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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:
A61K 39/395	A1	WO 91/14453
		(43) International Publication Date: 3 October 1991 (03.10.91)

(21) International Application Number: PCT/US91/02089

(22) International Filing Date: 27 March 1991 (27.03.91)

(30) Priority data:
499,790 27 March 1990 (27.03.90) US

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(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: IMPROVED METHOD TO DIRECTLY RADIOLABEL ANTIBODIES FOR DIAGNOSTIC IMAGING AND THERAPY

(57) Abstract

The invention is a novel method and kit for directly radiolabeling proteins such as antibodies or antibody fragments for diagnostic and therapeutic purposes. The method comprises incubating a protein-containing solution with a solution of sodium ascorbate; adding a required quantity of reduced radionuclide to the incubated protein. A kit is also provided wherein the protein and/or reducing agents may be in lyophilized form.

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IMPROVED METHOD TO DIRECTLY RADIOLABEL ANTIBODIES FOR DIAGNOSTIC IMAGING AND THERAPY

Introduction

The invention described herein was made in the course of work under a grant or award from the Department of Energy and an NIH Cancer Research training grant.

This invention was disclosed in Disclosure Document No. 232220 filed September 14, 1989.

Background of the Invention

The invention described herein was made in course of, or under U.S. Department of Energy Grant DE-FO-G2-85 and ER-60295 and a NIH Cancer Research Training Grant 05137-10.

The major thrust of the prior art concerning the use of radiolabeled monoclonal antibodies (Mabs) is focused on the development of their applications in imaging and therapy. Evidence suggests that Tc-99m possesses ideal characteristics for imaging and Re-186 or Re-188 for therapy. However, the procedures for labeling Mabs with these radionuclides remain inefficient and cumbersome. Unless the labeling processes are made simple, efficient and reliable, the use of Mabs for diagnostic imaging will remain restricted.

Technetium-99m possesses the attractive characteristics of convenience, inexpensiveness, low radiation dose, and suitability for planar and single

photon emission computerized tomography (SPECT) imaging. There has been a continuous development of technology to incorporate Tc-99m in all molecules intended for diagnostic imaging applications. Monoclonal antibodies are no exception. Since Tc-99m has only a 6 hour half-life, in most cases the biological half-life of Tc-99m labeled antibody will be determined by the half- life of Tc-99m and not by the clearance time of the IgM, IgG or fragmented antibody. It is for these reasons that TC-99m continues to be the favorite radionuclide for most imaging applications of Mabs.

Indirect labeling of Mabs with Tc-99m via bifunctional chelating agents (BFCA) such as DTPA, cyclam, and diaminodithio (N_2S_2) compounds is well known in the art. A number of investigators have labeled antibodies with Tc-99m via the use of BFCAs. Khaw, B.A., Strauss, W. and Carvalho, A. et al., Tc-99m Labeling of Antibodies to Cardiac Myosin Fab and to Human Fibrinogen, J. Nucl. Med. 23, 1011-109, 1982; Paik, C.H., Pham, L.N.B., Hong, J.J. et al., The Labeling of High Affinity Sites of Antibodies With Tc-99m, Int. J. Nucl. Med. and Biol. 12, 3-8, 1985; Thakur, M.L., Richard, M.D., and White, F.W., III, Monoclonal Antibodies as Agents For Selective Radiolabeling of Human Neutrophils, J. Nucl. Med. 29, 1817-1825, 1988; Franz, J., Volkert, W.A., Barefield, E.K., et al. The Production of Tc-99m Labeled Conjugated Antibodies Using a Cyclam-Based Bifunctional Chelating Agent, Int. J. Nucl. Med. Biol. 14, 569-572, 1987; and Fritzber, A.R., Kasina, S., Reno, J.M. et al., Radiolabeling Antibodies Using N_2S_2 Ligands, J. Nucl. Med. 27, 957-958, 1986. Perhaps the most prominent method among them uses the diaminodithio compound as a BFCA. However, even this method requires a lengthy and cumbersome procedure that has prompted researchers to investigate direct labeling methods.

Direct labeling methods offer the advantages of 1) providing higher specific activity (mCi/mg); 2)

eliminating the need for conjugation with a bifunctional chelating agent; 3) being less likely to alter the immunospecificity of the Mab; 4) being more likely to be adaptable to an instant "kit" labeling technique; and 5) being useful for labeling antibodies with radionuclides such as Re-186 or Re-188 for therapy.

Human serum albumin has been labeled with Tc-99m by the direct tin reduction method since 1971. The method was published by Lin et al., Use of Fe(II) or Sn(II) Alone for Technetium Labeling of Albumin, Journal of Nuclear Medicine, Vol. 12, No. 5, pp. 204-10, and modified by Eckelman et al., ^{99m}Tc-Human Serum Albumin, Journal of Nuclear Medicine, Vol. 12, No. 11, pp. 707-710 (1971). The method was adapted for labeling antibodies by Pettit Improved Protein Labeling By Stannous Tartrate Reduction of Pertechnetate, Journal of Nuclear Medicine, Vol. 21, No. 1, pp. 59-62 (1980); Huang et al. Detection of Bacterial Endocarditis with Technetium-99m-Labeled Antistaphylococcal Antibody, Journal of Nuclear Medicine, Vol. 21, No. 8, pp. 783-786 (1980); and Rhodes et al., Technetium-99m labeling of Murine Monoclonal Antibody Fragments, Journal of Nuclear Medicine, Vol. 27, No. 5, pp. 685-693 (1986). At least in one case (Rhodes) the resulting labeled product has been reported to be unstable, requiring an elaborate system for purification of the antibody. Sundrehagen et al., Formation of ^{99m}Tc-Immunoglobulin G Complexes Free from Radiocolloids, Quality Controlled by Radioimmunoelectrophoresis, European Journal of Nuclear Medicine, Vol. 7, pp. 549-552 (Spring 1982), appreciating that the tin used in all these methods leads to the formation of Tc-99m-Sn-colloid, used a concentrated hydrochloric acid and gentisic acid reduction method for labeling immunoglobulin with Tc-99m. In order to avoid the colloid formation of Tc-99m, Blok et al., A New Method for Protein Labeling With ^{99m}Tc, Nucl. Med. Biol., Vol. 16, No. 1, pp. 11-16 (1989), used a combination of

dimethylformamide and 5M HCl in which Tc-99m was heated at 140°C for 4 hours. The reduced Tc-99m was then extracted in $CHCl_3$, evaporated to dryness, and incubated with antifibrin antibody for 1 hour at 40°C. However, these methods are lengthy, cumbersome, and are not adaptable to an instant kit procedure.

Stiegman et al., The Importance of the Protein Sulfhydryl Group in HSA Labeling with Technetium-99m, Proceedings of 22nd Annual Meeting, J. Nucl. Med., Vol. 16, No. 6, suggested that the mechanism of Tc-99m binding was related to sulfhydryl groups. There are approximately 70 cysteine amino acid residues per IgG molecule, leading to approximately 175 disulfide bonds in an IgM molecule, approximately 35 in IgG, and approximately 25 in $F(ab')_2$. In the Fab fragment, there may be as many as 12 groups per molecule.

Liang et al., Serum Stability and Non-Specific Binding of Technetium-99m Labeled Diaminodithiol for Protein Labeling, Nucl. Med. Biol., Vol. 14, No. 6, pp. 555-561 (1987), suggested the importance of the helical structure of antibodies for the preservation of the Mab specificity and immunologic activity. However, Rhodes demonstrated that reducing disulfide bonds to sulfhydryl groups not only allowed them to label murine Mab fragments with Tc-99m, but also preserved the immunoreactivity of the protein. This reduction was achieved by incubating 600 μg of antibody with 5mM $SnCl_2$ for 21 hours. The product was then lyophilized, ready for adding Tc-99m pertechnetate. Presumably, the excess of $SnCl_2$ reduced Tc-99m for binding to the sulfhydryl groups. Following a 30 minute incubation, the reaction mixture was passed through a purification column designed to eliminate pertechnetate ions and other technetium impurities. Schwartz, A. and Steinstrabber, A., A Novel Approach to Tc-99m Labeled Monoclonal Antibodies, J. Nucl. Med. 28, 721, 1987, considered that an excess of $SnCl_2$ at pH 6-7, may lead Tc-

99m to the formation of colloid. Schwartz et al. achieved the reduction of antibody disulfide groups with 2-mercaptoethanol (ME). Excess reagent was eliminated by molecular gel filtration and the protein was lyophilized. Reduction of pertechnetate was achieved by the use of commercial pharmaceutical kits such as pyrophosphate, which contains $SnCl_2$. This solution was then added to the lyophilized protein to allow Tc-99m to exchange from the phosphate groups to the sulfhydryl groups.

In our hands, the Rhodes et al. and the Schwartz et al. methods result in a labeling efficiency of about 65% and 44%, respectively. Both methods also require further purification to eliminate unbound Tc-99m. These methods, therefore, are appealing and improvement over bifunctional chelating agent methods. In a routine radiopharmacy setting, however, these methods are less than ideal because of the required purification steps. Recently Pak et al., A Rapid and Efficient Method for labeling IgG Antibodies with Tc-99m and Comparison to Tc-99m FAB' Antibody Fragments, Journal of Nuclear Medicine, Vol. 30, p. 793 (1989) and Del Rosario et al., Site-Specific Radiolabeling of Monoclonal Antibodies with Biotin/Streptavidin, Nucl. Med. Biol., Vol, 16, pp. 525-527 (1989) used DTT (dithiothreitol) to reduced antibody disulfide groups and label them with Tc-99m. Pak et al. used a commercial glucoheptonate kit which contains $SnCl_2$ to reduce Tc-99m. DTT and its isomer DTE (dithioerrythritol) were prepared by Cleland, Dithiothreitol, A New Protective Reagent for SH Groups, Biochemistry, Vol. 3, pp. 480-482, 1966) in 1964, and used to reduce protein disulfide groups.

In addition 2-ME has a very unpleasant, strong smell of sulfur. DTT and DTE also contain sulfur but have a less intense smell. Some patients are allergic to such compounds. For this reason, unlike ascorbic acid excess of these compounds must be removed from the proteins prior to administration to a patient.

Labeling of Tc-99m to sulfhydryl groups of antibodies has been designated by Paik et al. as high affinity but low capacity binding. Neither Rhodes et al. or Schwartz et al. show any data as to how many disulfide groups, if any, are involved and how the reduction affects the structural integrity and immunoreactivity of the antibody. Relative stability of the labeled tracer with respect to other competing chelating agents was also undetermined. A recent article by Rhodes group (Hawkins et al., Resistance fo Direct Tc-99m-Protein Bond to Transchelation, Antibody, Immunoconjugates, and Radiopharmaceuticals, Vol. 3, No. 1 (1990)) show Tc-99m-s-protein bond is stable.

Jones, A.G., Orvig, C., Trop, H.S. et al., A Survey of Reducing Agents for the Synthesis of Tetraphenylarsonium-oxotechnetium (Ethanedithiolate) From Tc-99m Pertechnetate in Aqueous Solution, J. Nucl. Med. 21, 279-281, 1980, disclose that sodium dithionite in the range of pH 11-13 gives quantitative yields of the required technetium complex through the 100% reduction of Tc-99m rapidly at room temperature. He also reported that at pH 11-13, the results were consistently quantitative.

U.S. Patent No. 4,305,922 (Rhodes) teaches labeling proteins with Tc-99m by ligand exchange using a Sephadex column (chelating agent). A mixture of Tc-solution and stannous solution are poured into the top of the column. The protein to be labeled is then added and ligand exchanged occurs as the Tc-99m ions preferentially migrate from the chelating agent to the protein. The labeled protein is then washed through the column and recovered.

U.S. Patent No. 4,401,646 (Rhodes et al.) discloses a method and apparatus for purifying materials radiolabeled with Tc-99m by passage through a filtration column which contains a reducing agent, colloidal stannous phthalate, anti-oxidant and a particulate solid substrate

capable of binding reduced Tc and of retaining insoluble Tc. Useful anti-oxidants include gentisic acid, ascorbic acid, tartaric acid, or mixtures thereof. In this case, ascorbic acid is used to reduce the radioactive Tc only.

U.S. Patent No. 4,416,865 (Rhodes et al.) discloses urokinase, streptokinase or fibrinokinase labeled with Tc-99m, ^{131}I or ^{123}I for localization of thromboembolic diseases. The enzymatic protein is combined with the nuclide in a basic solution containing ferric chloride and ascorbic acid, and the pH adjusted to acidic conditions to produce a radiopharmaceutical. In this case, ascorbic acid is used to reduce the radioactive Tc only.

U.S. Patent No. 4,424,200 (Crockford et al.) discloses methods for radiolabeling proteins with Tc-99m in a reducing environment by incubating a source of stannous ion with a protein in the presence of a buffering composition (a mixture of an alkali metal biphtalate and alkali metal tartrate) at pH 4.5-8.5 for at least 15 hours at a temperature where the protein is not denatured.

U.S. Patent No. 4,478,815 (Burchiel et al.) discloses a composition and method for detecting cancer with technetium labeled antibody fragments. The composition is an $F(ab')_2$ or F(ab) Fragment of antibody to hCG, hCG-α, hCG-β or hCG-like material or other tumor specific or associated molecules including CEA, AFP, human melanoma associated antigens labeled with Tc-99m. The radiolabeled fragment is injected intravenously, accumulates at tumor sites, and is detected by scintigraphy. A double antibody approach is also taught wherein a tumor specific antibody in the form of IgG, $F(ab')_2$ or F(ab) is administered to a patient intravenously, followed by administration of a radiolabeled antibody in the form of $F(ab')_2$ or F(ab) which is reactive with the first antibody. The radiolabeled reagents are formed under reducing conditions to minimize or prevent the reversible reaction by which the Tc-99m becomes free of the antibody

fragment. The Tc-99m labeled antibody fragment may be prepared by acidic, basic or neutral (ligand exchange) radiolabeling techniques.

U.S. Patent No. 4,652,400 (Paik et al.) discloses a direct labeling method comprising reacting a reduced radioisotope of rhenium or technetium with an antibody in the presence of diethylebnetriaminepentaacetic acid (DTPA). The DTPA inhibits binding of the radioisotopes to nonstable binding sites.

U.S. Patent No. 4,472,371 (Burchiel et al.) discloses the labeling of radiolabeling antibodies with technetium. The Tc is reduced by stannous ions prior to labeling and the protein is also reduced using stannous chloride or stannous fluoride.

U.S. Patent No. 4,645,660 (Takahashi et al.) discloses the reduction of a radioisotope carrier with ascorbic acid in order to increase its chelating capacity. In this case, ascorbic acid is used to reduce the radioactive Tc only.

U.S. Patent No. 4,837,003 (Nicolotti) discloses a labeling process utilizing a coupling agent that complexes with a radionucleotide. The coupling agent is covalently bound to the Ab through sulfhydryl groups produced by mild reduction of the disulfide linkages. Reducing agents which are disclosed include 2-mercaptoethanol and, as the preferred agent, cysteine.

U.S. Patent No. 4,851,515 (Bonnyman et al.) discloses a labeling process using the nitrodotetrahalotechnetium-99m anion and at least partial reduction of the disulfide linkages to sulfhydryl residues by use of a reducing agent such as dithiothreitol (DTT).

Summary of the Invention

The present invention provides an improved method to directly label proteins such as antibodies or serum protein with a radiolabel such as Tc-99m, Re-186 or Re-188. The first step involves the reduction of the

protein's disulfide bonds to sulfhydryl groups that allow efficient bonding of the radiolabel. The disulfide reducing agent is ascorbic acid. The radiolabel is separatly reduced. In this method, a protein-containing solution is allowed to react with Na-ascorbate solution, pH 6.5 (AA), in the protein:AA molar ratio of 85-8500. Following incubation, a required quantity of a reducing agent, such as dithionite or stannous chloride, is used to reduce the radiolabel, at a basic pH. This method provides a number of advantages including: stability upon incubation with DPTA, human serum and cysteine; the immunospecificity of the protein as determined by specific antigen interaction assay is unchanged; and purification is not required. Ascorbic acid, in it's oxidized, dehydrogenated form is vitamin C, which is non-toxic, non-odorous and need not be eliminated. A kit suitable for preparation of the radiolabeled antibodies is also provided.

None of the prior art methods teach or suggest the direct labeling method of the present invention. The importance of a technique that allows labeling of proteins with a radionuclide quickly and reliably, by an instant kit type of procedure is a significant improvement in the art.

It is therefore an object of the invention to provide a method for directly radiolabeling proteins for diagnostic imaging and therapy.

It is a further object of the invention to provide a novel method to label an IgG, IgM or $F(ab')_2$ antibody fragment with Tc-99m, Re-186 or Re-188.

It is another object of the invention to provide a kit for labeling proteins useful for diagnostic imaging and therapy.

Further objects and advantages of the invention will become more apparent in light of the following detailed discussion of the invention.

Detailed Description of the Invention

To overcome the problems described above, the present invention 1) uses an agent which will effectively reduce the protein without the need to eliminate any excess and 2) uses an agent that will reliably reduce the radionuclide and will minimize colloidal formation.

The presence of excess of the disulfide bond reducing agent prevents the reoxidation of sulfhydryl groups, maintaining their ready availability for Tc-99m binding. At a 1:3500 ratio only 2.7±0.2% of the available S-S groups are reduced. Thakur et al., Determination of Reduced Disulfide Groups in Monoclonal Antibodies, BioTechniques, Vol. 8, No. 4 (1990). This excess of reducing agent, under appropriate chemical conditions, and with the correct oxidation-reduction potential, prevents reoxidation of reduced Tc-99m once it is introduced to the antibody.

Colloid formation not only prevents reduced Tc-99m from binding to the protein but also requires a step to eliminate it. An agent and the chemical conditions that will minimize colloid formation are of an obvious advantage.

The present invention satisfies both criteria resulting in enhanced labeling yields with elimination of the need for separation procedures. This method is adaptable to a kit comprising the reduced protein in lyophilized form and lyophilized chemicals ready to receive radionuclide solution to produce the reduced radionuclide. The reduced radionuclide is then added to the lyophilized antibody. Preferably, alternatively one vial containing reduced antibody and a radionuclide reducing agent ready to receive the radionuclide could be produced. Following incubation, (5-30 minutes, 22°C-37°C) and a quality control assay, the labeled antibody is ready for administration to a patient. The kit format and procedure is especially

useful for the preparation of Tc-99m, Re-186 or Re-188 labeled proteins.

The Disulfide Reducing Agent

In 1928, Szent-Gyorgyi, A Textbook of Pharmacology, Toxicology, and Therapeutics for Physicians and Medical Students, Third Edition, Chapter 74, Water-Soluble Vitamins, separated a "powerful reducing agent" in crystalline form from adrenal. Because of its physiological role in the prevention of scurvy, the compound was called ascorbic acid (AA). AA serves an important function in maintaining SH-activated enzyme systems in their reduced form. The agent also serves as a hydrogen donor and in its oxidized dehydrogenated form serves as a vitamin. In sodium salt form it can be administered to humans by intramuscular or intravenous injections. These properties make AA useful in this invention because this agent 1) can reduce the Mab disulfide bonds to sulfhydryl groups, 2) maintain the activated SH groups in their reduced from, and 3) does not require elimination because the excess or the oxidized portion of it can only serve a helpful role in the body.

The Tc-99m Reducing Agent

Sodium dithionite is the preferable agent to reduce Tc-99m. That is because the agent is readily soluble in water or normal saline (pH 5-6). At pH 11, the solution remains without colloid formation for a longer period of time than does $SnCl_2$, the commonly used reducing agent, under similar conditions. At pH 10-11, dithionite solutions ranging in concentrations from 1 mg to 50 mg per ml can be stored for longer than 48 hours without formation of colloid. The pH 11 buffer consists of 0.05M sodium bicarbonate solution, to which 0.1 M NaOH is added to adjust the pH. Sodium dithionite readily dissolves in this buffer and remains in solution without the formation of colloid for longer than 48 hours when protected from air or oxygen. This allows sufficient time for lyophilization and

incubation time. Up to 200 μg of Na-dithionite have been used to reduce several mCi of Tc-99m in up to 200 μl Tc-99m solution. Larger volumes of Tc-99m can be used. The incubation time at 22°C is no longer than 5 minutes. Since the antibody solution in reduced form is rendered at pH 5-6, the final pH after the addition of the reduced Tc-99m is 7.9 to 8.2. Na-dithionite in pH 11 buffer is thus the preferred agent to reduce Tc-99m for labeling with Mab.

The following examples illustrate the present invention and are not intended to limit the same.

EXAMPLES

Example 1 - Preparation of Labeled Antibodies

Three IgM neutrophil specific antibodies (anti-SSEA-1, B.40.1, B.37.2.1), three IgG and one $F(ab')_2$ antibodies were labeled. The SSEA-1 antibody was obtained from RhoMed, Albuquerque, NM and B.40.1 and B.37.2.1 from the Wistar Institute, Philadelphia, PA. Depending upon the number of tests to be performed, 25 to 100 μg antibody solution were incubated for one hour at 22°C with Na-ascorbate solution, pH 6.5 (AA), in the Mab:AA molar ratio of 85 to 8500. Following incubation, a required quantity of dithionite reduced Tc-99m, at pH 11 was added and the reaction mixture was further incubated for 1 hour at 22°C. The solutions were then subjected to quality control and immunospecificity tests as described in the following examples.

Example 2 - Reduction of Tc-99m

Tc-99m, eluted freshly from Medi-Physics generators was used. Typically, a known quantity of sodium dithionite ($Na_2S_2O_4$) was dissolved in a known volume of 0.05M sodium bicarbonate solution to which was added nitrogen purged 0.1 M solution of NaOH to adjust the pH to 11. The final concentration of dithionite solution was 10 mg/ml. This solution was added to a known volume of Tc-99m solution in such a way that the final concentration of dithionite was 0.6 to 0.8 μg/μl of the reaction mixture

which totaled 60-70 μl in volume. The reaction was allowed to proceed for 5 minutes at 22°C. Initially, the Tc-99m solution was checked for reduction using instant thin layer chromatography (ITLC). Strips (1 cm x 12 cm) cut from silica gel impregnated glass fiber sheets (Gelman Sciences Inc., Ann Arbor, MI) were used as a stationary phase and 0.15 M NaCl as a mobile phase. In this system, reduced Tc-99m remains at the point of application and the heptavalent, unreduced Tc-99m migrates to the solvent front. The radioactivity was then added to the antibody ready for labeling. The colloid formation, if any, of the Tc-99m was examined under a light microscope, by centrifugation, and by 0.22 μ Millipore filtration or using HSA impregnated ITLC and ethanol:ammonia:water (2:1:5) as a solvent. (Colloid Rf:0.0; bound Rf:1.0.) Thrall, et al., Clinical Comparison of Cardiac Blood Pool Visualization with Technetium-99m Red Blood Cells Labeled in Vivo and with Technetium-99m Human Serum Albumin, Journal of Nuclear Medicine. Vol. 19, pp. 796-803 (1978). These tests were performed at the end of the incubation period and, occasionally, 3 to 6 hours later.

Example 3 - Determination of Tc-99m Labeling Efficiency

The commonly used ITLC was used on each reaction mixture, as described above. Additionally, each mixture was also subjected to HPLC analysis. The HPLC system is equipped with a U.V. (280 nm) monitor, a NaI(Tl) radioactivity detector, and dual pen chart recorder. Water's protein Pak 300 SW column and 0.2M phosphate buffer in 0.15 M NaCl, pH 6.8 as an eluent were used. The flow rate was 0.5 ml/minute. The pressure varied between 100-300 PSI and the chart speed was 0.25 cm/minute. 2 to 10 μl samples were injected. Additionally, samples were filtered through 0.22 μ Cameo filters to eliminate any colloid, subjected to Centricon-30 filtration to eliminate any unreduced Tc-99m, and the protein bound activity collected was further analyzed on HPLC. On each occasion, the HPLC

samples gave a single radioactivity peak corresponding to the protein optical density peak. The results of the percentage of the protein bound radioactivity, with respect to the total present in the reaction mixture, indicated that even at the lowest protein to AA molar ratio (1:85), greater than 80% of the added reduced Tc-99m activity was protein bound, reaching 100% at the ratio of approximately 5000 and above. Greater than 95% labeling efficiency can be achieved with the protein to AA molar ratio of 1:3500 (35 μg AA for 50 μg IgM).

Example 4 - Stability of Tc-99m Labeled Antibody When Challenged With Cysteine, DPTA and Human Serum

The IgM antibodies were labeled with Tc-99m, as above, checked with ITLC and HPLC for labeling efficiency and freedom from unbound Tc-99m. The labeled Mabs were then challenged with such Tc-99m avid agents as cysteine, DTPA, and freshly prepared human serum. For each agent, 1:250, 1:500 and 1:1000 (antibody:agent) molar ratios were used and each incubated at 37°C for 30 minutes. Each sample was then subjected to HPLC analysis. The resulting elution patterns clearly separated Mab, each from cysteine, DTPA or serum indicating that all 100% Tc-99m remained bound to the antibody, except in the case of 1000 molar excess of cysteine. In that case, approximately 20% of the Tc-99m was bound to cysteine and approximately 80% remained protein bound.

Example 5 - Determination of Immunoreactivity

The immunoreactivity of the IgM antibodies labeled with Tc-99m by this technique was determined by the ability of the labeled antibodies to label freshly prepared

human neutrophils. Antibodies labeled with either In-111 or with Tc-99m via the c-DTPA bifunctional chelating agents served as controls. Antibodies labeled by art known techniques were also tested. For each antibody preparation, equal number of cells in duplicate and equal quality of antibody samples was used. The calculated quantities of antibody were added so that the total antibody molecules would not exceed 50% of the available cell surface antigens (5.1×10^5 / cell). Incubation was carried out at 22°C for 30 minutes, following which cells were washed twice with autologous plasma. Radioactivity associated with cells and remaining in the supernatant and the washing was counted, and the results expressed as % cell bound activity as follows:

Sample	**% Cells Bound**
111-In-c-DTPA (control)	75±2
Tc-99m - $SnCl_2$	66±1
Tc-99m - 2-ME	63±2
Tc-99m - DTT	62±3
Tc-99m - DTE	62±2
Tc-99m - AA	84±1

The association of Tc-99m activity bound to an equal number of platalets having nonspecific-antigen was only 16±0.5%. Similarly, only 8±1.5% of the nonspecific protein was bound to an equal number of human neutrophils.

Example 6 - Evaluation of Tc-99m Labeled Proteins for Localization of Inflammation: Comparison with I-125-TNT-1 and Ga-67 Citrate

Tc-99m labeled polyclonal IgG, antinucleus antibody TNT-1, and human serum albumin (HSA) were investigated in mice bearing inflammation in right thigh

induced by i.m. administered of 50 μl turpentine (TP) or 1:1 mixture of 10xE8 E. Coli and Enterococci (EC) using Ga-67, and I-125-TNT-1 as controls. Tc-99m was labeled by the above-described direct, disulfide reduction technique and I-125 labeled using Iodogen. Each preparation was analyzed by HPLC and ITLC and given IV (20 μg, known μCi) to groups of 5 mice with each type of inflammation. Four or 24 hours later mice were imaged, sacrificed and tissues dissected. At 4 hours TP abscess/muscle ratios for Ga were 4.8±2.1, I-125-TNT-1 4.3±1, Tc-99m-TNT-1 3.5±1.8, Tc-99m-IgG 3.9±0.6 and Tc-99m-HSA 4.3±1. With EC these were 2.6±0.6, 3.3±0.5, 3.4±.08, 3±1.1 and 4.1±0.6, respectively. At 4 hours liver uptake was highest (25.7±8.9 and 16.5±10.6) for Tc-99m IgG and lowest (6±5.1 and 4.8±3.20) for Tc-99m-TNT-1, which was similar to those of I-125-TNT-1. For 24 hour groups, no dramatic increase in inflammation/tissue ratios was noted. Results suggest a) distribution of I-125-TNT-1 is similar to Tc-99m-TNT-1 indicating no alteration in biological behavior of protein following Tc-99m labeling, b) Tc-99m-TNT-1, IgG and HSA are equivalent to Ga-67 for imaging inflammatory foci but may allow abscess imaging at 4 hour post administration.

	TNT-1-125		TNT-1-Tc-99m	
	TP	EC	TP	EC
Liver-	6.8±.9*	10.1±.85**	6±5.1*	4.8±3.2**
Abscess /Muscle-	4.3±1.1*	3.3±.5**	3.5±1.8*	3.4±.8**

TP=Turpentine
EC=Microorganic

Liver * P>0.76 ** P>004
Abscess/Muscle *,** P>0.75

Example 7 - Evaluation of Tc-99m-TNT-1-F(ab')$_2$ for Imaging Tumors: Comparison with I-125-TNT-1F(ab')$_2$

TNT-1 is a murine antibody that recognizes antigens released from degenerated cells. This has been labeled with I-131 and used for localization of tumors with necrotic centers (Feng-Ming Chen et al., Tumor Necrosis Treatment of ME-180 Human Cervical Carcinoma Model with ^{131}I-Labeled TNT-1 Monoclonal Antibody, Cancer Research, Vol. 49, pp. 4578-4585, August 15, 1989. F(ab')$_2$ fragmented TNT-1 was obtained from Dr. Epstein (USC-LA), labeled with Tc-99m using the ascorbic acid technique, and given i.v. (approximately 20 μg and 40 μCi) to a group of mice, bearing experimental teratocarcinoma grown to no more than 1 cm in size in the right thigh of BALB/c mice. TNT-1-F(ab')$_2$ labeled with I-125 was given to an additional group of tumor bearing mice served as a control. Four hours later, Tc-99m animals were imaged (I-125, low energy gamma cannot be imaged) with a gamma camera coupled to a pinhole collimator and a Microdot imager. All animals were then

sacrificed, tissues dissected and radioactivity associated with tissues was counted. Results were expressed as percent administered dose per gram of tissue and tumor to tissue ratios. With both agents, tumor/muscle ratios of 10.04±4.4 and 10.94±3.07 (P>0.5) were obtained and tumors were clearly delineated. Distribution of the two agents was similar (statistically insignificant) except that Tc-99m blood values were lower (P<0.001) and kidney values higher (P<0.005) than I-125-TNT-1. The rapid blood clearance may help enhance image quality and the rapid excretion further reduces the radiation burden which is already low because of the short half-life of Tc-99m.

Four Hour Tissue Distribution in Balb/c Mice Bearing Experimental Tumor

	TNT-1-F(ab')$_2$-Tc-99M %Ad. Dose/G	TNT-1-F(ab')$_2$-I-125 %Ad. Dose/G
Urine	129.3%±41.6	59.3%
Blood	5.1%±1.7*	12.8%±1.6*
Kidneys	25.3%±7.1*	7.2%±0.55*
Intestine	1.2%±0.54	2.7%±0.50
Muscle	0.5%±0.11	0.7%±0.06
Tumor	4.7%±1.4*	8%±2.5*
Spleen	14.8%±1.2	4.1%±0.21
Lungs	6.1%±1.7	9.4%±2.8
Liver	15.8%±10.9*	4%±0.66
Heart	2.1%±0.2	3.9%±0.25

*Blood P<0.0001; Kidneys P<0.005; Tumor P>0.11, Liver P>0.12

Tumor/Tissue Ratio

Tumor/Liver	0.20±0.07	1.99±0.43
Tumor/Blood	0.76±0.25	0.63±0.17
Tumor/Muscle	10.04±4.4	10.94±3.07

Obviously many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims the invention may be practiced otherwise than as specifically described.

What is claimed is:

1. A method for directly labeling proteins with radionuclides for use in diagnostic imaging and therapy comprising the steps of incubating a protein-containing solution with a solution of sodium ascorbate; adding a required quantity of reduced radionuclide to said incubated protein-containing solution and incubating.

2. The method of claim 1 wherein the protein to ascorbate ratio is between 1:85 and 1:8500.

3. The method of claim 2 wherein the protein to ascorbate ratio is 1:3500.

4. The method of claim 1 wherein said radionuclide is technetium.

5. The method of claim 4 wherein said technetium is technetium-99m.

6. The method of claim 1 wherein said radionuclide is rhenium.

7. The method of claim 6 wherein said rhenium is rhenium-186 or rhenium-188.

8. The method of claim 1 wherein said protein is an antibody or an antibody fragment.

9. The method of claim 8 wherein said antibody is an IgG antibody.

10. The method of claim 8 wherein said antibody is an IgM antibody.

11. The method of claim 8 wherein said antibody fragment is an $F(ab')_2$ or F(ab) fragment.

12. The method of claim 1 wherein said protein is serum protein.

13. The method of claim 1 further comprising the step of dissolving a known quantity of sodium dithionite in a known volume of sodium bicarbonate solution and adding a sodium hydroxide solution to adjust the pH to 11 and then adding a known volume of radionuclide solution to produce dithionite reduced radionuclide.

14. The method of claim 13 wherein the final concentration of dithionite is 0.1 μg to 10 μg per μl of the reaction mixture.

15. The method of claim 14 wherein said final concentration of dithionite is between 0.6 μg to 0.8 μg per μl of the reaction mixture.

16. The method of claim 13 wherein the reaction is allowed to proceed for 1-5 minutes at 22°C or 37°C.

17. The method of claim 1 wherein said radionuclide is reduced with stannous chloride.

18. The method of claim 1 wherein said first incubation step is performed for 1 hour at 22°C or 37°C.

19. The method of claim 1 wherein said second incubation step is performed at 22°C or 37°C.

20. The method of claim 1 wherein said sodium ascorbate solution is pH 6.5.

21. A kit suitable for directly labeling a protein with radionuclides for use in diagnostic imaging and therapy comprising a required quantity of protein and

sodium ascorbate; and a required quantity of reducing agent for reducing the labeling radionuclide.

22. The kit of claim 21 wherein said protein is an antibody or an antibody fragment.

23. The kit of claim 22 wherein said antibody is an IgG antibody.

24. The kit of claim 22 wherein said antibody is an IgM antibody.

25. The kit of claim 22 wherein said antibody fragment is an $F(ab')_2$ or F(ab) fragment.

26. The kit of claim 21 wherein said protein is serum protein.

27. The kit of claim 21 wherein said radionuclide reducing agent is sodium dithionite.

28. The kit of claim 21 wherein said radionuclide reducing agent is stannous chloride.

29. The kit of claim 21 wherein said protein and said sodium ascorbate are lyophilized.

30. The kit of claim 21 wherein said reducing agent is lyophilized.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/02089

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT CL(5) A61K 39/395
US CL 530/389

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	424/1.1 534/10,14 530/388,389,402

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	US, A, 4,645,660 (TAKAHASHI ET AL.) 24 FEBRUARY 1989	1,12,21,26 2-3,6-11,22-25 29-30
A	US, A, 4,401,646 (RHODES ET AL.) 30 AUGUST 1983	
A	US, A, 4,416,865 (RHODES ET AL.) 22 NOVEMBER 1983	

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
09 MAY 1991	03 JUN 1991
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	JOHN S. MAPLES

Form PCT/ISA/210 (second sheet) (May 1986)

DC 5-28-91