Published in final edited form as:
Methods Mol Biol. 2014 ; 1141: 147–157. doi:10.1007/978-1-4939-0363-4_9.

Antibody Labeling with Radioiodine and Radiometals

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1. Introduction

Radiolabeled antibodies have diverse applications in biomedical research and clinical practice. Due to their ability to selectively target tumor antigens, radiolabeled monoclonal antibodies (MAbs) are used for the delivery of both diagnostic and therapeutic radionuclides *in vivo* for radioimmunodiagnosis and radioimmunotherapy, respectively. Further, several radiolabeled antibodies serve as critical reagents in radioimmunoassays for quantitative estimation of biomarkers in serum. The selection of radionuclide for antibody conjugation depends on use of the radioimmunoconjugate and is dictated by the range of emission, emission type and half-life of radionuclide [1]. Various radionuclides decay by emitting γ-radiation, β^- particles or α-particles. Due to their greater emission range, considerable penetration, and low linear energy transfer rates, β^- emitters can kill surrounding cells by cross-fire effect and are thus used as therapeutic radionuclides [2]. ^{90}Y-a pure β-emitter, and I^{131}-a dual β and γ emitter, are the only FDA approved therapeutic radionuclides for conjugating antibodies for cancer therapy, while ^{111}In and ^{99m}Tc (γ emitters) labeled MAbs have been approved for diagnostic applications. Due to its relatively long half-life and ease of handling, ^{125}I, is also the radionuclide of choice for antibody-based radioimmunoassays, tracer studies for pharmacokinetics and biodistribution, and treatment of microscopic residual disease [3].^{177}Lu due to its short half-life (6.7d) ability to emit both gamma and beta radiation can be used simultaneously for therapy and diagnosis. Due to its shorter range of penetration than other β^- emitters, it has been explored for the treatment of smaller tumors in many clinical trials [4]. While predominantly intact IgGs are conjugated to radionuclides, various other formats including Fab′ and scFvs have been used for various clinical and preclinical applications [5,6].

The coupling of MAbs to a radionuclide depends upon the chemistry and half-life of radionuclide. Due to their easy availability, ease of handling and relatively longer half-lives, radioisotopes of iodine (^{123}I, ^{125}I ^{131}I) have been extensively used for labeling antibodies. The chemistry of iodine is well understood and it can form stable covalent bonds causing

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minimal alteration to the protein backbone. It is directly introduced by halogenation (in presence of enzymatic or chemical oxidants) of tyrosine and histadine residues of the MAbs [7]. Iodogen, and Chloramine-T are the most commonly used chemical oxidants used for direct labeling and convert sodium iodide to iodine form, which spontaneously incorporates into tyrosyl groups of the proteins. In order to achieve higher labeling efficiency the oxidant should be compatible with the aqueous solution of protein and should not affect the structure of the protein. In contrast to Chloramine-T, Iodogen method achieves lower specific activity, but exhibit relatively milder effect on protein stability. Unlike iodination, conjugation of metallic radionuclides such as ^{90}Y, ^{111}In, ^{177}Lu, ^{99m}Tc to antibodies requires a chelating agent. The selection of chelating agent largely depends on the physical properties and oxidation state of the radiometal ion to be conjugated. Usually, a bi-functional chelating agent (BFCA) is used which can bind covalently to MAbs on one hand and chelate radiometals on the other without affecting the kinetic and thermodynamic stability. The chelator provides the donor atoms which saturate the coordination sphere of the metal complex, thus stabilizing it. Several chelators like DOTA (1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetracetic acid), DTPA (NR-diethylenetriaminepentacetic acid), NOTA (1, 4, 7-triazacyclononane-1, 4, 7-acetic acid) have been used for radiolabeling antibodies for radioimmunotherapy and radioimmunodiagnosis. In this chapter, the labeling of antibody with heavy metal radionuclides (^{177}Lu, ^{99m}Tc) and radiohalogen (^{125}I) is described.

2. Materials Required (Note 1)

All solutions must be prepared in ultrapure water unless specified

2.1 Labeling with Radioiodine (Note 2)

1. Iodogen (Pierce Chemical, Rockford)
2. $Na^{125}I$ or $Na^{131}I$ (New England Reactor, Boston, Massachusetts)
3. 10 mM sodium phosphate buffer: Add 3.1 g of $NaH_2PO_4.H_2O$ and 10.9 g of Na_2HPO_4 to distilled water and make up the volume to 1 liter. Set the pH of the solution to 7.2 and store at 4°C.
4. 5 mM sodium Iodide: Dissolve 74.9 g of sodium iodide in 100 ml of ultrapure water and store at room temperature.
5. Chloroform

2.2 Radiolabeling with ^{99m}Tc (Note 3)

1. Tricine (Sigma Aldrich): Dissolve 1 mg of tricine in 1 ml of ultrapure water to attain a concentration of 1 mg/ml and store at room temperature.

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[1]Before using radioactive isotopes consult the radiation safety office for proper handling, usage and disposable of radionuclides.
[2]Free radioiodine (NaI) is volatile and should only be handled in a fume hood. In general all labeling reactions must be performed in fume hood with appropriate lead shielding.
[3]For labeling with radiometals, all reagents should be prepared in prepared in metal-free water using metal-free glassware and pipettes. Metal ions from water and reagents can be eliminated either by passing them through Chelex-100 column or by addition resin directly to the reagents.

2. Stannous Chloride dihydrate (Sigma Aldrich): Dissolve 1 mg of stannous chloride in 1 ml of 0.1 N HCl to attain a concentration of 1 mg/ml and store at room temperature.
3. N-hydroxy succinimide sodium salt (NHS) (Pierce), stored dry at ambient temperature.
4. 20x PBS (Phosphate buffer saline): Dissolve 160 g NaCl, 4 g KCl, 28.8 g NaH_2PO_4, 4.8 g KH_2PO_4 in 600 ml of ultrapure water. Mix well, set pH to 7.4 and make up the volume to 1 liter. For the working solution add 50 ml to 950 ml of ultrapure water. This will give a working concentration of 137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH_2PO4 and 1.4mM KH_2PO_4.
5. 10 mM sodium phosphate buffer: Add 3.1 g of $NaH_2PO_4.H_2O$ and 10.9 g of Na_2HPO_4 to distilled water and make up the volume to 1 liter. Set the pH of the solution to 7.8. The solution can be stored at 4°C for up to 1 month.
6. 20 mM sodium citrate: Dissolve 5.88 g of sodium citrate dihydrate in 1 liter of the water and store at room temperature.
7. 150 mM/L sodium acetate: Dissolve 12.30 g of anhydrous sodium acetate in 600ml of ultrapure water. Set the pH of the solution to 7.8 and make up the volume to 1 liter and store at room temperature.
8. 30 mM Dimethyl Formamide
9. ^{99m}Tc (supplied as pertechnate-$^{99m}TcO_4$, fresh from 99mTc generator)

2.3 Radiolabeling with $^{Lu}177$ (Note 3)

1. ITCB-DTPA (isothiocyanato-benzyl-diethylene penta-acetic acid) (Sigma, Poole, Dorset, UK): Prepare 5 mM aqueous solution
2. ^{177}Lu (usually supplied as $^{177}Lu_2O_3$) (Oak Ridge National Laboratory, Oak Ridge, TN)
3. Chelex-100 Resin (BioRad Laboratories, CA)
4. 20x PBS (Phosphate buffer saline): Dissolve 160 g NaCl, 4 g KCl, 28.8 g NaH_2PO_4, 4.8 g KH_2PO_4 in 600 ml of ultrapure water. Mix well, set pH to 7.4 and make up the volume to 1 liter. For the working solution add 50 ml to 950 ml of ultrapure water. This will give us 137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH_2PO4 and 1.4 mM KH_2PO_4.
5. 0.05 M sodium carbonate: Dissolve 5.29 g of sodium carbonate in 600 ml of ultrapure water. Set the pH of the solution to 8.3, adjust the volume to 1 liter and store at room temperature.
6. 0.06 M sodium citrate: Dissolve 17.64 g of sodium citrate dihydrate in 600 ml of ultrapure water. Set the pH of the solution to 5.5 with 1N HCl, adjust the volume to 1 liter and store at room temperature.

7. 0.6 M sodium acetate: Dissolve 49.21 g of sodium citrate dihydrate in 600 ml of ultrapure water. Set the pH of the solution to 5.3 with 1N HCl and adjust the volume to 1 liter. The solution can be stored at room temperature.

2.4 SDS-Polyacrylamide Gel Components

1. Resolving gel buffer (4x Tris HCl pH 8.8): Dissolve 182 g of Tris base in 600 ml of water. Adjust pH to 8.8 with 1N HCl and add water to make 1000 ml. Filter the solution through 0.45 μm filter, add 2 g of SDS (sodium dodecyl sulfate) and store at 4°C.
2. Stacking gel buffer (4x Tris HCl pH 6.8): Dissolve 60.5 g of Tris base in 600 ml of water. Adjust pH to 6.8 with 1 N HCl and add water to make 1000 ml. Filter the solution through 0.45 μm filter, add 4 g of SDS and store at 4°C.
3. 6x SDS sample buffer: To 7 ml of Tris HCl pH 6.8 add 3 ml of glycerol, 1 g of SDS and 0.5 ml of beta mercaptoethanol. Add12 mg of bromophenol blue and mix it well. Make up the volume to 10 ml by water and store in −20°C.
4. 30% acrylamide solution (National diagnostics)
5. 10% Ammonium persulfate (APS): Dissolve 100 mg of APS in 0.7 ml of water and adjust the volume to 1ml. Prepare fresh for each use.
6. N,N,N,N′-tetramethyl-ethylenediamine (TEMED) (Fischer Bioreageants)
7. SDS-running buffer: Add 12 g of Tris, 57.6 g of Glycine and 40 ml of 10% SDS in 2.5 liter of water and mix it well. Adjust volume to 4 liter with water.

2.5 Coomassie Staining Components

1. Staining solution: Add 100 ml of glacial acetic acid to 500 ml of water. With constant stirring add 400 ml of methanol and 1 g of Coomassie R250 dye and mix well. Filter with 0.45 μm filter and store at room temperature.
2. Destaining solution: Add 200 ml of methanol and 100 ml of glacial acetic acid in 700 ml of water and store at room temperature.

2.6 Instant Thin layer chromatography (ITLC) components

1. ITLC-SG strips (Silica impregnated glass fiber sheets)
2. Chromatography Chamber
3. Methanol
4. 150 mM Sodium acetate

2.7 Other Components

1. Fume hood (SEFA 1-2010)
2. Gamma counter
3. Dose Calibrator (Capintec, Inc, Ramsey, NJ)

4. Lead shielding
5. Gel Dryer
6. Sephadex G-10 column and G-25 column (Pharmacia)
7. Microseperation filter (Centricon 30)
8. pH meter
9. Sterile 12 × 75 mm glass tubes
10. Glass Beaker
11. Centrifuge
12. Eppendorf tubes
13. Glass plates
14. Whatmann filter paper 3
15. Kodak Film (Rochester, NY)
16. Light plus intensifying screen (Wilmington, DE)
17. X-Ray cassette

3. Methods

3.1 Labeling of Antibody with ^{125}I [8]

1. Dissolve Iodogen in chloroform to attain a concentration of 10mg/ml.
2. Dispense 200 μl of Iodogen solution in glass tubes and dry chloroform under a gentle stream of air while constantly swirling the tube to ensure uniform coating.
3. Cap the tube and store in −20°C till further use. Iodogen coated tubes can be stored for up to 1 year.
4. Equilibrate Sephadex G-25 10 ml column with 10 column volume of 0.1 M sodium phosphate buffer (or any desired buffer for downstream application of the antibody)
5. Place the Iodogen-coated tube in the fume hood and allow it to come to room temperature. Add 10 μl of 100mM sodium phosphate buffer (pH 8.0).
6. Adjust the concentration of antibody solution to 1mg/ml and add 50–200 μl of the antibody to Iodogen coated tube containing sodium phosphate.
7. Behind an appropriate lead shielding in a fume hood carefully open the vial containing radioiodine and determine the radioactivity/μl using a dose calibrator. Add 50–200 μCi of radioiodine (^{125}I or ^{131}I) to the bottom of the tube and gently swirl the tube. Typically 1 μCi radioiodine is added per μg of protein. However if higher specific activity is desired, the ratio can be adjusted by adding more radioiodine. (Note 4)
8. Measure the total radioactivity in the reaction tube using dose calibrator. After 2–3 min incubation at room temperature, load the samples on the buffer-equilibrated

Sephadex column to separate the iodinated antibody from the free iodine. Rinse the tube with 50–100 μl of sodium phosphate buffer and add the resulting solution to buffer.

9. Once the entire antibody-radioiodine reaction mixture has entered into the column matrix, add sodium phosphate buffer to fill the column reservoir and collect twenty 500 μl fractions in 5 ml (75 X 12 mm) plastic tubes. Measure the radioactivity in each fraction. The first peak represents radioiodinated protein while the subsequent flat peak represents free iodine.
10. Cap the column and measure the residual activity using dose calibrator.
11. Pool the fractions of the iodinated antibody and store the labeled antibody at 4°C.
12. Determine the efficiency of labeling from radioactivity measurements from steps 8–11 and perform ITLC to determine free radioiodine.
13. Calculate the specific activity of the radiolabeled antibody (Note 5)

3.2 Labeling of Antibody with ^{99m}Tc [9,10]

3.2.1 Preparation of Antibody-chelator conjugate

1. Dissolve succimidyl-6-hydrazinonicotinate hydrochloride (SHNH) in 30mM dimethyl formamide to prepare the hydrazinonicotinamide chelator at a concentration of 2–4 mg in 100–200 ul.
2. Dissolve 5 mg of IgG in 1ml of 0.1 M sodium phosphate buffer pH 7.8.
3. With constant stirring add 10 parts of modified SHNH to 1 part of IgG in 0.1 M sodium phosphate at 4°C in dark.
4. Allow the reaction to occur overnight.
5. Set up the Sephadex G-10 column and equilibrate with 10 column volumes of 0.1 M sodium phosphate.

[4]If dose calibrator is not available, radioactivity can be measured using gamma counter and CPM can be converted to Ci, mCi or μCi. First convert CPM (counts per minutes t to DPM (disintegrations per minute) as follows:

$$\mathrm{DPM}=\frac{\mathrm{CPM}_{\mathrm{sample}}-\mathrm{CPM}_{\mathrm{background}}}{\mathrm{Detector\ Efficiency}}$$

The background CPM and detector efficiency should be determined for gamma counter as per manufacturer's instructions.

$$1\,\mu\,\mathrm{Ci}=2.22\times10^{6}\,\mathrm{DPM}$$

[5] Specific activity is the amount of radioactivity per unit mass of protein. To determine specific activity, the amount of radioactivity in the radiolabeled protein must be measured using a gamma counter and the protein concentration should be determined using any standard protein estimation method (BCA, Bradford).

6. Purify the modified or bound protein from the unreacted fraction by loading the protein on column using 100 mM NaCl pH 5.2 buffered with 20 mM sodium citrate..
7. Collect the fractions as flow through in a fresh tube and pass through the column again.
8. Pool the fractions containing conjugated protein concentrate the pooled fractions to 1 mg/ml using Centricon 100 centrifugal filters.
9. Store the SHNH-antibody conjugate at 4°C till further use.

3.2.2 Radiolabeling of the Antibody

1. Aliquot 100 μg (100 μl) of tricine and 25 μg (25 μl) stannous chloride to fresh reaction tubes.
2. Using gamma counter measure 1 mCi of ^{99m}Tc (sodium pertechnate) and add to reaction tube described in step 1.
3. Allow the reaction to occur for 15 min at room temperature.
4. Add 400 μg of SHNH derivatized IgG to the reaction tube containing ^{99m}Tc tricine and stannous chloride.
5. Allow the reaction to occur for 45 min at room temperature.
6. Set up the Sephadex G-25 column and equilibrate with 10 column volume of 0.1 M sodium phosphate.
7. Load the sample in column to separate the radiolabeled IgG from free ^{99m}Tc.
8. Elute the column with buffer consisting of 100 mM NaCl pH 7.5 buffered with 20 mM sodium citrate and collect fractions as described in step 9 in section 3.1.
9. Pool fraction corresponding to the radiolabeled protein and concentrate the pooled fractions to 1mg/ml using a Centricon 100 by centrifugation.
10. Determine the labeling efficiency using ITLC.

3.3 Labeling of Antibody with ^{177}Lu [4]

3.3.1 Preparation of Antibody-chelator conjugate

1. Prepare the antibody in sodium carbonate buffer, pH 8.3 such that the final concentration of 5mg/ml is achieved.
2. Add 33 μl aqueous solution of ITCB-DTPA to the above tube.
3. Allow the reaction to proceed for 2 h at room temperature.
4. Equilibrate Sephadex G-25 column with 10 column volume of 0.05 M sodium carbonate.
5. Separate the ITCB-DTPA bound antibody fractions from the unreacted fractions by passing through the column.

6. Collect the fractions as flow through in a fresh tube and pass through the column again.
7. Pool the bound fractions by eluting with 100 mM PBS buffered with 20 mM sodium carbonate.
8. Adjust the immunoconjugate concentration to 10 mg/ml in PBS.
9. Aliquot the fractions into fresh tubes and store in −20°C till further use.

3.3.2 Radiolabeling of the Antibody

1. Thaw 1 mg of the immunoconjugate and allow it to reach room temperature.
2. Transfer the content to fresh reaction tube.
3. Add 50 μl each of 0.6 M sodium acetate and 0.06 M sodium citrate to the above tube.
4. Measure 1 mCi activity of ^{177}Lu using a dose calibrator and add to the tube using metal free pipette tips.
5. Allow the reaction to occur for 2 hours at room temperature.
6. Equilibrate Sephadex G-25 column with 10 column volume of 0.05 M sodium carbonate and load the sample to separate the ^{177}Lu bound hot fractions from the unbound one.
7. Collect the fractions as flow through in a fresh tube and pass through the column again as described in step 9 section 3.1.
8. Pool the bound fractions by eluting with 100 mM PBS buffered with 20mM sodium carbonate.
9. Concentrate the pooled fractions to 1mg/ml concentration using Centricon 100.
10. Check the labeling efficiency using ITLC.
11. Note 9

3.4 Assessment of Radiochemical purity using ITLC

1. Cut ITLC-SC sheet into narrow strips (1 cm X 10cm).
2. On each side ITLC strips mark with a soft pencil an origin (approximately 1 cm from the bottom of the strip).
3. Using a water-soluble marker place a small dot 1 cm below the upper edge of the ITLC strip (This helps to follow the progress of the elution: remove the strip from the developing chamber when the ink begins to run).
4. Place 1–2 ul of column purified radiolabeled antibody (from pooled fractions) at the origin of the ITLC strip and allow it to air dry. Run triplicate ITLC strips radioimmunoconjugate.

[9] Other chelators can also be used for radiometal labeling of antibodies [see refs [12,13] for details]

5. Place the strips carefully in chromatography chamber containing appropriate solvent (meniscus not higher than 0.5 cm from the bottom) such that the bottom touches the solvent and strip lies on the chamber wall. Cover the chamber with the lid. (Note 6)
6. Allow the solvent to reach the ink dot, remove strips from the developing chamber and allow to air-dry (approximately two minutes).
7. Cut the ITLC strip into two equal top and bottom parts. Bottom contains origin with protein bound radioactivity; while top contains solvent front with free radionuclide.
8. Place the top and bottom parts in in two separate tubes and measure radioactivity using gamma counter.
9. Calculate percent protein bound radioactivity according to the formula listed below: (Note 7)

$$\frac{\mathrm{CPM}^{\mathrm{bottom}} \times 100}{(\mathrm{CPM}^{\mathrm{top}} + \mathrm{CPM}^{\mathrm{bottom}})}$$

3.5 Gel Electrophoresis

1. Perform a SDS-Polyacrylamide gel electrophoresis (PAGE) under reducing and non-reducing conditions [11].
2. Following electrophoresis remove the gel from glass plate and rinse with ultrapure water.
3. Add coomassie staining solution and put for 1hr at room temperature under mild shaking conditions.
4. Add destaining solution, replacing the solution by every 15–20 mins until faint bands are seen. Continue destaining the gel till bands are clean.
5. Rinse the gel with ultrapure water once.
6. With the help of Whatman filter paper carefully remove the gel and place on a gel dryer.
7. Allow the gel to dry for 2hrs at 80°C.
8. Place the gel in an X-ray cassette and in expose the gel to an autoradiography film overnight.
9. Develop the film to visualize protein bands. A single band indicating intact antibody should be visible under non-reducing conditions while two bands corresponding to antibody heavy and light chains should be visible under reducing

[6] Methanol/water (1:4 v/v) is used as a solvent for ITLC of radiodinated antibody, and 0.15 mM sodium acetate is used for ^{99m}Tc. For ^{177}Lu, parallel ITLC strips should be run in Methanol/water and sodium acetate.

[7] The amount of free radionuclide should not exceed more than 5%. Excess free label should be removed using Sephadex 25 column.

conditions. There should be minimal signal near the dye front (indicating free radionuclide). (Note 8)

Acknowledgments

The authors on this work are supported, in part, by grants from the National Institutes of Health P20GM103480, R21 CA156037, U01CA111294, R03 CA 139285, R03 CA167342, P50 CA127297 and U54163120.

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[8] Immunoreactivity of the radiolabeled antibody should be ascertained using appropriate immunoassay established in the laboratory (solid phase RIA, ELISA or immunoblotting).