Synthetic Copolymer Kit for Radionuclide Blood-Pool Imaging

Alexei A. Bogdanov, Jr., Ronald J. Callahan, Robert A. Wilkinson, Christopher Martin, Jennifer A. Cameron, Alan J. Fischman, Thomas J. Brady and Ralph Weissleder

MGH-NMR Center, Department of Radiology and Division of Nuclear Medicine, Massachusetts General Hospital, Boston, Massachusetts

A synthetic blood pool imaging agent labeled with ^{99m}Tc is reported. **Methods:** The agent, methoxypolyethylene glycol-poly-L-lysyl-diethylenetriaminepentaacetate monoamide was synthesized from a covalent graft copolymer of methoxypolyethylene glycol succinate (molecular weight 5.1 kD) and poly-L-lysine (molecular weight average 35.6 kD) with subsequent modification of the product with diethylenetriamineacetyl residues. The polymer was formulated into a kit that contained Sn(II) and sodium acetate for radiolabeling with ^{99m}Tc. Biodistribution studies were performed in rats. Blood-pool imaging and blood clearance determination was carried out in rabbits and in a rhesus monkey. **Results:** The ^{99m}Tc-labeled agent [specific activity greater than 3.7 GBq/mg; radiochemical purity more than 98% by thin-layer and high-performance liquid chromatography (HPLC)] demonstrated remarkable stability in solution (pH 5.5–6.5) with no radioactive products of degradation detectable by HPLC even at 24 hr postlabeling. The agent exhibited prolonged circulation in the blood with a half-life of 31.5 hr in rabbits. Biodistribution in rats showed a lack of substantial accumulation of the agent in the reticuloendothelial system. Sequential acquisitions were performed in a rhesus monkey. The ^{99m}Tc-labeled polymer kit was compared with the ^{99m}Tc-red blood cells (RBCs) labeled in vitro. Both methods produced similar heart-to-lung ratios. The ratios remained essentially unchanged for up to 15 hr postinjection. **Conclusion:** The ^{99m}Tc-labeled methoxypolyethylene glycol-poly-L-lysyl-diethylenetriamine pentaacetate monoamide is an attractive alternative to radiolabeled RBCs for blood pool imaging applications.

Key Words: polyethylene glycol; poly-L-lysine; blood pool; imaging; kit

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Scintigraphic blood-pool imaging is a well-established diagnostic tool and is commonly performed in clinical practice (*1*). Major applications include a determination of myocardial ejection fraction (*2–6*) and detection of occult bleeding sites, usually from the gastrointestinal tract (*6–8*) (for other applications, see reference 9). Blood pool imaging is currently performed with ^{99m}Tc-red blood cells (RBCs) (*10*) labeled by a variety of methods. Although the RBC is an ideal natural carrier for a radionuclide, the preparation of ^{99m}Tc-RBCs is a complicated multistage procedure that requires the manipulation of blood products, which may contain hepatitis B or human immunodeficiency viruses. In addition, some in vitro labeling methods carry an increased risk of misadministration of blood products to the wrong patient with the possible transmission of blood-borne diseases. The relatively long time necessary for the preparation of an individual dose (20–30 min/patient) and the low efficiency of labeling because of the effect of some medications (*11*) are also drawbacks of RBC-based blood-pool imaging agents.

A prerequisite for an alternative blood-pool agent is a long circulation in the blood. Some proteins and other natural macromolecules meet this requirement; however, ideal preparations should be nontoxic, preferably nonimmunogenic and biodegradable. In addition, preparations should be available in a kit form to be labeled with readily available isotopes, such as ^{99m}Tc. Previous efforts to design non-RBC agents for blood-pool imaging concentrated on converting natural proteins [serum albumin (*12,13*)] or polysaccharides [dextran (*14,15*)] into molecules capable of efficient binding of ^{99m}Tc, either by formulating kits from native macromolecules (*12–17*) or by the use of additional chemical modification (*18–21*). As shown by several investigators, direct labeling of native macromolecules results in preparations with variable radiochemical purity, which all appear inferior to RBC in respect to their behavior in vivo (*13–15*). Chemically modified human serum albumin (HSA) [e.g., diethylenetriaminepentaacetic acid (DTPA-HSA)] (*17–19*) and mercaptoacyl-HSA (DMP-HSA) (*21*) have recently been shown to have a higher radiochemical purity and better blood retention than parent HSA in comparative ^{99m}Tc-labeling studies. In human trials, DTPA-HSA was used at the dose of 10 mg of DTPA-HSA (20 mCi of ^{99m}Tc) per patient. It was removed from the circulation rapidly with concomitant enhancement of the liver, supposedly because of extravasation of DTPA-HSA (*20*). Most kits that contain DTPA-HSA are thus useful only if imaging is performed within 5 to 10 min after intravenous injection, e.g., as in cardiac imaging (*20*). The fast removal

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For correspondence or reprints contact: Alexei A. Bogdanov, Jr, PhD, MGH-NMR Center, MGH-East, Room 5416, Bldg. 149, 13th St., Boston, MA 02129.

of an agent from the circulation decreases its diagnostic utility for applications such as gastrointestinal bleeding studies. On the other hand, DMP-HSA was reported to be comparable to ^{99m}Tc-RBCs at certain DMP:HSA ratios in animal studies (*21*). It should be noted that these results were reported for ^{99m}Tc-DMP-HSA purified by size-exclusion, high-performance liquid chromatography (HPLC). Moreover, mercapto derivatives of proteins are known to be very sensitive to oxidation (*22*). Therefore, storage stability and lack of batch-to-batch variability in ^{99m}Tc-DMP-HSA preparations have to be experimentally demonstrated before its potential for clinical use can be ascertained.

Although recent progress has been achieved to design better HSA-based macromolecular chelates for ^{99m}Tc, it would be desirable to eliminate the need for human blood products completely, e.g., by using recombinant autologous proteins such as HSA or synthetic carrier molecules.

Results with the latter approach are reported here. A synthetic macromolecular conjugate with a central poly-L-lysine (PL) backbone was used to which protective methoxypolyethylene glycol (MPEG) chains and chelating groups (DTPA moieties) were covalently attached (MPEG-PL-DTPA). The agent forms the central component of a kit formulation, which only requires the addition of ^{99m}Tc-pertechnetate solution before intravenous injection.

MATERIALS AND METHODS

Chemicals

MPEG [molecular weight (MW) 5000] was obtained from Polysciences (Warrington, PA). Succinic anhydride, 4-dimethylaminopyridine, poly-L-lysine hydrobromide (MW 41,000), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and trinitrobenzene sulfonic acid (TNBS) were obtained from Sigma Chemical (St. Louis, MO). N-hydroxysulfosuccinimide and diethylenetetraminepentaacetic acid anhydride were bought from Pierce Chemical (Rockford, IL). Peroxide-free dioxane, ethyl ether and dichloromethane were purchased from Aldrich (Milwaukee, WI). Thin-layer chromatography (TLC) plates (Kieselgel 60F254) were from EM Science (Gibbstown, NJ). The ^{99m}Tc-pertechnetate was obtained from Syncor (Chatsworth, CA).

Synthesis of MPEG Succinate N-Hydroxysulfosuccinimide Ester

MPEG was succinylated and converted into N-hydroxysulfosuccinimide ester essentially as described previously (*23,24*). Briefly, MPEG was treated with a 10-fold molar excess of succinic anhydride in dioxane in the presence of 4-dimethylaminopyridine as a catalyst. Purification was accomplished by recrystallization from ethyl acetate-ether (1:1) with subsequent treatment with AG 50 W X8 resin (Bio-Rad, Hercules, CA). Purified MPEG succinate was reacted with N-hydroxysulfosuccinimide and EDC (*24*).

Conjugation of MPEG Succinate to PL: Preparation of Chelating Polymer

PL was dissolved in 0.1 *M* sodium carbonate buffer (pH 8.5) at concentration of 25 m*M* lysine monomers. MPEG succinate N-hydroxysulfosuccinimidyl ester was added to PL to achieve a concentration of 5 m*M*. The remaining amino groups were modified by adding an excess of DTPA cyclic anhydride [5-fold molar excess over free amino groups determined by TNBS titration (*25*)], added in three portions after solubilization in dimethyl sulfoxide. The rest of the unmodified amino groups were blocked by succinylation. The modified polymer was then purified using diafiltration on a YM 100 (Amicon, Beverly, MA) membrane and was filtered through 0.2-μ nylon filter membrane.

Final purification was performed by passing the polymer solution (200 mg, 50 mg/ml) through a column packed with Sephadex A-25 (1 × 20 cm), equilibrated with water. The flow-through portion was collected and analyzed by HPLC using Hydropore-5-SEC (Rainin Instrument, Woburn, MA) columns with 0.05 *M* sodium phosphate buffer, pH 6.8, as an eluent. The PEG content was determined by the I/KI colorimetric assay (*26*). Elemental analysis was performed at Galbraith Labs (Knoxville, TN).

Kit Preparation

MPEG-PL-DTPA was dissolved at a concentration of 1 mg/ml in deoxygenated sodium acetate solution (0.2 *M*, pH 10) prepared using sterile apyrogenic water. $SnCl_2$ solution in concentrated HCl was added to the solution to achieve a Sn/DTPA molar ratio of 1:1. The resultant pH of the buffer after the addition of the reducer in HCl was 5.1 to 5.2. The kit was formulated by the addition of 1 ml of solution to sterile vials in an atmosphere of inert gas (argon). The vials were then capped, sealed and stored at −70°C.

Labeling Efficiency Determination

To the kit, 5–200 mCi of ^{99m}Tc-pertechnetate was added and TLC was performed with acetone as the mobile phase. Alternatively, samples were analyzed by HPLC with a Zorbax GF-250 (Rockland Technologies, Chadds Ford, PA) column with sodium phosphate buffered saline as the eluent.

Animal Experiments

Biodistribution studies were performed with male Sprague-Dawley rats (n = 6 for each time point) at a dose of 20–22 μCi of ^{99m}Tc-labeled MPEG-PL-DTPA per animal. One group of rats (n = 6) was kept in metabolic cages for 24 hr to determine the amount of radioactivity excreted with urine and feces. The animals were killed at 1, 6 and 24 hr to determine the distribution of the agent in the blood and major organs.

Male New Zealand rabbits (n = 5) were used for gamma camera imaging. The animals were anesthetized with ketamine (40 mg/kg) and xylazine (5 mg/ml) injected subcutaneously. A dose of 3–5 mCi of ^{99m}Tc-labeled MPEG-PL-DTPA in 1–2 ml of saline was injected into the rabbits through an ear vein. In one experiment, a rabbit was injected with 2.21 mCi of ^{99m}Tc-labeled MPEG-PL-DTPA and 105 μCi of ^{111}In-labeled MPEG-PL-DTPA. Both animals were injected with 1 mg of polymer per animal. Blood samples were taken from a central ear artery at the time intervals indicated. Anterior gamma camera images (model 410, Technicare, Solon, OH) were obtained at different time intervals up to 27 hr after injection. Gated cardiac images were acquired at 1, 1.5, 2 and 2.5 hr after the injections with a 3-mm pinhole collimator. Both 70° and 45° LAO images gated for 1200 sec were obtained. The left ventricular ejection fraction (LVEF) was determined using routine software. Nonlinear regression modeling was performed for blood elimination data with Systat 5 for Macintosh software (Systat, Inc, Evanston, IL).

In a male rhesus monkey that weighed 9.8 kg, anterior whole-body images were obtained with a Technicare Gemini gamma camera. The images were acquired on two separate occasions

separated by 7 days. During the first session, the animal was injected with 10 mCi of ^{99m}Tc-labeled MPEG-PL-DTPA. On the second occasion, the animal was injected with 10 mCi of ^{99m}Tc-labeled autologous RBCs labeled with a commercially available in vitro kit (Ultra-Tag, Mallinckrodt Medical, St. Louis, MO).

In both experiments, imaging was performed at 1, 2 and 15 hr after intravenous injection. In addition, anticoagulated whole blood samples were obtained at 5, 15, 30 and 45 min and 1, 2 and 14 hr postinjection. The percent of injected dose per milliliter of blood was determined, and blood clearance rates were calculated.

RESULTS

Polymer

The purified copolymer (MPEG-PL-DTPA) eluted as a single peak by HPLC. This peak represented a population of molecules with an average hydrodynamic radius corresponding to a protein with a molecular weight of 1400 kD (Fig. 1A). The quantitative chemical analysis gave the following results: C, 50.3 ± 2.2 (53.0); O, 34.5 ± 1.7 (35.0); N, 4.5 ± 0.6 (4.0); and H, 8.6 ± 0.3 (8.4). The values in parentheses correspond to theoretic calculations made with the following assumptions: (1) the PL used for the synthesis had a mean of 279 lysine residues (polydispersity 1.1); (2) the degree of modification of PL with MPEG was 32%, as determined by TNBS titration and MPEG quantitative determination; and (3) 96% of the remaining amino groups were substituted with DTPA. These results indicate a molecular structure of $MPEG_{89}$-PL_{279}-$DTPA_{190}$ and an average molecular weight of 550 kD.

To test the availability of DTPA for the chelation of ^{99m}Tc, each batch of the blood-pool agent was tested for chelation of $^{111}In^{3+}$ cations. Gel filtration of the reaction products showed that approximately 98% of the label coeluted with the polymer in the void volume of the Sephadex G-25 column (not shown).

Labeling

The addition of $[^{111}In]^{3+}$ to MPEG-PL-DTPA in the presence of citrate (pH 5.5) resulted in instantaneous high-efficiency labeling. However, attempts to label the copolymer with ^{99m}Tc-pertechnetate in the presence of Sn(II) and citrate/ascorbate or citrate/paraaminobenzoate (pH 5.0) were not successful. The latter preparation contained more than 30% of low-molecular weight impurities. Superior labeling efficiency was achieved when the procedure was carried out in the absence of antioxidants and with sodium acetate as the buffering salt. These results (size-exclusion HPLC and TLC) showed that the labeling efficiency exceeded 98% (specific activity exceeded 3.7 GBq/mg of copolymer, Fig. 1B). The labeled agent did not show any decomposition for at least for 4 hr postlabeling. Even after prolonged incubation (24 hr), the ^{99m}Tc-labeled copolymer eluted as a single peak (Fig. 1C).

Kit Preparations

In all further experiments, kits formulated from 1 mg of polymer in 1 ml of sodium acetate buffer that contained Sn(II) were used. Subsequent labeling was achieved by combining pertechnetate solutions (5–200 mCi) with the contents of the kit. The biodistribution experiments in rats (n = 6 per time point) indicated excellent retention of the agent in the blood pool (Fig. 2), with 50% of the dose in the circulation at 6 hr postinjection. The accumulation of the agent in the liver progressively decreased from 10.6% ± 1.5% dose/organ to 5.9% ± 1.2% dose/organ at 24 hr. The kidneys showed a slightly increased accumulation at 24 hr

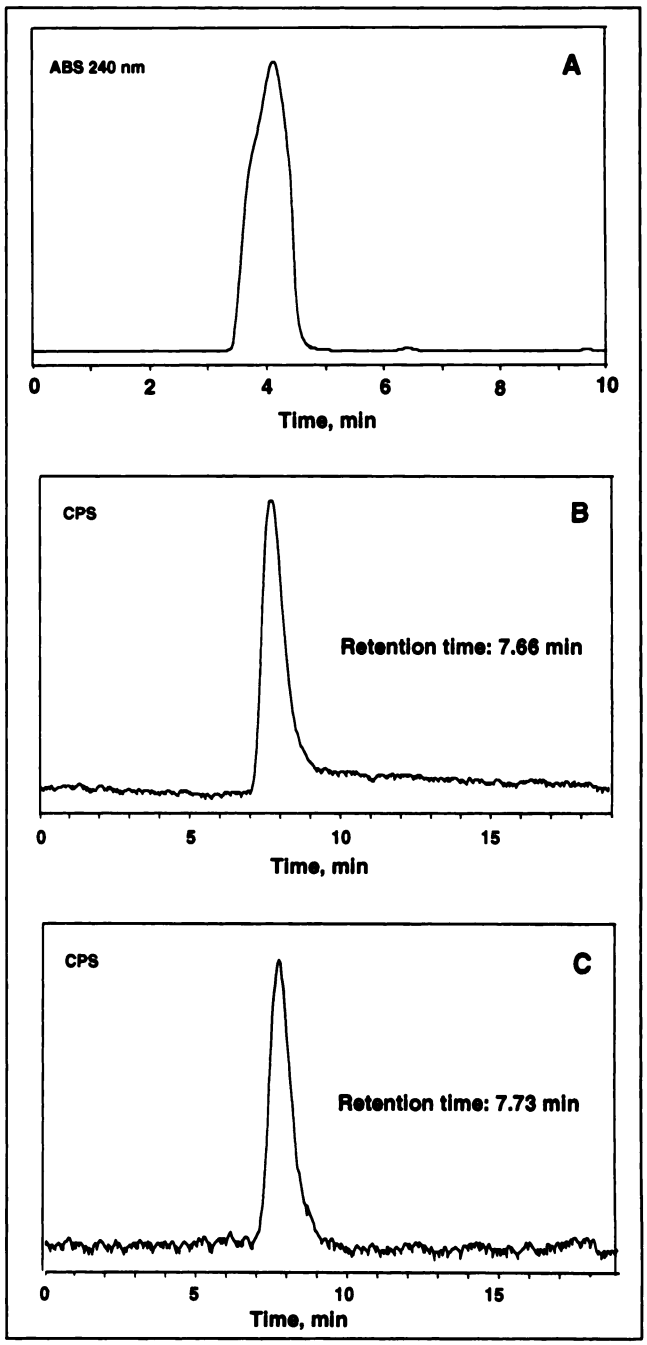

FIGURE 1. Size-exclusion chromatography of MPEG-PL-DTPA. (A) Hydropore-5-SEC chromatography profiles. Elution was done with 0.05 *M* sodium phosphate, pH 6.8, at 0.25 ml/min. (B,C) HPLC profiles of ^{99m}Tc-labeled polymer obtained using a Zorbax-GF-250 column. Polymer was labeled at 200 mCi (3.7 GBq)/mg and analyzed at 1 hr (B) and 24 hr (C) after addition of ^{99m}Tc-pertechnetate.

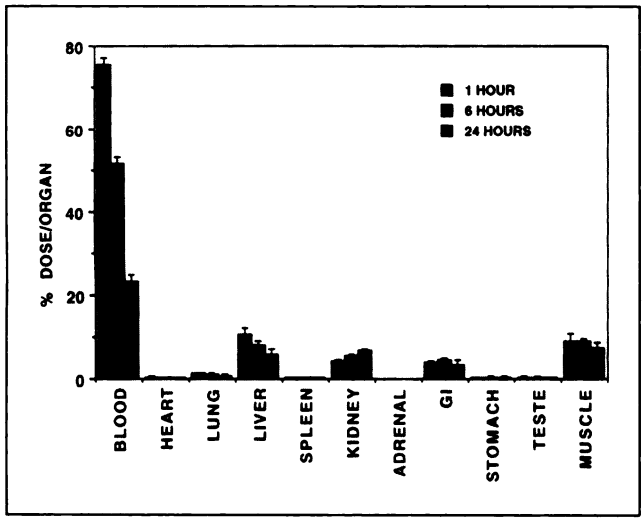

FIGURE 2. Biodistribution of MPEG-PL-DTPA in rats. MPEG-PL-DTPA kit (1 mg of polymer) was labeled with 5 mCi of ^{99m}Tc-pertechnetate. Three groups of rats (n = 6) were injected intravenously with 20 μCi of the polymer per rat at t = 0. Animals were sacrificed at 1, 6 and 24 hr postinjection and biodistribution in the major organs was determined. Results are presented as mean ± s.d.

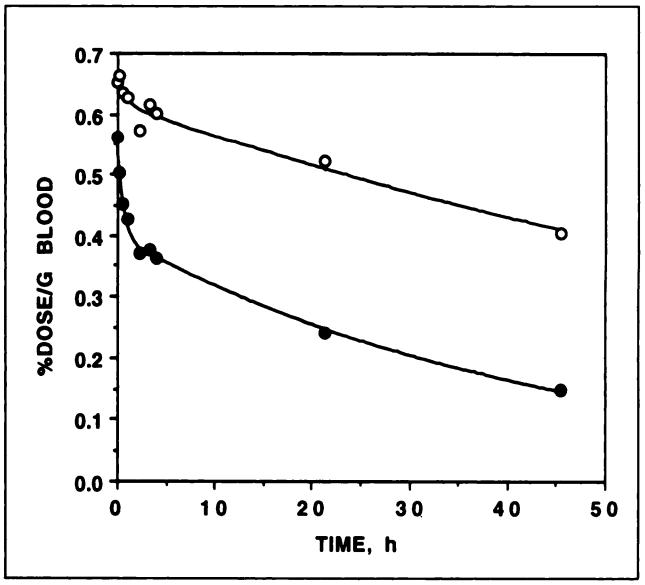

FIGURE 3. Blood elimination of ^{99m}Tc- and ^{111}In-labeled MPEG-PL-DTPA in a rabbit. The rabbit was injected with 2.21 mCi of ^{99m}Tc-labeled MPEG-PL-DTPA and 105 μCi of ^{111}In-labeled MPEG-PL-DTPA (both, 1 mg of polymer per animal). Blood samples were collected at time intervals indicated from central artery. Open symbols = ^{111}In radioactivity; closed symbols = ^{99m}Tc radioactivity.

(6.9% ± 0.2% dose/organ) compared with the 6-hr time point (4.1% ± 0.5% dose/organ). Other organs did not accumulate significant amounts of the injected dose. Approximately 25% of dose was recovered in urine (20.0% ± 2.7%) and feces (4.4% ± 1.3%) during 24 hr postinjection.

Experiments in rabbits performed by measuring the radioactivity in serial blood samples demonstrated a biexponential clearance of ^{99m}Tc-labeled polymer with a fast initial minor component ($T_{1/2}$ = 21.6 min) and a long major second component ($T_{1/2}$ = 31.5 hr, Fig. 3). For the ^{111}In-labeled agent included in the study as a control, the blood half-life was substantially longer (approximately 72 hr), presumably because higher stability of ^{111}In-DTPA complex. The calculated volume of distribution of ^{99m}Tc was 300 ml, a value equivalent to the intravascular volume of the rabbit. Region of interest analysis resulted in the following heart-to-lung ratios (mean ± s.d., n = 2): 2.1 ± 0.3, 2.1 ± 0.3, 2.4 ± 0.2 and 1.9 ± 0.7 at 0.3, 2, 3.4 and 26 hr after injection. The heart-liver ratios were 1.7 ± 0.4, 2.1 ± 0.3, 2.4 ± 0.2 and 1.9 ± 0.5 at the same time points, respectively. Gated images of the heart are shown in Figure 4. The mean initial LVEF was 47.7% ± 3.4%. This value did not change significantly for at least 2.5 hr after injection.

Two studies in a rhesus monkey were performed to compare blood-pool images obtained with the ^{99m}Tc-labeled polymer kit and the standard labeling procedure (^{99m}Tc-RBCs). Both techniques produced high-quality images of the heart, which did not deteriorate significantly over time. The image quality was similar with the two agents, even 15 hr after the injection (Fig. 5). Immediately after the injection, the activity of the liver was notably higher in the case of the labeled polymer than with the RBCs. However, gated cardiovascular images were of similar quality with both labeled agents (Fig. 6). Although heart-to-liver ratios were lower for the ^{99m}Tc-labeled polymer than for RBCs (1.1 versus 1.5), the ratio remained largely unchanged for at least 15 hr postinjection. Heart-to-lung ratios were similar for both labeling methods (Fig. 7).

DISCUSSION

The goal of this study was to synthesize a blood-pool imaging kit that contained an organic copolymer that ful-

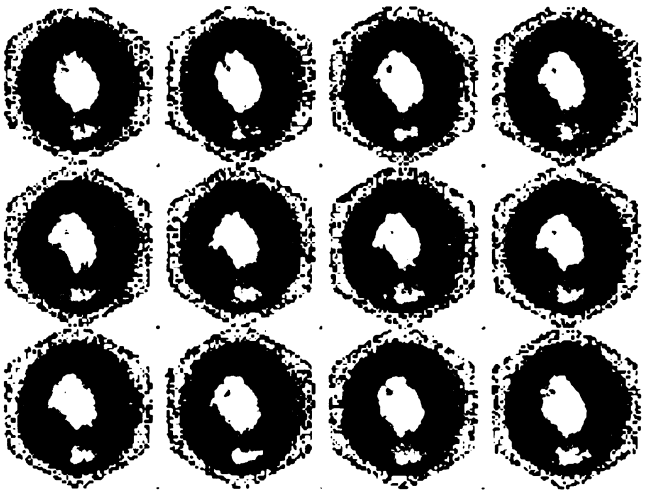

FIGURE 4. Twelve gated blood-pool images of the heart of a rabbit acquired in LAO projection 2 hr after injection of 4 mCi of ^{99m}Tc-labeled MPEG-PL-DTPA. LVEF calculated using these images was 47.7% ± 3.4%.

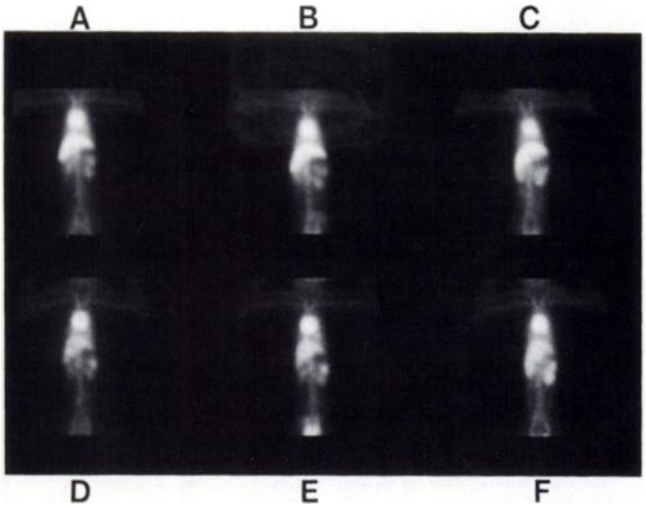

FIGURE 5. Anterior whole-body images obtained in a rhesus monkey after administration of ^{99m}Tc-labeled MPEG-PL-DTPA (A–C) or ^{99m}Tc-RBC (D–F). Images were obtained 1 hr (A and D), 5 hr (B and E) and 15 hr (C and F) after intravenous administration of the radiotracers. Note the excellent delineation of the intravascular blood pool (heart or large arteries) with either agent.

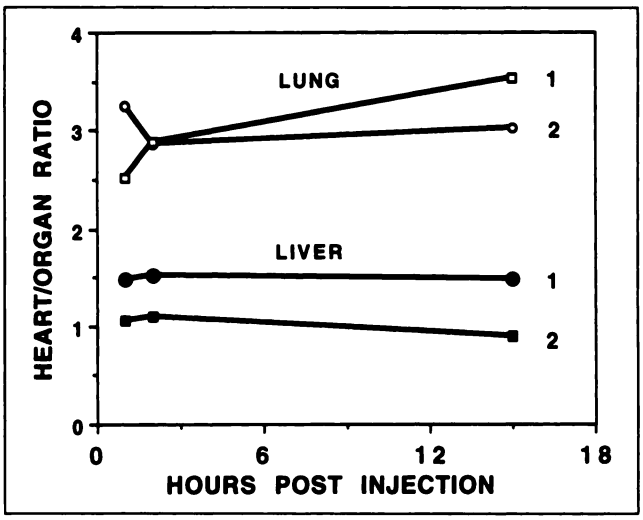

FIGURE 7. Heart-to-lung and heart-to-liver ratios in rhesus monkey determined by ROI analysis in a comparative study. (1) Results obtained with ^{99m}Tc-RBC. (2) Results obtained with ^{99m}Tc-labeled MPEG-PL-DTPA. Closed symbols = heart-liver ratios; open symbols = heart-lung ratios.

filled the prerequisites of long blood half-life, low immunogenicity and low toxicity. It was originally hypothesized that a graft copolymer of MPEG and PL modified with DTPA would have favorable characteristics because MPEG chains are expected to protect the polyamino acid from rapid removal from circulation. The central polyamino acid in turn serves as a carrier for the numerous DTPA residues necessary for the binding of cationic ^{99m}Tc.

PEG is well known to be a nontoxic and biocompatible polymer (*27*) that dramatically reduces the immunogenicity of proteins when covalently bound to their surface amino groups (*28*). In many instances, PEG greatly increases the circulation times of protein conjugates (*29*) and liposomes (*30,31*). Protective properties of PEG may arise from the ability to create a "hydrated shell" in a close proximity to the polypeptide chain or phospholipid membrane and thus mask molecules from recognition and removal from the circulation by the defense systems of the organism. PEG conjugation to a radioisotope carrier ((PL)-DTPA) favorably improves the biologic behavior by (1) increasing the molecular weight and apparent hydrodynamic radius as a result of the presence of highly hydrated polymeric chains, (2) decreasing the recognition of the macromolecule by the reticuloendothelial system and (3) decreasing the immunogenicity of the conjugate (including DTPA residues). Although chelate-bearing polyamino acids [e.g., PL-DTPA, MW 70 kD (*32*)] are known to induce a weak immune response after intravenous injection (*33*), such a response is typically absent after conjugation to MPEG (*23*)].

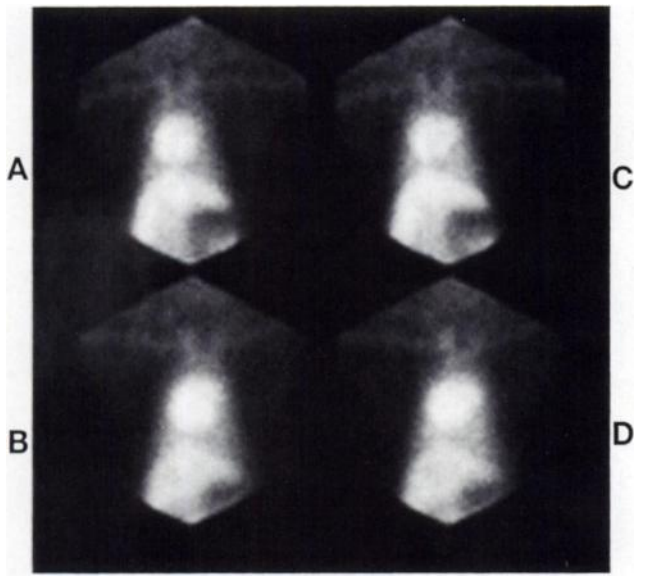

FIGURE 6. Gated cardiac images were obtained in LAO projection (rhesus monkey) 3 hr after administration of ^{99m}Tc-labeled MPEG-PL-DTPA (A and C) or ^{99m}Tc-RBC (B and D). Images were obtained in either end-diastole (A and B) or end-systole (C and D).

Size-exclusion HPLC (Fig. 1A) demonstrates that the synthesized copolymer elutes as a narrow single peak. This copolymer was formulated into a kit that contained a buffering solution and Sn(II) to enable the reductive conversion of pertechnetate to a technetium oxocomplex. To estimate the radiochemical purity and stability of the labeled preparation, further HPLC analysis was done. Chromatography of the ^{99m}Tc-labeled conjugate showed that the copolymer and more than 98% of the radioactivity coeluted as the same peak even 24 hr after the labeling was performed (Figs. 1B and C), i.e., the labeling efficiency was 98%. Moreover, this result implies that the ^{99m}Tc-labeled copolymer prepared from the kit is stable against hydrolytic degradation and radiolysis.

Animal experiments from current and earlier studies show that the copolymer remains in the circulation for long periods and is retained to a large extent in major organs (*23*). A similar biologic behavior of the agent is observed in rats (Fig. 2) and rabbits (Fig. 3). Interestingly, the blood half-life of the ^{99m}Tc-labeled copolymer was approximately twofold lower compared with the ^{111}In-labeled preparation. This may be attributed to a lower affinity of DTPA for reduced ^{99m}Tc with slow rechelation of ^{99m}Tc to some plasma components. These data indicate that at least 25% of MPEG-PL-DTPA is excreted during the first day after injection. A similar excretion pattern has been observed with the ^{111}In-complex of MPEG-PL-DTPA.

In an effort to obtain information on the preclinical utility of the kit for cardiac imaging, rabbit and monkey experiments were performed. Two distinct phases of elimination could be determined in the rabbit with nonlinear-regression analysis of the blood elimination curves. The first component, with a $T_{1/2}$ of 20 min, most likely represents distribution of a minor fraction (approximately 20% of the injected dose) to the extravascular space and the kidneys. The remaining dose (80%) is eliminated from the blood with a $T_{1/2}$ of 31.5 hr. The slow rate of elimination of the agent allows acquisitions of high-quality whole-body planar and gated images at 0.5–4 hr postinjection (Fig. 4). A comparative imaging experiment between the copolymer and labeled RBCs was performed in a rhesus monkey. These data indicate that image quality (Fig. 5) is similar for both agents. Corresponding heart-to-lung and heart-to-liver ratios measured at 1, 2 and 15 hr postinjection showed little changes in time for both agents. However, ^{99m}Tc-labeled RBCs were less actively trapped in the liver than MPEG-PL-DTPA, resulting in a higher heart-to-liver ratio (1.5 versus 1.1, Fig. 7). This did not impair the ability to obtain high-quality gated images at 3 hr postinjection (Fig. 6).

From the results of the experiment presented here, it was concluded that the synthetic copolymer (MPEG-PL-DTPA) may be formulated in a kit and that it can be efficiently labeled with ^{99m}Tc. The unique combination of excellent blood-pool retention and simple labeling procedure and stability make this compound an attractive substitute for blood products in blood-pool imaging.

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