Video Article

Cell Labeling and Targeting with Superparamagnetic Iron Oxide Nanoparticles

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Abstract

Targeted delivery of cells and therapeutic agents would benefit a wide range of biomedical applications by concentrating the therapeutic effect at the target site while minimizing deleterious effects to off-target sites. Magnetic cell targeting is an efficient, safe, and straightforward delivery technique. Superparamagnetic iron oxide nanoparticles (SPION) are biodegradable, biocompatible, and can be endocytosed into cells to render them responsive to magnetic fields. The synthesis process involves creating magnetite (Fe_3O_4) nanoparticles followed by high-speed emulsification to form a poly(lactic-co-glycolic acid) (PLGA) coating. The PLGA-magnetite SPIONs are approximately 120 nm in diameter including the approximately 10 nm diameter magnetite core. When placed in culture medium, SPIONs are naturally endocytosed by cells and stored as small clusters within cytoplasmic endosomes. These particles impart sufficient magnetic mass to the cells to allow for targeting within magnetic fields. Numerous cell sorting and targeting applications are enabled by rendering various cell types responsive to magnetic fields. SPIONs have a variety of other biomedical applications as well including use as a medical imaging contrast agent, targeted drug or gene delivery, diagnostic assays, and generation of local hyperthermia for tumor therapy or tissue soldering.

Video Link

The video component of this article can be found at https://www.jove.com/video/53099/

Introduction

Targeted delivery and capture of cells to specific sites within the body is desirable for a variety of biomedical applications. Delivery of neural stem cells to the brain by MRI-guided focused ultrasound has been proposed as a possible treatment option for neurodegenerative disease, traumatic brain injury, and stroke[1]. Mesenchymal stem cells are being studied for their ability to deliver anti-cancer drugs to tumors due to their natural tumor-tropic properties[2,3]. Cardiac stem cells have been delivered to the heart as a possible treatment for myocardial infarction[4,5]. Vascular stents have been developed with CD34 antibodies to capture circulating progenitor cells[6]. While promising, these cell targeting approaches present drawbacks including lack of cell specificity, inconsistent cell retention, and off-target cell delivery.

The overall goal of the current method is to enable magnetically directed targeting of cells for a variety of cell delivery and sorting applications. Magnetic targeting allows for controlled delivery of specific cells to a specific target site with minimal off-target effects[7]. The magnetic fields can be generated by implanted or external devices to safely direct the movement of magnetically-labeled cells within the body[8]. Numerous research efforts have focused on magnetically directed targeting of stem cells to injured tissues such as the heart[9-14], retina[15], lung[16], skin[17], spinal cord[18,19], bone[20], liver[21], and muscle[22,23] in order to improve regeneration outcomes.

Magnetic targeting of cells has also been studied extensively as a means to endothelialize implantable cardiovascular devices. A uniform and complete endothelium provides a barrier between the device and circulating blood elements to mitigate thrombosis and inflammation. Endothelial cells can be delivered to the device either prior to implantation or via the vascular system following implantation. In both cases, magnetic fields are used to capture cells to the surface of the device and retain the cells when subjected to the shear stress generated by circulating blood. Magnetic vascular stents[24-27] and vascular grafts[28] have both been fabricated and tested for this purpose.

Magnetic cell targeting requires a strategy for labeling cells with magnetic carrier particles. These particles can be bound to the surface of cells via antibodies or ligand/receptor pairs or they can be endocytosed into the cells. Superparamagnetic iron oxide nanoparticles (SPION) are biodegradable, biocompatible, and readily endocytosed by a variety of cell types[29]. These particles effectively render a cell responsive to magnetic fields and are naturally degraded over time. SPIONs provide a straightforward and safe means of magnetically labeling cells in culture

for a variety of magnetic targeting and sorting applications. A method for synthesizing SPIONs with a magnetite (Fe_3O_4) core and poly(lactic-co-glycolic acid) (PLGA) shell is provided. In addition, a method for labeling cells in culture with SPIONs is provided.

Protocol

1. Synthesis of Magnetite Gel

1. Wash all glassware by using concentrated hydrochloric acid followed by deionized water followed by ethyl alcohol. Allow to dry O/N, preferably in a drying oven.
   CAUTION! hydrochloric acid is harmful – wear personal protective equipment and work in a fume hood; ethyl alcohol is harmful – wear personal protective equipment.
2. Use a Dreschel bottle to de-gas 500 ml of deionized H_2O by gently bubbling N_2 gas for 30 mins.
3. Set-up the magnetite synthesis apparatus within a chemical fume hood.
   1. Place a 500 ml three-neck round-bottom flask within an isomantle heater and secure the center neck using a clamp and stand.
   2. Install a rubber septum into one of the round-bottom flask's side necks and a reflux condenser with a rubber septum into the remaining side neck. Continuously run cold water through the reflux condenser.
   3. Puncture the round-bottom flask's rubber septum with a needle connected to an N_2 gas line and puncture the reflux condenser's rubber septum with a needle connected to a gas line running to a bubbler (*i.e.*, flask with water) to visualize gas outflow.
   4. Install a blade paddle into the round-bottom flask's center neck via a paddle adapter. Attach the blade paddle's shaft to an overhead stirrer mounted onto a stand.
4. Purge the round-bottom flask with N_2 gas and leave N_2 gas flowing at a low but detectable rate.
5. Remove the reflux condenser from the round-bottom flask and add 1.000 g of iron(III) chloride, 0.6125 g of iron(II) chloride tetrahydrate, and 50 ml of de-gassed H_2O.
   CAUTION! iron(III) chloride and iron(II) chloride tetrahydrate are harmful – wear personal protective equipment.
6. Replace the reflux condenser and stir at 1,000 rpm while heating to 50 °C. Stirring under these conditions produces 10 nm diameter magnetite nanoparticles.
7. Once at 50 °C, add 10 ml of 28% ammonium hydroxide solution by injecting through the rubber septum in the round-bottom flask while still stirring.
   CAUTION! ammonium hydroxide is harmful – wear personal protective equipment.
   NOTE: The ammonium hydroxide solution is used to precipitate the magnetite and the solution should turn black.
8. Remove the rubber septum and N_2 gas line from the round-bottom flask and heat to 90 °C to boil off the ammonia gas while still stirring.
   NOTE: It is optional to maintain the flow of N_2 into the round-bottom flask by puncturing the reflux condenser's rubber septum, however, oxidation of magnetite to maghemite is negligible during this step.
9. Once at 90 °C, add 1 ml of oleic acid to the round-bottom flask while still stirring. The oleic acid is used to coat the magnetite nanoparticles to form magnetite gel.
   CAUTION! oleic acid is harmful – wear personal protective equipment.
10. Replace the rubber septum and N_2 gas line onto the round-bottom flask and remove the reflux condenser.
11. Turn off heat and stir at 500 rpm for 2 hr.
12. Remove the round-bottom flask from the isomantle heater and decant any remaining liquid while using a strong magnet held against the bottom of the flask to retain the magnetite gel.
    CAUTION! handle the strong magnet with extreme care to avoid damage or injury.
13. Allow magnetite gel to air-dry O/N (optional).

2. Purification of Magnetite Gel

1. Add 40 ml of hexane into the round-bottom flask to dissolve the magnetite gel
   CAUTION! hexane is harmful – wear personal protective equipment and work in a fume hood.
2. Use a separatory funnel with 40 ml of de-gassed H_2O to remove residual H_2O from the magnetite solution.
   1. Slowly pour the magnetite solution onto the H_2O within the separatory funnel and gently swirl the two-phase liquid for 5 mins.
   2. Drain out and discard the lower aqueous fraction.
   3. Slowly add 40 ml of de-gassed H_2O to the separatory funnel such that it settles beneath the magnetite solution and gently swirl and drain as before.
   4. Repeat to wash for a third time.
3. Transfer magnetite solution to an Erlenmeyer flask, add a few spatulas worth of anhydrous sodium sulfate, and swirl to remove any remaining residual H_2O from the magnetite solution.
4. Filter the magnetite solution through 1 µm filter paper in a filter funnel to remove the sodium sulfate and residual H_2O.
   NOTE: Vacuum assistance is recommended.
5. Transfer the magnetite solution to a 50 ml evaporating flask and use a rotary evaporator to evaporate the hexane for 2 h with the following conditions: moderate rotation speed, vacuum applied, evaporating flask in a 50 °C water bath, and 24 °C water circulating through the condenser.
   NOTE: Optionally, store the magnetite gel prior to coating with PLGA.

3. Coating of Magnetite Nanoparticles with PLGA Shell

1. Dissolve 3.60 g of PLGA (75/25 blend) in 240 ml of ethyl acetate to create a 1.5% (m/v) solution. CAUTION: ethyl acetate is harmful – wear personal protective equipment and work in a fume hood.
2. Dissolve 25.00 g of Pluronic F-127 in 500 ml of de-gassed H_2O using a magnetic stirrer to create a 5.0% (m/v) solution.
   NOTE: Pluronic F-127 is a non-ionic amphiphilic block copolymer that acts as a biocompatible surfactant. It helps to stabilize the oil-in-water emulsion in step 3.3.2.
3. Using a microspatula, collect the magnetite gel into six 0.040 g aliquots within weighted glass vials. Perform the following coating and washing process for each aliquot.
   NOTE: The aliquots are necessary to ensure efficient handling and magnetic decantation, which will maximize purity and yield while minimizing degradation prior to freeze-drying in step 4.
   1. Add a 0.040 g aliquot of magnetite gel and 40 ml of the PLGA solution to a plastic beaker and sonicate in an ultrasonic cleaner for 10 mins.
   2. Add 80 ml of the Pluronic solution to the plastic beaker and immediately emulsify with a laboratory mixer at the highest setting for 7 mins to form the PLGA coating on the magnetite nanoparticles as an oil-in-water emulsion.
   3. Immediately dilute the SPION solution in 1 L of deionized H_2O and sonicate for 1 h in a chemical fume hood to evaporate the ethyl acetate.
   4. Place a strong magnet next to the SPION solution and gently stir to collect brownish SPIONs at the magnet.
      NOTE: It may be necessary to intermittently stir for several hours before the solution turns whitish indicating that most of the SPIONs have been collected.
   5. Decant the aqueous solution while retaining the SPIONs in the beaker with the magnet.
   6. Wash the SPIONs three times as follows.
      1. Suspend the SPIONs in 1 L of deionized H_2O.
      2. Sonicate the SPION solution for 20 mins.
      3. Place a strong magnet next to the SPION solution and gently stir to collect brownish SPIONs at the magnet. It may be necessary to intermittently stir for several hours before the solution turns clear indicating that most of the SPIONs have been collected.
      4. Decant the aqueous solution while retaining the SPIONs in the beaker with the magnet.
4. Collect the SPIONs synthesized from each of the six magnetite gel aliquots into a single weighted glass vial as an aqueous suspension. Optionally decant excess water magnetically as needed.

4. Freeze-drying of SPIONs

1. Freeze the SPION solution.
2. Freeze-dry the SPION solution O/N in a lyophilizer.
3. Weigh the freeze-dried SPIONs. Freeze-dried SPIONs can be stored at -20 °C until used for cell labeling.
   NOTE: Storage at -20 °C dramatically reduces degradation kinetics and increases shelf life.

5. Labeling of Cells with SPIONs

1. Suspend an aliquot of SPIONs in phosphate-buffered saline (PBS) at a concentration of 40 mg/ml and sonicate for 30 mins.
2. Add the SPION solution to a nearly confluent flask of cells at a concentration of 5 µl/ml of cell culture medium. Ensure even distribution by gently rocking the flask.
3. Incubate the cells for 16 hr at 37 °C.
4. Gently aspirate culture medium and wash cells twice with PBS.
5. Collect magnetically-labeled cells and use for experiments.
6. Unused SPION solution can be stored at 4 °C and should be used within a few months. Sonicate for 30 mins before each use.

Representative Results

Magnetite nanoparticles are approximately 10 nm in diameter as a result of stirring an aqueous solution of iron(III) chloride and iron(II) chloride tetrahydrate at 50 °C and 1,000 rpm **(Figure 1)**. These results demonstrate successful synthesis of magnetite nanoparticles. It is important to verify the size and shape of magnetite nanoparticles taken from a small sample of the batch when attempting the synthesis for the first time. Transmission electron microscopy (TEM) is the preferred method for visualizing these particles. The batch should be discarded and the synthesis should be attempted again if the magnetite nanoparticles are not approximately 10 nm in diameter and spherical as shown in **Figure 1**.

Coating the magnetite nanoparticles with PLGA using a high-speed emulsifier results in PLGA-magnetite SPIONs with a diameter of 120 nm **(Figure 2)**. These results demonstrate successful synthesis of PLGA-magnetite SPIONs. It is important to verify the size and shape of PLGA-magnetite SPIONs taken from a small sample of the batch when attempting the synthesis for the first time. Scanning electron microscopy (SEM) is the preferred method for visualizing these particles. The batch should be discarded and the synthesis should be attempted again if the PLGA-magnetite SPIONs are not approximately 120 nm in diameter and spherical as shown in **Figure 2**. While larger or smaller particles may be desirable for certain applications, the composition will be unknown and therefore cell labeling, magnetic susceptibility, and cytotoxicity will be unpredictable.

Incubation of blood outgrowth endothelial cells with SPIONs for 16 hr results in endocytosis of the nanoparticles **(Figure 3)**. These results demonstrate successful labeling of cells with SPIONs. It is important to verify the presence of SPIONs within cells taken from a small sample of the batch when attempting the labeling for the first time. Transmission electron microscopy is the preferred method for visualizing these SPION-

labeled cells. The cells should be discarded and the labeling should be attempted again if the PLGA-mangetite SPIONs do not appear as round black particles clustered together within cytoplasmic endosomes as shown in **Figure 3**. Furthermore, lower concentrations of SPIONs may fail to enable magnetic cell targeting and higher concentrations of SPIONs may be cytotoxic. If necessary, the concentration of SPIONs used to label cells can be adjusted accordingly.

The quantity of iron loaded into the cells is sufficient to achieve magnetic capture of viable cells to ferromagnetic implantable medical devices **(Figure 4)**. These results demonstrate successful SPION-mediated magnetic cell targeting. If a stronger cell targeting effect is desired, the preferred strategy is to increase the strength or gradient of the generated or applied magnetic field[8,30]. Increasing the concentration of SPIONs used to label cells should only be tried as a last resort due to cytotoxicity concerns. If improved cell viability is desired, the concentration of SPIONs used to label cells should be decreased.

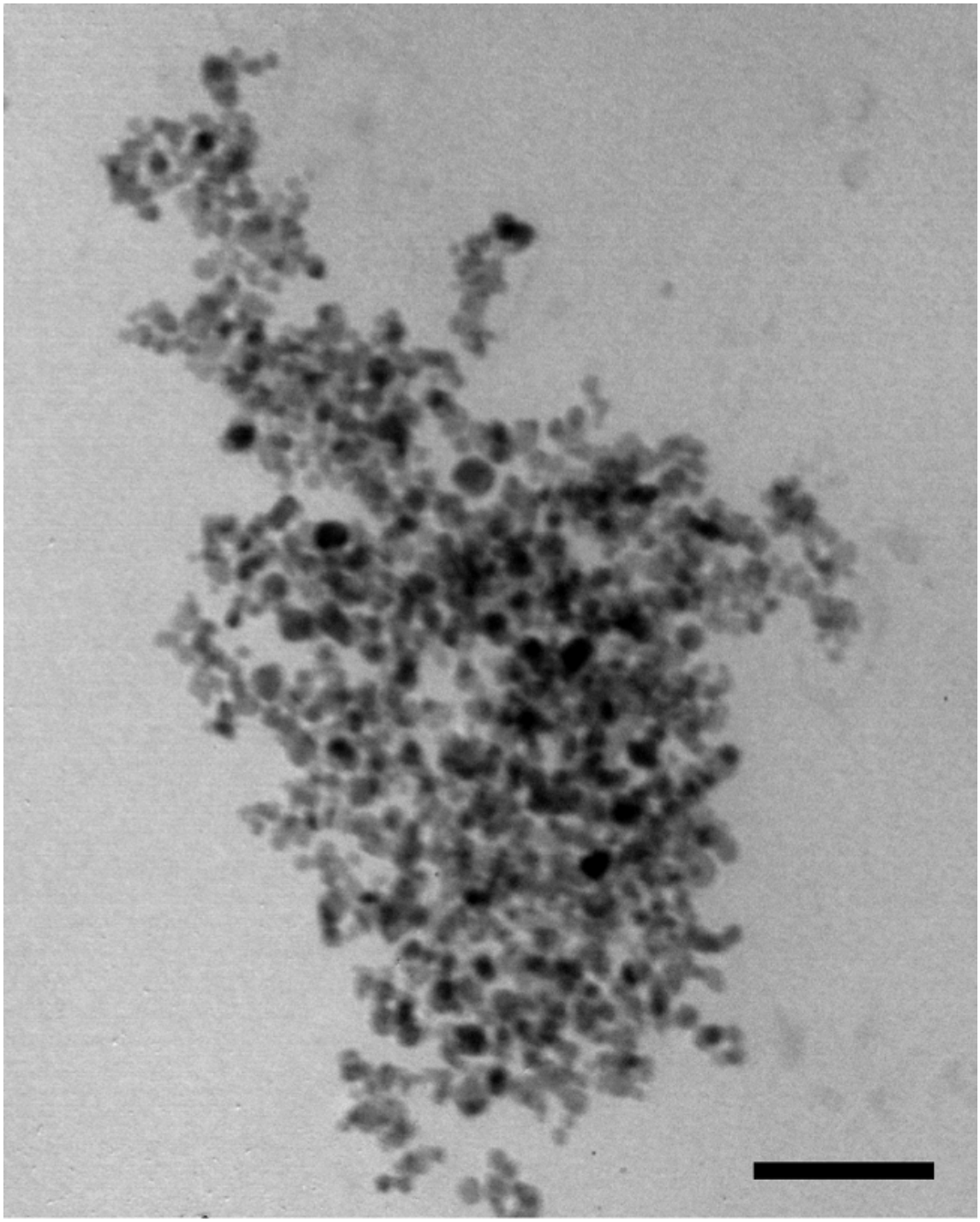

Figure 1. TEM image of magnetite nanoparticles. Magnetite nanoparticles are approximately 10 nm in diameter as seen by transmission electron microscopy (TEM). Particles are spherical and uniform in size. Scale bar = 100 nm. Please click here to view a larger version of this figure.

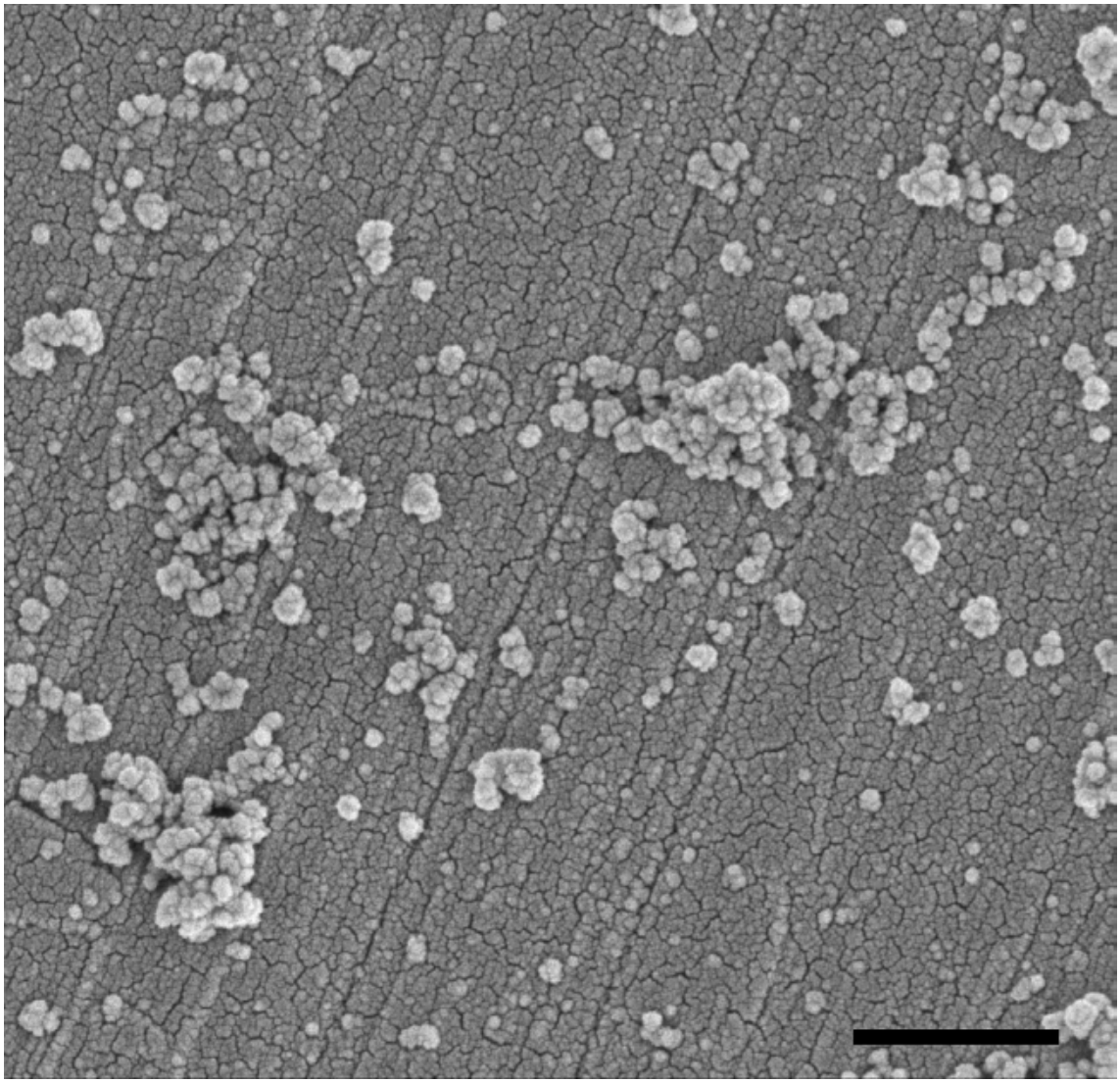

Figure 2. SEM image of PLGA-magnetite SPIONs. PLGA-coated magnetite SPIONs are approximately 120 nm in diameter as seen by scanning electron microscopy (SEM). Particles are spherical and uniform in size. Scale bar = 500 µm. Please click here to view a larger version of this figure.

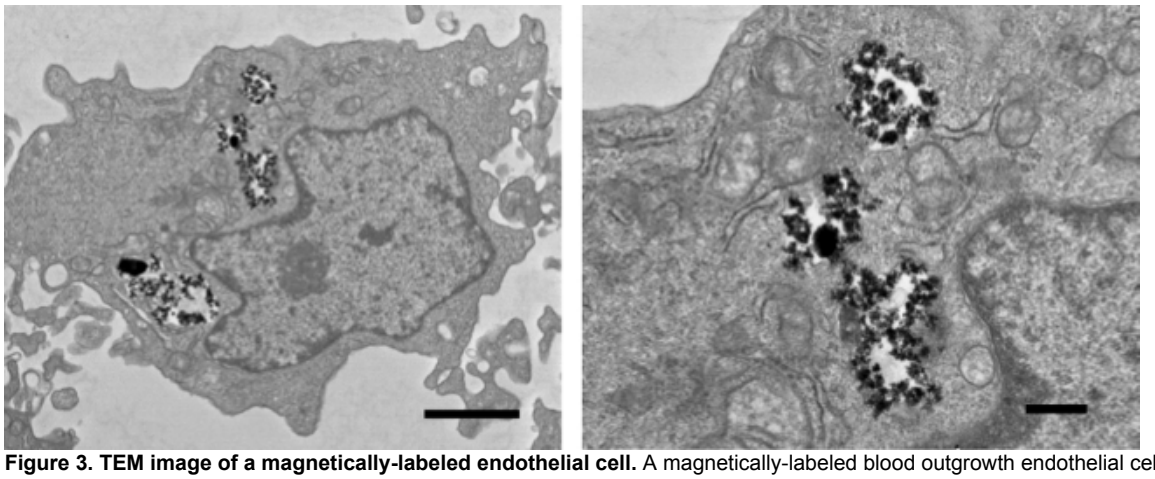

Figure 3. TEM image of a magnetically-labeled endothelial cell. A magnetically-labeled blood outgrowth endothelial cell visualized by transmission electron microscopy (TEM). The SPIONs are naturally endocytosed by the cells and stored in small clusters within cytoplasmic endosomes. Left scale bar = 2 µm and right scale bar = 0.5 µm. Re-print with permission from[24]. Please click here to view a larger version of this figure.

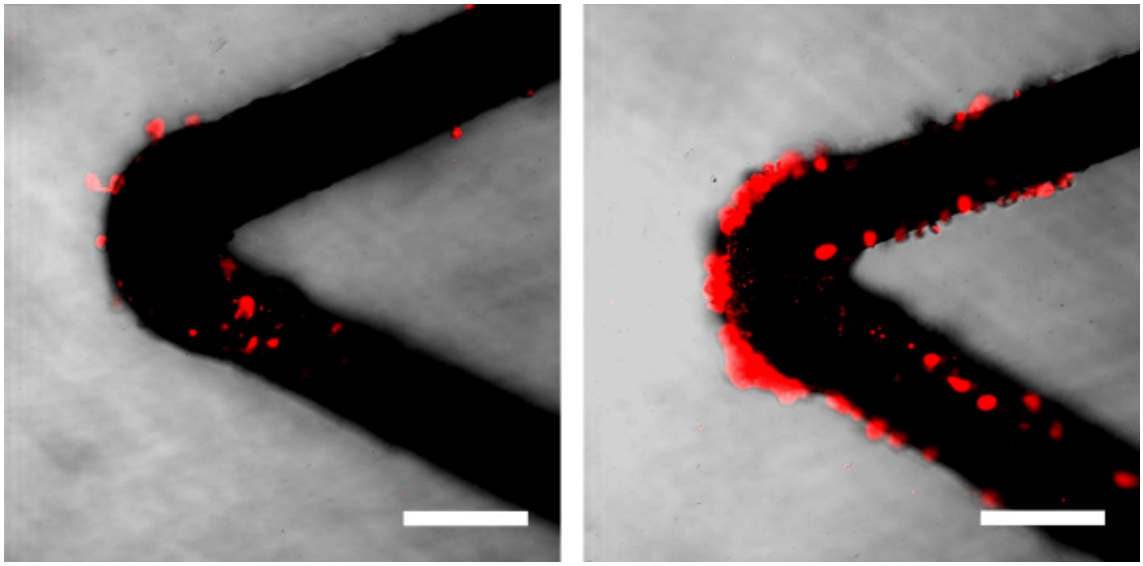

Figure 4. Fluorescence microscopy image of magnetic cell capture. Magnetically-labeled endothelial cells are attracted to a ferromagnetic vascular stent (right) at a significantly higher rate than a non-magnetic stent (left). Scale bars = 100 µm. Re-print with permission from[24]. Please click here to view a larger version of this figure.

Discussion

As with any nanoparticle synthesis protocol, the purity of the reactant chemicals is critical for achieving high quality SPIONs that will have minimal cytotoxic effects. It is therefore important to purchase very pure reagents including oleic acid (≥99%), iron(II) chloride tetrahydrate (≥99.99%), iron(III) chloride (≥99.99%), ethyl acetate (HPLC grade, ≥99.9%), hexane (HPLC grade, ≥97.0%), ammonium hydroxide (≥99.99%), and sodium sulfate (≥99.0%). It is of particular importance to purchase very pure and high quality PLGA, which can be relatively expensive. In addition, all glassware must be thoroughly washed with hydrochloric acid, deionized water, and ethyl alcohol and allowed to dry before use.

Similarly, the purification and washing steps within the protocol are critical to ensure the final SPIONs will be of high quality and have minimal cytotoxic effects. The magnetite gel must be free of as much ammonium hydroxide, water, and hexane as possible before coating with PLGA. Accordingly, much of the protocol is devoted to ensuring the purity of the magnetite gel. Subsequently, the PLGA-magnetite SPIONs must be free of ethyl acetate, Pluronic, and excess PLGA. The final SPION washing steps are the most time consuming portion of the protocol, but must be completed to ensure high purity. Specifically, magnetic collection of the particles during each washing step can be very time consuming. Stirring the solution can greatly increase the speed of particle collection, but magnetic stir bars cannot be used. Overhead stirrers operating at a low speed are the most effective means for rapid particle collection. Ensure a large brownish collection of SPIONs appears at the magnet and the solution appears white or clear before decanting. This can often require several hours of stirring, but will result in a higher final yield. The magnetic decantation steps also serve to ensure only magnetic particles are retained while all non-magnetic materials are discarded.

Excessive iron levels can be cytotoxic, so the amount of magnetic mass that can be imparted to a cell using this technique is limited. The concentration of iron may need to be decreased for particularly sensitive cell types or increased for particularly weak magnetic fields, but the protocol described here provides a proven starting point to balance safety and efficacy. The SPIONs synthesized by this protocol are made from a solution with a 1:15 ratio by mass of magnetite to PLGA and the SPIONs are introduced to cells at a concentration of 200 µg/ml of cell culture medium. Either of these parameters can be adjusted to alter the quantity of iron endocytosed by each cell as necessary.

SPIONs are safe for human implantation and will biodegrade over time (half-life of approximately 40-50 days)[31]. Both the magnetite and PLGA form harmless degradation products and are cleared from the body via natural pathways[32]. The biodegradable nature of the SPIONs means any cytotoxic effects will diminish with time, but also limits the potential applications to those that do not require cells to maintain their magnetic properties beyond a few months. SPIONs also have the advantage of labeling cells and imparting their magnetic effects without the need for surface proteins nor targeting ligands that are susceptible to the formation of a protein corona upon exposure to the biological milieu[33,34].

Imparting magnetic properties to cells is useful for a broad array of biomedical applications requiring targeted cell delivery or sorting[29]. A variety of cell types have demonstrated the ability to safely endocytose SPIONs including mesenchymal stem cells[35], endothelial progenitor cells[36], beta islet cells[37], and neural stem cells[38]. Magnetic cell targeting may be preferred over other cell targeting techniques when a high degree of control over the delivery conditions is necessary.

Disclosures

The authors declare that they have no competing financial interests.

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