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Polyaspartic Acid Coated Iron Oxide Nanoprobes for PET/MRI Imaging

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Abstract

Iron oxide nanoparticles, due to their exceptional magnetic property, biocompatibility, and biodegradability, have long been studied as contrast agents for magnetic resonance imaging (Xie et al., *Curr Med Chem* 16(10):1278–1294, 2009; Xie et al., *Adv Drug deliv Rev* 62(11):1064–1079, 2010). While previous applications mostly target reticuloendothelial system (RES) organs such as liver and lymph nodes, recent efforts have been made to impart targeting peptides or antibodies onto particle surface to enable site-specific targeting after systemic administration (Xie et al., *Adv Drug Deliv Rev* 62(11):1064–1079, 2010; Cai and Chen, *Small* 3(11):1840–1854, 2007; Corot et al., *Adv Drug Deliv Rev* 58 (14):1471–1504, 2006; Xie et al., *Acc Chem Res* 44(10):883–892). Moreover, other imaging functionalities can be loaded onto nanoparticles to achieve multimodality imaging probes (Cai and Chen, *Small* 3(11):1840–1854, 2007; Lee et al., *J Nucl Med Soc Nucl Med* 49(8):1371–1379, 2008). In this protocol, we describe the procedure of constructing an iron oxide nanoparticle (IONP)-based probe with high affinity towards integrin $\alpha_v\beta_3$ for positron emission tomography (PET) and magnetic resonance imaging (MRI) dual modality imaging. The related characterizations and validation experiments, including particle concentration determination, Prussian blue staining, animal model preparation, and in vivo PET/MRI imaging will also be discussed.

Keywords

IRON oxide nanoparticles; Integrin $\alpha_v\beta_3$; Multimodal imaging; Positron emission tomography; Magnetic resonance imaging; Tumor angiogenesis

1 Introduction

Nanomaterials, especially nanoparticles, have been extensively studied in the recent decades for their potentials in improving the current clinic diagnostic and therapeutic techniques [2, 3]. These materials usually have unique physical or physiochemical properties, and when conjugated with targeting molecules, become “smart” gadgets that can pinpoint and/or kill diseased tissues in a living subject [7]. One of the most studied nanomaterials in the field has been IONPs [4, 5]. With strong magnetic property, biocompatibility, and biodegradability, IONPs are candidate nanoplatforms to construct functional nanogadgets for both imaging and therapy purposes [2, 3]. One of the primary applications of IONPs is to work as contrast

agents for MRI. Several IONP formulations have been approved by the Federal Drug Administration (FDA) and used in the clinics to improve imaging quality [1].

While MRI provides high resolution and good anatomical details, its signal-to-background ratio, even with contrast agents, is suboptimal. In fact, none of the current imaging techniques are perfect and there are always trade-offs, for instance, between resolution and sensitivity. To address this issue, efforts have been made to develop multimodality imaging systems and imaging probes that allow simultaneous interrogation with two or even more imaging methods [6, 8–10]. Such an approach can greatly improve the diagnosis quality and lower misdiagnosis rate. For instance, positron emission tomography/computed tomography (PET/CT) systems have been developed and become an important diagnostic tool. PET/MRI is another promising technique under intensive studies and is expected to be implemented nationwide soon. Engineering IONPs and loading onto them radioisotopes is a common way to achieve PET/MRI dual functional imaging probes.

In the current protocol, we describe the detailed procedure for preparing tumor targeting IONPs with both PET and MRI imaging functionalities. IONPs are made from a high temperature thermal decomposition method. They participate in a ligand exchange with polyaspartic acid (PASP), a biocompatible and biodegradable polymer with high affinity toward IONP surface. Subsequently, a cyclic peptide, c(RGDyK), and a macrocyclic chelating agent, 1,4,7,10-tetraazacyclododecane- *N*, *N'*, *N''*, *N'''*-tetraacetic acid (DOTA), are both covalently conjugated onto the PASP coating. Here c(RGDyK) works as targeting a motif for its high affinity toward integrin $\alpha_v\beta_3$, a cell adhesion mediating receptor that is highly expressed on many types of tumor cancer cell surface, and more importantly, on tumor vasculature [10, 11]. This makes integrin $\alpha_v\beta_3$ a tumor biomarker. Imaging probes and drug delivery vehicles using RGD or its derivatives as tumor targeting motifs have been widely reported [12–15]. Due to the fact that multiple RGD can be loaded onto a single particle surface, RGD-nanoparticle conjugates usually show even higher affinity toward integrin $\alpha_v\beta_3$, the so-called multivalency effect [15]. DOTA is introduced onto IONP surface as a metal chelator. DOTA can form serum-stable complexes with many types of transition metals and is widely used in PET or single-photon emission computed tomography (SPECT) imaging to assist radioisotope labeling [16]. In the current protocol, we load ^{64}Cu , a radioisotope commonly used for PET imaging, onto IONPs.

2 Materials

2.1 Reagents

1. Iron chloride.
2. Sodium oleate.
3. Ethanol.
4. Hexane.
5. 1-octadecene.
6. DOTA.

7. 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.
8. *N*-hydroxysulfonosuccinimide (SNHS, Sigma).
9. Polyaspartic acid (PASP) (SPECTRUM).
10. O-[*N*-(3-Maleimidopropionyl)aminoethyl]-O'-[3-(*N*-succinimidyl)-3-oxopropyl]heptacosaneglycol (NHS-PEG-MAL, Sigma).
11. 4-Maleimidobutyric acid *N*-succinimidyl ester (Sigma).
12. PBS buffer.
13. RGD peptide (c(RGDyK); Peptides International).
14. *N*-Succinimidyl *S*-acetylthioacetate (SATA, Pierce Biotechnology Inc.).
15. DMSO.
16. Acetonitrile.
17. Trifluoroacetic acid.
18. Hydroxylamine hydrochloride.
19. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl; Pierce Biotechnology Inc.).
20. Sodium hydroxide.
21. Hydrochloric acid.
22. U87MG human glioblastoma cells.
23. MCF-7 human breast cancer cells.
24. Low-glucose DMEM (GIBCO).
25. Minimum essential medium (GIBCO).
26. Fetal bovine serum (FBS; GIBCO).
27. Cell-binding buffer (20 mM Tris, 150 mM NaCl, 2 mM CaCl₂, 1 mM MnCl₂, 1 mM MgCl₂, 0.1 % (wt/vol) bovine serum albumin; pH 7.4).
28. ¹²⁵I-Echistatin (GE Healthcare).
29. Isoflurane (RxElite Inc.).
30. U87MG tumor-bearing athymic nude mice (Harlan).

2.2 Equipment

1. HPLC (Dionex).
2. Analytical HPLC column (Vydac).
3. Semi-preparative HPLC column (Vydac).
4. Freeze-dry system (Labconco Corp.).

5. PD-10 column (GE Healthcare, GE Healthcare).
6. Centrifuge (Allegra X-15R, Beckman-Coulter).
7. Refrigerated microcentrifuge (Eppendorf).
8. Rotary vaporizer (Buchi, RII).
9. 96-well plate (Thermolab Systems).
10. Vacuum manifold (Millipore).
11. Vacuum pump (VWR).
12. Dry bath incubator (Fisher Scientific).
13. Polystyrene culture test tube (Fisher Scientific).
14. γ -counter (Packard).
15. GraphPad Prism (GraphPad Software Inc.).
16. 4-well chamber slides (Thermo-Fisher).
17. ICP-MS (VG Elemental).
18. Rodent anesthesia system (Summit Anesthesia Solutions).
19. 7 T small animal MRI system (Varian).
20. microPET R4 rodent scanner (Siemens Medical Solutions).

3 Methods

3.1 Preparation of Iron-Oleate Precursor

1. 10.8 g of iron chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 40 mmol) and 36.53 g of sodium oleate (120 mmol) are dissolved in a mixture solvent composed of 80 ml ethanol, 60 ml distilled water, and 140 ml hexane.
2. The resulting solution is heated up to 80 °C and kept at that temperature for 4 h.
3. The organic (top) layer is washed three times with 30 ml distilled water and collected by a separatory funnel.
4. Use a rotary evaporator to remove hexane and ethanol. The final product is in a waxy solid form.

3.2 Synthesis of IONPs

1. 9.0 g iron-oleate and 1.58 ml oleic acid is dissolved in 35 ml 1-octadecene with magnetic stirring (*see Note 1*).
2. The solution is heated stepwise at 150 °C for 45 min, 200 °C for 45 min, and finally to reflux at ~320 °C for 45 min (*see Note 2*).
3. Cool down the mixture to room temperature. Add 30 ml ethanol to precipitate IONPs. Collect IONPs by centrifuge at 8,000 rpm ($7,700 \times g$) (*see Note 3*).

4. Decant the supernatant and redissolve the nanoparticles in 15 ml hexane with sonication. Add 30 ml ethanol to precipitate the nanocrystals. Repeat the washing step for three times (*see Note 4*).

3.3 Preparation of PASP Coated IONPs

1. 20 mg IONPs are dissolved in 5 ml hexane and added onto 5 ml 25 mg/ml PASP solution in water to form a two phase mixture.
2. Sonicate the mixture for 5 min and then centrifuge. Wash the aqueous phase three times with hexane.
3. Collect the aqueous phase and run it through a PD-10 column for purification and solvent exchange. Briefly, load the solution onto a column and wait until all the solution is in. Add 3.5 ml borate buffer (50 mM, pH 8.5) and collect only the deepest-colored fractions at the bottom. The product is PASP-coated IONPs (PASP-IONPs).

3.4 Preparation of Thiolated RGD

1. Mix c(RGDyK) (5 mmol) in 1 ml of borate buffer (pH 8.5) with 100 ml DMSO solution containing 6 mmol of SATA. Incubate at room temperature with gentle shaking.
2. Monitor the reaction by analytical RP-HPLC (reverse phase-high performance liquid chromatography). The mobile phase is changed from 95 % solvent A (0.1 % TFA in water) and 5 % solvent B (0.1 % TFA in acetonitrile) (0–2 min) to 35 % solvent A and 65 % solvent B at 32 min. The flow rate is 1 ml/min and the UV absorbance is monitored at 218 nm.
3. When the reaction comes to completion, quench with 100 ml of 2 % TFA in water.
4. Freeze-dry the crude mixture. Redissolve the product in 1 ml water.
5. Add 100 ml of 0.5 M hydroxylamine solution. Adjust the pH to 6.0 with 0.5 M sodium hydroxide (*see Note 5*).
6. Use RP-HPLC to monitor the reaction. When the reaction comes to completion, purify the thiolated c(RGDyK) (RGD-SH) by semi-preparative RP-HPLC. The gradient is the same as described above. The flow rate is changed to 5 ml/min. Collect the fractions containing pure RGD-SH and freeze-dry (Fig. 1).
7. Store RGD-SH under acidic condition (pH 3–4) in water to prevent disulfide formation (*see Note 6*).

3.5 Conjugation of DOTA and RGD onto PASP-IONPs

1. DOTA is activated by 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC) and *N*-hydroxysulfonosuccinimide (SNHS) at pH 5.5 for 30 min with a DOTA:EDC:SNHS molar ratio of 10:5:4.

2. 0.8 μmol activated DOTA and 1.2 μl NHS-PEG-MAL (4.1 mg) are added to a 600 μl PASP-IONP solution in borate buffer (2.5 mg Fe/ml, pH = 8.5). Incubate the mixture at 4 °C for 1 h.
3. Add 1.0 mg RGD-SH (1.5 μmol) to the solution. Adjust the pH to 7.0 and incubate the mixture overnight at 4 °C (*see Note 7*).
4. Run the raw product through a PD-10 column to remove unreacted species. Briefly, load the solution onto a column and wait until all the solution is in. Add PBS buffer (pH 7.4) as eluent and collect only the deepest-colored fractions at the bottom (*see Note 8*).
5. The product, RGD and DOTA modified IONPs (RD-IONPs), is stored at 4 °C and is stable for up to 4 weeks (Fig. 2).

3.6 Determination of IONP Concentration

Take 20 μl of the particle stock solution and decompose the particles in 10 % HNO_3 with heating. Add water to adjust the final volume to 10 ml. The iron content is to be analyzed by ICP-MS. The results will be converted to obtain the iron concentration in the original solution (*see Note 9*).

3.7 Cell Culture

U87MG cells are cultured in DMEM (low glucose) supplemented with 10 % (vol/vol) FBS at 37 °C. MCF-7 cells are cultured in MEM supplemented with 10 % (vol/vol) FBS at 37 °C. The cells are used when they reach 70–85 % confluency.

3.8 Binding Affinity Assay

1. Prepare ^{125}I -echistatin solution in cell-binding buffer (20 mM Tris, 150 mM NaCl, 2 mM CaCl_2 , 1 mM MnCl_2 , 1 mM MgCl_2 , 0.1 % (wt/vol) bovine serum albumin; pH 7.4; 0.5 $\mu\text{Ci/ml}$). It is estimated that about 50 μl solution is needed for each well (*see Notes 10 and 11*).
2. Prepare three stock solutions of different concentrations of RD-IONPs in cell-binding buffer. Parallel analyses with free RGD peptide can be carried out as a comparison. Triplicate samples are recommended.
3. Add 0.02–0.03 μCi of ^{125}I -echistatin and appropriate amount of the RD-IONP stock solution to each well of a 96-well plate. The final concentrations should range from 1.0 $\mu\text{g Fe/ml}$ to 1.0 mg Fe/ml.
4. Prepare a U87MG stock in cell-binding buffer at two million cells/ml concentration. Add 1×10^5 U87MG cells (50 μl) to each well and adjust the total volume to 200 μl per well with cell-binding buffer. Incubate for 2 h at room temperature.
5. Use a vacuum manifold to remove the incubation buffer from the 96-well plate and wash three times with the cell-binding buffer (100 μl per well).

6. Heat-dry the 96-well plate in a dry bath incubator. It usually takes 15 min to dry the membrane.
7. Collect the membranes from each well and put them into polystyrene culture test tubes.
8. Measure the radioactivity on each membrane with a γ -counter.
9. Fit the data by nonlinear regression and calculate the best-fit IC₅₀ (inhibitory concentration of 50 %) values. RD-IONPs should have much lower IC₅₀ values (higher binding affinity) than the unconjugated RGD peptide due to the multivalent efficiency.

3.9 Cell Staining

1. Seed U87MG (integrin $\alpha_v\beta_3$ -positive) or MCF-7 ($\alpha_v\beta_3$ -negative) cells (1×10^5 cells per well) onto 4-well cell culture chambers and incubate overnight.
2. Aspirate the cell culture medium from the dish. Wash the cells twice with PBS.
3. Fix the cells with ice-cold 95 % EtOH for 15 min.
4. Wash the cells 2–3 times with 1 ml cell-binding buffer (3–5 min each time).
5. Add PASP-IONP or RD-IONP solutions into each well. Adjust the final particle concentration to 0.02 mg Fe/ml. Incubate at 37 °C for 1 h with gentle shaking (*see Note 12*).
6. Remove the particles and wash the cells three times with PBS buffer.
7. Incubate the cells with Prussian blue staining solution, which is a 1:1 mixture of 20 % hydrochloric acid and 10 % potassium ferrocyanide solution, for 40 min at room temperature. Wash cells with PBS twice.
8. Incubate cells with fast red nuclear staining solution for 10 min.
9. Consecutive dehydrations with 70, 90, and 100 % EtOH.
10. Remove the chamber and mount the slide for microscopy.

3.10 Preparation of U87MG Xenograft Models

The animal models will be prepared by injecting 5×10^6 U87MG cells subcutaneously into the right flank of athymic nude mice. Imaging studies will be performed when the tumor size reaches $\sim 500 \text{ mm}^3$, which usually takes 3–4 weeks. The tumor size will be calculated as $a \times b^2/2$, where a represents the longer dimension and b represents the shorter dimension of the tumor.

3.11 Loading ^{64}Cu onto RD-IONPs

1. Before in vivo PET imaging, incubate RD-IONPs with ^{64}Cu (185 GBq/g Fe) in 0.1 N sodium acetate (pH 6.5) buffer for 45 min at 40 °C (*see Note 13*).
2. Run the mixture through a PD-10 column with PBS buffer (pH 7.4) as the mobile phase. Collect the fractions with the deepest color.

3.12 In Vivo PET Studies

1. Prepare U87MG xenograft models as above mentioned. Wait until the tumor size reaches $\sim 500 \text{ mm}^3$.
2. Label IONPs with ^{64}Cu by following the above mentioned procedure.
3. Anesthetize the animals using the rodent anesthesia system with isoflurane (2 % (vol/vol) isoflurane in 0.2 l/min O_2 flow).
4. Inject 100–200 μl ^{64}Cu labeled RD-IONPs intravenously into the U87MG tumor-bearing mice at a dose of 10 mg Fe/kg. For the control group, free c(RGDyK) (10 mg/kg) is coinjected with the particle probes.
5. Perform PET scans on a microPET R4 rodent scanner at selective time points post injection.
6. For each scan, 3-dimensional ROIs are drawn over the tumor and organs on decay-corrected whole-body coronal images. The average radioactivity is obtained from the mean pixel values within the ROI volume, and is converted to counts per milliliter per minute by using a predetermined conversion factor.
7. Given a tissue density of 1 g/ml, the counts per milliliter per minute are converted to counts per gram per minute, and the values are divided by the injected dose to obtain the imaging-ROI-derived percentage injected dose per gram (% ID/g).
8. If needed, harvest the tumor and major organs. Take PET images again with the organs. Subsequent ex vivo tissue staining can be performed when after the reactivity is decayed.

3.13 In Vivo MRI Studies

1. Prepare U87MG xenograft models as described above. Wait until the tumor size reaches $\sim 500 \text{ mm}^3$.
2. Anesthetize the animals using the rodent anesthesia system with isoflurane (2 % (vol/vol) isoflurane in 0.2 l/min O_2 flow).
3. RD-IONPs are intravenously injected through the tail vein (10 mg Fe/kg per mouse). For the control group, c(RGDyK) (10 mg/kg) is co-injected with the particles.
4. T_2 -weighted fast spin-echo images are acquired on a 7.0 T small animal MRI system, before and at selective time points post injection. The following are suggested parameters: TE 40 ms, TR 3,000 ms, thickness 1 mm, FOV 6×6 , NEX 1.0, Echo 1/1 (*see Note 14*).

4 Notes

1. Iron-oleate is a very viscous compound and can prove difficult to handle. It is best to keep the spatula slightly moist with hexane to help transfer the metal oleate into the reaction flask.

2. Any smoke observed during the refluxing period is due to the lack of a closed system and the solvent is being removed. In that case, cease the heating and repair any leak in the system.
3. IONPs can be split into multiple tubes to ensure good purification.
4. Small portion of aggregations may be observed. To remove these, centrifuge at low speed (2,500 rpm ($460 \times g$)) and dispose the aggregates at the bottom.
5. If the pH is too low, it may result in incomplete de-protection of the thiol. If the pH is too high, disulfide bond may form when the thiol group is released.
6. RGD-SH can be stored for up to a few months under an acidic condition.
7. It is highly recommended to check the purity of the RGD-SH by analytical HPLC before conjugation. If significant amount of disulfide already formed, some TCEP. HCl (1 mg or less) can be added.
8. NAP-5 columns can be used when dealing with smaller amount of samples.
9. Remember that 2 % HNO_3 is commonly used in ICP analysis as the eluent. Estimate the iron content and make sure it lies within the range of the standards.
10. It is imperative to obtain appropriate training and abide by all regulatory rules when handling radioactivity. The radioactive waste needs to be collected and disposed under the guidance of the institutional radiation safety office.
11. The presence of certain metal ions (e.g., Mn^{2+} and Mg^{2+}) is essential for integrin $\alpha_v\beta_3$ binding. Binding buffer without these ions will result in much lower readings.
12. Aggregation may form during particle aging. The aggregates may stick onto the slides and interfere with the staining. Use fresh samples when possible. Sonication prior to the staining may also help.
13. It is imperative to obtain appropriate training and abide by all regulatory rules when handling radioactivity. The radioactive waste needs to be collected and disposed under the guidance of the institutional radiation safety office.
14. A monitoring system should be used to monitor physiological signals of mice such as their heart rate, respiration, and body temperature. Respiration rate is usually obtained from a small pneumatic pillow sensor placed next to the animal's abdomen. Heart rate is monitored using ECG by placing three leads with subdermal needle electrodes. Temperature is monitored with a rectal thermometer or a fiber optic sensor.

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Fig. 1.
Converting c(RGDyK) to RGD-SH. Modified with permission from ref. 16

Fig. 2. Schematic diagram of the ^{64}Cu loaded RD-IONPs for PET and MRI imaging. Modified with permission from ref. 7