardized Solution	Alfa Aesar	35640	
Iron(III) acetylacetonate (> 99.9%)	Sigma Aldrich	517003	
ITS Premix Universal Culture Supplement (20x)	Corning	354352	
L-Ascorbic Acid 2-phosphate	Sigma	A8960	Prepare a fresh concentrated solution (25 mM) diluted in distilled water
L-Proline	Sigma	P5607	Prepare a 175 mM stock solution diluted in distilled water and store at 4°C
Mesenchymal Stem Cell (MSC)	Lonza	PT-2501	
Monowave glass vial	Anton Paar	82723_us	
Microwave reactor	Anton Paar	Monowave 300	
MSCGM BulletKit medium	Lonza	PT-3001	For the complete medium, add the provided BulletKit (containing serum, glutamine and antibiotics) to the MSCGM medium
PBS w/o $CaCl_2$ w/o $MgCl_2$	Life Technologies	14190-094	

Penicillin (10.000U/mL)/Streptomicin (10.000µg/mL)	Life Technologies	15140-122	
Poly(acrylic acid, sodium salt)	Sigma Aldrich	416010	MW = 1200 g/mol
RPMI medium 1640, no Glutamine	Life Technologies	31870-025	No sodium pyruvate, no HEPES
Sodium hydroxide, 1.0N Standardized Solution	Alfa Aesar	35629	
Sodium pyruvate solution 100mM	Sigma	S8636	
Sterile conical centrifuge tube	Falcon	352097	15 mL tubes
Trypsin-EDTA (0.05%), phenol red	Thermo Fisher Scientific	25300054	
Tri-sodium citrate	VWR	33615.268	Prepare a 1 M stock solution diluted in distilled water and store at 4°C
Tri-Sodium Citrate Dihydrate, Certil	Sigma Aldrich	10396430	
Ultra centrifugal filter	Amicon	AC S510024	

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been carefully proofread and a number of errors have been corrected.

2. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

The table has been revised and reorganized to follow the alphabetical order

3. Figure 1: Please use word labels for min and temperature (°C).

The labels have been modified as required.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

All trademark and registered symbols have been removed from the manuscript and the products are appropriately referenced in the Table.

5. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

More details have been added to the protocol.

6. 1.1.1: Dissolve in what container?

It is now specified

7. 1.1.3: Transfer to a glass vial of what size/volume? Separate with the magnet for how long?

It is now specified

8. 1.1.4: Wash using the magnet?

It is now specified

9. 1.1.5: Centrifuge at what speed for how long?

It is now specified

10. 2.1: Amplify the cells how?

It is now specified

11. 3.2: Please quantitate a small volume.

It is now specified

12. 3.3: When/How are the cells counted?

It is now specified in 3.2.

13. 3.4: What happens after centrifugation?

After centrifugation, a cell pellet is formed at the bottom of the tube, which is now better explained.

14. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

The figures have been removed from the manuscript and uploaded separately.

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The mentioned points have been addressed in the discussion, which has been increased with two new paragraphs.

Reviewers' comments:
Reviewer #1:

We will consider publishing your paper entitled "Monitoring the cellular incorporation and degradation of magnetic nanoparticles by 6 magnetometry"; after minor revisions based on the below review(s).

1. The title is not catchy and does not reflect essential contents.

The title has been modified to better reflect all main content of the manuscript, as follows:

"Magnetometry as a tool to monitor cellular incorporation and subsequent biodegradation of chemically synthetized iron oxide nanoparticles"

2. The quality of figure 2 is not acceptable.

We agree that the quality of figure 2 was low, we solved this issue by saving the figure in a better format.

3. I was surprised to see that the synthesis method, according to the manuscript (discussion/conclusion), has no major limitations. Is this correct? If not, please specify in the manuscript.

The reviewer is right that we had justified the use of our non-aqueous sol-gel synthesis but not discussed its limitations. In our opinion, the biggest limitation of the non-aqueous sol gel route is the limited size increase of the nanoparticles, which is up to 12 nm. This information is now added to the discussion as follows: "Nevertheless, we can notice that with such microwave assisted non-aqueous sol-gel route, the upper size limit of the obtained nanoparticles is around 12 nm. This procedure would not be adapted for applications using ferromagnetic particles at room temperature."

4. In the case of particle size, please give size± in nm. Concern approximately 10 particles and average their size.

The average nanoparticles size is obtained by measuring 200 nanoparticles and the standard deviation in nm has been added to the text: "8.8 ± 2.5 nm"

5. It is better to present a Table that compares obtained values with values reported by others.

The present paper is not focusing on the synthesis of magnetic nanoparticles themselves and, from pubmed, you find 14144 items about "iron oxide synthesis" which does not appear suitable for a table comparison within an experimental article.

Nevertheless, in accordance with remark 3, we briefly describe the main ways of iron oxide nanoparticles synthesis as follows: "Each protocol presents advantages and limitations. Iron oxide nanoparticles were usually synthesized in aqueous solutions via coprecipitation of iron ions[24]. Other synthesis methods such as polyol-mediated sol-gel have then been developed[25]. To overcome the limitations of nanoparticles size polydispersity control several nonaqueous approaches have been developed for the production of well-calibrated iron oxide nanoparticles[26]. However, the use of massive amounts of surfactants like oleylamine or oleic acid complicates their functionalization and water transfer for biomedical applications."

6. Several references, including synthesis of nanoparticles for drug delivery, should be added. Some of the related references are given: Colloids and Surfaces B: Biointerfaces 172

(2018) 244-253, Journal of Molecular Liquids 249 (2018) 1151-1160, Materials Science and Engineering: C 76 (2017) 1085-1093, Rsc Advances 4 (2014, 49), 25993-26001; Materials Science and Engineering: C 2014, 45, 29-36; Chemical Engineering Journal 252, 2014, 173-184; Materials Science and Engineering: C 2014, 40, 288-298; Journal of nanoparticle research 16 (2014, 9), 2604; Scientific reports 6, 2016, 32539; Materials Science and Engineering: C 76, 2017, 1085-1093; Microchimica Acta 184 (2017, 3), 825-833; International Journal of Hydrogen Energy 42 (2017, 39), 24846-24860; Journal of Molecular Liquids 249, 2018, 1151-1160; Colloids and Surfaces B: Biointerfaces 172, 2018, 244-253;

Consequently we added the possibility of using magnetic nanoparticles as drug delivery systems within the introduction: "The range of possible of these nanoparticles is also extended to drug delivery systems[7,8] or to magnetic and photo-induced hyperthermal treatment for killing cancerous cells[9-11]"

Fairly good paper. However, many technical and formal flaws. If these are eliminated, the paper will be much more attractive.

Reviewer #2:

Manuscript Summary:
The manuscript describes a reliable techniques for monitoring in cellulo degradation of magnetic nanoparticles. Such assay can be suitable for future experiments performed by scientists in the field.

Minor Concerns:
Authors should maintain the same sentence time (either present or past).

The sentences have been corrected such as maintaining a constant time.

References for VSM measurements should be provided.

References are now provided.

Grammar of sentence 1.2.1 should be revised.

We thank the reviewer for pointing it out, the grammar of the sentence has been revised.

In 2.1., please provide the passage ratio.

The cells are amplified until passage 4 or 5, it is now specified in the manuscript.

Why authors wash with RPMI and then use DMEM for spheroid seeding?

The washes in the nanoparticle incubation steps are performed using RMPI media as its ionic force is lower than DMEM and it prevents the aggregation of the nanoparticles. It is now specified in the manuscript as follows:

"RPMI is used for nanoparticle incubation (and the related rinsing steps) as it has a lower ionic strength than DMEM and it better prevents nanoparticle aggregation events."

IN 2.4. how many times spheroids are washed?

The spheroids are washed once, it is now specified in the manuscript.

Is there any way to monitore the proper formation of spheroids during the 21 days of incubation?

The monitoring of the proper formation of the spheroids over the 21 days of culture is by simple observation. Indeed, it is possible to see the spheroid transitioning from a cell pellet to a spheroid. Photographs that illustrate this transition have been added to the Figure 3 as follows:

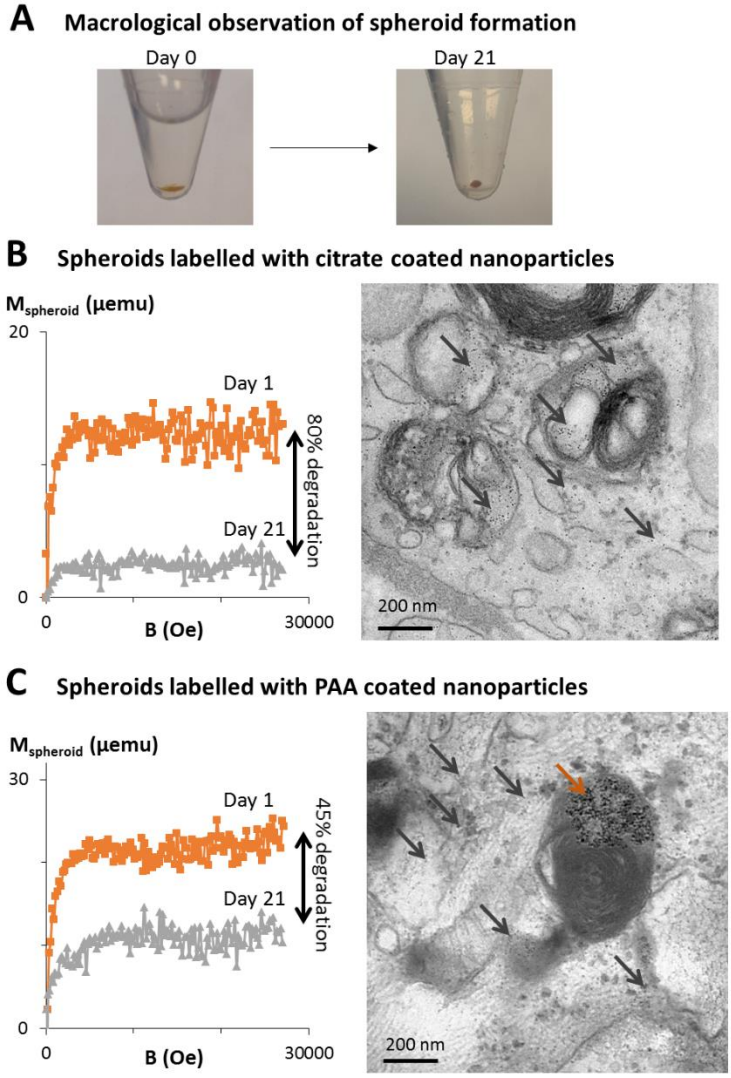


Figure 3: Quantification of magnetic nanoparticle degradation in cellulo via VSM. (A) Upon cell labeling with magnetic nanoparticles, a cell pellet is formed by centrifugation (day 0). The cells then form a cohesive structure resulting in an easy to handle cell-spheroid that can be kept in culture without cell loss for extended time periods (months). (B, C) Herein, two types of magnetic nanoparticles, coated with citrate (B) or PAA (C), are internalized in stem cells and the cells are cultured as spheroids for up to 21 days. Magnetism of spheroids cultured for 1 day (orange curves) and 21 days (grey curves) are measured with the VSM, with a decrease in magnetism indicating a degradation of the nanoparticles. Representative

TEM images taken at day 21 show light grey dots about 6 nm is size within the endosomes and the cytoplasm of the cells, typical size and shape of ferritin, the iron storage protein (black arrows). Some intact nanoparticles can also be observed, mostly for the PAA-coated nanoparticles (brown arrow).

During these 21 days cells devide. How much this VSM decrease have to do with a dilution of nanoparticles as cells devide?

The reviewer is right to point out that, in case of cell proliferation, the nanoparticles would be diluted from mother to daughter cell as the cells divide. In the spheroid model used here, the cells are cultured in a specific medium that stops their proliferation, thus avoiding cell division and nanoparticle dilution. It was already specified in the manuscript with the following sentence

“Upon internalization of magnetic nanoparticles into stem cells, spheroids can be formed via a simple centrifugation followed by culture in a medium that stops the proliferation of the cells and drives extracellular matrix production.”

and it is known more thoroughly explained for a better comprehension, as follows

“By stopping cell division, there is no dilution of the nanoparticles from mother to daughter cell, moreover, it has been verified that, with this cell-spheroid model, there is no iron escape over a month of culture[19, 20, 26]. As a consequence, a decrease in magnetism values can only correspond to a degradation of the nanoparticles and not to iron being exported out of the cells.”