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Development and application of a multimodal contrast agent for SPECT/CT hybrid imaging

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Abstract

Hybrid or multi-modality imaging is often applied, in order to take advantage of the unique and complementary strengths of individual imaging modalities. This hybrid non-invasive imaging approach can provide both critical information about anatomical structure in combination with physiological function or targeted molecular signals. While recent advances in software image fusion techniques and hybrid imaging systems have enabled efficient multimodal imaging, accessing the full potential of this technique requires development of a new toolbox of multimodal contrast agents that enhance the imaging process. Toward that goal, we report the development of a hybrid probe for both single photon emission computed tomography (SPECT) and X-ray computed tomography (CT) imaging that facilitates high sensitivity SPECT and high spatial resolution CT imaging. In this work, we report the synthesis and evaluation of a novel intravascular, multimodal dendrimer-based contrast agent for use in pre-clinical SPECT/CT hybrid imaging systems. This multimodal agent offers a long intravascular residence time ($t_{1/2} = 43$ min) and sufficient contrast-to-noise for effective serial intravascular and blood pool imaging with both SPECT and CT. The co-localization of the dendritic nuclear and X-ray contrasts offers the potential to facilitate image analysis and quantification by enabling correction for SPECT attenuation and partial volume errors at specified times with the higher resolution anatomic information provided by the circulating CT contrast. This may allow absolute quantification of intramyocardial blood volume and blood flow and may enable the ability to visualize active molecular targeting following clearance from the blood.

Introduction

Hybrid or multi-modality imaging is often applied in the evaluation and management of cardiovascular disease, in order to take advantage of the unique and complementary strengths of individual imaging modalities¹⁻⁵. This has become particularly critical when

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Supporting Information

Experimental details regarding the efficiency of ^{99m}Tc reduction and assessment of radiopurity of ^{99m}Tc-G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂] are presented. This material is available free of charge via the internet at <http://pubs.acs.org>.

combining targeted molecular or physiological imaging with anatomic imaging. Toward this goal, efforts have been directed at combining modalities like magnetic resonance imaging (MRI) or X-ray computed tomography (CT) possessing high spatial resolution for evaluation of anatomical structure with those that possess high sensitivity like single photon emission tomography (SPECT) and positron emission tomography (PET) for detection of molecular or physiological signals. This has been accomplished, both by design and implementation of hybrid imaging systems⁶⁻¹¹ and/or use of software fusion of images collected with multiple independent imaging systems¹²⁻¹⁴. Multimodal imaging with hybrid imaging systems combine multiple, independent probes to achieve high sensitivity and high-resolution anatomic visualization with multiple modalities (e.g. SPECT/CT). On the other hand, software image fusion methodologies use a single probe incorporating multiple forms of contrast media along with multiple imaging modalities to adequately obtain desired information (e.g. MRI/optical). With recent advances in both hardware and software, hybrid imaging systems, specifically, PET/CT, PET/MRI, and SPECT/CT have seen widespread implementation in pre-clinical and clinical applications.

Despite these technological advances, the development of multimodal contrast agents has lagged behind, necessitating the use of multiple independent imaging agents and application of software image fusion techniques^{12, 13, 15, 16}. The limiting factors in the design of multimodal contrast agents for hybrid imaging systems are two-fold: (1) sensitivity differences inherent to the imaging modalities and (2) temporal restrictions related to image acquisition. The main challenge in developing these agents manifests itself within the sensitivities of the modalities¹⁷. Because the modalities providing high spatial resolution often exhibit poor sensitivity, and vice versa, it is essential to achieve the appropriate balance required for the selected application. The other significant challenge arises with the selection of the appropriate radionuclide for incorporation in a radiotracer based imaging approach. The decay half-life governs not only the chemistry, but also the imaging protocols.

For targeted molecular imaging, one needs to design a single cardiovascular contrast agent that possess long enough intravascular residence time to allow sufficient binding to the molecular target, with appropriate blood clearance to enable imaging of both the blood pool and the specific molecular target. This might require use of radioisotopes with relatively long decay half-life. Radiotracer based dynamic imaging approaches have remained the mainstay for evaluation of a physiological parameter like absolute regional myocardial blood flow or receptor binding. However, the lower resolution of SPECT and PET approaches require knowledge of anatomical structure in order to correct for attenuation and partial volume errors associated with limited resolution of these radiotracer approaches. To date, assessment of cardiovascular structure and function requires either software image fusion of multiple modalities with single multimodal probes^{16, 18-20} or hybrid system imaging with multiple probes, for example, Omnipaque and ^{99m}Tc-NC100692 for CT and SPECT, respectively²¹. An integrated imaging approach that employs a hybrid probe, incorporating contrast moieties to achieve both high spatial resolution (e.g. MRI or CT active) and high sensitivity (e.g. PET or SPECT active) with hybrid imaging systems, has the potential to efficiently and non-invasively generate the essential complementary sets of information required for diagnosis with a single dose and one imaging session.

Incorporation of the different contrast enhancing moieties within a single agent is greatly facilitated by core molecules manifesting multifunctional architectures. Satisfying this criterion, dendrimers, a class of hyperbranched polymers²², possess a high degree of surface functionality and have been routinely utilized as macromolecular contrast agents for many pre-clinical imaging modalities, including MRI^{15, 23-28}, CT^{12, 29-31}, PET³² and SPECT³³⁻³⁶.

Though multimodal dendrimer-based contrast agents have been developed, their utility has been limited to software image fusion techniques (e.g. MRI/optical).

In this work, we report the synthesis and evaluation of a novel, multimodal blood pool dendrimer-based contrast agent designed for use in pre-clinical hybrid microSPECT/CT imaging systems with direct applicability to hybrid clinical imaging systems. This long-circulating dendrimer, comprising both triiodinated moieties and chelated ^{99m}Tc , provides effective, simultaneous contrast enhancement in both CT and SPECT, respectively.

Materials and Methods

Synthesis of G4-[Ac]

Five hundred milligrams of ethylenediamine-core PAMAM generation 4 dendrimers (PAMAM(G4)) [Sigma Aldrich] were desiccated in a 100 mL round bottom flask and subsequently dissolved in 100 mM sodium bicarbonate buffer (pH 9.0) to a final concentration of 10 mg/mL with magnetic stirring. To this dendrimer solution, 36 molar equivalents of sulfosuccinimidyl acetate [Thermo Scientific] were added as a solid and allowed to dissolve. The pH of the reaction mixture was immediately adjusted to 8.5 with 1N NaOH and the reaction was allowed to proceed for 2 hours at 25°C. The partially acetylated PAMAM(G4) product was purified by ultrafiltration with deionized water using 10K MWCO Amicon Ultra-15 filtrators [Millipore] and lyophilized to obtain a white crystalline solid (yield: 97%). The product was characterized by proton nuclear magnetic resonance (^1H NMR) spectroscopy (D_2O , 400 MHz); δ = 1.91 (105H, COCH_3), 2.28-2.42 (248H, C- CH_2CONH), 2.50-2.61 (124H, N- $\text{CH}_2\text{CH}_2\text{NH}$), 2.68-2.80 (248H, N- $\text{CH}_2\text{CH}_2\text{CO}$), 3.14-3.36 (318H, CONH-CH_2). Extent of surface acetylation was determined according to the method of Majoros *et al.*³⁷

Synthesis of G4-[[Ac]-TIBA]

Activation of 2,3,5-triiodobenzoic acid (TIBA)—Two hundred eighty-seven milligrams of 2,3,5-triiodobenzoic acid (18 mol eq.) were added to a 20 mL glass scintillation vial and dissolved in anhydrous dimethyl sulfoxide (DMSO) [Sigma Aldrich] to a final concentration of 50 mg/mL with magnetic stirring. To the solution of TIBA, 2 molar equivalents of both N-hydroxysuccinimide [Thermo Scientific] and N,N-dicyclohexylcarbodiimide [Thermo Scientific] were added and allowed to dissolve. The reaction was allowed to proceed for 1 hour at 25°C in the absence of light. The reaction mixture was then filtered with a 0.22 μm PTFE syringe filter to remove the dicyclohexylurea (DCU) reaction byproduct and the filtrate was retained.

Coupling of G4-[Ac] to TIBA—A three-necked round-bottom flask was charged with 500 milligrams of G4-[Ac] and purged with argon. The G4-[Ac] was then dissolved in 60 mL anhydrous DMSO with magnetic stirring under an argon atmosphere. To this solution, the entire contents of the filtered activated TIBA solution were added via syringe without further purification. The reaction proceeded for 24 hours at 25°C under an argon atmosphere in the absence of light. The reaction mixture was then diluted with 10 volumes of deionized water and subsequently filtered with a 0.22 μm PES vacuum filtration system. The filtrate was purified into deionized water by ultrafiltration using 10K MWCO Amicon Ultra-15 filtrators and lyophilized to obtain a light brown solid (yield: 82%). The product was characterized by ^1H NMR spectroscopy (D_2O , 400 MHz); δ = 1.91 (105H, COCH_3), 2.28-2.42 (248H, C- CH_2CONH), 2.50-2.61 (124H, N- $\text{CH}_2\text{CH}_2\text{NH}$), 2.68-2.80 (248H, N- $\text{CH}_2\text{CH}_2\text{CO}$), 3.14-3.36 (348H, CONH-CH_2), 7.49 (15H, aromatic), 8.21 (15H, aromatic).

Synthesis of G4-[[[Ac]-TIBA]-DTPA]

Six hundred milligrams of G4-[[Ac]-TIBA] were added to a 100 mL round bottom flask and subsequently dissolved in 100 mM sodium bicarbonate buffer (pH 9.0) to a final concentration of 10 mg/mL with magnetic stirring. To this dendrimer solution, 4 molar equivalents of 2-(4-isothiocyanatobenzyl)-diethylenetriaminepentaacetic acid [Macrocyclics] dissolved in anhydrous DMSO were added. The pH of the reaction mixture was immediately adjusted to 8.5 with 1N NaOH and the reaction proceeded for 18 hours at 25°C in the absence of light. The product was purified by ultrafiltration with deionized water using 10K MWCO Amicon Ultra-15 filtrators and lyophilized to obtain a pale yellow solid (yield: 89%). The product was characterized by ¹H NMR spectroscopy (D₂O, 400 MHz); δ = 1.91 (105H, COCH₃), 2.28-2.42 (248H, C-CH₂CONH), 2.50-2.61 (124H, N-CH₂CH₂NH), 2.68-2.80 (248H, N-CH₂CH₂CO), 3.14-3.36 (348H, CONH-CH₂), 7.18 (8H, aromatic), 7.27 (8H, aromatic), 7.49 (15H, TIBA aromatic), 8.21 (15H, TIBA aromatic).

Synthesis of G4-[[[[Ac]-TIBA]-DTPA]-mPEG₁₂]

Four hundred ninety milligrams of G4-[[[Ac]-TIBA]-DTPA] were added to a 100 mL round bottom flask and subsequently dissolved in 100 mM sodium bicarbonate buffer (pH 9.0) to a final concentration of 10 mg/mL with magnetic stirring. To this dendrimer solution, 8 molar equivalents of succinimidyl-(N-methyl-dodecaethyleneglycol) ester [Thermo Scientific] dissolved in anhydrous DMSO were added. The pH of the reaction mixture was immediately adjusted to 8.5 with 1N NaOH and the reaction proceeded for 12 hours at 25°C in the absence of light. The product was purified by ultrafiltration with deionized water using 10K MWCO Amicon Ultra-15 filtrators, filtered with a 0.22 μm MCE syringe filtered, and lyophilized to obtain a pale yellow solid (yield: 83%). The product was characterized by ¹H NMR spectroscopy (D₂O, 400 MHz); δ = 1.91 (105H, COCH₃), 2.28-2.42 (248H, C-CH₂CONH), 2.50-2.61 (124H, N-CH₂CH₂NH), 2.68-2.80 (248H, N-CH₂CH₂CO), 3.14-3.36 (360H, CONH-CH₂), 3.5-3.76 (336H, -OCH₂CH₂), 7.18 (8H, aromatic), 7.27 (8H, aromatic), 7.49 (15H, aromatic), 8.21 (15H, aromatic).

¹H NMR spectroscopy

All samples were dissolved in D₂O (20 mg/mL) for characterization by proton nuclear magnetic resonance (¹H NMR) spectroscopy on a 400 MHz Bruker spectrometer with the solvent as a reference. Extent of surface conjugation was determined by analysis of peak integrations.

Dynamic light scattering (DLS)

PAMAM(G4) and G4-[[[[Ac]-TIBA]-DTPA]-mPEG] were dissolved in sterile 1x PBS (pH 7.4) at a final concentration of 5 mg/mL and filtered with a 0.22 μm MCE syringe filter. Following filtration, the hydrodynamic diameter of the solutions was assessed with a Zetasizer Nano ZS-90 (Malvern Instruments) in triplicate sequential runs. The hydrodynamic diameter was obtained from volume distribution fitting averaged over the triplicate runs.

X-ray attenuation measurements

To evaluate the X-ray attenuation properties of G4-[[[[Ac]-TIBA]-DTPA]-mPEG₁₂], 50 milligrams of G4-[[[[Ac]-TIBA]-DTPA]-mPEG₁₂] dissolved in 0.3 mL of deionized water were serially diluted. Nine hundred projections of the serially diluted phantoms were acquired at 90 kVp and 40 mA on a pre-clinical microCT scanner [Explore CT120, GE Healthcare] with 16 msec exposure time in the presence of an external reference containing bone, air and water for subsequent calibration. Three-dimensional (3D) CT images were reconstructed through a filtered back-projection algorithm and transferred to an image

processing workstation [Advantage Windows 4.4, GE Healthcare] for analysis. X-ray attenuation values were obtained in Hounsfield units (HU) averaged over the 3D-based region of interest (ROI).

Animal protocol

All animal experiments were performed in accordance with the regulations set by the Yale University Institutional Animal Care and Use Committee (IACUC). Healthy, wild-type C57BL/6 mice fed on a normal diet (n=29, female, 6-8 weeks of age, 19-20 grams, Jackson River Laboratories) were used in these studies. All surgical catheterizations with PE-10 polyethylene tubing and injections were performed under anesthesia (1-2% isoflurane/100% oxygen). All serial in vivo imaging studies were performed under anesthesia (1-2% isoflurane/100% oxygen) with electrocardiographical (ECG) monitoring [AccuSync 71, AccuSync Medical Research Corporation].

Preparation of small-volume reduction ampoules

Small-volume reduction ampoules were prepared according to a previously established method³⁸ with slight modifications. A 20 mL screw-cap glass scintillation vial was charged with 10 mL deionized water and purged with nitrogen for 30 minutes. To the purged deionized water, sodium bicarbonate (450 mg, 5.36×10^{-3} moles) [Sigma Aldrich], sodium carbonate (60 mg, 5.65×10^{-4} moles) [Sigma Aldrich], and sodium p-aminobenzoate (20 mg, 1.26×10^{-4} moles) were added as solids and mixed with magnetic stirring in a closed system. Tin(II) chloride (1.6 mg, 7×10^{-6} moles) [Sigma Aldrich] and methylenediphosphonic acid (2.5 mg, 1.42×10^{-5} moles) [Sigma Aldrich] were weighed together and added to the buffer salt solution as solids with magnetic stirring. The buffered solution was stirred until a clear solution was obtained. From this solution, either 10, 20, 30 or 40 μ L volumes were aliquotted to 1.7 mL Eppendorf ampoules labeled A, B, C or D, respectively, flash frozen, and subsequently lyophilized. Reduction efficiency was assessed by thin layer chromatography (Supporting Information). The assembled reduction ampoules were stored at -20°C.

Radiolabeling for biodistribution and pharmacokinetics

Sodium pertechnetate ($\text{Na}[^{99\text{m}}\text{TcO}_4]$) was eluted from a $^{99\text{m}}\text{Tc}/^{99}\text{Mo}$ generator using 0.9% saline and diluted in deionized water. For each group (n=4 per time point), 500 μCi of $[\text{Na}[^{99\text{m}}\text{TcO}_4]]$ in 100 μL deionized water was added to a 40 μL assembled reduction ampoule, vortexed and allowed to equilibrate for 2 minutes at 25°C. The entire contents of the ampoule were then combined with a solution of G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂] (20 mg dissolved in 600 μL sterile 1x PBS), vortexed and allowed to equilibrate and chelate for 10 minutes at 25°C. One hundred seventy-five microliters of the final radiolabeled construct (radiopurity >79.7%, Supporting Information) was loaded into 1 mL syringes without further purification.

Radiolabeling for microSPECT/CT imaging

Sodium pertechnetate ($\text{Na}[^{99\text{m}}\text{TcO}_4]$) was eluted from a $^{99\text{m}}\text{Tc}/^{99}\text{Mo}$ generator using 0.9% saline and diluted in deionized water. 500 μCi of $[\text{Na}[^{99\text{m}}\text{TcO}_4]]$ in 50 μL deionized water was added to a 20 μL assembled reduction ampoule, vortexed and allowed to equilibrate for 2 minutes at 25°C. The entire contents of the ampoule were then combined with a solution of G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂] (50 mg dissolved in 250 μL sterile 1x PBS), vortexed and allowed to equilibrate and chelate for 10 minutes at 25°C. Three hundred microliters of the final radiolabeled construct (radiopurity >80%, Supporting Information) was loaded into 1 mL syringes without further purification.

Biodistribution and pharmacokinetics

The biodistribution and pharmacokinetics of ^{99m}Tc -labeled G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ were evaluated in healthy, wild-type C57BL/6 mice (female, 6-8 weeks). Each mouse received a 115 ± 3 μCi dose of the radiolabeled dendrimer construct (^{99m}Tc chelated to 5 mg G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ dissolved in 0.175 mL chelation solution) injected intravenously via the left jugular vein ($n=4$ per time point). Following suturing of the injection site, anesthesia was removed and the mice were euthanized at specific time points (0.5, 1, 2, 4 and 6 hours). Blood samples were collected by cardiac puncture and organs (heart, lungs, liver, spleen and kidneys) were harvested. The blood samples and organs were weighed and counted [Gamma Counter, Cobra Packard] with an appropriate energy window for ^{99m}Tc (full width at half-maximum of 140 keV photopeak) to determine their respective radioactivity counts per minute per gram (cpm/g). The obtained radioactivity counts (cpm/g) were decay corrected to the time of injection and normalized to the injected dose.

MicroCT imaging

Serial in vivo imaging of healthy, wild-type C57BL/6 mice (female, 6-8 weeks) was performed on a pre-clinical microCT scanner [Explore CT120, GE Healthcare] using the CT Fast Scan protocol (220 projections, 70 kVp, 32 mA, and a 16 msec exposure time). Following acquisition of a baseline, pre-contrast scan, either 0.15 mL ($n=2$) or 0.30 mL ($n=2$) of 166.7 mg/mL solutions of G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ dissolved in sterile 1x PBS were injected via left jugular vein catheter over a 10 minute duration to assess optimal dosage to achieve sufficient contrast-to-noise. Following injection of the contrast, mice were imaged serially from 0-90 minutes. Three-dimensional CT images were reconstructed through a filtered back-projection algorithm and further processed with MicroView software [GE Healthcare] and BioImage Suite³⁹.

MicroSPECT/CT imaging

The multimodal properties of ^{99m}Tc -labeled G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ were evaluated in five healthy, wild-type C57BL/6 mice (female, 6-8 weeks) using a pre-clinical microSPECT/CT hybrid system equipped with a gamma-camera having a 2 mm pinhole collimator [X-SPECT, Gamma Medica-Ideas]. Each mouse received a 440 ± 26 μCi dose of the radiolabeled dendrimer construct (^{99m}Tc chelated to 50 mg G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ dissolved in 0.3 mL chelation solution) intravenously via a left jugular vein catheter over a duration of 10 minutes. Following the injection and subsequent 5 minutes of circulation time, the mice were euthanized to eliminate motion artifacts from the chest cavity and imaged in the presence of two porous silica point sources to assist in image fusion. CT scans were acquired with 1024 projections at 75 kVp, 305 μA , 50 μm spot size and magnification factor of 3. Nuclear scans were acquired with 360° counter-clockwise (CCW) rotation, 64 projections, 40 seconds per projection, ROR of 2.7 cm, FOV of 3.6 cm and a 20% energy window centered around 140 keV. CT images were reconstructed with FLEX X-O CT software [Gamma Medica-Ideas] using a filtered back-projection algorithm. Nuclear images were reconstructed using FLEX SPECT software (8 iterations, 4 subsets) [Gamma Medica-Ideas], filtered with a Butterworth filter (Nyquist frequency=0.5, order=6) and subsequently fused with the CT images and visualized using Inveon Research Workstation [Siemens, USA].

Results

Chemistry

Ethylenediamine-core, generation 4 poly(amidoamine) dendrimers (64 terminal amines) exhibit a highly cationic surface with limited water solubility. To increase the water solubility and decrease the overall surface charge arising from residual primary amines, we chose to partially acetylate the surface of PAMAM(G4) dendrimers^{35, 37, 40} with sulfosuccinimidyl acetate (Figure 1). Partial acetylation was necessary to ensure the aqueous solubility of the subsequent intermediate and final products. ¹H NMR characterization of the isolated product G4-[Ac] shows the appearance of a peak at 1.91 ppm corresponding to the methyl protons of the acetyl surface groups (Figure 2a). The degree of acetylation was determined with ¹H NMR according to the method of Majoros *et al.*³⁷, by calculating the ratio of the integrals obtained from the methyl protons of the acetyl groups and the sum of all the methylene protons of the dendrimer (-CH₃/-CH₂ ratio). The number of acetyl groups coupled to the dendrimer surface was determined to be 35 (Table 1).

To transform the partially acetylated dendrimer into a radio-opaque construct, we chose to couple 2,3,5-triiodobenzoic acid to 64% of the remaining dendrimer primary surface amines (Figure 1). To achieve the desired extent of conjugation, a slight molar excess of TIBA was converted to an amine-reactive NHS ester with DCC and NHS. The active NHS ester was retained after filtration to remove insoluble DCU and coupled immediately to the dendrimer surface without further purification. The product G4-[[Ac]-TIBA] showed the appearance of two broad peaks at 7.49 and 8.21 ppm corresponding to the two aromatic protons of TIBA in the ¹H NMR spectrum (Figure 2b). Further, the product showed an increase in the CONH-CH₂ integral (3.14-3.36 ppm) corresponding to the coupling of ~15 TIBA moieties (Table 1). The degree of acylation was further corroborated by comparing the integral values obtained for the aromatic protons of the TIBA moieties and the core methylene protons of the dendrimer (N-CH₂CH₂-NH, 2.50-2.61 ppm). The changes in the dendrimer peak integrations and inherent line-broadening of the aromatic protons indicated covalent attachment of TIBA, rather than complexation. Further, no reaction was observed in the absence of DCC and NHS.

Similar to the work of Zhang *et al.*^{35, 36}, we chose to couple p-SCN-Bn-DTPA, an acyclic chelator, to roughly 28% of the remaining dendrimer primary surface amines for subsequent chelation of the nuclear probe, ^{99m}Tc (Figure 1). The product G4-[[[Ac]-TIBA]-DTPA] showed the appearance of two overlapping broad peaks at 7.18 and 7.27 ppm in the ¹H NMR spectrum (Figure 2c) corresponding to the aromatic protons of the benzyl linker of p-SCN-Bn-DTPA. By comparing the integral values for the aromatic protons of the benzyl linker and the core methylene protons of the dendrimer (N-CH₂CH₂-NH, 2.50-2.61 ppm), the extent of conjugation via isothiocyanate linkage was determined to be ~4 (Table 1). Again, the inherent line-broadening of the aromatic protons indicated covalent attachment of the DTPA via isothiocyanate linkage.

To further enhance the water solubility and intravascular residence time, we chose to PEGylate the remaining primary surface amines with mPEG₁₂-NHS (Figure 1). The product G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ showed the presence of a peak 3.5-3.76 ppm in the ¹H NMR spectrum (Figure 2d) corresponding to the methylene protons (-OCH₂CH₂-) of the 12 ethylene oxide repeat units. By comparing the integral values for these methylene protons and the core methylene protons of the dendrimer (N-CH₂CH₂-NH, 2.50-2.61 ppm), the extent of conjugation was determined to be ~7 (Table 1). The final product, G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂, was reproducibly attained both in terms of extent of surface conjugation and yield.

In vitro evaluation of G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂

To evaluate the X-ray attenuation properties of the dendrimer construct, we acquired images of serially diluted G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ in deionized water (Figure 3) and subsequently converted them to Hounsfield maps for quantitative analysis. Iodine concentrations of the serially diluted dendrimer construct were extrapolated from the linear attenuation versus iodine concentration calibration curve generated from the serially diluted 20% Omnipaque 350 solution (Figure SI.2) for comparison to theoretical iodine concentrations estimated from ¹H NMR. A linear attenuation versus iodine concentration curve, an essential criterion for X-ray contrast agents, was obtained for G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂. The extrapolated iodine concentrations for G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ were observed to be consistent with the estimated iodine weight percent (~19% wt.) determined with ¹H NMR.

Blood clearance and biodistribution

To assess the potential of the multimodal probe for blood pool imaging, we examined both the blood clearance pharmacokinetics and biodistribution of ^{99m}Tc-labeled G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂. Following intravenous injection of the radiolabeled dendrimer construct, the blood and organs were collected from healthy, female C57BL/6 mice (6-8 weeks, n=4 per time point) and subsequently analyzed by gamma counting. To determine the circulation half-life of the construct, a blood clearance curve (Figure SI.5) was generated and fit to the mono-exponential decay equation

$$A=A_0e^{-kt}+C \quad (1)$$

where k is the rate constant. From the curve-fitting analysis, the circulation half-life was calculated to be 0.72 hours (43 minutes). Analysis of the biodistribution showed that the construct was cleared from the blood pool predominantly by the kidneys (Figure 4), as expected given the small hydrodynamic diameter (12.37 ± 6.09 nm) of the final dendrimer construct (Table 1).

Serial blood pool imaging with microCT

The efficacy of G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ as a blood pool contrast agent was assessed in healthy, female C57BL/6 mice (6-8 weeks) with serial cardiac microCT following intravenous injection. We observed intravascular and intraventricular enhancement with an excellent contrast-to-noise ratio in the right and left ventricles of the heart across both doses. This enhancement persisted longer than 90 minutes post-injection (Figure 5a).

Multimodal blood pool imaging with microSPECT/CT

To assess the multimodal capabilities of the dendrimer construct, ^{99m}Tc-labeled G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ was prepared and injected intravenously into healthy, female C57BL/6 mice (6-8 weeks, n=5). Post-mortem microSPECT/CT imaging revealed substantial co-localization of the radioactivity and the dendritic iodinated contrast within the intravascular and intraventricular cavity in the absence of motion artifacts (Figures 5b-d). The intravascular retention, minimal liver accumulation and co-localization of the different contrast media provides evidence for the formation of a sufficiently pure and stable chelate, as either colloidal or free hydrolyzed/reduced ^{99m}Tc would be cleared predominantly by the liver^{41, 42}.

Discussion

With increasing desire to extract both structural and functional information with a single probe, the necessity for development of multimodal probes is evident. Along these lines, multimodal probes have been designed for software image fusion techniques, such as, PET-MRI¹³ CT-MRI^{12, 43, 44} and optical-MRI^{15, 16} however, the design of multimodal probes for hybrid imaging systems is relatively unexplored⁴⁵. Thus, the primary objective of this work was to synthesize a single, multimodal blood pool agent designed to simultaneously offer complementary forms of contrast enhancement for small animal hybrid imaging systems, specifically, SPECT/CT.

We chose generation 4 PAMAM dendrimers as the core structure of the contrast agent for the following reasons: (1) the well-defined, multifunctional surface (64 primary amines) offers the ability to conjugate several different moieties with negligible steric hinderance and (2) dendrimer-based contrast agents have been successfully designed and examined in single, non-invasive imaging modalities, including MRI^{15, 23-28}, CT^{12, 29-31} and SPECT³³⁻³⁶. Partial acetylation of the PAMAM(G4) surface was performed to enhance the aqueous solubility^{35, 37, 40} and abrogate the potential of cytotoxicity and hemolysis derived from the inherent positive surface charge^{40, 46}. We chose to couple TIBA to ~25% of the initial surface amines to obtain sufficient iodine weight percent necessary for effective X-ray attenuation without sacrificing aqueous solubility. Further, TIBA was selected because it imparts similar X-ray attenuation properties to the FDA-approved, small-molecule triiodobenzoic acid derivative (Omnipaque 350, GE Healthcare) routinely used in clinical applications. To generate the multimodal character of the agent, the acyclic chelator, DTPA, was chosen to chelate tin(II)-reduced ^{99m}Tc because of its established chemistry and stability^{35, 36, 47}. Design of effective macromolecular, polymeric contrast agents for blood pool imaging requires long intravascular residence times. To further enhance the circulation half-life and limit clearance by the reticulo-endothelial system (RES)⁴⁸, we chose to PEGylate the remainder of the surface with methoxy-terminated PEG.

The final dendrimer construct, G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂, offers both sufficient X-ray contrast enhancement and sufficiently long circulation half-life. for blood pool imaging with microCT. Following chelation of ^{99m}Tc, demonstration of the multimodal character of ^{99m}Tc-labeled G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ was successfully achieved with sequential cardiac microSPECT/CT image acquisition. This showed significant co-localization of nuclear and X-ray contrast in the heart. While others have reported long-circulating iodine-based contrast agents (e.g. Fenestra and liposomal formulations) and nuclear probes (e.g. ^{99m}Tc-Sestimibi), as well as, dendrimers, for CT and SPECT imaging, combining these forms of contrast into a single probe for hybrid imaging is virtually unexplored. Thus, this work represents the first report of a multimodal dendrimer-based contrast agent designed specifically for use with hybrid imaging systems to acquire blood pool images that exhibit both high spatial resolution and high sensitivity derived from a single probe. Further, the inherent long intravascular residence time offers the potential to enable assessment of cardiac function with both ECG and respiratory-gated microSPECT and microCT, which often imparts long image acquisition times.

Aside from integrating both the high spatial resolution of CT and the high sensitivity of nuclear imaging, the ability to combine the CT contrast and radionuclide in a single construct offers a great advantage for image processing and quantification. The fusion of sequentially obtained SPECT and CT images to gain information regarding anatomical structure and physiological function, such as absolute myocardial blood flow, relies heavily upon the ability to accurately correct the SPECT images for attenuation and partial volume effects. Specifically, this hybrid intravascular SPECT and CT probe would facilitate

determination of intramyocardial blood volume and absolute myocardial blood flow using sequential, hybrid SPECT/CT imaging. Further, the probe would allow correction of SPECT partial volume errors by defining the endocardial and epicardial surfaces with CT imaging, while not compromising the ability to correct non-uniform attenuation within the chest. The multimodal properties of the contrast agent therefore offer the ability to perform these corrections directly with the circulating contrast agent at the precise time of image acquisition.

While the blood residence time is sufficiently long for blood pool imaging applications, simple synthetic modifications to the dendrimer and incorporation of targeting moieties would extend the utility of this construct to targeted imaging applications. For example, changing the acyclic chelator, DTPA, to the cyclic chelator, DOTA, would enable chelation of longer-lived radionuclides like ^{111}In for SPECT and ^{64}Cu for PET. Incorporation of these longer-lived radionuclides would provide sufficiently long nuclear half-lives for detection of the probe at active target sites following clearance from the blood. This would be essential for demonstrating site-specific targeting in vascular models such as angiogenesis and atherosclerosis.

Conclusions

We have successfully demonstrated the synthesis, characterization and application of a radiolabeled, multimodal dendrimer-based contrast agent for pre-clinical microSPECT/CT imaging. The long intravascular residence time and predominant renal clearance of the agent provide optimal characteristics for intravascular and blood pool imaging with microSPECT/CT. The co-localization of the dendritic nuclear and X-ray contrast offers the potential to facilitate image analysis and quantification by enabling correction for SPECT attenuation and partial volume errors at specified times with the higher resolution anatomic information provided by the circulating CT contrast. This may allow absolute quantification of intramyocardial blood volume and blood flow. Finally, the chelation of longer-lived SPECT (e.g. ^{111}In) or PET (e.g. ^{64}Cu) radionuclides might enable the ability to visualize active molecular targeting following sufficient clearance from the blood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic of the synthesis of ^{99m}Tc -labeled G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂].

Figure 2.

^1H NMR characterization of the synthetic route to G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂. ^1H NMR spectra were acquired at 400 MHz for 20 mg/mL solutions of (a) G4-[Ac], (b) G4-[[Ac]-TIBA], (c) G4-[[[Ac]-TIBA]-DTPA], and (d) G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ in D₂O. Peaks are labeled according to the following: (1) 1.91 (COCH₃), (2) 2.28-2.42 (C-CH₂CONH), (3) 2.50-2.61 N-CH₂CH₂NH), (4) 2.68-2.80 (N-CH₂CH₂CO), (5) 3.14-3.36 (CONH-CH₂), (6) 7.49 and 8.21 (TIBA aromatic), (7) 7.18 and 7.27 (DTPA aromatic), and (8) 3.5-3.76 (-OCH₂CH₂).

Figure 3.

X-ray attenuation measurements. Phantom images of serial dilutions of G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ in deionized H₂O acquired at 90 kVp. The intensity values obtained in Hounsfield units averaged over 3D-based ROIs were plotted against iodine concentration. Iodine concentrations for G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ were extrapolated from the linear attenuation calibration curve for Omnipaque 350 (Supporting information) and compared to those estimated from ¹H NMR.

Figure 4. Biodistribution of ^{99m}Tc -labeled G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂. The biodistribution of ^{99m}Tc -labeled G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ revealed relatively long blood residence with predominant renal clearance and excretion.

Figure 5.

Serial microCT and multimodal microSPECT/CT blood pool imaging. (a) Following acquisition of a pre-contrast image and subsequent intravenous jugular vein injection of 50 mg of G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂ dissolved in 0.3 mL sterile 1x PBS (10 minute duration), serial short axis images of the RV/LV blood pool showing significant contrast enhancement were acquired from 0-90 minutes post-injection. Following intravenous jugular vein injection (10 minute duration) of a 440 ± 26 μ Ci dose of the radiolabeled dendrimer construct (50 mg of ^{99m}Tc-labeled G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂) dissolved in 0.3 mL chelation solution), post-mortem microSPECT/CT images of the heart were acquired. The fusion of the (b) microCT and (c) microSPECT coronal images presented in (d) shows significant co-localization of the nuclear and X-ray contrast components.

Table 1Physicochemical properties of G4-[[[Ac]-TIBA]-DTPA]-mPEG₁₂].

Compound	NH ₂	NHCOCH ₃	TIBA	DTPA	mPEG	Molecular Weight	d _{hyd} (nm)
G4	64	n/a	n/a	n/a	n/a	14214 ¹	4.47 ± 1.45
G4-[[[Ac]-TIBA]-DTPA]-mPEG ₁₂	3	35	15	4	7	29762 ²	12.37 ± 6.09

¹ Obtained from Sigma Aldrich.² Obtained from ¹H NMR.