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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 both primary nanocrystal and cluster dimension as well as the iron to manganese ratio. Magnetic separation allows for rapid sample purification while surface functionalization using a sulfonated polymer ensures that the materials are non-aggregating even in biologically relevant aqueous solutions. The resulting products are well positioned for applications in biotechnology and medicine.

**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 well as 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 copolymers 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 both 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 multicore nanoparticles or alternatively nanoflowers, 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 isolated nanocrystals are well developed, clusters of assembled nanocrystals of the type of interest in this work are not easily produced in a uniform and controlled fashion. [15, 16] Additionally, the 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 precipitate thereby limiting their routes of exposure to animals or people. [21, 22 20, 21] 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 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 until completely dry.

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

1.2 Transfer 20 mL ethylene glycol into a 50 mL beaker with a magnetic stir bar..

1.3 Add 1.3 mM of iron(III) chloride ($FeCl_3 \cdot 6H_2O$, solid) to the beaker and place on a stir plate 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 Add 250 mg polyacrylic acid (PAA, Mw ~6000, powder) to the beaker. The red?orange?brown? solution becomes opaque and slightly lighter in color.

1.5 Add 1.2 g urea [$CO(NH_2)_2$, powder] to the beaker

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

1.7 Add 0.9 mL milli-Q water to the beaker.

1.8 Allow the beaker to stir for 30 minutes. It will present as a translucent, dark orange color.

1.9 Transfer the reaction mixture into a Teflon-lined steel reactor of xxx volume.

1.9.1 First, use a handheld magnet 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 Pour the solution 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. 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 hours at 215 °C.

1.11 Remove the reactor from the oven. Let the reactor cool to room temperature. The pressure of the oven will enable the reactor to be opened by hand. At this point, the reactor will contain the MFC product dispersed in ethylene glycol with other impurities, such as unreacted polymer. 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 the glass vial. Fill the glass vial halfway with the reaction mixture. Fill the rest of the vial with acetone.

2.2 Shake well and place on a cubic permanent rare earth magnet for precipitation to occur. 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.

2.3 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 Place the vial on top of the magnet and wait for precipitation to occur. Then pour off the clear supernatant solution.

2.5 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 xx amount of ethylene glycol, approximately 80 mg MFC product will be obtained.

3. Surface functionalization of MFCs to ensure good colloidal stability

3.1 Poly(AA-co-AMPS-co-PEG) copolymer is made through free radical polymerization. Add 0.20 g 2,2'-Azobis(2-methylpropionitrile) (AIBN), 0.25 g acrylic acid (AA), 0.75 g 2-Acrylamido-2-methylpropane sulfonic acid (AMPS) and 1.00 g Poly(ethylene glycol) methyl ether acrylate (PEG) in 10 mL N,N-Dimethylformamide (DMF). Heat the mixture in a 70 °C water bath for 1 hour and transfer it to a dialysis bag in water. The weight ratio of AA, AMPS, and PEG is 1:3:4. Note how do you get to the concentration of the polymer here? Do you ever weigh it out as a solid or do you calculate it from this?

3.2 Combine 10 mL of purified nanoparticles (around 100 mg) in a 20 ml vial with 10 mL saturated N-[2-(3,4-dihydroxyphenyl)ethyl]nitramide (nitro-dopamine) solution (~1 mg/mL). Wait for 5 min. Note: The synthesis of nitro-dopamine can be found in our previous work.[16]

3.3 Wash the nitro-dopamine coated MFCs using magnetic separation. To accomplish this, place the vial on the magnet to precipitate and 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 precipitate in the vial.

3.4 Prepare a 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) aqueous solution with a concentration of 20 mg/mL, a 2-morpholin-4-ylethanesulfonic acid (MES) buffer solution with a concentration of 100 mg/mL, and a poly(AA-co-AMPS-co-PEG) copolymer solution with a concentration of 20 mg/mL. See yellow above? How do you know the polymer concentration?

3.5 Mix 1 mL EDC solution, 1 mL MES buffer, and 3 mL of the polymer solution described above and let this mixture sit for approximately min. It should be a clear and colorless solution when fully combined.

3.6 Add this mixture to the MFC precipitate 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)

3.6.1 After a five minute sonication treatment, add roughly 5 mL of milli-Q water to the vial while the sonicator is still in operation. 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.

3.6.2 Allow the mixture to sonicate for an additional 25 minutes, for a total of 30 minutes.

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

4. Removal of aggregated clusters.

4.1 Wash the modified MFCs with deionized water several times. First fill the vial with deionized water and place on top of a magnet to collect the product. Pour out the supernatant solution while holding the magnet to the bottom of the vial. Repeat this several times.

4.2 Fill the vial with the MFCs with milli-Q water. Pipette this fluid into a vacuum filtration system with a 0.1 µm filter to remove any irreversibly aggregated MFCs.

4.2.1 Make sure to flush the walls of the funnel to minimize any loss of product.

4.3 Run the vacuum to filter through the solution. Repeat this process 2-3 times. 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 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), collecting into a large 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, while the composition is only determined by the precursor ratio. 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 execution of the method can lead to failed products. **Figure 3** depicts samples with irregular MFC morphologies. In Figure 3A, odd-shaped MFC 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 shape.[16] The samples shown in Figure 3B had insufficient reaction time (xxxx min) 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 consistently successful results.

Upon the 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 be then decanted to yield pure and monodispersed MFCs.[27] **Figure 4** displays the time frame it took for nearly complete precipitation of the MFCs to occur with and without the steel wool inside. The steel wool placed in the vial during magnetic separation increases the magnetic field strength 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 ultracentrifuge (C and D). With a similar average cluster diameter, magnetic separation, however, can achieve a significantly lower spread in size than that of ultracentrifuge separation. The result indicates magnetic separation is more beneficial for the purification of the as-synthesized MFCs.

The as-synthesized MFCs with a surface coating of PAA are only stable in pure water due to their reliance on charge repulsion to prevent interparticle aggregation, as seen in **Figure 6A**.. However, utilizing a ligand replacement reaction with nitrodopamine (depicted in **Figure 6B)**, replaces the PAA coating with a copolymer coating of P(AA-co-AMPS-co-PEG) and allows for greater stability in a wide range of solution conditions. **Figure 7** depicts this surface medication progression. In **Figure 8,** we demonstrate the colloidal stability of the MFCs dissolved in PBS buffer. For the as-synthesized MFCs coated with PAA, the MFCs rapidly aggregated to the extent that they were visible to the eyes and precipitated out of the solution completely in 30 minutes, rendering them of little use in biological applications. The functionalized MFCs with polysulfonate coating remained well dispersed in this solution for over two days without any sign of aggregation. The post-synthesis surface modification could allow for stability in biological media over a drastically longer timeframe.

**DISCUSSION (VC stopped here):**

This work demonstrates a modified polyol synthesis of manganese ferrite nanocrystals clustered together into uniform nanoscale aggregates.[CITE] 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 then 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 chemical reactions and assembly process can complete yielding product cluster that of a uniform spherical diameter and comprised of 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 response even to small external magnetic fields.[27] As a result, these materials have great potential for applications in drug delivery, magnetic hyperthermia, magnetic resonance imaging, and magnetic particle imaging.

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 0.3 to 2 mL which gives clusters of sizes from <<large diameter>> to <<small diameter>>. Water has an important role in that it hydrolyzes the metal precursors, and the more this process is favored with 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 and affect the subsequent dimensions and morphology of the produce. While the humidity control in most research laboratories (e.g., 30 – 60% RH) is sufficient to minimize this possibility, it may be necessary to reduce the amount of added water to account for this possibility. 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] Nevertheless, among the conditions of our synthetic method, we found the product composition is well predict 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. In this synthesis, ultracentrifugation produces relatively polydisperse nanomaterials of many different dimensions. To overcome this problem, we take advantage of the magnetic response of these materials to improve the uniformity and purity of the final product. The magnetic separation is affected by creating very high gradients of magnetic fields within macroscopic vial using steel wool and a rare-earth permanent magnet. This permits uniform samples to be recovered in under 30 minutes with high yields (> 90%). Depending on the dimensions of the final product, we vary the amount of steel wool applied to the solution. For example, a MFC with an average diameter of 40 nm requires between 100 to 200mg of steel wool, while larger materials may require much less or even no steel wool less to no steel wool. It is well established that smaller diameters 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 materials with greater uniformity.

Although the as-synthesized MFCs form stable suspensions in pure water, they exhibit poor colloidal stability under 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 and the electrostatic repulsion that maintains the homogeneous MFC dispersion is removed; alternatively in higher ionic strength the charge repulsion is shielded which has a similar effect.

Aggregation of the MFCs creates macroscopic materials that are not homogeneously dispersed in solution making it virtually impossible to introduce the materials into biological samples or exploit their high surface areas. 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. 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 the samples are effectively coated. For example, the method requires constant monitoring of the reaction mixture while it is treated with a probe sonicator to ensure a homogeneous and complete replacement of the initial polyacrylate coating. It is also important to use appropriate glassware to minimize any product loss during vigorous sonication and use an ice bath to minimize degradation of the polymers as probe sonication does evolve heat.

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 materials must be non-aggregating in solution in order to be applied in diverse applications including targeted drug delivery, water remediation, and advanced imaging systems.

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**DISCLOSURES:**
The authors have nothing to disclose.

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