Development of Radiochemically Pure Antibodies

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Abstract

There are presently three approaches to radiolabeling antibodies: direct radiolabeling; radiolabeling of a chelating agent conjugated to an antibody; and radiolabeling of a chelating agent before conjugation to an antibody. Using either the direct radiolabeling or radiolabeling of a chelating agent conjugated to an antibody has not led to a radiochemically pure \(^{99m}Tc\)-labeled protein. High radiochemical purity is obtained using a prelabeled ligand; therefore this method is preferred.

One of the major efforts since the first Conference on Radioimmunodetection and Radioimmunotherapy of Cancer has been the development of antibodies that are radiochemically pure in vitro and remain so in vivo. Since radioiodine has been one of the first radionuclides used to label antibodies, many studies have centered around the preparation of radiochemically pure antibodies either by direct iodination or by indirect labeling through a reagent such as the Bolton-Hunter ligand. The current research emphasis is on in vivo stability using various iodinated species less susceptible to enzymatic deiodination (1, 2).

There have been two approaches to radiolabeling antibodies with metallic radionuclides using bifunctional chelates; either the chelating agent is first attached to the antibody followed by radiolabeling or the chelating agent is first radiolabeled and the resulting chelate is bound to the antibody. Very few experiments have shown that first attaching the chelating agent to the antibody results in an "instant" kit in which all the radiolabel is bound via the chelating agent. For \(^{99m}Tc\), the indirect labeling methods have mostly involved the use of DTPA bound to the protein. DTPA is considered a bifunctional chelate in that it binds to the protein and to the \(^{99m}Tc\). Other chelating agents, derivatives of DADS (3), derivatives of bis-N-methylsemicarbazone (4), and metallothionein (5), have been conjugated to antibodies for \(^{99m}Tc\) labeling. Although \(^{99m}Tc\) labeling conditions and biodistribution studies of the labeled products were reported, a systematic investigation of the effectiveness of this latter chelator for complexing \(^{99m}Tc\) in competition with antibody has been reported only in abstract form (6).

Hnatowich's group (7, 8) has carried out various experiments to determine the expected ratio of \(^{99m}Tc\) binding to the chelating agent in the bifunctional chelate compared to direct binding to the antibody. Using 50 \(\mu\)g/ml DTPA as the molar equivalent to 20 mg/ml IgG, (0.13 \(\mu\)mol/ml) various binding conditions were tested. Without protein present, DTPA complexed reduced \(^{99m}Tc\) efficiently, but not quantitatively (Table 1). The DADT-3C-OH in which the ethylene backbone is replaced with a propylene backbone has the highest percentage binding. This appears to be the only example of a chelating agent that can compete quantitatively with an equimolar concentration of IgG.

If the source of \(^{99m}Tc\) is \(^{99m}Tc\)-EDTA at 100 \(\mu\)g/ml, then the requirement for chelator concentration is less (Table 4). Using 150,000 as the molecular weight of IgG there are 33 \(\text{nmmol/ml}\) of IgG. If the molecular weight of the free amine is used (236), then 3 \(\mu\)g of DADT are 13 \(\text{nmol/ml}\) and 8 \(\mu\)g of DADT are 34 \(\text{nmol/ml}\). These molar values do not agree with the molar ratios stated by Liang et al. (9) to determine if they had advantages over DTPA as bifunctional chelates of antibodies (Table 2). Optimal concentrations of tin and chelator were determined to be 2.5 to 5 \(\mu\)g/ml SnCl\(_2\)-2H\(_2\)O and 50 \(\mu\)g/ml of chelator. The optimal pH range was 5 to 8. Labeling in the presence of 5 mg/ml of IgG was used to determine the percentage expected to be labeled via the \(\mathrm{NiS}_2\) chelating agent and the percentage expected to be directly bound to the antibody (Table 3).

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Yokoyama's group (10) has developed the neutral \(^{99m}Tc\) chelate ketobisethiosemicarbazone for use in bifunctional chelates. Various derivatives have been prepared including the \(p\)-carboxyethylphenylglycol derivative, which has been conjugated to antibody by activating the acid group using the phosphorylazide method. Stannous ascorbate was used as the reducing agent and \(^{99m}Tc\)-ascorbate as the transfer agent. As in most of these studies the optimal pH is between 4.5 and 6.2. The labeling experiments were carried out to determine what percentage of the \(^{99m}Tc\) binds to the chelating agent and what percentage binds directly to the antibody. At ratios of chelating agent to antibody that preserve the immunological activity (i.e., 1:1 molar ratio), about 15-20% of the \(^{99m}Tc\) is directly bound to the protein. In vitro and in vivo stability of the derivatized antibody is high. Although direct binding of \(^{99m}Tc\) to antibodies has been reported (11–14) the stability and inertness of the bond are in question. There appear to be two binding sites on the antibody, one with high affinity and one with lower affinity.

Steigman et al. (15) were the first to suggest that sulfhydryl groups were responsible for the direct binding of \(^{99m}Tc\). Paik et al. (16, 17) substantiated this hypothesis by carrying out a number of competitive experiments and by titrating the sulfhydryl groups using Ellman's reagent. The available sulfhydryl groups were determined after 30 min incubation of stannous chloride with antibody. The stannous chloride was present in 10-fold excess over the protein. The results obtained are reported in Table 5.

The presence of both high affinity sites and low affinity sites on the protein was defined by competition studies using DTPA. The high affinity sites were determined as those which bound \(^{99m}Tc\) even at large molar excesses of DTPA. The percentage bound to protein was determined at various ratios of DTPA to protein.

These results are obtained only under conditions of direct competition between DTPA and antibody. If the protein is labeled first, then DTPA is effective only at high pH. One
hypothesis is that the high affinity binding to IgG involves a Tc(V) species whereas the binding to DTPA involves a Tc(III) or Tc(IV) species. Competitive experiments indicate that the distribution of 99mTc between the competing ligands is determined by the forward rate constants but not by the equilibrium constants for these reaction conditions. This direct labeling approach has been refined by others to maximize the percentage of technetium on the high affinity sites. Distribution studies in mice showed similarities among the antibodies labeled by different techniques at the high affinity sites (16, 17). The generally lower % D/g values for the antibody containing both strongly and weakly bound 99mTc indicates that a soluble chelate rather than pertechnetate may be the major radiochemical impurity. The development of an "instant kit" will be difficult with this approach. Fritzberg (18, 20) has compared the use of direct labeling a bifunctional chelate and a preformed chelate to determine relative radiochemical purity and stability. He used the 2,5-dimercaptosuccinimide as a 99mTc substitute for hippuran. In the case of bifunctional chelate, the ligand was conjugated to antibody using an activated ester. The antibody-ligand complex was then radiolabeled by ligand exchange. For the prelabeled ligand, the diamidodithio ligand was radiolabeled first, the acid was activated to the ester, and then the prelabeled ligand was reacted with the antibody. The relative stabilities were tested by incubation of each radiolabeled antibody type with either DTPA or excess diamidodithio ligand (Table 6). These experiments show one of the problems with using prelabeled ligands. Only 4% of the 99mTc becomes bound to the ligand. For the prelabeled ligand, the diamidodithio ligand was radiolabeled first, the acid was activated to the ester, and then the prelabeled ligand was reacted with the antibody. The relative stabilities were tested by incubation of each radiolabeled antibody type with either DTPA or excess diamidodithio ligand (Table 6).

It is clear from this experiment that the prelabeled ligand forms the most stable bond. Esterification of the preformed technetium complex went in about 70% yield. Conjugation yields using 2.5 mg/ml F(ab')2 at pH 9 were between 50 and 70%.

Franz et al. (19) have used a derivative of the well characterized cyclam to avoid direct antibody binding. Cyclam was derivatized at the 1-nitrogen with a 3-aminopropyl derivative. The cyclam derivative was treated with 2-iminothiolate to form the imidate. This was reacted with activated IgG to form the covalently bound chelating agent, do not achieve high radiochemical purity, investigators have turned to prelabeling the ligand and then reacting this 99mTc chelate with the antibody. Fritzberg et al. (3, 18) have prelabeled the DADS ligand and then bound the ligand to the antibody via an activated ester. Likewise Franz et al. (19) have prelabeled the N-propylamine derivative of cyclam with 99mTc and then reacted that chelate with an activated antibody. This method assures that the 99mTc is bound to the chelating agent and, therefore, that a single radiochemical should be produced. On the other hand, the development of an "instant kit" will be difficult with this approach. Fritzberg (18, 20) has compared the use of direct labeling a bifunctional chelate and a preformed chelate to determine relative radiochemical purity and stability. He used the 2,5-dimercaptopropanoate as the ligand. The core ligand (Tc-DADS) has been suggested as a 99mTc substitute for technetium went in about 70% yield. Conjugation yields using 2.5 mg/ml F(ab')2 at pH 9 were between 50 and 70%.

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<table>
<thead>
<tr>
<th>Challenge</th>
<th>Direct labeled</th>
<th>Bifunctional chelate</th>
<th>Prelabel ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM DTPA</td>
<td>0.5</td>
<td>0.7</td>
<td>0</td>
</tr>
<tr>
<td>1 mM N2S2</td>
<td>54.0</td>
<td>32.0</td>
<td>0</td>
</tr>
<tr>
<td>6 m urea</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Taken from Fritzberg (20).

Table 2 Various diaminodithiol derivatives*  
<table>
<thead>
<tr>
<th>Compound</th>
<th>molar ratio</th>
<th>% 99mTc chelate</th>
</tr>
</thead>
<tbody>
<tr>
<td>DADT</td>
<td>1:1</td>
<td>98</td>
</tr>
<tr>
<td>DADT</td>
<td>2:1</td>
<td>94</td>
</tr>
<tr>
<td>DADT</td>
<td>3:1</td>
<td>99</td>
</tr>
<tr>
<td>DADT</td>
<td>5:1</td>
<td>92</td>
</tr>
<tr>
<td>DADT</td>
<td>10:1</td>
<td>96</td>
</tr>
</tbody>
</table>

* Taken from Liang et al. (9).

Table 4 Labeling of DADT by trancomplexation from 99mTc-EDTA in the presence of 5 mg/ml IgG*  
<table>
<thead>
<tr>
<th>DADT (μg/ml)</th>
<th>DADT-IgG (molar ratio)</th>
<th>% 99mTc as Tc-DADT</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2</td>
<td>85</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>88</td>
</tr>
</tbody>
</table>

* Mean ± SD.
coping group, then optimal radiolabeling conditions may not be available. On the other hand, if the technetium complex is activated toward conjugation after formation, another pro-
cedural step is required. This balance must be dealt with to obtain maximum efficiency.

The ideal nuclear properties of $^{99m}$Tc warrant further definition of the chemistry of technetium. To date, the labeling of antibodies with $^{99m}$Tc using an “instant kit” has not resulted in a product with high radiochemical purity. High radiochemical purity is obtained using the prelabeled ligands, but this necessitates a multistep kit. However, in light of the large number of variables involved in each experiment with radiolabeled monoclonal antibodies, it seems prudent to use only preformed ligands, not only with $^{99m}$Tc, but with all metallic radionuclides, until such time that an ideal combination of radionuclide and antibody can be identified. The use of directly labeled antibodies or antibodies labeled nominally via bifunctional chelates has added an unnecessary complication to the already complicated experiment involving the search for the ideal diagnostic or therapeutic antibody.

Since the First Conference many new labeling techniques and chelating agents have been developed. One purpose of this conference is to investigate these new techniques and chelating agents to determine if radiochemically pure antibodies have been developed.

References

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