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Synthesis of ⁶⁴Cu-Labeled Magnetic Nanoparticles for Multimodal Imaging

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Abstract

Complementary imaging modalities provide more information than either method alone can yield and we have developed a dual-mode imaging probe for combined magnetic resonance (MR) and positron emission tomography (PET) imaging. We have developed dual-mode PET/MRI active probes targeted to vascular inflammation and present synthesis of (1) an aliphatic amine polystyrene bead and (2) a novel superparamagnetic iron oxide nanoparticle targeted to macrophages that were both coupled to positron-emitting copper-64 isotopes. The amine groups of the polystyrene beads were directly conjugated with an amine-reactive form (isothiocyanate) of aza-macrocyclic 1,4,7,10-tetraazacyclo-dodecane-1,4,7,10-tetraacetic acid (DOTA). Iron oxide nanoparticles are dextran sulfate coated, and the surface was modified to contain aldehyde groups to conjugate to an amine-activated DOTA. Incorporation of chelated Cu-64 to nanoparticles under these conditions, which is routinely used to couple DOTA to macromolecules, was unexpectedly difficult and illustrates that traditional conjugation methods do not always work in a nanoparticle environment. Therefore, we developed new methods to couple Cu-64 to nanoparticles and demonstrate successful labeling to a range of nanoparticle types. We obtained labeling yields of 24% for the amine polystyrene beads and 21% radiolabeling yield for the anionic dextran sulfate iron oxide nanoparticles. The new coupling chemistry can be generalized for attaching chelated metals to other nanoparticle platforms.

1. INTRODUCTION

Challenges in diagnostic imaging have led to an explosion of interest in combining modalities to more accurately interpret disease and abnormalities in vivo. Combinations of imaging modalities are being investigated for a synergistic effect, and the combination of positron emission tomography/magnetic resonance imaging (PET/MRI) has raised expectations for highly sensitive and high-resolution imaging (1, 2). PET and MRI are complementary to each other given the high anatomical spatial resolution of MRI and the unparalleled sensitivity and functional imaging of PET (3). Noninvasive monitoring of physiological abnormalities in vivo promises to greatly enhance our knowledge and treatment of diseases.

Concomitant with the development of dual-mode imaging systems has been an increase in interest for dual-mode molecular imaging probes that are targeted for very specific clinical applications, e.g., imaging of tumors and atherosclerotic plaques (4-7). Most of the reported dual-mode agents have been combinations of either PET or MRI agents with optical agents (6, 8-12). Reports of SPIOs with dual-modality properties are increasing in the literature, with the focus primarily on probes with magnetic and optical properties suitable for imaging (13-18). Very recently, a trimodal PET/CT/optical agent based on iron oxide nanoparticles

was developed (19). In this work, we describe development of a combined MRI/PET agent composed of iron oxide nanoparticles coupled to chelated positron emitting ions.

A commonly used method to radiolabel macromolecular targeting agents for imaging with any PET active metal is to chelate the metal to a bifunctional polyazacarboxylate- (20) or a tetraaza-macrocycle (21). The isotope ^{64}Cu , with a half-life of 12.7 h, has expanded in use as a positron emitter (22) for PET and metabolic radiotherapy (23, 24) due to the additional high-energy β^- particle and Auger electron emissions of this nucleus. The ligand–cage pairs of polyazacarboxylate- or tetraaza-macrocycle form thermodynamically stable complexes with Cu^{2+} ($\log K = 20\text{--}28$) (25, 26). The copper chelate could then be conjugated to the superparamagnetic iron oxide nanoparticle to develop a dual-modality probe for MRI and PET imaging. We found that the development of an iron oxide conjugated to a copper chelate was synthetically difficult using traditional bioconjugation methods.

Herein, we report ^{64}Cu radiolabeling of dextran sulfate coated superparamagnetic iron oxide nanoparticles; these are targeted toward inflammatory events, such as atherosclerotic plaques. The labeling was done by coordination of the ^{64}Cu to the chelating bifunctional ligand *S*-2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (*p*-SCN-Bz-DOTA) and then conjugation to the nanoparticle. We also describe a synthetic route for a ^{64}Cu -radiolabeled model imaging probe utilizing an aliphatic amine polystyrene microsphere as another example for radiolabeling of nanoparticles.

2. EXPERIMENTAL PROCEDURES

2.1. General

Ferric chloride hexahydrate (99%, Acros Organics, (Geel, Belgium)), ferrous chloride tetrahydrate (>99%, Fluka (Buchs, Switzerland)), dextran sulfate, sodium salt (Fluka (Buchs, Switzerland)), ammonium hydroxide solution (~30%, EM Science (Lawrence, KS)), ethylenediamine (>99.8%, Fluka (Buchs, Switzerland)), sodium cyanoborohydride (95%, Fluka (Buchs, Switzerland)), periodic acid (reagent grade, Fisher Scientific (Waltham, MA)), surfactant-free aliphatic amine polystyrene beads (1 μm , 2.0% w/w, Molecular Probes, Invitrogen (Carlsbad, CA)), *S*-2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (*p*-SCN-Bz-DOTA, Macrocylics (Dallas, TX)), *S*-2-(4-aminobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (*p*-NH₂-Bz-DOTA, Macrocylics (Dallas, TX)), cupric chloride (97%, Aldrich (St. Louis, MO)), and cupric-64 chloride solution (MDS Nordion (Ottawa, Canada)) were used as purchased. Nanopure water (18.0 MΩ·cm) was from a Barnstead nanopure filtration unit. Dextran (10 095 MW, Sigma (St. Louis, MO)) was reduced and purified according to Paul et al. (27).

2.2. Preparation of Polystyrene Beads and Dextran Sulfate Coated Iron Oxide Nanoparticles Labeled with “Cold” Copper

2.2.1. Prechelation of “Cold” Copper into *p*-SCN-Bz-DOTA Followed by Conjugation to Polystyrene Beads— CuCl_2 (1.29 mg; 9.57 μmol in 20 μL nanopure H_2O) was added to *p*-SCN-Bz-DOTA (5.26 mg; 7.656 μmol in 1.0 mL nanopure H_2O) and stirred for 2 h. The reaction solution was then purified by size exclusion chromatography (SEC) on a Sephadex G-25 column. UV–vis spectra of the purified product were used to determine the fraction corresponding to the *p*-SCN-Bz-DOTA- Cu^{2+} complex based on the absorption of the benzyl group and copper ion. UV–vis was not used to assess the purity of the product. The *p*-SCN-Bz-DOTA-Cu solution was then added to tetramethylammonium phosphate solutions (pH 8) of both 78 nm and 1 μm sized polystyrene beads in molar ratios of approximately 5000:1 and 218 000:1, respectively. The reactions were stirred at room temperature overnight and purified by dialysis against 4 L nanopure water for ~2 days with

6 changes of water. Inductively coupled mass spectrometry (ICP-MS) was used to determine amounts of conjugated copper.

2.2.2. Labeling of ADIO Nanoparticles with “Cold” Copper to Determine Affect of Labeling on Particle Size

—Dextran sulfate coated iron oxide nanoparticles (anionic dextran sulfate iron oxide, ADIO) were synthesized as described previously (28). It was found that amination of the ADIO nanoparticles leads to cross-linking as the ethylenediamine is bifunctional (Scheme 1); therefore, we sought to avoid particle cross-linking by attaching the ethylenediamine to the *p*-SCN-Bz-DOTA instead. In this way, any dimer formation of *p*-SCN-Bz-DOTA-amine complexes would be unreactive and not contribute to particle aggregation. Synthesis of copper-labeled dextran sulfate nanoparticles was achieved by conjugation of an amine reactive *p*-SCN-Bz-DOTA with the dextran sulfate coating.

The *p*-SCN-Bz-DOTA (9.82 mg; 16.70 μ mol in 14.28 mL DMSO), was mixed in a 1:1 molar ratio with a solution of CuCl₂ (1.92 mg of CuCl₂ in 0.465 mL nanopure water) and incubated at room temperature for 15 min. Next, the *p*-SCN-Bz-DOTA-Cu was amine-activated by coupling ethylenediamine to the *p*-SCN-Bz-DOTA to form a urea bond (Scheme 2). A 0.1 M triethanolamine acetate (TEAA) (pH 8) buffer was added to the *p*-SCN-Bz-DOTA-Cu solution (7 mL added, 0.032 M TEAA final) and ethylenediamine (1 μ L) was mixed in a 1:1 molar ratio with the *p*-SCN-Bz-DOTA and incubated at room temperature for 15 min. To couple the amine group to the particle surface (Scheme 3a), the dextran sulfate particles were prepared by the ring-opening reaction with periodic acid (29). Depending on the amount of labeling desired, 1% periodic acid was mixed with a 1.41 mM Fe solution (2 mL particles) of dextran sulfate particles in an Fe/HIO₄ molar ratio of 15.2:1, 5:1, or 2.5:1 (4.25, 12.75, 25.5 μ L HIO₄, respectively). Then, 0.404 mL, 1.212 mL, or 2.424 mL of the amine-activated *p*-SCN-Bz-DOTA-Cu (Fe/*p*-SCN-Bz-DOTA molar ratio of 11.4:1, 3.8:1, 1.9:1) was added to the particles and stirred at room temperature for 2 h. Next, 0.6 mL ethylene glycol was added to quench the reaction, followed by 15 min of stirring. This was followed by the addition of ~5 to 15 mg solid sodium cyanoborohydride to reduce the imine bond (Schiff base) and stirred for an additional 20 min. The product was purified by gel chromatography on a Sephadex G-25 (fine bead size, Sigma (St. Louis, MO)) column equilibrated with 1 $\times$ phosphate buffered saline (PBS). Fractions corresponding to the particles were collected and pooled together.

2.3. Preparation of Copper-64 Labeled Polystyrene Beads and Dextran Sulfate Coated Iron Oxide Nanoparticles (ADIO-(DOTA-Cu))

2.3.1. Labeling of Polystyrene Beads with Copper-64—⁶⁴CuCl₂ (2.35 mCi, approximately 0.6 nmol, in 50 μ L of 10 mM HCl) was buffered with 1 M TEAA buffer, pH 8 (5 μ L), then added to *p*-SCN-Bz-DOTA (0.120 μ mol in 6 μ L of 0.1 M TEAA buffer, pH 8). Pure triethanolamine (1 μ L) was added to adjust the final pH to 8. This reaction mixture was then incubated for 20 min at 40 °C and added to a solution of polystyrene beads (1 μ m; 5.2 mg in 440 μ L of 0.1 M TEAA buffer, pH 8), and incubated for 2 h at 40 °C. After incubation, 10 mM EDTA (100 μ L) was added to scavenge any free metal ions. To purify, the beads were centrifuged at 7500 rpm for 5 min, the supernatant was drawn off, and the beads were resuspended in water (300 μ L). The purification procedure was performed three times.

2.3.2. Labeling of ADIO Nanoparticles with Copper-64 for Multimodal Imaging

—The protocol described for the “cold” copper in section 2.3.1 was used with the following modifications. Ethylenediamine (at least 1 μ L was used) was mixed in a 1:1, 2:1, or 30:1 molar ratio with the *p*-SCN-Bz-DOTA and incubated at room temperature for 4 h to

generate amine-Bz-DOTA (2-*p*-(3-aminopropylthioureido)benzyl-1,4,7,10-tetraazacyclododecane-tetraacetic acid). If a 2:1 or 30:1 molar ratio was used, the product was purified by size exclusion gel chromatography (SEC) on a Sephadex G-25 column equilibrated with 50 mM ammonium bicarbonate. To couple the amine group to the particle surface (Scheme 3b), the dextran sulfate particles were prepared by the ring-opening reaction with periodic acid (29).

Briefly, amine-Bz-DOTA was synthesized by combining a buffered solution of *p*-SCN-Bz-DOTA (2.8 mg; 4.07 μ mol dissolved in 408 μ L 0.1 M carbonate buffer (pH 9.2)) with ethylenediamine. Within 1 min of preparation of the aqueous *p*-SCN-Bz-DOTA, ethylenediamine (8.2 μ L; 122.4 μ mol, 30 equiv) was added and stirred at room temperature for 4 h. The product was then purified by SEC on a Sephadex G-25 column equilibrated with 50 mM ammonium bicarbonate. Fractions corresponding to amine-Bz-DOTA were pooled (confirmed spectrophotometrically by a broad absorbance at 260–280 nm). Second, the ADIO polysaccharide surface was modified with aldehyde groups by mixing 0.5 mL ADIO (5.64 mM Fe) with 0.25 mL 1% HIO₄ (3.9 equiv) at room temperature for 4 h. The modified particles were then purified by SEC on a Sephadex G-25 column equilibrated with nanopure water. The purified amine-Bz-DOTA was then mixed with 930 μ Ci (~0.2 nmol) ⁶⁴CuCl₂ at room temperature for 20 min. The amine-Bz-DOTA(⁶⁴Cu) was then added to the aldehyde-ADIO particles and stirred at room temperature for 1.5 h. The reaction was completed by the addition of ~3 mg solid sodium cyanoborohydride to reduce the imine bond formed. The labeled nanoparticles were then purified by SEC on a Sephadex G-25 column equilibrated with 1× PBS.

2.3.3. Comparison of *p*-NH₂-Bz-DOTA with the Synthesized Amine-Bz-DOTA for Labeling of ADIO Nanoparticles with Copper-64—*S*-2-(4-

Aminobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (*p*-NH₂-Bz-DOTA) was used in parallel with amine-Bz-DOTA for comparison of labeling efficiency. Amine-Bz-DOTA was synthesized by reacting *p*-SCN-Bz-DOTA (1.09 μ mol, 10 mM) with 30 mol equiv of ethylenediamine in 0.1 M sodium bicarbonate buffer (pH 9.0) for 3 h at room temperature (RT). The amine-Bz-DOTA product was then purified by SEC on a Sephadex G-25 column equilibrated with 50 mM ammonium bicarbonate using the Panofsky method (centrifuge at 2000 rpm for 2 min). This was repeated 2 times to yield amine-Bz-DOTA.

Next, aldehyde-terminated ADIO nanoparticles were generated by incubating 1 mol equiv (Fe/HIO₄) of 1% HIO₄ with ADIO nanoparticles at RT for 3 h (6.36 μ mol Fe, 85 μ L of a 74.8 mM Fe solution in 0.9% NaCl). The product was purified by SEC on a Sephadex G25 column equilibrated with nanopure water using centrifugation (2000 rpm, 2 min). This was repeated 2 times to yield the aldehyde-modified ADIO nanoparticles.

Then, copper-64 was chelated by *p*-NH₂-Bz-DOTA or amine-Bz-DOTA by first mixing ⁶⁴CuCl₂ in 10 mM HCl (30 μ L) with 1 M triethanolamine acetate (pH 8, 7 μ L) to form a ⁶⁴Cu-acetate complex in a solution with a pH of 8.0. Then, the *p*-NH₂-Bz-DOTA (1.07 μ mol) in 50 mM ammonium bicarbonate or amine-Bz-DOTA (~1.09 μ mol) in 50 mM ammonium bicarbonate was mixed with 37 μ L of the copper acetate solution at RT for 30 min.

The aldehyde-ADIO nanoparticles (110 μ L) were then reacted with either *p*-NH₂-Bz-DOTA(⁶⁴Cu) (263 μ Ci) or amine-Bz-DOTA(⁶⁴Cu) (246 μ Ci) at RT for 2 h. Then, 0.5 mg solid NaCNBH₃ was added and allowed to incubate with the nanoparticles for an additional 20 min at RT to reduce the imine bond formed between the chelator and the nanoparticle to form a stable product. The nanoparticles were then purified by SEC on a Sephadex G25 column equilibrated with 0.9% NaCl using centrifugation (2000 rpm, 2 min). This was

repeated 3 times to yield ADIO-DOTA(^{64}Cu) using either *p*-NH₂-Bz-DOTA or amine-Bz-DOTA. The activity was then measured and the radiolabeling yield determined.

2.4. Characterization

2.4.1. Iron and Copper Quantification—The iron and copper content of the labeled ADIOs was determined by atomic absorbance spectroscopy (AAS) using a Varian SpectrAA 220FS (Varian (Palo Alto, CA)) to verify iron concentration and the number of “cold” copper coordinated *p*-SCN-Bz-DOTA ligands were conjugated to each ADIO particle. The AAS samples were prepared in 3% HCl and five standard solutions with known iron and copper concentrations were used to generate standard curves.

Copper content of the polystyrene bead comparable experiments with “cold” copper were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) on an Agilent 7500a (Agilent Technologies (Santa Clara, CA)) quadrupole ICP-MS. The ICP-MS samples were prepared as 10 mL triplicates in 3% HNO₃. In order to correct for instrumental drift during analysis, germanium solution was mixed with all sample and standard solutions as an internal standard.

2.4.2. Characterization of Amine-Bz-DOTA (2-*p*-(3-aminopropylthioureido)Benzyl-1,4,7,10-Tetraazacyclododecane-Tetraacetic Acid)—Amine-Bz-DOTA was generated by reacting 8.26 mg *p*-SCN-Bz-DOTA with 30 mol equiv of ethylenediamine in 0.1 M sodium carbonate buffer (pH 9.0, 10 mM DOTA solution). After purification by SEC on a Sephadex G-25 column equilibrated with 50 mM ammonium bicarbonate, the amine-Bz-DOTA product was then dialyzed (MWCO = 100) against 4 L of nanopure water for 18 h with 2 water changes to remove salts. The product (~3.3 mg, 40% yield) was then lyophilized (−48 °C, 0.002 mTorr, 3 days) and analyzed by ¹H NMR and mass spectrometry. A small portion was dissolved in nanopure water for ESI⁺ MS. The instrument was a Thermo LCQ ion trap (San Jose, CA) operated with an electrospray source in positive ion mode under standard conditions. The remaining sample (3.3 mg) was resuspended in D₂O (750 μL) for ¹H NMR (500 MHz, 300 K, Bruker, Billerica, MA). The *p*-SCN-Bz-DOTA was also dissolved in nanopure water for ESI⁺ MS and DMSO-*d*₆ for ¹H NMR.

The ¹H NMR spectra for the final product, amine-Bz-DOTA (2-*p*-(3-aminopropylthioureido)benzyl-1,4,7,10-tetraazacyclododecane-tetraacetic acid), is presented in Supporting Information Figure S1. Amine-Bz-DOTA, ¹H NMR (D₂O): δ 1.9–2.4 (4H, m, CH₂), δ 2.4–2.7 (4H, m, CH₂), δ 2.745 (1H, s, CS-NH-Ar), δ 2.78–2.9 (4H, m, N-CH₂ and Ar-CH₂), δ 2.9–3.9 (21H, m, CH₂), δ 7.128 (2H, s, ArH), δ 7.225 (2H, s, ArH). ESI⁺ MS (100% H₂O): *m/z* found 612.5 (100%, calc. M + H = 612.67), 634.5 (12%, M + Na⁺, calc. 635.66), 306.8 (22%, [M + H]²⁺, calc. 306.34), 552.5 (5%, *p*-SCN-Bz-DOTA calc. 552.4), 1223.8 (6%, dimer + NaCl, calc. 1221.77). The ¹H NMR of *p*-SCN-Bz-DOTA is presented in Supporting Information Figure S2. *p*-Bz-DOTA, ¹H NMR (DMSO-*d*₆): δ 2.6–4.2 (43H, m, CH₂), δ 7.424 (4H, s, ArH), δ 12.5–14 (3H, m, COOH). ESI⁺ MS (100% H₂O): *m/z* found 552.4 (100%, calc. M + H = 552.4).

2.4.3. Size Measurements—Dynamic light scattering (DLS) was used to determine the overall particle size in solution (expressed as the volume weighted diameter) with a Nanotracer 150 particle size analyzer (Microtrac, Inc. (Montgomeryville, PA)). A geometric eight-root regression, with no residuals, was used to fit the data. The Nanotracer 150 has a built-in thermometer to measure the cell temperature, from which the viscosity is calculated; the nanorange option was enabled and a scan time of 30 s was used. Particle size is expressed as the mean volume diameter ± one standard deviation.

3. RESULTS AND DISCUSSION

In previous work, the synthetic strategy for conjugating fluorescent dyes to nanoparticles has been to form an amide bond between the dye and the amine sites on the coating of the SPIO, or a linker connecting to the SPIO through a disulfide or a thioether linkage (9, 13). Our initial labeling approach was based on a similar theory. For the dextran sulfate coated nanoparticles, the particle surface is modified to contain aldehyde groups, which can be conjugated to the amine-reactive *p*-SCN-Bz-DOTA chelator. For the polystyrene beads, the chelating ligand is directly conjugated to the aliphatic amine groups on their surfaces.

3.1. Nanoparticles are Not Labeled if DOTA is Conjugated to the Particle before Chelation of Copper

3.1.1. Polystyrene Beads As a Model Compound: Conjugation of DOTA to the Particle Surface—To conveniently explore and develop the chemistry of the labeling of surface-modified nanoparticles, we utilized a system consisting of aliphatic amine polystyrene beads. The polystyrene beads are inexpensive, commercially available, and mimic the surface environment of amine-functionalized ADIO nanoparticles, or alternatively, different functionality if the ADIO particles are aldehyde-terminated. Because the polystyrene beads are fundamentally different from the aldehyde-modified ADIO nanoparticles (size, surface charge, and functional groups), labeling of these particles presents an opportunity to create a labeling method that can be applied to a broad range of nanoparticle systems.

The amine-reactive, metal chelating ligand (*p*-SCN-Bz-DOTA) was attached by direct linkage to the amine groups of the bead by the formation of a thiourea bond (Figure 1) described earlier by Mirazdeh et al. (30). We found for the aliphatic amine polystyrene bead system that labeling is not achieved if the chelator is conjugated to the nanoparticles before the copper is introduced. This has previously been observed for the 1,4,8,11-tetraazacyclotetradecane-*N,N',N'',N'''*-tetraacetic acid (TETA) chelator by other researchers (31). In the study, by Moi et al. labeling antibodies with copper-64 TETA, it was found that labeling was only achievable if the copper was preinserted into the TETA before conjugation to the antibody (31).

In our polystyrene system, for example, if 0.5 mL of the 1 μ m PS beads were first coupled to *p*-SCN-Bz-DOTA (~20 400 DOTA molecules per bead), purified and then mixed with a Cu²⁺ chloride solution (molar ratio of Cu²⁺/*p*-SCN-Bz-DOTA = 2.1:1), no copper was detected in the product after purification. We believe that that lack of coupling is due to competition for binding of copper by the numerous free amines available at the surface. Each nonconjugated 1 μ m sized bead had an average of 157 000 amines on the surface. Even with a 100% conjugation of the *p*-SCN-Bz-DOTA (in this case 20 400 per bead), the free surface amine groups remaining on each bead could bind up to approximately 130 000 copper(II) ions and might thereby compete with, and prevent, chelation by the DOTA groups. Therefore, prechelation of the copper ion must first be performed in a separate step.

3.1.2. Conjugation of DOTA to the ADIO Particles before Chelation of Copper Did Not Result in Labeling—The ADIOs were previously shown to have small size and good magnetic properties to work as a MR imaging contrast enhancing agent. The relaxivities were determined to $r_1 = 14.46 \text{ mM}^{-1} \text{ s}^{-1}$ and $r_2 = 72.55 \text{ mM}^{-1} \text{ s}^{-1}$ at 60 MHz, 37 °C, and the overall particle diameter was measured as $32.3 \pm 11.8 \text{ nm}$ (28).

A literature procedure for coupling fluorescein isothiocyanate was modified to couple *p*-SCN-Bz-DOTA to the nanoparticles after amination of the dextran (sulfate) coating

(Scheme 1) (29). After amination of the ADIO particles, *p*-SCN-Bz-DOTA was conjugated to the nanoparticles through the amine groups.

We found that, for both nanoparticle systems, aliphatic amine polystyrene beads and dextran sulfate coated iron oxides, labeling with copper was not possible if the metal chelator was conjugated to the nanoparticle surface before chelation; only after preinsertion of the metal into the chelator before conjugation to the nanoparticle surface was labeling achievable. As observed for the polystyrene bead system, if the *p*-SCN-Bz-DOTA was first conjugated to the particle surface before the chelation of copper, there was inhibition of the labeling (Table 1). In agreement with the previous study by Moi et al., for our nanoparticle systems, we found that prechelation of copper-64 was necessary for both polystyrene beads and dextran sulfate coated iron oxide nanoparticles (31).

3.2. Chelation of the Copper by the DOTA before Conjugation to the Nanoparticles Results in Some Labeling at Low Yield

3.2.1. Preinsertion of Copper into the *p*-SCN-Bz-DOTA Resulted in Labeling of Polystyrene Beads—By first chelating the copper ion in the DOTA complex, and then conjugating the *p*-SCN-Bz-DOTA(Cu) to the PS, bead labeling was achieved (Table 2). The higher degree of labeling for the 1 μ m PS beads (18%) compared to the 78 nm beads (3%) could have been due to several factors. First, the amount of *p*-SCN-Bz-DOTA was 43 times greater for the 1 μ m beads, and this may have resulted in the higher labeling efficiency. Another possible explanation for the higher labeling efficiency of the larger beads could have been due to the surface density of the amine groups. For a 1 μ m particle (surface area of 3 141 593 $\text{\AA}^2$), the surface density for 157 000 amines would be approximately 1 amine group every 20 $\text{\AA}^2$. For the 78 nm beads (1 911 345 $\text{\AA}^2$), the surface density of 54 000 amines would be 1 amine every 36 $\text{\AA}^2$. The surface charge of the larger particles has a greater charge density and therefore may result in a more efficient attraction and subsequent coupling between the chelator and the bead.

3.2.2. Preinsertion of Copper into the *p*-SCN-Bz-DOTA Resulted in Labeling of ADIO Nanoparticles, But Cross-Linking Lead to Aggregation—A literature procedure for coupling fluorescein isothiocyanate was modified to couple *p*-SCN-Bz-DOTA to the nanoparticles after amination of the dextran (sulfate) coating (Scheme 1) (29). After amination of the ADIO particles, *p*-SCN-Bz-DOTA was conjugated to the nanoparticles through the amine groups. We found that amination of the ADIO particles led to irreversible cross-linking and flocculation. The cross-linking is most likely due to the ethylenediamine being equally reactive at both ends. The ADIO nanoparticles were 32 nm before surface modification and 133 nm after amination.

Particle size plays an integral role in clearance from the body, and iron oxide particles larger than 50 nm have been shown to be rapidly cleared by the liver (32-34). To target specific sites of interest, particle size is to be kept small, smaller than ~30 nm, to increase circulation time to allow the particles to reach targets of interest (32, 35, 36). To avoid the irreversible cross-linking of the ADIO nanoparticles, we opted to modify the synthesis by introducing amine groups to the *p*-SCN-Bz-DOTA rather than the ADIO nanoparticles (Scheme 2). In this revised chemistry, we attach the ethylenediamine to the *p*-SCN-Bz-DOTA rather than the ADIO surface. If dimers of the DOTA form, they cannot react with the nanoparticles, and can be removed by purification, and thus the aggregation seen with functionalizing the ADIO nanoparticles was avoided.

3.2.3. Cross-Linking of ADIO Nanoparticles AVOIDed by Amination of the *p*-SCN-Bz-DOTA but Cu Labeling Yield Remains Low—After preinsertion of the

copper into the *p*-SCN-Bz-DOTA (Scheme 2), the chelator was conjugated to the nanoparticle surface (Scheme 3a). The *p*-SCN-Bz-DOTA was modified to have an amine group and this could then react with the aldehyde group on the ADIO surface (Scheme 3a). By coupling the ethylenediamine to the *p*-SCN-Bz-DOTA instead of the ADIO nanoparticles, we were able to avoid the cross-linking of the particles. Results for the degree of “cold” copper labeling of the ADIO nanoparticles are shown in Table 3. As expected, the degree of labeling increased with additional HIO₄ (more binding sites) and the addition of extra *p*-SCN-Bz-DOTA(Cu²⁺). Approximately 7 *p*-SCN-Bz-DOTA(Cu²⁺) molecules were conjugated to the particle surface with a Fe/*p*-SCN-Bz-DOTA molar ratio 11.4:1 and the number of conjugated *p*-SCN-Bz-DOTA(Cu²⁺) molecules increased to 328 with a Fe/*p*-SCN-Bz-DOTA molar ratio of 1.9:1 (Table 3). Interestingly, a 3-fold increase in the *p*-SCN-Bz-DOTA, from 11.4:1 to 3.8: 1, did not result in an increased labeling yield (Table 3); only when a 6-fold increase in *p*-SCN-Bz-DOTA (1.9:1) was used did the labeling yield increase from 0.8% to 6%. The DOTA/HIO₄ was kept constant in all three instances, approximately 1:1; therefore, the increase in labeling yield (6%) for the highest concentration of HIO₄ (25.5 μL HIO₄, Fe/*p*-SCN-Bz-DOTA = 1.9:1) must have been more efficient compared to lower concentrations of HIO₄ for creating more reaction sites in the dextran coating on the nanoparticles. The higher acid concentration (25.5 μL HIO₄) may have been necessary to decrease the pH significantly to drive the ring opening reaction (on the polysaccharide nanoparticle coating) (Scheme 3a).

The particle size increased slightly as the amount of *p*-SCN-Bz-DOTA added increased (Table 3). The size of the particles with 7 DOTA(Cu²⁺) moieties attached was 31.6 nm and was 36.5 nm for particles with 329 DOTA(Cu²⁺) attached. This was most likely the result of an added hydration layer due to the nitro and carboxyl groups added by the additional DOTA moieties. Dynamic light scattering measures the hydrodynamic radius, the particle size plus surface-bound water, and therefore as the number of DOTA moieties increased the hydration layer also increased, resulting in a larger particle diameter (Table 3).

3.3. Modification of the Chemistry Resulted in Greater Labeling Yields for Both Nanoparticle Systems

Initially, by attempting conjugation of *p*-SCN-Bz-DOTA chelator to an amine-coated nanoparticle, polystyrene beads or dextran sulfate coated nanoparticles, before insertion of the metal (Cu²⁺) no labeling was achievable. By preinserting the copper into the DOTA chelator before conjugation to the nanoparticle surface, labeling was accomplished. However, for each system, there were limitations in labeling efficiency that warranted improved chemistry. In the polystyrene bead system, labeling with copper took several days, which is not desirable for a radionuclide with a limited lifetime. Therefore, we sought to obtain similar labeling yields (at least 18%) of the polystyrene beads with more rapid chemistry and purification. For the ADIO nanoparticle system, labeling was achievable and particle cross-linking avoidable by generating an amine-Bz-DOTA(Cu) complex to conjugate to the ADIO surface; however, the yields were very low, <1% for ⁶⁴Cu, and therefore we sought to improve the chemistry to obtain higher labeling yields.

3.3.1. Radiolabeling of Polystyrene Beads with Improved Chemistry—The radiolabeling for the polystyrene beads with copper-64 was modified from the “cold” copper labeling to minimize the reaction time to avoid unnecessary decay of the radionuclide. For the “cold” copper labeling, the copper was incubated with the DOTA at a 1.25:1 molar ratio for 2 h in diH₂O at room temperature and then purified by SEC. As for the copper-64, it was supplied in a 10 mM HCl solution, and we therefore adjusted the pH to 8 with TEAA and incubated at 40 °C for 20 min (compared to 2 h). The solution was buffered, as it is known that the stability of the copper-DOTA complex decreases as the pH decreases (37, 38).

Furthermore, no SEC was used for purification after chelation of the copper-64 by DOTA as the *p*-SCN-Bz-DOTA was in excess. The use of an acetate buffer, TEAA, in this work is similar to the work by Sun et al. in which copper-64 was chelated by 1,4,8,11-tetraazacyclododecane-1,4,8,11-tetraacetic acid (TETA) in ammonium acetate (pH 7) at 43 °C for 2 h (39).

The conjugation of the *p*-SCN-Bz-DOTA(⁶⁴Cu) to the polystyrene beads was done at 40 °C and the incubation was 2 h compared to overnight for labeling the PS beads with the “cold” copper. Finally, similar to the work by Sun et al. (39), we used centrifugation to purify the radiolabeled beads in favor of dialysis.

These time reduction steps, utilizing an elevated temperature, resulted in a radiolabeling yield for the [(*p*-SCN-Bz-DOTA)-⁶⁴Cu] on the polystyrene beads of 7% to 24% (Table 4). The radiolabeling for the 78 nm particles increased to 22–24% (Table 4), compared to the 3% labeling with the “cold” copper (Table 2). This increase in labeling yield was most likely due to the increased *p*-SCN-Bz-DOTA/bead ratio for the radiolabeling. However, the radiolabeling of the 1 μm PS beads was only ~7% compared to 18% for the “cold” copper (Tables 1 and 3). This may have been due to a large change in pH during the addition of the buffer with a large amount of the copper-64, 4.02 mCi, compared to the smaller amount used for the 78 nm PS beads.

3.3.2. Labeling of ADIO with Modification of the Chemistry Resulted in Greater Labeling Yields—As was the case with the “cold” copper labeling of ADIO particles and PS beads, if the copper-64 was not chelated by the *p*-SCN-Bz-DOTA before conjugation to the nanoparticle there was no labeling achieved (Table 1). Following the preinsertion scheme and molar ratios of starting materials developed during the cold copper experiments (ratio of ethylenediamine/*p*-SCN-Bz-DOTA = 1:1) (Schemes 2 and 3a), labeling the ADIO nanoparticles with copper-64 had a yield of 1% (Table 1). This was most likely due to the dimerization of *p*-SCN-Bz-DOTA and was alleviated by increasing the amount of ethylenediamine.

The amine-Bz-DOTA was purified by SEC (Schemes 2 and 3b) and excess ethylenediamine removed before addition to the aldehyde-modified nanoparticles or the excess ethylenediamine would react with adjacent nanoparticles and cause flocculation. A molar ratio of the ethylenediamine/*p*-SCN-Bz-DOTA of 2:1 and purification with SEC increased the labeling yield from 1% to ~5% (Table 1). By further increasing the ratio to 30:1, the labeling yield increased to ~20% (Table 1). After the radioactivity had decayed, the particles that had the ~20% yield were sized by DLS and found to be 26.6 ± 7.3 nm in diameter, which is of similar size to the starting ADIO material (32 ± 11.8 nm). This demonstrates that the radiolabeling did not affect the overall particle size. Due to the low yields, the other labeled particles were not sized by DLS.

The increase in yields using SEC to purify the aldehyde-modified ADIO nanoparticles and amine-Bz-DOTA (Scheme 3b) had a strong affect on radiolabeling. The amine-Bz-DOTA was purified by SEC purification not only to remove excess ethylenediamine, but also for the removal of sodium to avoid competition between the copper-64 and sodium by the DOTA macrocycle. Additionally, by using SEC to purify the aldehyde-modified ADIO nanoparticles, the HIO₄ could be removed without the use of ethylene glycol, which could have had two effects on the outcome of the labeling.

Chromatography ensured the removal of all HIO₄ so that the solution was not acidic during the conjugation of the *p*-SCN-Bz-DOTA complex to the nanoparticle. It is known that if the pH drops below about 4 the DOTA can no longer conjugate the copper as the amines in the

ring become protonated. Second, by avoiding ethylene glycol any dioxane formation at the aldehyde groups on the nanoparticle surface was avoided, which would block reaction sites for the amine-Bz-DOTA(^{64}Cu).

Low labeling yields, around 1–5%, were observed for both the cold-copper and copper-64 labeling in TEAA buffer and a 1:1 ethylenediamine/*p*-SCN-Bz-DOTA molar ratio (Tables 2 and 4). The reaction between the aldehyde group on the nanoparticle and the amine of the amine-Bz-DOTA is more efficient at a higher pH, around 9, compared to neutral or slightly basic (conditions seen with the TEAA, pH 8). We therefore used a more basic buffer, sodium carbonate buffer, pH 9.2 (versus pH 8 for the TEAA), for ethylenediamine/*p*-SCN-Bz-DOTA molar ratios of 2:1 and 30:1 (Scheme 3b). By increasing the ratio of ethylenediamine to *p*-SCN-Bz-DOTA and increasing the pH of the reaction during the copper-64 labeling, the highest labeling yield, ~20%, was achieved without an increase in particle size.

3.4. Labeling of ADIO with the Synthesized Amine-Bz-DOTA Resulted in a Greater Labeling Yield Compared to the Commercially Available *p*-NH₂-Bz-DOTA

The two chelators, amine-Bz-DOTA and the commercially available *p*-NH₂-Bz-DOTA, were incubated with ^{64}Cu and then conjugated to the ADIO nanoparticles in parallel (Scheme 3b). The radiolabeling using each of the DOTA derivatives is shown in Table 5, and the labeling efficiency for the amine-Bz-DOTA was ~32% greater than that of the *p*-NH₂-Bz-DOTA. This could have been due to the ethylenediamine linker, which may decrease the steric hindrance between the DOTA and the nanoparticle.

4. CONCLUDING REMARKS

From iron oxides for magnetic resonance imaging (MRI) to quantum dots for optical imaging, nanotechnology has come to play an important role in many imaging applications. The highly complementary nature of MRI and PET has resulted in great interest in combined MR/PET imaging (2, 40); however, there are very few reports of multimodal PET/MRI contrast agents (5, 19). Our recent successes in synthesizing dextran sulfate coated iron oxide nanoparticles (ADIO) (28) and acquiring simultaneous PET and MR images (1), as well as the scarcity of suitable imaging probes for PET/MR imaging made it desirable to synthesize nanoparticles labeled with ^{64}Cu . Labeling was recalcitrant to traditional coupling methods and new syntheses had to be developed to achieve labeling of nanoparticles with ^{64}Cu .

In summary, radiolabeling was not achievable for either nanoparticle system if the metal (Cu^{2+}) was not first chelated in the DOTA before conjugation. Radiolabeling yields for the polystyrene beads was 24% and was achieved by conjugating the *p*-SCN-Bz-DOTA(Cu^{2+}) to the amines on the polystyrene surface. For the ADIO nanoparticles, however, amine groups had to be introduced onto the *p*-SCN-Bz-DOTA to avoid the cross-linking that occurred when the bifunctional ethylenediamine was used to functionalize the ADIO surface. Radiolabeling of the aldehyde-functionalized ADIO nanoparticles with amine-Bz-DOTA(Cu^{2+}) was improved from 1% to 5% by using SEC instead of ethylene glycol to remove the HIO_4 during purification of the aldehyde-functionalized ADIO nanoparticles. Further improvement of radiolabeling of the ADIO nanoparticles was achieved by increasing the molar ratio of ethylenediamine to *p*-SCN-Bz-DOTA to 30:1 from 1:1. Furthermore, the synthesized amine-Bz-DOTA [2-*p*-(3-aminopropylthioureido)benzyl-1,4,7,10-tetraazacyclododecane-tetraacetic acid] had a radiolabeling yield that was 32% more efficient compared to the commercially available *p*-NH₂-Bz-DOTA. This increase in labeling of the amine-Bz-DOTA was most likely due to the extended linker that the ethylenediamine provided, compared to the NH₂ group directly

off of the benzyl group of the commercial compound, and thus resulted in less steric hindrance. The radiolabeling yield for the *p*-SCN-Bz-DOTA on the ADIO was ~21%, which is ample for our imaging applications; due to the extreme sensitivity of PET, even modest levels of labeling are sufficient for detection of the compound.

It is important to note that the presence of amines on a surface does not guarantee that traditional amine-coupling methods will work, as we found with the nanoparticles of two different types. The nanoparticle environment posed an unexpected challenge for conjugation. For example, compared to the dextran-coated particles described by Nahrendorf et al., where the authors first chelate the DTPA to the nanoparticle surface and then purify the nanoparticle-DTPA(⁶⁴Cu) by centrifugation (19), we found that, if the DOTA was chelated to the nanoparticle (e.g., polystyrene) and then incubated with ⁶⁴Cu, centrifugation alone did not remove surface-bound (not chelated) ⁶⁴Cu. In our polystyrene system, this surface-bound ⁶⁴Cu was easily removed by chromatography. The stability constants for DTPA or DOTA complexes with copper are similar, 21.5 and 22.25, respectively (25, 41), but slightly higher for DOTA. It is possible for the polystyrene bead system that the large number of amine groups at the particle surface presented a competition binding site for the Cu²⁺; if the metal was not preinserted into the chelator before conjugation to the nanoparticle surface, then the Cu²⁺ bound to the surface of the nanoparticle rather than the DOTA and could be removed by chromatography. Similarly, the Cu²⁺ could have interacted with high negative charge on the surface of dextran sulfate coated particles (ADIO) rather than the DOTA, which could explain why in our ADIO system we had to preinsert the Cu²⁺ into the DOTA before conjugation compared the method used by Nahrendorf et al. with the more neutral charge of the dextran-coated particles.

Additionally, for the polysaccharide-coated (dextran sulfate) iron oxide nanoparticles there were several parameters that had to be considered, including the affect of surface modification on particle aggregation (e.g., functionalizing ADIO surface with ethylenediamine) and preinsertion of the metal into the chelator before conjugation to the nanoparticle surface. The ethylenediamine used here to introduce amine groups most likely resulted in cross-linking between nanoparticles as a result of the bifunctionality of the ethylenediamine and numerous aldehyde groups present on the nanoparticle surface.

The methods developed here can be applied to other nanoparticle systems with amine-functionalized surfaces, or it can be used with other functional groups such as *N*-hydroxysuccinimide esters, thiol, or carboxyl functional groups. One can envision the use of the multimodal contrast agent developed here for whole body screening using PET to identify potential lesions, then applying MRI to obtain detailed information on plaque structure and composition. We are currently applying these probes for multimodal imaging in an animal model of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A schematic illustration of the conjugation of the metal chelating ligand to the surface of a polystyrene bead.

Scheme 1. Amination of ADIO Nanoparticles^a

^aThe dextran coating was opened with periodic acid and the aldehyde group conjugated to ethylenediamine to generate amine functionalized nanoparticles. The bifunctionality of the ethylenediamine lead to cross-linking of the nanoparticles. R = OSO₃⁻ or OH.

Scheme 2.

Amine “Activation” of the *p*-SCN-Bz-DOTA for Coupling to the Dextran Sulfate Coated Nanoparticles

Scheme 3. Conjugation of *p*-SCN-Bz-DOTA-Copper to the Dextran Sulfate Coated Nanoparticles^a

^a (a) Initial synthesis where ethylene glycol was used to quench the HIO₄ (b) No ethylene glycol is used and SEC is used to purify the aldehyde modified nanoparticles and the final product after reduction of the imine bond between the amine-Bz-DOTA(⁶⁴Cu) and the nanoparticles.

Table 1Radiolabeling of ADIO Nanoparticles^a

particle ^b	E.D.: <i>p</i> -SCN-Bz-DOTA	% yield (⁶⁴ Cu)
ADIO	1:1	0 ^c
ADIO	1:1	1
ADIO	2:1	5.2
ADIO	30:1	21.5

^aThe yield was determined by measuring the activity level before conjugation and then after purification of the final product. Size was determined by DLS.

^bParticle size of the ADIO before labeling was 32 ± 11.8 nm as determined by DLS and was 26.6 ± 7.3 nm after labeling with copper-64 (for the product that had a yield of 21.5%).

^cCopper-64 was not prechelated into the *p*-SCN-Bz-DOTA and no labeling was achieved.

Table 2Conjugation of *p*-SCN-Bz-DOTA-Cu to the Polystyrene Bead Surface^a

size of PS-bead	<i>p</i> -SCN-Bz-DOTA:bead	number DOTA-Cu/bead	% yield (Cu)
78 nm	4 968: 1	150	3
1000 nm	218 000:1	40 000	18

^aThe amount of Cu was determined by ICP-MS after particle purification. Particle size was given by the manufacturer and confirmed by DLS.

Table 3Conjugation of *p*-SCN-Bz-DOTA-Cu to ADIO Surface^a

molar ratio Fe/ <i>p</i> -SCN-Bz-DOTA	particle size	number <i>p</i> -SCN-Bz-DOTA-Cu/ADIO	% yield (Cu)
11.4: 1	31.6 ± 9.1	7.0	0.8
3.8: 1	33.1 ± 8.4	16.1	0.6
1.9: 1	36.5 ± 10.4	328.8	6.0

^aThe amount of Fe and Cu were determined by AAS after particle purification. The amount of iron per core was calculated based on an average particle size of 7.9 nm and one core per ADIO particle was assumed. Particle size in solution was determined by DLS.

Table 4Radiolabeling of Polystyrene Beads^a

size of PS bead	<i>p</i> -SCN-Bz-DOTA:bead	activity used (mCi ⁶⁴ Cu)	labeled activity (mCi ⁶⁴ Cu)	% yield (⁶⁴ Cu)
78	1.13 × 10 ⁶ : 1	2.35	0.571	24
78	1.31 × 10 ⁶ : 1	2.9	0.639	22
1000	1.54 × 10 ⁸ : 1	4.02	0.272	6.8

^aThe yield was determined by measuring the activity level before conjugation and then after purification of the final product.

Table 5Radiolabeling Yield for ADIO-DOTA(⁶⁴Cu)^a

chelator	labeling yield (%)
<i>p</i> -NH ₂ -Bz-DOTA	6.2
amine-Bz-DOTA	9.1

^aRunning the labeling procedure in parallel using the same batch of ⁶⁴CuCl, the radiolabeling was more efficient (32%) using the synthesized amine-Bz-DOTA.