Specific and stable labeling of antibodies with technetium-99m with a diamide dithiolate chelating agent

(immunomunoimaging/active ester/preformed chelate conjugation to antibody fragments)


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ABSTRACT  Technetium-99m labeling of antibodies has been suboptimal because of low affinity adventitious binding, nonspecific labeling, and loss of immunoreactivity. The diamide dithiolate ligand system (N₂S₂) forms highly stable, well-defined tetradeutate complexes with Tc(V). Antibodies and their fragments have been labeled by conjugation of preformed ⁹⁹ᵐTc-4,5-bis(thioacetamido)pentanoate active ester to protein amine groups to give a chemically known ⁹⁹ᵐTc-N₂S₂ complex covalently linked to antibody. Evaluations of the ⁹⁹ᵐTc-N₂S₂-bound antibodies and their fragments have shown high stability and retained immunoreactivity.

Successful targeting of diagnostic radionuclides to tumors not only provides a tool to diagnose and stage cancer but also demonstrates feasibility for therapy where ligand systems can be applied to therapy radionuclides. Early studies with radiolabeled antibodies utilized radiodiode (¹³¹I/¹³¹I) because of extensive experience in protein radioiodination, covalent attachment, and ready availability of the radionuclide (1, 2). Improved tumor-to-nontumor ratios were achieved with ¹¹¹In compared to ¹³¹I by using diethylenetriaminepentaacetate (DTPA) bifunctional chelating agent technology (3–5). ¹³¹I and ¹¹¹In have long half-lives of 8 days and 67 hr, respectively. Recent studies utilizing antibody fragments have shown that tumor uptake and background tissue clearance can take place more rapidly with fragments, allowing the use of shorter half-life radionuclides such as ⁹⁹ᵐTc (6).

⁹⁹ᵐTc is the ideal single-photon radionuclide with a 6-hr half-life, 140 keV γ radiation, no particulate radiation, and inexpensive, convenient availability. These attributes allow the routine administration of doses of 30 mCi (1 Ci = 37 GBq) that result in high photon-flux levels facilitating lesion detection by single-photon-emission computerized tomography. Despite these attractive aspects, the numerous oxidation levels available and the proclivity of ⁹⁹ᵐTc for nonspecific binding to proteins preclude easy adaptation to antibody labeling processes developed for other radionuclides such as ¹¹¹In.

Antibodies have been labeled with ⁹⁹ᵐTc both directly and through bifunctional chelating agents, but with suboptimal results. Direct labeling involves metal binding to donor atoms on the protein, resulting in multiple binding sites of low metal affinity (7). Bifunctional chelating agents, including DTPA (8, 9) and bis(semithio carbazones) (10), have been studied for ⁹⁹ᵐTc labeling but have been found to suffer from problems of (i) nonspecific uptake because of the requirement for several ligands per antibody, (ii) adventitious binding of the metal to the protein, (iii) formation and binding of colloids to the antibody (11), (iv) control of the oxidation level of Tc, and (v) low yields of specifically bound ⁹⁹ᵐTc (9).

We have applied a diamide dithiolate ligand system (N₂S₂) to ⁹⁹ᵐTc labeling of antibody fragment. This ligand system forms highly stable tetradeutate complexes with Tc (⁹⁹ᵐTc-N₂S₂) at the +5 oxidation level (12). Studies of a large number of compounds containing the N₂S₂ donor group showed that high yields of ⁹⁹ᵐTc complexes of the predicted structure were obtained (13). We have shown that other potential metal-binding groups, such as the carboxylate group present in the N₂S₂ ligands, do not participate in metal binding (14). This property enables labeling of antibody by formation of the technetium complex, then chemical conversion of the carboxylate group to an active ester, and finally conjugation of the preformed complex to antibody. Preliminary results of studies of this approach were included in a review of ⁹⁹ᵐTc labeling of antibodies (15).

MATERIALS AND METHODS

Preparation of ⁹⁹ᵐTc-4,5-bis(thioacetamido)pentanoyl (N₂S₂)-Conjugated Anti-Melanoma 9.2.27 F(ab)₂ Fragment:

To a mixture of 25 μl of 4,5-bis(phenylthioacetamido)pentanoic acid (1.0 mg/ml solution in 90% CH₃CN) and 100 μl of 1 M NaOH was added 100 mCi of sodium [⁹⁹ᵐTc]pertechnetate in 1.0 ml of saline (0.9% NaCl). Then 1.0 mg of sodium dithionite (0.10 ml of a freshly prepared 10 mg/ml solution) was added, and the mixture was heated at 75°C for 15 min. The pH was brought to about 6 with 0.10 ml of 1 M HCl and 0.30 ml of 0.2 M sodium phosphate buffer (pH 6.0). Then 10.0 mg of 2,3,5,6-tetrafluorophenol (0.10 mg of a 100 mg/ml solution in 90% CH₃CN) and 12.5 mg of 1-(3-dimethylamino)propyl)-3-ethylcarbodiimide (0.10 ml of a 125.0 mg/ml solution in 90% CH₃CN) were added, and the solution was heated at 75°C for 30 min. The resulting tetrafluorophenyl ester active ester derivative of ⁹⁹ᵐTc-4,5-bis(thioacetamido)pentanoic acid was purified by loading the reaction mixture on a conditioned C₁₈ cartridge (J. T. Baker), washing with 2.0 ml of 20% (vol/vol) ethyl alcohol/0.01 M sodium phosphate, pH 7.0, eight times, and eluting with 100% CH₃CN. The solvent was evaporated under a stream of N₂. Then 0.5 ml of the 2.2.27 F(ab)₂ fragment (16) at 2.5 mg/ml and 0.50 ml of 0.2 M sodium phosphate (pH 9.0) were added for conjugation. After 15 min at room temperature, 25 μg of lysine (0.25 ml of a 250-mg/ml solution at pH 9.0) was added to quench unreacted ester. The ⁹⁹ᵐTc-N₂S₂-2.2.27 F(ab)₂ was purified by passage through a G-25 Sephadex column (Pharmacia) equilibrated with phosphate-buffered saline.

Abbreviations: N₂S₂, diamide dithiolate chelating system; ⁹⁹ᵐTc-N₂S₂-9.2.27 F(ab)₂, ⁹⁹ᵐTc-4,5-bis(thioacetamido)pentanoyl-9.2.27 F(ab)₂ fragments; DTPA, diethylenetriaminepentaacetate.

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An idiotypic antibody (NR-2-AD) specific to an individual's human B-cell lymphoma (17) was used in F(ab')₂ form as a control antibody for the in vivo biodistribution studies. It was labeled as described for 9.2.27 F(ab')₂.

**Evaluation of ⁹⁹ᵐTc-N₂S₂-9.2.27 F(ab')₂ Stability.** The stability of the ⁹⁹ᵐTc binding to the antibody fragment was tested by incubation at 37°C in the presence of various challenge agents, including human serum, 10 mM DTPA, 10 mM 2,3-bis(thioacetamido)propionate, and 6 M urea. Samples were removed at various times and analyzed by HPLC, isoelectric focusing electrophoresis, and NaDodSO₄/PAGE.

**Radiolabeled Cell Binding Assay.** The immunoreactivity of ⁹⁹ᵐTc-N₂S₂-9.2.27 F(ab')₂ was assessed in an antigen-excess cell binding assay (18). Radioactivity bound to cells was determined at increasing FMX-met (metastatic; ref. 19) melanoma cell levels until maximum binding was shown (antigen excess); then correction was made for nonspecific binding by blocking of radiolabeled binding with excess unlabeled antibody.

**In Vivo Evaluation of ⁹⁹ᵐTc-Labeled Antibody F(ab')₂ Fragments in Tumored Nude Mice.** Nude mice (mean weight, 28.2 g ± 1.7 SD) were subcutaneously implanted with human melanoma xenograft A-375 m/m in the left flank or side. Imaging and biodistribution studies were conducted in groups of three and six mice, respectively, with ⁹⁹ᵐTc-labeled F(ab')₂ fragments of melanoma-specific 9.2.27 antibody and nonspecific NR-2-AD antibody. Tumor weights were 335 mg ± 185 SD for the 9.2.27 F(ab')₂ biodistribution and 222 ± 67 mg for the NR-2-AD F(ab')₂. Tumor weights were 619 mg for the 9.2.27 F(ab')₂ and 543 mg (range, 543–720 mg) for the NR-2-AD F(ab')₂ imaging studies. Imaged mice each received 50 μg of protein labeled with 1–1.5 mCi in an intravenous administration of 100 μl via the tail vein. Mice were imaged serially for 16 hr after injection with a small-field-of-view (200 mm) scintillation camera (General Electric). In the biodistribution studies, mice each received 10 μg of protein labeled with 100 μCi in an intravenously administered dose of 100 μl. The animals were sacrificed 20 hr after injection; blood, tumor, tail (injection site), skin, heart, bone, lung, liver, spleen, stomach, neck (thyroid), kidneys, and intestine were isolated, blotted when appropriate, weighed, and assayed in a crystal scintillation detector well counter. Mean percent injected dose per g of body weight was calculated by comparison with radioactivity of standard samples of injectate.

![Diagram of radiolabeled antibody synthesis](image1)

**FIG. 1.** Synthesis of ⁹⁹ᵐTc-N₂S₂-antibody by formation of ⁹⁹ᵐTc-4,5-bis(thioacetamido)pentanoate, which is converted to an active ester by water-soluble carbodiimide and finally conjugated to antibody via acylation of amino groups.

![Diagram of HPLC chromatograms](image2)

**FIG. 2.** HPLC chromatograms of carboxylate (A) and active ester (B) forms of ⁹⁹ᵐTc-4,5-bis(thioacetamido)pentanoate (A) and its 2,3,5,6-tetrafluorophenyl derivative (B). The column used was reversed-phase Ultrasphere ODS (5 μm; Beckman). Conditions: 10% (vol/vol) CH₃CN/0.01 M phosphate, pH 7.0 (A); and 34% CH₃CN/0.01 M phosphate, pH 7.0 (B), at 1.0 ml/min. Pairs of peaks result from chelate ring epimers with respect to syn or anti alkyl carboxylate functionality relationship to the Tc oxo group (20). The decreased polarity because of esterification is evident by the increase in organic solvent necessary to elute the complexes.
RESULTS

Starting material, [99mTc]-pertechnetate, was reduced by dithionite in basic solution in the presence of the N₂S₂ ligand to give 99mTc-[4,5-bis(thioacetamido)pentanoate], which was converted to the 2,3,5,6-tetrafluorophenyl active ester by reaction with the phenol and water-soluble carbodiimide (Fig. 1). HPLC chromatograms of carboxylate and active ester forms of the Tc complex are shown in Fig. 2. The reduced polarity of the ester allowed purification by a low-pressure reversed-phase cartridge system. Conjugation to antibody lysine amino groups was carried out by addition of anti-melanoma 9.2.27 F(ab')₂ and buffer to adjust the pH to 9.0. HPLC chromatograms of crude product and 99mTc-N₂S₂-9.2.27 F(ab')₂ after gel filtration purification are shown in Fig. 3. The yields of the carboxylate form of the complex were essentially quantitative. The ester yields were about 70%. Conjugation yields ranged from 50% to 70%. Thus, overall yields were about 35% to 50%. Total time for the procedure was about 3 hr.

Support for the aminocyclation reaction to give an amide conjugate was provided by pH dependency of the reaction (Fig. 4). The yield of 99mTc-labeled antibody was low at neutral pH and increased as the pH increased in a manner expected for reaction-rate acceleration because of increased availability of deprotonated amino groups. Routinely, conjugations were run at pH 9 as a trade-off between yield and concern for maintaining mild conditions with respect to the relatively unstable F(ab')₂ fragments.

The stability of the 99mTc label was evaluated by challenge under several conditions. These included human serum, chelating agents 10 mM DTPA and 10 mM 2,3-bis(thioacetamido)propanoate as the free thiol form of the propanoate form of the N₂S₂ ligand, and 6 M urea for denaturation of the antibody fragment. The challenge with the propanoate form of the ligand allowed identification of released 99mTc radioactivity as (i) non-N₂S₂-associated, (ii) propanoate N₂S₂-chelated if exchangeable to an N₂S₂ complex, or (iii) simply nonspecifically associated pentanooate N₂S₂, the ligand form used for labeling. The results in Table 1 show insignificant losses of 99mTc radioactivity under all challenge conditions and, thus, high stability of the 99mTc label consistent with the metal bound in a stable complex and the complex covalently attached to the antibody.

The effects of antibody derivatization by preformed 99mTc-N₂S₂ (pentanoate form) complex conjugation on 9.2.27 F(ab')₂ were assessed by isoelectric focusing electrophoresis (Fig. 5). The protein mass was detected by Coomassie blue stain, and radioactivity was detected by autoradiography. No change in migration after labeling was seen in bands by staining. However, autoradiography indicated a shift to lower pI of the radiolabeled species. The covalent attachment of 99mTc would be expected to give a charge shift because of the net -1 charge on the 99mTc-N₂S₂ complex.

Immunoreactivity was assessed by measuring the fraction of 99mTc radioactivity bound to FMX-met melanoma cells. With 99mTc-N₂S₂ (pentanoate form) attached to whole 9.2.27, a mean immunoreactivity of 77 ± 4.0% SD (6 preparations) was found. Immunoreactivity appeared to be insignificant.

![Fig. 3. HPLC chromatograms of starting 9.2.27 F(ab')₂ (spectrophotometric trace in A), crude 99mTc conjugation product (radio- metric trace in B), and purified 99mTc-N₂S₂-9.2.27 F(ab')₂ (radio- metric trace in C).](image)

![Fig. 4. The dependency of the reaction of the 2,3,5,6- tetrafluorophenyl active ester derivative of 99mTc-4,5-bis(thioacetamido)pentanoate with 9.2.27 Fab antibody fragment on pH. The conjugation reaction was carried out at 1.0 mg/ml antibody fragment concentration with pH buffered as shown by phosphate. Nonspecific association of the active ester complex was minimized by addition of 25 mg of lysine after 30 min at room temperature.](image)

Table 1. Radiolabel stability studies of 99mTc-N₂S₂-9.2.27 F(ab')₂ by challenge with various agents

<table>
<thead>
<tr>
<th>Challenge</th>
<th>1 hr</th>
<th>3 hr</th>
<th>9 hr</th>
<th>24 hr</th>
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<td>97.4</td>
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<td>97.0</td>
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<td>98.1</td>
<td>97.2</td>
<td>96.5</td>
</tr>
<tr>
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<td>100.0</td>
<td>—</td>
<td>100.0</td>
</tr>
<tr>
<td>Urea</td>
<td>98.1</td>
<td>98.2</td>
<td>—</td>
<td>97.5</td>
</tr>
</tbody>
</table>

* N₂S₂ propanoate form.
† Serum stability was studied with a separate preparation. No change was observed in 24 hr by HPLC, NaDODSO₄/PAGE, or isoelectric focusing techniques.
comparable or better than that found with previous labeling methodologies using 9.2.27—e.g., 68% for 111In-DTPA-conjugated (21) and 59% for 125I- and 131I-labeled by the chloramine-T method (22). Cell binding immunoreactivity of 99mTc-N2S2-9.2.27 F(ab')2 was 74 ± 4.9% (11 preparations).

In vivo tumor targeting of 99mTc-N2S2-9.2.27 F(ab')2 was assessed in nude mice bearing melanoma A-375/m/m xenografts. The uptake in tumor and other organs is shown in Fig. 6 at 20 hr after injection. The concentration, as shown by the percent dose per g of body weight, was highest in tumor, with good clearance from all other organs indicated. Also shown for comparison are concentration values for 99mTc-N2S2-NR-2-AD F(ab')2, an irrelevant idiotypic antibody to an individual B-cell lymphoma. Values are somewhat higher in blood and much lower in tumor for the control antibody fragment. Other organ concentrations are comparable for each. The localization index as indicated by the tumor-to-blood ratio for specific antibody 9.2.27 divided by the ratio of tumor to blood for nonspecific antibody NR-2-AD was 25.2. Images of 99mTc-N2S2-9.2.27 F(ab')2, recorded 1, 4, and 16 hr after injection are shown in Fig. 7. Also included is an image of 99mTc-N2S2-NR-2-AD F(ab')2, recorded 16 hr after injection for comparison. Uptake in tumor is seen on early images with most of body background radioactivity except for kidneys clearing by 16 hr after injection. Kidneys are the primary route of excretion. Images of the kidneys are seen intensely at early times with significant clearing by 16 hr. The irrelevant F(ab')2 showed relatively increased blood pool activity at the 16-hr time point.

**FIG. 5.** Isoelectric focusing electrophoresis analysis of 99mTc-N2S2-9.2.27 F(ab')2. Lanes show markers and Coomassie blue staining of antibody F(ab')2, corresponding radioactivity of 99mTc-N2S2-conjugated F(ab')2, and a sample [lanes serum and 99mTc-N2S2-F(ab')2] in which the preparation was mixed 1:1 with serum. The shift in the autoradiography bands compared to protein staining can be attributed to the minus charge associated with the Tc complex. The isoelectric focusing was wide-gradient pH 3.5-9.5 in a 0.5-mm thick agarose gel (0.9% isogel, LKB) containing 2.5% (wt/vol) Ampholines (LKB) and 10% sorbitol. Samples applied were 10 µg of 9.2.27 F(ab')2 and 1:1 serum and labeled F(ab')2, diluted 1:9 with phosphate-buffered saline before application. Volumes were 15 µl. Specific activity of labeled F(ab')2 was 6.9 mCi/mg, and concentration was 1.8 mg/ml.

**DISCUSSION**

The results of these studies indicate that it is practical to label antibodies or their fragments with 99mTc by using well-
defined chemistry. The chemistry involves forming a stable complex with $^{99m}$Tc and subsequently conjugating the complex by using the standard active-ester approach under mild conditions. This has been done with a diamide dithiolate ligand system. High stability and retention of immunoreactivity has been demonstrated for $^{99m}$Tc-labeled antibodies and their fragments by this approach. Cleavage of F(ab')$_2$ to Fab' was not seen, which has been problematic for other approaches that involved the reducing agent stannous ion (23). Other work in our laboratory has shown similar labeling results for Fab or Fab'. The challenge studies of antibody fragments labeled with the N$_2$S$_2$ complexes indicate that the label was stably bound. Together with rapid clearance of antibody fragments and lack of normal tissue accumulation, optimal imaging can be achieved within periods appropriate for the short half-life of the isotope. Encouraging results of imaging sites of metastatic melanoma in patients by using the $^{99m}$Tc–N$_2$S$_2$ preformed chelate approach have been reported (24).

Rhenium has virtually identical structural properties in its complexes compared to Tc (25). We have demonstrated this with penicillamine, an N,S ligand (26). Application of the N$_2$S$_2$-metal complex conjugation technology for radiotherapy is thus apparent from these studies, since isotopes of rhenium, $^{186}$Re and $^{188}$Re, have favorable therapy properties because of their $\beta$ radiations. Preliminary studies of $^{186}$Re and $^{188}$Re indicate that the same N$_2$S$_2$ diamide dithiolate technology is applicable to the rhenium labeling of proteins and gives biodistribution properties that are virtually identical to those of $^{99m}$Tc (27).

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