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[^{99m}Tc]MAG$_3$-*mannosyl*-dextran: a receptor-binding radiopharmaceutical for sentinel node detection

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Abstract

Technetium-99m-labeled benzoyl-mercaptoacetylglycylglycyl-glycine-*mannosyl*-dextran ([^{99m}Tc]MAG$_3$-*mannosyl*-dextran) is a receptor-binding radiotracer that binds to mannose-binding protein, a receptor expressed by recticuloendothelial tissue. This agent is composed of a 10.5-kilodalton molecule of dextran and multiple units of mannose, and benzoyl-mercaptoacetylglycylglycyl-glycine (BzMAG$_3$). The tetraflorophenol-activated ester of BzMAG$_3$ and the imidate of thiomannose were used to covalently attach BzMAG$_3$ and mannose to an amino-terminated conjugate of dextran. This yielded a 19-kilodalton macromolecule consisting of 3 BzMAG$_3$ and 21 mannose units per dextran. Dynamic light scattering was used to measure a mean diameter of 5.5 nanometers for BzMAG$_3$-*mannosyl*-dextran and 0.28 microns for filtered Tc-99m sulfur colloid. A preliminary sentinel node detection study employing right fore and hind footpad injections of [^{99m}Tc]MAG$_3$-*mannosyl*-dextran and left fore and hind footpad injections of filtered Tc-99m sulfur colloid demonstrated greater sentinel lymph node uptake by the receptor-binding agent.

Keywords: Receptor-binding; Technetium-99m; MAG$_3$; Sentinel node detection

1. Introduction

Lectins [26] are receptors that recognized and bind specific carbohydrate bearing macromolecules. At the time of the first Receptor-Binding Radiotracer Workshop [10] only a small number of mammalian lectins have been identified. Figure 1 contains a list of mammalian lectins that we considered [35] as candidate targets for a receptor-binding radiotracer. These included lectins specific to hepatocytes [36], recticuloendothelial cells [25], myocardial cells [21, 37], and thrombin-activated platelets [14]. We selected the hepatic binding protein receptor as the molecular target for [^{99m}Tc]galactosyl-neoglycoalbumin [30]. A common theme of this early list was the simplicity of the carbohydrate specificity—all of the lectins recognized a monosaccharide. During the intervening twenty years lectins have been discovered with carbohydrate specificities of greater complexity. Some examples are: fucose-terminated tri-saccarharide-specific lectins involved in cell-cell interactions of the early mammalian embryo [34], and sialyl-terminated tetra-saccarharde-specific selectins, which recruit neutrophils to sites of endothelial inflammation [20]. Additionally, knowledge of their tissue distribution has been extended to include the immune system [33], the central and peripheral nervous system [45], and malignantly transformed cells [18].

Sentinel node biopsy is gradually replacing lymph node dissection in breast cancer patients [28]. The sentinel node is the first lymph node that receives drainage from a given anatomic area of the body [32]. Its location is determined by injection of a radiopharmaceutical [2] around the tumor site as well as a peritumoral injection of isosulfan blue dye [1,15]. Lymphoscintigraphy is often performed preoperatively to localize the sentinel node on the day of surgery. The blue dye is typically injected at the beginning of surgery to facilitate the actual visualization of the sentinel node in the operative field. A multitude of studies have been performed whereby the sentinel node was removed and examined pathologically [29]. Patient's in these studies then proceeded to standard levels I and II axillary node dissection. The sentinel node technique has been found to be an accurate predictor of lymph node status [4,8,17], with a false negative rate between zero and 12%. The sentinel node

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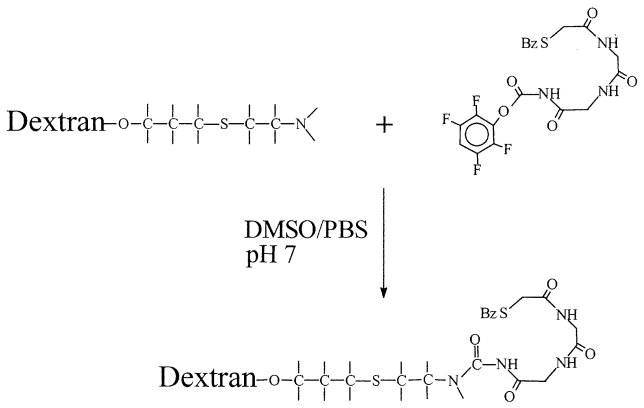

Fig. 1. Covalent attachment of $BzMAG_3$ to an amino-terminated leash of dextran.

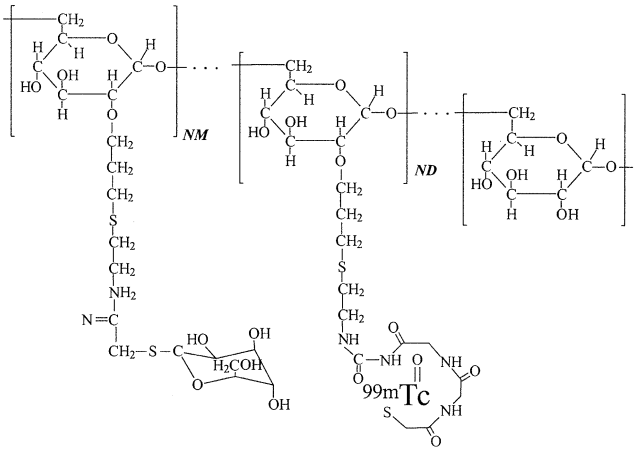

Fig. 2. [^{99m}Tc]MAG_3-*mannosyl*-dextran dextran consisted of 21 mannose units and 3 MAG_3 units per dextran. The molecular weight was 19,389 g/mole.

technique has also allowed for a more detailed pathologic evaluation of one specific lymph node via serial sectioning and immunohistochemistry. As such, micrometastatic disease is being discovered that previously was unrecognized. Therefore, the technique offers a smaller surgical dissection, minimal reduction in diagnostic accuracy compared to a standard axillary dissection, and the ability to focus detailed histopathological analyses to a single node [7,43].

Numerous radiopharmaceuticals are presently utilized for sentinel node detection: Tc-99m-sulfur colloid, filtered Tc-99m-sulfur colloid, Tc-99m-albumin colloid, Tc-99m-antimony trisulfide, and Tc-99m-human serum albumin. The colloids, especially unfiltered Tc-99m-sulfur colloid, exhibit slow injection site clearance [16]. Consequently, scatter from the injection site can mask the sentinel node, which can often be only a few centimeters away. Tc-99m-human serum albumin, with no affinity for lymph node tissue, can travel through the lymph node chain, making sentinel node identification difficult. Because of the different agents in use, injection techniques are not standardized. The overall result is a long learning curve for both the radiologist and the surgeon, which can limit the success rate of sentinel node detection, and overall diagnostic accuracy.

This paper describes the continued development of a receptor-binding radiopharmaceutical for sentinel node detection. In 1998 we demonstrated the ability to image lymph nodes via the mannose binding protein receptor [40]. Recently, we described the synthesis of a dextran-based glycoconjugate, which displayed rapid injection site clearance [39]. Here, we present the synthesis and preliminary testing of a Tc-99m-labeled MAG_3-*mannosyl*-dextran conjugate. Our goal is an instant labeling kit, which utilizes the high stability exhibited by the MAG_3 chelation system.

2. Materials and methods

2.1. Reagents

All aqueous solutions were prepared using deionized water (NANOpure Infinity, Barnstead-Thermolyne, Dubuque IA). Amino-terminated dextran was synthesized as previously described [39]. All dextran conjugates were lyophilized and stored at −80°C. Benzoyl-mercaptoacetylglycylglycyl-glycine ($BzMAG_3$) was synthesized as described by Fritzberg et al. [13]. Cyanomethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-mannoside was synthesized by published methods [6,27,31]. Methanol was purchased as ACS grade, stored over 4A molecular sieves, and distilled immediately prior to use. All other reagents were purchased from commercial suppliers and used without purification. A 90% (v/v) solution of DMSO/PBS was prepared by combining 9 volumes of DMSO with 1 volume of phosphate buffered saline (0.1 M, 0.15 M NaCl, pH 5.5). Particle-size standards of known diameter (NIST/BCR traceable) were purchased from Duke Scientific (Palo Alto, CA).

2.2. Synthesis

Benzoyl-mercaptoacetylglycylglycyl-glycine-*mannosyl*-dextran ($BzMAG_3$-*mannosyl*-dextran) was synthesized in two steps: first, the covalent attachment of mannose to the amino-terminated leashes of the dextran conjugate, and second the covalent attachment of benzoyl-mercaptoacetylglycylglycyl-glycine to the remaining amino-terminated leashes is illustrated in Figure 1.

The covalent attachment of mannose used the bifunctional reagent 2-imino-2-methoxethyl-1-thio-β-D-mannoside (IME-thiomannose) [31]. Freshly distilled methanol (100 ml) was added to a flame-dried 500 ml recovery flask. Four grams of CNM-thiomannose was added to the flask, which was placed under hot tap water to dissolve the carbohydrate. Sodium methoxide (55 mg) was then added and the flask was placed on a gyratory shaker for incubation at room temperature. After 24 hours, ten milliliters of the product was transferred to a dried recovery flask and a sample (0.25 ml) removed for measurement of the IME-thiomannose yield [38]. Removal of the methanol by rotary evaporation (60°C) produced a thick golden syrup. Imme-

Table 1
Mammalian lectins

Receptor	Carbohydrate	Cell/Tissue
Hepatic binding protein	Galactose	Hepatocyte
Mannose binding protein	Mannose	RE Cells, bacteria
Electrolectin	Lactose	Heart
Thrombospondin	Mannosamine	Activated platelets
Ligatin	Mannose 6-P	Brain, ileum, heart

diately after addition 0.10 g amino-terminated-dextran (25 amines/dextran, 13,460 g/mole) in a 10 mg/ml solution of Clark's Borate buffer (0.2 M, pH 9.0) the flask was swirled rapidly to produce a clear golden solution, which was agitated (150 rpm) at room temperature for 24 hours. After filtration (0.4 um) the product was transferred into an ultrafiltration cell (Model 8010, Millipore Corp, Bedford MA) fitted with a ultrafiltration membrane (YM01, Millipore Corp) and dialyzed with ten exchange volumes of deionized water, the retentate was concentrated, and lyophilized. A sample was assayed for amine density.

Covalent attachment of benzoyl-mercaptoacetylglycyl-glycyl-glycine began with the activation [24] of $BzMAG_3$ with tetraflorophenol (TPF). A 100 ml recovery flask was charged with 100 mg $BzMAG_3$ (0.27 mmole), 10 ml N,N-dimethylformamide, 66 mg tetraflorophenol (0.40 mmole), and 76 mg 1,3 dicyclohexylcarbodiimide (0.37 mmole). The solution was complete within a few minutes of swirling without heating. The flask was then placed on a gyratory shaker. After 18 hours (150 rpm) at room temperature, the solution was rotory evaporated without heat, dried under high vacuum, redissolved in chloroform, and filtered. This resulting solution was rotory evaporated without heat and dried under high vacuum. Lastly, to remove, any urea by-products, the product was dissolved in dichloromethane and filtered. After rotory evaporation without heat the product, the TFP-ester of $BzMAG_3$, was stored at −20°C.

$BzMAG_3$-dextran and $BzMAG_3$-*mannosyl*-dextran were prepared in the following manner. Two ml of DMSO/PBS (10:1 v/v) were added to a 15 ml polypropylene centrifuge tube containing 0.5 mg of the TFP-$BzMAG_3$ product. After adjusting the pH to 7.3 with 0.5 N NaOH, 20 mg of *mannosyl*-dextran (18,288 g/mole) having 3 amino and 21 mannose units per dextran or amine-terminated dextran (24 amines per dextran, 13,332 g/mole) were added in small portions. This solution was shaken (200 rpm) overnight at room temperature after which it was transferred to a diafiltration cell (Model 8010, Millipore Corp) fitted with a ultrafiltration membrane (YM01, Millipore Corp) that was pretreated with the DMSO/PBS solution. After diafiltration with 2 exchange volumes DMSO/PBS, the retentate was diluted to 10 ml with 0.1 M PBS (pH 5.5) and diafiltered for 10 exchange volumes. Because the resulting retentate was slightly opaque, its volume was doubled with DMSO and diafiltered with 10 exchange volumes of 0.1 M PBS (pH 5.5). After diafiltration, the clear retentate was concentrated, transferred to a 50 ml polypropylene centrifuge tube, and freeze dried. A sample was assayed for amine concentration.

The concentration of free amino groups in the $BzMAG_3$-dextran and $BzMAG_3$-*mannosyl*-dextran was measured by trinitrobenzylsulfonic assay [11]. The carbohydrate concentration of each conjugate was measured by the sulfuric acid method [9] using a known concentration of dextran T10 (Amersham-Pharmacia Biotech, Piscataway NJ) as a standard. Molecular weights were calculated using the following values: T10-dextran, 10,500 g/mole (Amersham-Pharmacia Biotech); amino-terminated leash, 118 g/mole [39]; coupled thiomannose, 236 g/mole; and $BzMAG_3$, 367 g/mole.

The size distribution of $BzMAG_3$-*mannosyl*-dextran and filtered technetium-99m sulfur colloid was measured (10 min) by dynamic light scattering (*UPA-150*, Honeywell-Microtrac, Clearwater FL). Latex particles standards of three different sizes gave weight-averaged diameters that were within 5% of their reported diameters (19 ± 1.5 nm, 102 ± 3 nm, 0.993 ± 0.021 um). Values were calculated from volume distribution data without the assumption of a gaussian distribution.

2.3. Radiolabeling

We employed a standard tin reduction method to radiolabel $BzMAG_3$-dextran and $BzMAG_3$-*mannosyl*-dextran with technetium-99m. Briefly, in a 2 ml glass vial fitted with a rubber septum, $BzMAG_3$-dextran (1.5×10^{-7} mole) or $BzMAG_3$-*mannosyl*-dextran (2.0×10^{-8}) was combined with a 0.30 mL isotonic saline solution of sodium pertechnetate (11 mCi) and 0.02 ml sodium gluconate (0.1 mg/ml). After purging with nitrogen for 10 minutes, a fresh solution of (0.010 ml) $SnCl_2$ [5.0 mg $SnCl_2$ in 0.175 mL HCl (37%) followed by 0.25 ml saline (0.9%)] was added and the reaction incubated for 60 minutes at 60°C.

Radiochemical yield was assessed by electrophoresis (250 V, 20 min) using 2 × 25 cm polyacetate strips (Gelman Sciences, Ann Arbor MI) in barbital buffer (pH 8.6, 25°C). The strips were scanned with a collimated sodium iodide detector [22]. Technetium-99m-labeled MAG_3-*mannosyl*-dextran was also assessed by fast protein liquid chromatography (G25, 1 × 8 cm, 2 ml/min isotonic saline) with radioactivity (100–200 keV) and absorbance (226 nm) detection.

Filtered technetium-99m sulfur colloid was obtained from a centralized radiopharmacy. The labeling protocol followed the package insert (CIS-US, Bedford MA) using the minimum volumes and maximum radioactivity specified. A 0.22 micron syringe filter (Millex GV, Millipore Corp, Bedford MA) was used to prepare the filtered agent.

2.4. Sentinel node detection

The rabbit sentinel node detection study was performed in compliance and approval of the Institutional Animal Care and Use Committee of the University of California, San Diego. After anesthesia by intramuscular injections of ketamine (25 mg/kg) and xylazine (25 mg/kg), 0.50 ml of [^{99m}Tc]MAG_3-*mannosyl*-dextran dextran (2.5 nmole) was injected to the right front (0.21 mCi) and right rear (0.22 mCi) foot pads and 0.50 ml of filtered Tc-sulfur colloid to the left front (0.18 mCi) and left rear (0.19 mCi) foot pads. The rabbit was hydrated by I.V. administration (1 drop/min) of saline into the marginal ear vein. After injection of each radiopharmaceutical, the foot pads were massaged for 10 minutes. The lower limbs were manually exercised for 10 minutes at 60-minute intervals. Lymph flow in the rabbit hind leg increases linearly with the log of the massage or leg rotation frequency [19]. The rabbit was euthanized 3.5 hours after injection. With the aid of a hand-held gamma detector (Model 1000; Neoprobe, Dublin OH) each sentinel node was excised and assayed for radioactivity with diluted counting samples of the [^{99m}Tc]MAG_3-*mannosyl*-dextran or filtered TcSC dose. These measurements were used to calculate the percent-of-injected dose (*%ID*) in each sentinel node at the time of sacrifice. A signal-to-background ratio was calculated by dividing the probe's count rate for the excised lymph node by the count rate when the probe was placed over adjacent tissue [3].

3. Results

3.1. Synthesis

The synthesis yielded a dextran conjugate with a mean mannose density of 21 mannose units per dextran molecule and a mean $BzMAG_3$ density of 3 chelators per dextran. The mean molecular diameter was 5.5 ± 2.4 nanometers. The calculated molecular weight was 19,389 g/mole. The $BzMAG_3$ density of the $BzMAG_3$-dextran was not calculated.

3.2. Radiolabeling

Labeling of $BzMAG_3$-dextran was quantitative; the electrophoresis scans displayed a single peak at the origin. An attempt to label amino-terminated dextran without $BzMAG_3$ produced an electrophoresis with a peak at 12 mm toward the cathode. Pertechnetate produced a peak at 14 mm from the origin. Labeling of $BzMAG_3$-mannose-dextran typically produced yields in the 80–90 percent range when assayed by electrophoresis and 90–95 percent range when assayed by FPLC. The labeled product migrated 5 mm toward the anode. The yield measurements for both labeled compounds were stable for 6 hours.

Size distribution measurements of filtered Tc-99m sulfur colloid typically yielded values corresponding to the size cut-off of the 0.22-micron filter. However, one preparation, which was not used for the imaging study, had a bimodal distribution at 4.60 ± 0.97 and 0.277 ± 0.063 microns. The relative areas under each distribution were 4 and 96 percent, respectively.

Table 2
Sentinal node detection study

Lymph node	Radiopharmaceutical			
	[^{99m}Tc]MAG_3-*mannosyl*-Dx		Filtered Tc-SC	
	%ID (%)	S/B	%ID (%)	S/B
Axillary	3.95	5.3	2.89	6.9
Popliteal	4.33	5.6	1.95	2.6

3.3. Sentinel node detection

The percent-of-injected dose demonstrated greater lymph node uptake of [^{99m}Tc]MAG_3-*mannosyl*-dextran in both axillary and popliteal sentinel nodes over a 3.5-hour period. Table 2 lists the percent-of-injected dose (%ID) for each lymph node and agent. The single-to-background ratios (S/B) ranged from 2.6 to 6.9.

4. Discussion

To our knowledge this is the first demonstration that a receptor-binding radiotracer or a macromolecule can be directly labeled with Tc-99m via the MAG_3 ligand system. Since the introduction of preformed chelates for technetium-99m labeling of antibodies [12] and antibody fragments [23], the high temperature and pH requirements of the DADS and MAG_3 ligand systems have prevented the development of instant labeling kits. Consequently, the labeling of macromolecules, such as F_{ab} [24] and F_v fragments [44], human serum albumin [41], and interleukin-2 [5], require up to 23 separate steps [42]. These multiple steps include labeling of the activated ligand, purification of the labeled chelate, changes of buffer and solvent systems, and finally conjugation of the preformed radioligand to the macromolecule. Our goal is an instant single-step kit that simply requires an injection of pertechnetate into the labeling vial followed by a one-hour incubation in a 50°C heater block.

An instant single-step kit will require a quantitative labeling yield. This was achieved by $BzMAG_3$-dextran. However, the labeling yields for $BzMAG_3$-*mannosyl*-dextran were typically in the 90–95 percent range. One possible solution to this problem is an increase in $BzMAG_3$-*mannosyl*-dextran concentration and pertechnetate activity. The

concentration of BzMAG-dextran was 7 times that of the $BzMAG_3$-[^{99m}Tc]-dextran concentration. A 10-fold increase in concentration during labeling will easily provide an adequate specific activity for human sentinel node studies with 0.5 nanomole injections. A second approach would be an increase in the $BzMAG_3$ density of the conjugate.

The preliminary in vivo study supports our hypothesis that injection site clearance, and sentinel node uptake of [^{99m}Tc]MAG_3-*mannosyl*-dextran are superior to the filtered Tc-99m sulfur colloid. One of our design goals for a sentinel node agent [40] is a rapid injection site clearance. Two factors, low molecular weight and low charge density, will promote a high clearance rate. A low molecular weight will permit diffusion into the blood capillaries, as well as, the lymph channels. A charge density that approaches neutrality will also enhance capillary and lymph channel diffusion by minimizing any electrostatic barrier during transport across the blood capillary and lymph channel endothelium. Additionally, a low charge density will permit a more compact folding of the macromolecule, which will result in a smaller molecular diameter. The formal charge of negative one for a [^{99m}Tc]MAG_3-conjugate minimizes the charge density of [^{99m}Tc]MAG_3-*mannosyl*-dextran when compared to of [^{99m}Tc]DTPA-*mannosyl*-dextran of similar DTPA and mannose densities.

In conclusion, our preliminary studies demonstrate the potential of a instant single-step labeling kit of [^{99m}Tc]MAG_3-*mannosyl*-dextran for sentinel node detection via receptor-targeting.

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