Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Methods Article - Author Produced Video
Manuscript Number:	JoVE63140R3
Full Title:	Stable Aqueous Suspensions of Manganese Ferrite Clusters with Tunable Nanoscale Dimension and Composition
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TITLE:

Stable Aqueous Suspensions of Manganese Ferrite Clusters with Tunable Nanoscale Dimension and Composition

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SUMMARY:

We report a one-pot hydrothermal synthesis of manganese ferrite clusters (MFCs) that offers independent control over material dimension and composition. Magnetic separation allows rapid purification while surface functionalization using sulfonated polymers ensures the materials are non-aggregating in biologically relevant medium. The resulting products are well positioned for biomedical applications.

ABSTRACT:

Manganese ferrite clusters (MFCs) are spherical assemblies of tens to hundreds of primary nanocrystals whose magnetic properties are valuable in diverse applications. Here we describe how to form these materials in a hydrothermal process that permits the independent control of product cluster size (from 30 to 120 nm) and manganese content of the resulting material. Parameters such as the total amount of water added to the alcoholic reaction media and the ratio of manganese to iron precursor are important factors in achieving multiple types of MFC nanoscale products. A fast purification method uses magnetic separation to recover the materials making production of grams of magnetic nanomaterials quite efficient. We overcome the challenge of magnetic nanomaterial aggregation by applying highly charged sulfonate polymers to the surface of these nanomaterials yielding colloidally stable MFCs that remain non-aggregating even in highly saline environments. These non-aggregating, uniform, and tunable materials are excellent prospective materials for biomedical and environmental applications.

INTRODUCTION:

The inclusion of manganese as a dopant in an iron oxide lattice can, under the appropriate conditions, increase the material's magnetization at high applied fields as compared to pure iron oxides. As a result, manganese ferrite ($Mn_xFe_{3-x}O_4$) nanoparticles are highly desirable magnetic nanomaterials due to their high saturation magnetization, strong response to external fields, and low cytotoxicity[1–5]. Both single domain nanocrystals as well as clusters of these nanocrystals, termed multidomain particles, have been investigated in diverse biomedical applications, including drug delivery, magnetic hyperthermia for cancer treatment, and magnetic resonance imaging (MRI)[6–8]. For example, the Hyeon group in 2017 used single domain manganese ferrite nanoparticles as a Fenton catalyst to induce cancer hypoxia and exploited the material's T_2 contrast for MRI tracking[9]. It is surprising in light of these and other positive studies of ferrite materials that there are few *in vivo* demonstrations as compared to pure iron oxide (Fe_3O_4) nanomaterials, and no reported applications in humans[9,10].

One immense challenge faced in translating the features of ferrite nanomaterials into the clinic is the generation of uniform, non-aggregating, nanoscale clusters[11–14]. While conventional synthetic approaches to monodomain nanocrystals are well developed, multidomain clusters of the type of interest in this work are not easily produced in a uniform and controlled fashion[15,16]. Additionally, ferrite composition is usually non-stoichiometric and not simply related to the starting concentration of the precursors and this can further obscure systematic structure-function characterization of these materials[9,12,13,17]. Here, we address these issues by demonstrating a synthetic approach that yields independent control over both the cluster dimension and composition of manganese ferrite nanomaterials.

This work also provides a means to overcome the poor colloidal stability of ferrite nanomaterials[18-20]. Magnetic nanoparticles are generally prone to aggregation due to strong particle-particle attraction; ferrites suffer more from this problem as their larger net magnetization amplifies particle aggregation. In relevant biological media, these materials yield large enough aggregates that the materials rapidly collect, thereby limiting their routes of exposure to animals or people[20–22]. Hilt et al. found another consequence of particle-particle aggregation in their study of magnetothermal heating and dye degradation[23]. At slightly higher particle concentrations, or increased time of exposure to the field, the effectiveness of the materials was reduced as materials aggregated over time and the active particle surface areas decreased. These and other applications would benefit from cluster surfaces designed to provide steric barriers that precluded particle-particle interactions[24,25].

Here we report a synthetic approach to synthesize manganese ferrite clusters (MFCs) with controllable dimensions and composition. These multidomain particles consist of an assembly of primary manganese ferrite nanocrystals that are hard aggregated; the close association of the primary nanocrystals enhances their magnetic properties and provides for an overall cluster size, 50–300 nm, well matched to the optimum dimensions for a nanomedicine. By changing the amount of water and manganese chloride precursor, we can independently control the overall diameter and composition. The method utilizes simple and efficient one-pot hydrothermal reactions that allow for frequent experimentation and material optimization. These MFCs can be easily purified into a concentrated product solution, which is further modified by sulfonated

polymers that impart colloidal stability. Their tunability, uniformity, and solution phase stability are all features of great value in applications of nanomaterials in biomedical and environmental engineering.

PROTOCOL:

1. Synthesis of MFCs with control over MFCs' overall diameter and ferrite composition

1.1. Wash and thoroughly dry all glassware to be used in the synthesis. The amount of water in the synthesis impacts the dimensions of the MFCs, so it is crucial to ensure the glassware has no residual water in it[16,26].

1.1.1. To wash the glassware, rinse with water and detergent and scrub with a flask brush to remove debris. Thoroughly rinse to remove all detergent and finish with a rinse of deionized water.

1.1.2. To dry the glassware, shake water droplets off the surface of the glassware and place into an oven at 60 °C until completely dry.

1.1.3. Rinse the polyphenylene-lined (PPL) reactors with 37% hydrochloric acid to remove any debris from previous use. To do this, place the reactors and their caps in a large beaker and fill with hydrochloric acid until the reactors are completely submerged. Let this sit for 30 min before pouring out the hydrochloric acid. Continuously rinse the beaker containing the reactors with water for 1–2 min, and then place the reactors in the oven to dry.

1.2. Use an automatic pipette to transfer 20 mL of ethylene glycol into a 50 mL beaker with a magnetic stir bar.

1.3. Weigh out the required amount of iron(III) chloride ($FeCl_3 \cdot 6H_2O$, solid) to achieve a final concentration of 1.3 mM and add it to the beaker. Put the beaker on a stir plate and turn it on at 480 rpm to begin continuous stirring of the beaker.

NOTE: As this is a hydrate, it must be measured and added quickly to avoid unwanted absorption of water from the ambient air.

1.4. Weigh 250 mg of polyacrylic acid (PAA, Mw ~6,000, powder) and add it to the beaker. After the addition of PAA, the solution becomes opaque and slightly lighter in color.

1.5. Weigh 1.2 g of urea ($CO(NH_2)_2$, powder) and add it to the beaker.

1.6. Using a pipette, add 0.7 mM manganese(II) chloride ($MnCl_2 \cdot 6H_2O$ aq, 3.5 M, 0.2 mL) to the beaker.

1.7. Finally, using a pipette add the required amount (0.5 mL) of ultra-pure water to the

beaker.

1.8. Let the solution stir for 30 min and notice the color change. It will present as a translucent, dark orange color.

1.9. Transfer the reaction mixture into the polyphenylene lined (PPL) reactor. Note that after the solution has stirred some solids may have accumulated on the sides of the beaker.

1.9.1. Use a magnet (cubic permanent rare earth magnet, 40 x 40 x 20 mm, hereafter referred to as a "magnet" for all separation and magnetic collection procedures) to drag the stir bar around the walls of the beaker to ensure any solids that have accumulated on the sides are dispersed into the reaction solution.

1.9.2. Once the solution is mixed and ready, transfer it into the 50 mL PPL lined reactor.

1.9.3. Use a clamp and lever to seal the reactor in the stainless-steel autoclave as tightly as possible. Clamp the reactor vessel to a stable surface, and using a rod inserted into the cap as a lever, push the reactor to seal. Note that the sealed reactor should not be able to be opened by hand. This is crucial as the high-pressure environment of the oven requires a tight seal on the reactor.

1.10. Place the reactor into an oven for 20 h at 215 °C.

1.11. After the hydrothermal reaction is done, remove the reactor from the oven and allow it to cool down to room temperature. The pressure of the oven will enable the reactor to be opened by hand. Note that at this point, the reactor will contain the MFC product dispersed in ethylene glycol with other impurities, such as unreacted polymer, and will be an opaque black solution. The product will be isolated in the following steps.

2. Magnetic separation and purification of MFCs

2.1. Place 200 mg of steel wool into a glass vial. Fill the glass vial halfway with the reaction mixture from the reactor. Fill the rest of the vial with acetone and shake well. Note that the steel wool increases the magnetic field strength in the vial and will help magnetic separation of the nanoclusters from the solution.

2.2. Place the vial on a magnet for magnetic collection to occur. The result will be a translucent solution with precipitate at the bottom.

2.2.1. Pour off the supernatant solution while the MFCs are magnetically trapped by the steel wool by holding the magnet to the bottom of the vial while pouring. Ethylene glycol will be mostly removed in this step.

2.2.2. Start washing with the low ratio of acetone to water and increase the ratio in subsequent

washes until pure. Do this 3–4 times.

2.3. Remove the vial from the magnet and fill it with water. Shake well to dissolve the MFCs. Now the product will be fully dispersed in water.

2.4. Repeat the previous two steps several times until the aqueous solution of the MFCs produces no bubbles when shaken. The result will be a dark, opaque ferrofluid that will respond strongly to magnets.

NOTE: In a typical synthesis with 20 mL of ethylene glycol, approximately 80 mg of MFC product will be obtained.

3. Surface functionalization of MFCs toward ultra-high colloidal stability

NOTE: The synthesis of nitro-dopamine and Poly(AA-co-AMPS-co-PEG) can be found in our previous work[16]. The copolymer is made through free radical polymerization. Add 0.20 g of 2,2'-Azobis(2-methylpropionitrile) (AIBN), 0.25 g of acrylic acid (AA), 0.75 g of 2-Acrylamido-2-methylpropane sulfonic acid (AMPS), and 1.00 g of Poly(ethylene glycol) methyl ether acrylate (PEG) in 10 mL of N,N-Dimethylformamide (DMF). Heat the mixture in a 70 °C water bath for 1 h and transfer it to a dialysis bag (Cellulose Membrane, 3 kDa) in water. The weight ratio of AA, AMPS, and PEG is 1:3:4. Polymerization for these monomers has a 100% conversion rate as confirmed by freeze drying and weighing.

3.1. Combine 10 mL of purified nanoparticles (around 100 mg) in a 20 mL vial with 10 mL of saturated N-[2-(3,4-dihydroxyphenyl)ethyl]nitramide (nitro-dopamine) solution (~1 mg/mL). Wait for 5 min.

3.2. Wash the nitro-dopamine coated MFCs using magnetic separation. Pour out the pale-yellow supernatant. Add water and shake vigorously. Then, pour out water using the magnet to retain the product. Repeat this washing several times leaving the dark brown collection in the vial.

NOTE: Prepare a aqueous solution with a concentration of 20 mg/mL, a buffer solution with a concentration of 100 mg/mL, and a poly(AA-co-AMPS-co-PEG) polymer solution with a concentration of 20 mg/mL.

3.3. Mix 1 mL of EDC solution, 1 mL of MES buffer, and 3 mL of the polymer solution. Lightly stir by swirling the mixture, and let it sit for approximately 5 min. It should be a clear and colorless solution when fully combined.

3.4. Add this mixture to the MFC collection and place the vial in an ice bath. Lower the probe sonicator into the solution, and then turn it on (250 watts of power at 20 kHz).

3.4.1. After a 5 min sonication treatment, add roughly 5 mL of ultra-pure water to the vial while

the sonicator is still running. Continue monitoring the vessel to ensure that no product spills. Maintain the ice in the ice-water mixture as some of the initial ice will melt due to the intensity and heat of the sonication.

3.4.2. Allow the mixture to sonicate for an additional 25 min, for a total of 30 min.

3.5. Place the vial on top of a magnet to separate the MFCs and pour out the supernatant solution.

3.6. Wash the modified MFCs with deionized water several times.

3.7. Fill the vial containing the MFCs with ultra-pure water. Pipette this fluid into a vacuum filtration system with a 0.1 µm polyethersulfone membrane filter to remove any irreversibly aggregated MFCs. Make sure to flush the walls of the funnel to minimize any loss of product.

3.8. Vacuum filter the solution. Repeat this process 2–3 times. The result will be a purified aqueous solution of monodispersed MFCs.

NOTE: Roughly 10% of the product will be irreversibly aggregated and this material will remain on the filter and should be discarded.

REPRESENTATIVE RESULTS:

After hydrothermal treatment, the reaction mixture turns into a viscous black dispersion as can be seen in **Figure 1**. What results after purification is a highly concentrated MFC solution that behaves like a ferrofluid. The fluid in the vial responds within seconds when placed near a handheld magnet (<0.5 T), forming a macroscopic black mass that can be moved around as the magnet is placed at different locations.

This synthesis yields products whose dimension and ferrite composition depend on the amount of water added and the ratio of manganese to iron precursor in the reaction mixture. **Figure 2** illustrates how the cluster morphology depends on water and precursor concentration; it also details the reaction conditions used to obtain the samples listed in **Table 1**. We find that the MFC diameter is affected by the amount of water added, and the MFC composition depends on the ratio of iron and manganese in the precursors. Both parameters can thus be independently controlled to make a library of MFCs with distinct dimensions and manganese content.

While this is a very simple synthetic procedure, errors in the method execution may lead to failed products. **Figure 3** depicts samples with irregular MFC morphologies. In **Figure 3A**, odd-shaped MFCs result if water is completely excluded from the reaction environment. The lack of water hinders the dynamic assembly of the primary nanocrystals and results in a very broad distribution of nanocluster dimension and non-spherical shapes[16]. The samples shown in **Figure 3B** had insufficient reaction time (6–12 h) and as a result did not have sufficient primary nanocrystal growth. These poor results demonstrate that an appropriate amount of the reactant, as well as reaction time, is necessary to achieve consistent and uniform clusters.

After completion of the hydrothermal synthesis, the ferrite MFCs were separated and purified using magnetic separation. A magnet was placed under the solution to force their collection at the bottom of the vessel. Impurities and non-magnetic by-products formed in the synthesis, along with the excess solvent, could then be decanted to yield pure and monodispersed MFCs[27]. **Figure 4** illustrates the time required for nearly complete magnetic collection of the MFCs with and without the addition of steel wool. The steel wool placed in the vial during magnetic separation increases the gradient of the magnetic field within the vial, allowing for a much faster separation[28].

The MFCs purified using magnetic separation show a high degree of uniformity compared to those purified using a more conventional ultracentrifugation process. **Figure 5** shows the size distribution of MFCs obtained using magnetic separation (A and B) compared to those using ultracentrifugation (5,000 g for 30 min) (C and D). Magnetic separation results in a narrower cluster diameter distribution as compared to ultracentrifugation and is the preferred purification strategy for the MFCs.

The as-synthesized MFCs are coated with polyacrylate (PAA), which provides a negatively charged surface and some degree of interparticle repulsion that prevents interparticle aggregation (**Figure 6A**). However, by performing a ligand replacement reaction with nitrodopamine (**Figure 6B)**, we can replace the PAA coating with a copolymer coating of P(AA-co-AMPS-co-PEG), which allows for greater stability in higher ionic strength solutions. **Figure 7** shows the schematic of this surface functionalization process. The colloidal stability of the MFCs dispersed in a PBS buffer is apparent in **Figure 8**. As-synthesized MFCs coated with PAA rapidly aggregate and separate from the solution within 30 min and are of little use in biological applications. In contrast, MFCs functionalized with a polysulfonate coating remained well dispersed in this solution for over 2 days without any sign of aggregation. The post-synthesis surface modification described here provides a route for forming homogeneous solutions of MFCs appropriate for introduction into biological environments.

FIGURE AND TABLE LEGENDS:

Figure 1: **The schematic for the synthesis of manganese ferrite nanoclusters.** The reagents, iron(III) chloride, manganese(II) chloride, polyacrylic acid (PAA), urea, water and ethylene glycol are combined under hydrothermal conditions to produce the manganese ferrite nanoclusters. This product forms a stable colloidal solution in pure water as shown in the middle. The amount of water added in the synthesis and the ratio of manganese to iron in the precursors is used to tune the cluster size and ferrite composition, respectively. After magnetic separation, the nanoclusters form a ferrofluid as shown in the right, indicating they are highly responsive to even small applied magnetic fields.

Figure 2: Transmission electron microscope (TEM) images of the manganese ferrite nanoclusters and their diameter distributions. In images A–D, the cluster diameter (Dc) increases as a result of reducing the amount of water added in the synthesis. The average cluster diameter is 31, 56, 74, and 120 nm for A, B, C, and D, respectively, with a constant composition

of $Mn_{0.15}Fe_{2.85}O_4$. In images E–H, the ferrite composition monotonically changes in proportion to the Mn/Fe ratio of the precursors. Despite their different compositions, a nearly equivalent cluster diameter is achieved. Our synthesis allows for independent control over both the cluster diameter and ferrite composition, both features that are important for the magnetic properties of nanoscale ferrites.

Table 1: **Reaction conditions for the synthesis of the nanocluster samples shown in Figure 2.** Other synthesis parameters are: 20 mL ethylene glycol, 250 mg PAA, and 1.2 g urea. The reaction mixtures are hydrothermally heated at 200 °C for 20 h. For A, B, C, and D, decreasing the water content while keeping other parameters constant resulted in clusters of larger diameters. For E, F, G, and H, increasing the ratio of $MnCl_2$ to $FeCl_3$ in the initial reaction mixture resulted in clusters with higher proportions of manganese in the cluster structure. Varying the amount of water E, F, G, and H at the same time allows for clusters of different composition but near equivalent diameters.

Figure 3: TEM images of failed and incomplete reactions. The small, low contrast features observed in these images are primary nanocrystals that have not developed into nanoclusters. The sample in **Figure 3A** was prepared with no additional water, while the material shown in **Figure 3B** had an insufficient, four-hour, reaction time.

Figure 4: Comparison of magnetic separation of nanoclusters. Comparison of magnetic separation of nanoclusters without (**A**) and with (**B**) the addition of steel wool in the container. Steel wool increases gradient of the magnetic field inside the vial to allow for faster magnetic separation of the nanoclusters. As a result, it is possible to scale-up the production of nanoclusters efficiently without sacrificing sample quality.

Figure 5: **Comparison of ultracentrifugation and magnetic separation**. Comparison of ultracentrifugation (**A,B**) and magnetic separation (**C,D**) and their impact on the uniformity of the purified clusters. A and C are the TEM images of the purified clusters, and B and D are the size distributions of the clusters in A and C, respectively. The y axis represents the number of clusters counted, and for each sample, a total of 150 clusters were surveyed.

Figure 6: **The structure of poly(acrylic acid) (PAA) (A) and nitro-dopamine (B) used in the surface modification step.** The initial PAA coating used in synthesis is not ideal in biological or acidic media due as the carboxylic acid is easily protonated. Nitro-dopamine is used to replace the PAA coating creating a functional group to anchor a sulfonated copolymer.

Figure 7: **Schematics of the cluster surface modification process**. (**A**) original PAA coating, (**B**) intermediate nitro-dopamine coating, and (**C**) the final P(AA-co-AMPS-co-PEG) coating. In (C), the blue, red, and green curves represent the AA, AMPS, and PEG units, respectively. The composition of the cluster can be either Fe_3O_4 or $Mn_xFe_{3-x}O_4$.

Figure 8: **Surface functionalization of the nanoclusters with polysulfonate leads to materials that are colloidally stable under many different aqueous conditions.** Clusters with two different

surface coatings, as-synthesized PAA coated (**A**) and P(AA-co-AMPS-co-PEG) surface functionalized (**B**) are dissolved in the PBS buffer solution that is relevant for biological settings and observed for their colloidal stability over time.

DISCUSSION:

This work demonstrates a modified polyol synthesis of manganese ferrite nanocrystals clustered together into uniform nanoscale aggregates[29]. In this synthesis, iron(III) chloride and manganese(II) chloride undergo a forced hydrolysis reaction and reduction, forming molecular $Mn_xFe_{3-x}O_4$. These ferrite molecules form primary nanocrystals under the high temperature and high pressure in the reactors, ultimately assembling into spherical aggregates termed here magnetite ferrite clusters (MFCs). Without sufficient reaction time or sufficient water, the aggregation process cannot fully complete leading to non-uniform, poorly formed particles. Conversely, given enough time and enough water, the metal oxide crystallization and assembly process is complete and yields a uniform spherical cluster comprising tens to hundreds of primary nanocrystals. The primary nanocrystals in these materials are hard aggregated, sharing some crystalline interfaces, leading to a high initial susceptibility, and pronounced magnetic response even to the small fields available from handheld permanent magnets[27]. As a result, these materials have great potential for applications in drug delivery, magnetic hyperthermia, magnetic resonance imaging, and magnetic particle imaging[30–32].

We find that the amount of water added to the initial reaction mixture controls the diameter of the assembled clusters. As the water content in the reactants is increased, the diameter of the clusters and the number of aggregated primary nanocrystals decreases. The optimal range is 0.8 M to 5.0 M water, conditions that yield, respectively, cluster diameters ranging from 150 nm to 30 nm. Water has an important role in this process because it is necessary to ensure rapid hydrolysis of the metal precursors, more rapid aggregation of primary crystallites, and consequently smaller clusters[16]. Because the synthesis is extraordinarily sensitive to water, reactants handled under ambient conditions of variable humidity could absorb different amounts of water from the air. This could affect the subsequent dimensions and morphology of the product. While the humidity control in most research laboratories (e.g., 30%–60% RH) is sufficient to minimize this issue, this is one source of systematic error in the reported procedure. Control of the manganese to iron ratio in the product is achieved by varying the ratio of manganese to iron precursors. This is surprising as in many hydrothermal reactions the doping level of products is often not simply related to the stoichiometry of the starting materials[4,6,8,12,13,17]. For these conditions, however, the product composition is well predicted by the ratio of the metal precursors. Taken together, independent control of both the cluster diameter as well as its composition is possible through straightforward manipulation of the starting reactant mixtures.

Often the purification of nanoparticles from the reaction media is the most time-consuming and intricate step in generating high quality materials. Ultracentrifugation is often applied for this purpose and while this is effective at separating nanoparticles from molecular by-products, it is poorly suited for removing unwanted solid products. When applied here to the purification of nanomaterials, the ultracentrifugation produces relatively polydisperse particles with variable

dimensions and shapes. It is far more efficient to take advantage of the magnetic response of these materials by applying magnetic separation to improve the uniformity and purity of the final product. We speed up magnetic separation by creating very high gradients of magnetic fields within macroscopic vial using steel wool immersed in the solution and a rare-earth permanent magnet applied outside of the sample containers. This arrangement permits uniform samples to be recovered in under thirty minutes with high yields (~90%). It is important to match the amount of steel wool introduced to the solution to the anticipated MFC cluster diameters. For example, a MFC with an average diameter of 40 nm requires between 100 to 200 mg of steel wool for a rapid separation, while larger materials may require much less or even no steel wool. It is well established that smaller of magnetic nanoparticles are less responsive to applied fields by virtue of their smaller magnetic volume[15,17,26]. The magnetic separation process thus provides a means to sharpen the uniformity of these materials as smaller clusters are not retained as efficiently by the process[16]. Using this magnetic separation method not only saves time in the laboratory, but it also results in products with greater uniformity in diameter.

Although the as-synthesized MFCs are stable in pure water, they exhibit poor colloidal stability in solutions with lower pH or higher ionic strength. Manganese ferrites have large magnetization densities, and as a result for these diameters the clusters possess magnetic dipoles that lead to interparticle attraction. The native polyacrylate coating used during the formation of the materials imparts a negative charge to the particle surfaces and helps to prevent particle-particle aggregation. However, at lower pH the carboxylic groups are fully protonated in effect removing the electrostatic repulsion needed to maintain homogeneous MFC dispersions; alternatively, in higher ionic strength media, the charge repulsion is reduced leading to more particle aggregation. Aggregation of the MFCs creates macroscopic materials that are not homogeneously dispersed in the solution making it challenging to use the materials *in vivo* or in applications that require large and available nanoparticle surfaces. For these reasons, we introduce a second polymer into the reaction to replace the original PAA coating. The copolymer, P(AA-co-AMPS-co-PEG), includes neutral polyethylene glycol (PEG) to provide biocompatibility and some degree of steric hindrance. Additionally, the polysulfonate component (PAMPS) offers both a greater charge density than the polyacrylate as well as a functional group that has a much lower pKa and consequently a greater working pH range (pKa ~ 1.2)[24]. Manganese ferrite clusters modified with these surface coatings show dramatically increased stability in acidic and biological media. The procedure to ensure the correct surface modification is detailed, however, and must be followed carefully to ensure that the samples are effectively coated. Specifically, the method requires constant monitoring of the reaction mixture while it is treated with a probe sonicator to ensure homogeneous, complete replacement of the initial polyacrylate coating. It is also important to use appropriately sized glassware to minimize any product loss during vigorous sonication and apply an ice bath to the sonication mixture to minimize thermal degradation of the polymers caused by probe sonication.

In conclusion, this method allows for the quick and efficient production of manganese ferrite clusters (MFCs) with tunable diameters and manganese to iron compositions. Reactant water content as well as the ratio of iron to manganese are important parameters in defining the material product characteristics. A simple magnetic separation technique utilizing a handheld

magnet and steel wool provides an efficient means to purify the product after synthesis yielding more uniform clusters. Finally, a sulfonated PEG copolymer is applied to the materials to ensure they remain non-aggregating in a variety of different pH and ionic strength media. The increased magnetic responsiveness of these manganese-doped iron oxides compared to pure iron oxide (Fe_3O_4) nanomaterials makes it simpler, cheaper, and easier to develop devices for applying external fields to manipulate the materials *in vivo*. Their improved surface coatings are also important as applications for magnetic nanoparticles in drug delivery, water remediation, and advanced imaging systems all require materials that are non-aggregating and homogeneous in a variety of biological and environmental media.

ACKNOWLEDGMENTS:

This work was generously supported by Brown University and the Advanced Energy Consortium. We gratefully thank Dr. Qingbo Zhang for his established synthetic method of iron oxide MFCs.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Makridis, A. et al. In vitro application of Mn-ferrite nanoparticles as novel magnetic hyperthermia agents. *Journal of Materials Chemistry B*. **2** (47), 8390–8398 (2014).
2. Nelson-Cheeseman, B., Chopdekar, R., Toney, M., Arenholz, E., Suzuki, Y. Interplay between magnetism and chemical structure at spinel-spinel interfaces. *Journal of Applied Physics*. **111** (9), 093903 (2012).
3. Otero-Lorenzo, R., Fantechi, E., Sangregorio, C., Salgueiriño, V. Solvothermally driven Mn doping and clustering of iron oxide nanoparticles for heat delivery applications. *Chemistry–A European Journal*. **22** (19), 6666–6675 (2016).
4. Mohapatra, J. et al. Enhancement of magnetic heating efficiency in size controlled MFe 2 O 4 (M= Mn, Fe, Co and Ni) nanoassemblies. *Rsc Advances*. **5** (19), 14311–14321 (2015).
5. Qi, Y. et al. Carboxylic silane-exchanged manganese ferrite nanoclusters with high relaxivity for magnetic resonance imaging. *Journal of Materials Chemistry B*. **1** (13), 1846–1851 (2013).
6. Anandhi, J. S., Jacob, G. A., Joseyphus, R. J. Factors affecting the heating efficiency of Mn-doped Fe3O4 nanoparticles. *Journal of Magnetism and Magnetic Materials*. **512**, 166992 (2020).
7. Del Bianco, L. et al. Mechanism of magnetic heating in Mn-doped magnetite nanoparticles and the role of intertwined structural and magnetic properties. *Nanoscale*. **11** (22), 10896–10910 (2019).
8. Padmapriya, G., Manikandan, A., Krishnasamy, V., Jaganathan, S. K., Antony, S. A. Enhanced catalytic activity and magnetic properties of spinel Mn x Zn 1− x Fe 2 O 4 (0.0≤ x≤ 1.0) nano-photocatalysts by microwave irradiation route. *Journal of Superconductivity and Novel Magnetism*. **29** (8), 2141–2149 (2016).
9. Kim, J. et al. Continuous O2-evolving MnFe2O4 nanoparticle-anchored mesoporous silica nanoparticles for efficient photodynamic therapy in hypoxic cancer. *Journal of the American Chemical Society*. **139** (32), 10992–10995 (2017).

10. Silva, L. H., Cruz, F. F., Morales, M. M., Weiss, D. J., Rocco, P. R. Magnetic targeting as a strategy to enhance therapeutic effects of mesenchymal stromal cells. *Stem Cell Research & Therapy*. **8** (1), 1–8 (2017).
11. Otero-Lorenzo, R., Ramos-Docampo, M. A., Rodriguez-Gonzalez, B., Comesaña-Hermo, M., Salgueiriño, V. Solvothermal clustering of magnetic spinel ferrite nanocrystals: a Raman perspective. *Chemistry of Materials*. **29** (20), 8729–8736 (2017).
12. Aghazadeh, M., Karimzadeh, I., Ganjali, M. R. PVP capped Mn2+ doped Fe3O4 nanoparticles: a novel preparation method, surface engineering and characterization. *Materials Letters*. **228**, 137–140 (2018).
13. Li, Z. et al. Solvothermal synthesis of MnFe 2 O 4 colloidal nanocrystal assemblies and their magnetic and electrocatalytic properties. *New Journal of Chemistry*. **39** (1), 361–368 (2015).
14. Guo, P., Zhang, G., Yu, J., Li, H., Zhao, X. Controlled synthesis, magnetic and photocatalytic properties of hollow spheres and colloidal nanocrystal clusters of manganese ferrite. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. **395**, 168–174 (2012).
15. Pardo, A. et al. Synthesis, characterization, and evaluation of superparamagnetic doped ferrites as potential therapeutic nanotools. *Chemistry of Materials*. **32** (6), 2220–2231 (2020).
16. Xiao, Z. et al. Libraries of uniform magnetic multicore nanoparticles with tunable dimensions for biomedical and photonic applications. *ACS Applied Materials & Interfaces*. **12** (37), 41932–41941 (2020).
17. Choi, Y. S., Young Yoon, H., Sung Lee, J., Hua Wu, J., Keun Kim, Y. Synthesis and magnetic properties of size-tunable Mn x Fe3− x O4 ferrite nanoclusters. *Journal of Applied Physics*. **115** (17), 17B517 (2014).
18. Creixell, M. et al. The effect of grafting method on the colloidal stability and in vitro cytotoxicity of carboxymethyl dextran coated magnetic nanoparticles. *Journal of Materials Chemistry*. **20** (39), 8539–8547 (2010).
19. Latorre, M., Rinaldi, C., Applications of magnetic nanoparticles in medicine: magnetic fluid hyperthermia. *Puerto Rico Health Sciences Journal*. **28** (3) (2009).
20. Yeap, S. P., Lim, J., Ooi, B. S., Ahmad, A. L. Agglomeration, colloidal stability, and magnetic separation of magnetic nanoparticles: collective influences on environmental engineering applications. *Journal of Nanoparticle Research*. **19** (11), 1–15 (2017).
21. Lee, S.-Y., Harris, M. T. Surface modification of magnetic nanoparticles capped by oleic acids: Characterization and colloidal stability in polar solvents. *Journal of Colloid and Interface Science*. **293** (2), 401–408 (2006).
22. Yeap, S. P., Ahmad, A. L., Ooi, B. S., Lim, J. Electrosteric stabilization and its role in cooperative magnetophoresis of colloidal magnetic nanoparticles. *Langmuir*. **28** (42), 14878–14891 (2012).
23. Wydra, R. J., Oliver, C. E., Anderson, K. W., Dziubla, T. D., Hilt, J. Z. Accelerated generation of free radicals by iron oxide nanoparticles in the presence of an alternating magnetic field. *RSC Advances*. **5** (24), 18888–18893 (2015).
24. Bagaria, H. G. et al. Iron oxide nanoparticles grafted with sulfonated copolymers are stable in concentrated brine at elevated temperatures and weakly adsorb on silica. *ACS Applied Materials & Interfaces*. **5** (8), 3329–3339 (2013).

25. Park, J. C., Park, T. Y., Cha, H. J., Seo, J. H. Multifunctional nanocomposite clusters enabled by amphiphilic/bioactive natural polysaccharides. *Chemical Engineering Journal*. **379**, 122406 (2020).
26. Hemery, G. et al. Tuning sizes, morphologies, and magnetic properties of monocore versus multicore iron oxide nanoparticles through the controlled addition of water in the polyol synthesis. *Inorganic Chemistry*. **56** (14), 8232–8243 (2017).
27. Lartigue, L. et al. Cooperative organization in iron oxide multi-core nanoparticles potentiates their efficiency as heating mediators and MRI contrast agents. *ACS Nano*. **6** (12), 10935–10949 (2012).
28. Yavayo, uz, C. T. et al. Low-field magnetic separation of monodisperse Fe3O4 nanocrystals. *Science*. **314** (5801), 964–967 (2006).
29. Matijević, E., Scheiner, P. Ferric hydrous oxide sols: III. Preparation of uniform particles by hydrolysis of Fe (III)-chloride,-nitrate, and-perchlorate solutions. *Journal of Colloid and Interface Science*. **63** (3), 509–524 (1978).
30. Weizenecker, J., Gleich, B., Rahmer, J., Dahnke, H., Borgert, J. Three-dimensional real-time in vivo magnetic particle imaging. *Physics in Medicine & Biology*. **54** (5), L1 (2009).
31. Zhu, X., Li, J., Peng, P., Hosseini Nassab, N., Smith, B. R. Quantitative drug release monitoring in tumors of living subjects by magnetic particle imaging nanocomposite. *Nano Letters*. **19** (10), 6725–6733 (2019).
32. Tay, Z. W. et al. Magnetic particle imaging-guided heating in vivo using gradient fields for arbitrary localization of magnetic hyperthermia therapy. *ACS Nano*. **12** (4), 3699–3713 (2018).

Figure 1

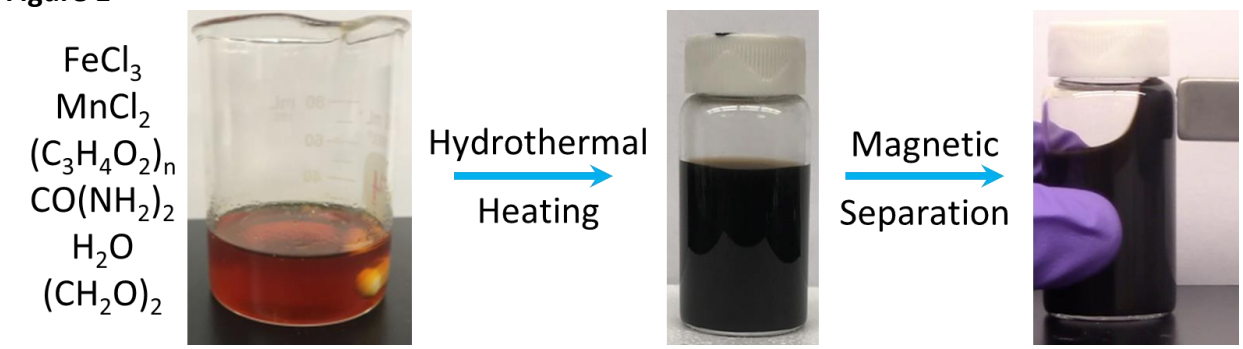


Figure 2

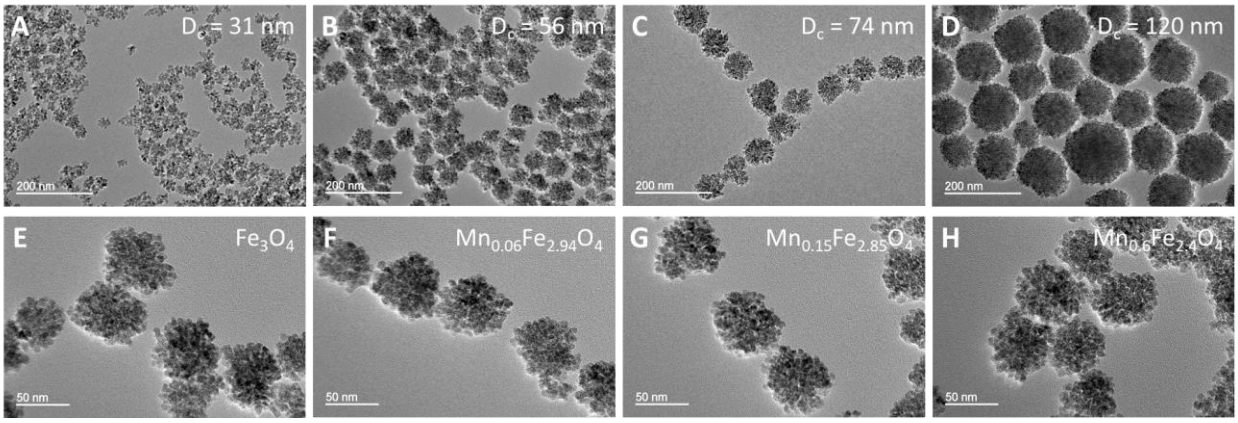


Figure 3

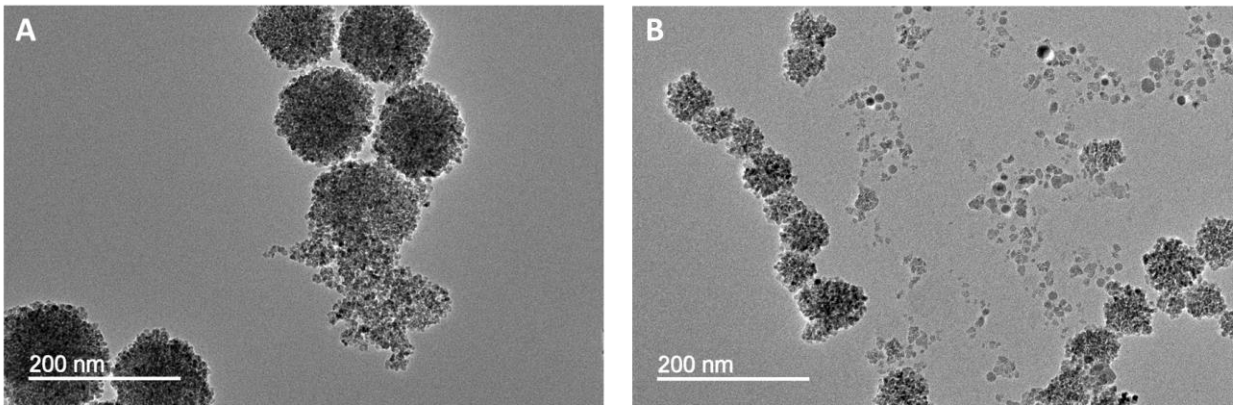


Figure 4

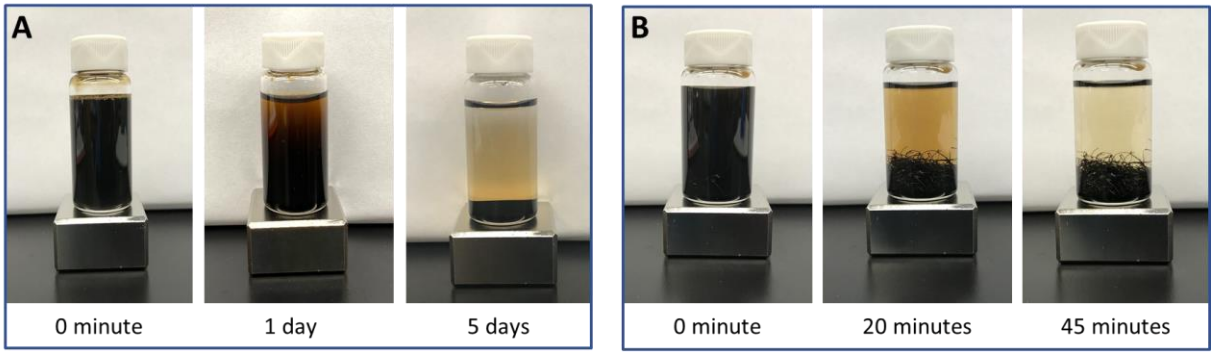


Figure 5

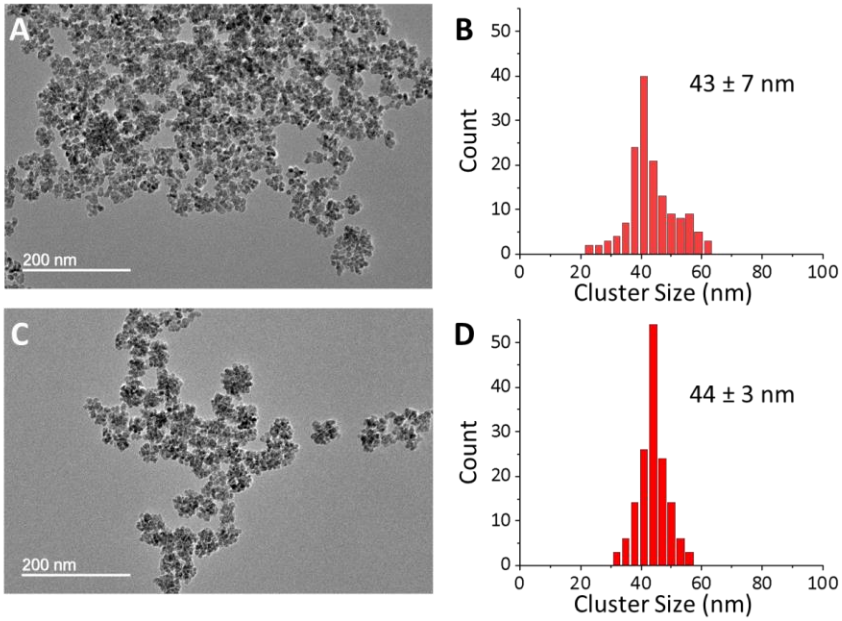


Figure 6

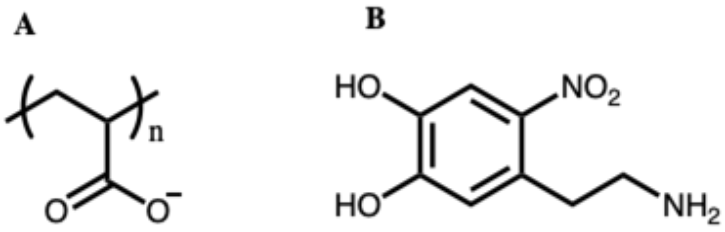


Figure 7

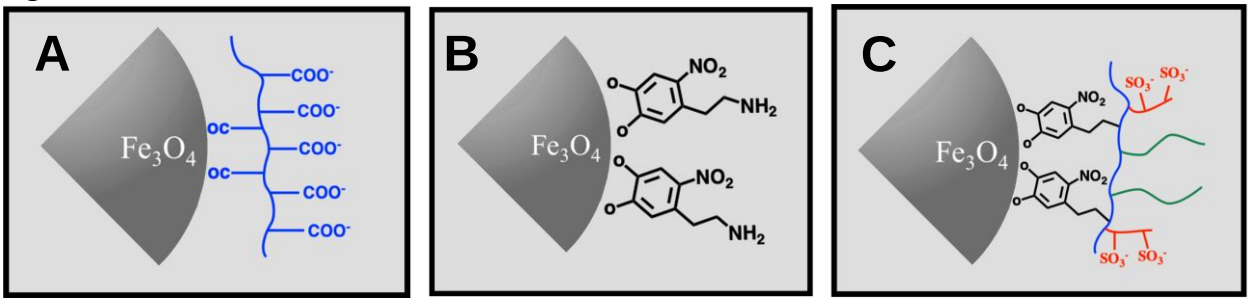
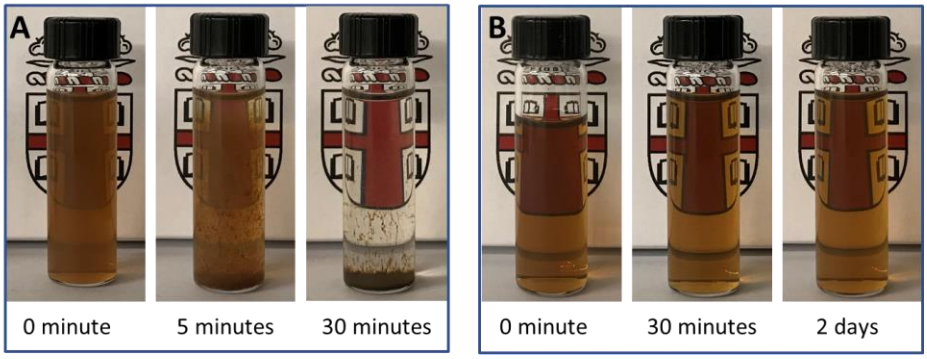


Figure 8



Table

Label in Figure 2	H_2O (mL)	$FeCl_3$ (mmol)	$MnCl_2$ (mmol)
A	1.5	1.3	0.7
B & G	0.7	1.3	0.7
C	0.5	1.3	0.7
D	0.1	1.3	0.7
E	1.3	2	0
F	0.6	1.5	0.5
H	2	1	1

Ferrite Composition	Dc (nm)
$Mn_{0.15}Fe_{2.85}O_4$	34
$Mn_{0.15}Fe_{2.85}O_4$	56
$Mn_{0.15}Fe_{2.85}O_4$	74
$Mn_{0.15}Fe_{2.85}O_4$	120
Fe_3O_4	56
$Mn_{0.06}Fe_{2.94}O_4$	56
$Mn_{0.6}Fe_{2.4}O_4$	55

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Table of Materials

Materials.xlsx

We are deeply appreciative of the positive feedback from the reviewers and the editor. Our revision is greatly improved because of these contributions as is the clarity of our video presentation. We have revised our manuscript and the video accordingly and addressed all the comments from the editor and the reviewers (in blue). Below we describe our responses and further note how then manuscript changed as a result. Thank you again for considering our paper for publication in your journal and offering us the opportunity to improve it.

Editorial and production comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We appreciate the note and have thoroughly checked our spelling and grammar in our manuscript. As a result much of the text has changed as we have carefully considered the presentation though our basic findings and paragraph organization remains the same.

2. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

We thank the reviewer for reminding us of the necessary formatting. The numbering in the protocol section of the manuscript was changed as a result. Instead of the previous A, B, C... lettering scheme, the current scheme corresponds with that of our video, and is now 1 followed by 1.1 and then 1.1.1 and 1.1.2 and so on.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

We thank the reviewer for reminding us of this guidance. Edits were made to sections 1.3, 2.5, 3.1, 3.2 to use the imperative tense. Phrase such as “it should be measured…” were replaced with “it must be measured…” to coincide with the necessary tense of the protocol.

4. Please ensure you answer the “how” question, i.e., how is the step performed?

We appreciate the reminder to elaborate on steps that we may have not considered and need further explanation. To ensure clarity, details were added in sections 1.1, 1.9, 1.11, and 3.6 to answer “how” to perform the steps. Extra steps were added to clarify how to wash the glassware, seal the reactor, remove the reactor from the oven, wash the clusters, and apply the sonicator. We believe these changes will provide further detail for users and eliminate any confusion.

5. C3: How do you perform the washing steps?

We appreciate the reminder to elaborate on the washing steps. The washing process involves placing the vial on a magnet and decanting the supernatant, followed by filling the vial with water. In this way, non-magnetic impurities, including the original solvent, excessive polymers, and other reagents, will be disposed and only the manganese ferrite clusters will be retained. We have changed our protocols accordingly to clarify this and avoid confusion.

6. Please ensure the results are described in the context of the presented technique e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

We thank the reviewer for this reminder and explanation of the requirements of the results section. We provide guidance on how to identify correct procedures by describing how the solution will look after the hydrothermal reaction and then again after purification. We then provide examples of improperly synthesized particles along with TEM images, which demonstrates how to identify issues with the process. Here we have added figures from our video that previously did not match with the manuscript, and ensured they were referenced in the paragraph text.

7. As we are a methods journal, please ensure that the Discussion 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.

We thank the reviewer for reminding us that these topics need to be covered in our discussion section and not just the manuscript in general. We added modifications in the technique, for example we have clarified the amount of steel wool needed for magnetic separation. We also noted possible issues, such as relative humidity variations affecting the cluster diameter of the product, and suggested ways to troubleshoot. Finally, we expanded upon future applications of the technique to fields in which our particles will be useful, such as drug delivery, water remediation, and advanced imaging systems. The discussion section should now explicitly and comprehensively cover the necessary topics.

8. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.

We thank the reviewer for pointing out the expectations for reference formatting. All the references have been edited to put the year at the end and in parentheses to reflect the JoVE citation scheme.

9. Please include all the Figure/Table Legends together at the end of the Representative Results in the manuscript text.

We thank the reviewer for reminding us of the format. We have attached the figure/table legends at the end of the Results in the manuscript text.

10. Please upload each figure individually (with all panels combined) to your editorial manager account as.pdf file. Please do not include the legends here.

We thank the reviewer for reminding us of the format. We have uploaded each figure individually as a pdf file. The legends have been excluded.

11. Please upload each table individually as .xlsx file.

We thank the reviewer for reminding us of the format. We have uploaded each table individually as a xlsx file.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.

We appreciate the reminder to make our manuscript and video more consistent. We have updated our video according to the revised manuscript. Figures shown in the video that were previously not present in the manuscript have been added to our figure list. These include Figures A, B and C.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol

We appreciate the reminder to revise the narration of our video. We have updated our video narration and made it consistent to the written protocol.

3. Please ensure that the title of the submission is same in both the text manuscript and the video.

We appreciate the reminder of the title in the manuscript and the video. We have changed them, and they are now the same.

4. Please ensure that the protocol section subheadings are the same in the text and the video.

We appreciate the reminder of the subheadings in the manuscript and the video. We have changed them, and they are now the same.

5. Please place and discuss all the figures in the representative result section after the protocol section.

We appreciate the reminder to further polish our video. We have updated our video and added discussions on the representative result after the protocol section.

6. Format:

• As per JoVE criteria, "Each section or chapter should have its own chapter title card, clearly separating the sections of the video". Please consider adding chapter title card in the beginning of each section which separates the title with video (Use slides for title cards).

We appreciate the reminder to add chapter title card in the video. We have updated our video and added title card in the beginning of each section and separated the different sections.

7. Results and Conclusion Card seems to be missed, please consider adding Results and Conclusion card.

Thank you and we have added the Results and Conclusion card in the video

8. Many Chapter Cards are visible on the screen for very short duration, please consider increasing the duration of the cards to at least for 0:05 seconds.

Thank you and we increased the duration of the cards for 5 seconds in the video.

9. Video Comments:

• As per the criteria, JoVE videos avoid jump cuts (Sudden change of footage/image) whenever possible, as they can be jarring and confusing to viewers. Consider changing these transitions to cross dissolves. Mentioning some time stamps for reference:

• 0:12

• 0:31

• 1:00

• 1:12

• 1:46

• All the on screen text comes in and goes out suddenly which can be jarring and distracting for the viewers, please consider putting cross dissolve to the text in the beginning and in the end.

We appreciate the reminder to further polish our video. We have changed all the jump cuts in the video and put cross dissolve to the text at the beginning and in the end of the video. The time stamps listed by the editor have been mentioned in our manuscript.

10. • Please consider adding a dark and less transparent background to on screen texts for better visibility of viewers.

• 4:01 - 4:09 The picture looks blurry, please consider replacing the image with high quality image.

Thank you and we have added a dark and less transparent background to on screen texts in our video. Picture between 4:01-4:09 has been replaced with a high quality image.

11. Audio Comments:

• Audio seems to be high in some places, consider keeping the volume level for whole video between -12 to -6 dB

Thank you and we have changed our video and made sure the volume level is consistent.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors present a hydrothermal method to produce spherical assembly of magnetic nanoparticles.

Major Concerns:

I recommend the authors to refrain from using the term "nanocluster" to describe the aggregated nanoparticles. These clustered nanoparticles have been described for example as "spherical assembly of nanoparticles" in J. Am. Chem. Soc. 2002, 124, 4958-4959. "Nanocluster" often refers to sub-nanometer scale particles, e.g. in Chem. Soc. Rev. 2012, 41, 3594-3623.

We thank the reviewer for bringing this important terminology to our attention. The term “nanocluster” was replaced with “manganese ferrite clusters,” abbreviated to MFC, throughout the paper. We have also added explanation in both the manuscript and the video that these MFCs are the spherical assembly of primary manganese ferrite nanocrystals that form hard aggregates. However, as our project has always used the term “cluster” to indicate the special structures of our materials, and has been well recognized by our collaborators, we now refer them only as “clusters” throughout the video.

Minor Concerns:

It would be useful if the authors could comment on critical or difficult steps (if any) in the synthesis and/or surface modification.

We thank the reviewer for pointing out the need to include this in our manuscript. We have fixed this issue by making comments in our discussion section on the difficulty of the probe sonication during the surface modification protocol. We highlighted the importance of ensuring the sample is fully sonicated without excess vibration of the vial that can cause loss of product. We have also point out that excess heat can cause degradation of the polymer coating so the ice bath should be monitored closely.

Reviewer #2:

Manuscript might be accepted as it is.

We appreciate the reviewer's time and positive feedback on our paper.

Video Produced by Author: Less than 50 MB. If your video is greater than 50 MB, click "offline" as the delivery method and our

This piece of the submission is being sent via mail.