RADIOCHEMISTRY AND RADIOPHARMACEUTICALS

Factors Influencing DTPA Conjugation with Antibodies by Cyclic DTPA Anhydride

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Diethylenetriaminepentaacetic acid (DTPA) was conjugated with a practical concentration (300 μg/ml) of antibody to human serum albumin (Ab) and 1083 17-1A monoclonal colorectal antibody (MAb-17-1A) via an acylation reaction using cyclic DTPA anhydride (cDTPAA). The conjugation reaction was favored as pH increased. Bicarbonate buffer at pH 8.2 was chosen for studies of the effect of the cDTPAA-to-antibody ratio on DTPA conjugation with antibody because of its good buffer capacity at that pH. The reaction of cDTPAA with Ab at molar ratios of 2000, 1000, 500, and 100 in the bicarbonate buffer gave rise to 11, 9, 8, and 2 indium atoms incorporated per Ab with 47%, 55%, 59%, and 77% retention of the binding activity. For the conjugation reaction of MAb-17-1A, 29, 28, 31, 11, 4, and 1 indium atoms were incorporated, with the retention of <5%, <5%, <5%, 12%, 60%, and 93% of binding activity when the molar ratio was 5000, 2000, 1000, 500, 100, and 50.

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Carboxylic acid anhydrides are common acylating agents for the modification of proteins at the amino group of lysine residues and at the hydroxyl group of tyrosine residues (*1,2*). There are two reported anhydride methods for conjugation of DTPA to proteins: the DTPA carboxycarbonic mixed anhydride method (*3–9*), and the cyclic DTPA anhydride method (*10–12*). The former, however, requires extreme caution because the DTPA carboxycarbonic mixed anhydride is thermally unstable and hydrolyzes very rapidly. On the other hand, cyclic DTPA anhydride is sufficiently stable to be isolated for identification. Hnatowich et al. (*10*) reported a successful conjugation of DTPA to human serum albumin (18.8 mg/ml, $2.81 \times 10^{-4} M$) using cDTPAA as an acylating agent. We have used this agent to conjugate DTPA to a practical concentration (300 μg/ml) of antibody (Ab) to human serum albumin and of 17-1A monoclonal colorectal carcinoma antibody (MAb-17-1A). With antibody at a low concentration, the conjugation yield is very low because it cannot compete effectively with the hydrolysis reaction of the DTPA anhydride with water. We have attempted to optimize the DTPA conjugation to a practical concentration of antibody (300 μg/ml) by varying the pH values of aqueous media and also by varying the molar ratios of cyclic DTPA anhydride to antibody.

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MATERIALS

The following reagents were procured: rabbit antibody (Ab) to human serum albumin*, 1083-17-1A monoclonal colorectal carcinoma antibody† (MAb-17-A, for details see Ref. *13*), human serum albumin (HSA), Sephadex G-150, acetic anhydride, pyridine, dimethyl sulfoxide, and DTPA. The Ab was purified using an

affinity column containing Sepharose-4B-conjugated HSA. Monomeric HSA was isolated from the HSA by a gel filtration column containing Sephadex G-150. DTPA, pyridine, and acetic anhydride were used without further purification. Dimethyl sulfoxide was purified by fractional freezing at 18.4°C.

EXPERIMENT

Preparation of cyclic DTPA anhydride. This was carried out by the method of Eckelman et al. (*14*). Dry and ground diethylenetriaminepentaacetic acid (DTPA, 19.75 g, 0.05 mole) was placed in a 250-ml three-neck flask equipped with a condenser, a magnetic stirrer, a thermometer, and a heating mantle. Acetic anhydride (37.8 ml, 0.4 mole) and pyridine (25.5 ml, 0.32 mole) were added to the flask and the solution was stirred for 24 hr at 65 ± 4°C. The resulting anhydride was washed thoroughly with acetic anhydride and with dry diethyl ether. The cream-colored powder was then freeze-dried. Decomposition point was 179–185°C. Element analysis calculated for $C_{14}H_{19}N_3O_8$: C = 47.06, H = 5.36, N = 11.76, O = 35.82. Found: C = 46.95, H = 5.48, N = 11.76, O = 35.86. IR spectrum showed absorption bands at 1825 and 1780 cm^{-1}, characteristic stretching vibrations for the anhydride carbonyl group. It also showed an absorption at 1640 cm^{-1} indicating the presence of a carboxylate group. This spectrum agrees with those reported by Hnatowich et al. (*10*) and Karesh (*15*).

Conjugation of DTPA with anti human serum albumin antibody. cDTPAA and Ab (300 μg/ml) at the molar ratio of 5000:1 were reacted to ensure DTPA conjugation with Ab sufficient to obtain a high specific activity of In-111 labeled Ab. Moreover, the pH effect is better determined with a higher conjugation. Since *N*-acylation is pH-dependent, we have chosen four different buffer solutions (0.1*M* Hepes buffer at pH 7; 0.1*M* phosphate buffer at pH 7; 0.1*M* borate buffer at pH 8.6; and 0.1*M* bicarbonate buffer at pH 8.2) in order to find an optimum condition for DTPA conjugation to Ab. The best buffer system was then applied for the further studies on the conjugation to MAb-17-1A.

Affinity-purified antibody (300 μg, 2.0 nmol) was dissolved in 1 ml of 0.1*M* buffer solution (Hepes buffer at pH 7, phosphate at pH 7, borate at pH 8.6, or bicarbonate at pH 8.2) in a 2.5-ml vial. To the antibody solution was added solid cyclic DTPA anhydride at a given molar ratio (Table 1). The solution was shaken gently to dissolve the anhydride, then let stand for 1 hr at 24°C.

For the reaction at cDTPAA-to-Ab ratios less than 500, cDTPAA (35.7 mg, 0.1 mmol) was dissolved in 5 ml of dry dimethyl sulfoxide. Fifty, 10, or 5 μl of this solution was immediately added to 1 ml of the antibody solution in order to produce a cDTPAA-to-Ab ratio of 500, 100, or 50. The percentage of dimethyl sulfoxide for the reaction at the cDTPAA-to-Ab ratio of 500 was 5%. Other reaction conditions are the same as for those at the higher molar ratio.

The reaction mixture from each of the conjugation reactions was cooled on an ice bath and acidified with 0.4 ml of 0.2*M* citrate buffer to pH 5. The solution was then reacted for 30 min with 0.2 ml of 0.1*M* $InCl_3$ containing a tracer amount of In-111 in 0.01*M* citric acid at pH 2.2. The pH of the final solution was 4.8. Unconsumed indium ion was then complexed with 0.3 ml of 0.1*M* DTPA to prevent the formation of insoluble indium hydroxide. The solution was immediately neutralized by addition of 0.4 ml of 0.2*M* borate buffer at pH 10.5. Half of the solution was then put on a 0.9-by 60-cm column containing 56 cm of Sephadex G-50 topped by 2 cm of Sepharose-4B-conjugated HSA. The

TABLE 1. pH EFFECT ON DTPA CONJUGATION WITH RABBIT ANTIBODY TO HSA (Ab) BY THE CYCLIC DTPA ANHYDRIDE METHOD

cDTPAA: Ab ratio	Buffer solution	pH		Active Ab	
		Initial	Final	%	In/Ab*
—	—	—	—	91.0(88.5–93.1)†	—
5000	0.1*M* Hepes	7.0	4.3	48.7(41.0–54.0)	6.8(6.6–7.5)
2000	0.1*M* Hepes	7.0	6.4	64.3(57.0–75.0)	4.1(3.4–5.0)
5000	0.1*M* Borate	8.6	4.8	45.5(38.0–50.0)	12.1(9.9–14.0)
5000	0.1*M* Bicarbonate	8.2	7.3	50.4(44.0–60.0)	10.9(9.3–11.7)
2000	0.1*M* Bicarbonate	8.2	7.5	47.3(38.0–55.0)	10.8(9.5–12.4)
1000	0.1*M* Bicarbonate	8.2	8.2	54.9(46.0–60.4)	9.0(7.6–9.6)
500	0.1*M* Bicarbonate	8.2	8.2	59.1(54.1–68.8)	7.5(5.4–8.6)
100	0.1*M* Bicarbonate	8.2	8.2	77.0(64.5–85.5)	1.7(1.3–1.9)

* Indium atoms per antibody molecule.

† Percentage of active antibody in stock anti-HSA solution; concentration of antibody was 300 μg/ml; data are averages from triplicate experiments.

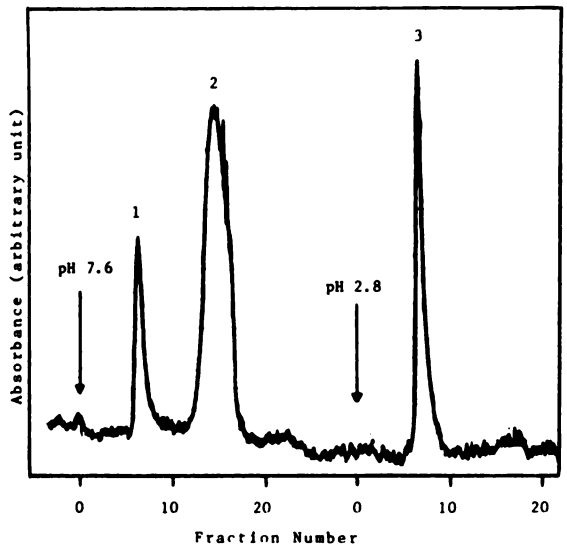

FIG. 1. Elution patterns of DTPA-conjugated antibody. Column was first eluted with 0.02*M* phosphate/0.5*M* NaCl at pH 7.6, then with 0.2*M* glycine/0.5*M* NaCl at pH 2.8. Each fraction contains 2.5 ml. 1 = deactivated In-DTPA-Ab; 2 = In-DTPA; 3 = active In-DTPA-Ab.

column was eluted with 0.02*M* phosphate buffer/0.5*M* NaCl at pH 7.6 to separate inactive antibody and unconjugated DTPA from active antibody. The active antibody was then dissociated with 0.2*M* glycine HCl/0.5*M* NaCl at pH 2.8. Active and inactive antibody were detected by a UV monitor recording on a strip chart (Fig. 1). The percentages of active and inactive antibody were calculated from the peak intensities. The number of indium atoms incorporated into each antibody fraction was calculated from the indium-111 activity associated with the fraction.

Conjugation of DTPA to monoclonal colorectal carcinoma antibody (MAb-17-1A). The reaction condition of DTPA conjugation to MAb-17-1A and its indium incorporation reaction are the same as those for Ab. For the determination of indium atoms incorporated into total MAb-17-1A, we have used a 0.9- by 60-cm column containing 54 cm of Sephadex G-50 with 2 cm of Sepharose-4B-conjugated protein A. Unconjugated DTPA and unbound antibody (negligible amount) were eluted with 0.02*M* phosphate/0.5 *M* NaCl at pH 7.6. The bound antibody was then dissociated from the column upon elution with 0.2*M* glycine/0.5*M* NaCl at pH 2.8. The number of indium atoms incorporated into the antibody fraction was calculated from the indium-111 activity associated with the fraction. The binding activity was determined as follows: The cold antibody (50 μg/ml) or its DTPA conjugate (50 μg/ml) at increasing dilutions (1:1, 1:5, 1:10, 1:50, 1:100, and 1:500) was incubated with 250,000 colon cancer cells for 1 hr. Antibody not bound to the cells was washed off. A second antibody against mouse immunoglobulin labeled with I-125 (50,000 cpm) was then added. After 1 hr of incubation, unbound second antibody was washed off and the activity on the cells was counted. The activity of I-125 bound to the cells was then plotted against the logarithm of antibody concentration (Fig. 2). The concentration of the control antibody (MAb-17-1A) giving 50% bound radioactivity was divided by the concentration of the DTPA-conjugated MAb-17-1A giving the same I-125-bound activity, to obtain the fractional antibody-binding activity of the conjugated antibody.

Electrophoresis. The method of Laemmli (*16*) was used. Electrophoresis was performed with 10% polyacrylamide gel, 1.5 mm thick, in the presence of 2% sodium dodecylsulfate. For the reduction of interchain disulfide bonds, the DTPA-conjugated MAb-17-1A was treated with 5% β-mercaptoethanol before electrophoresis. Standards for molecular weight were: myosin (200,00), phosphorylase B (92,000), bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen

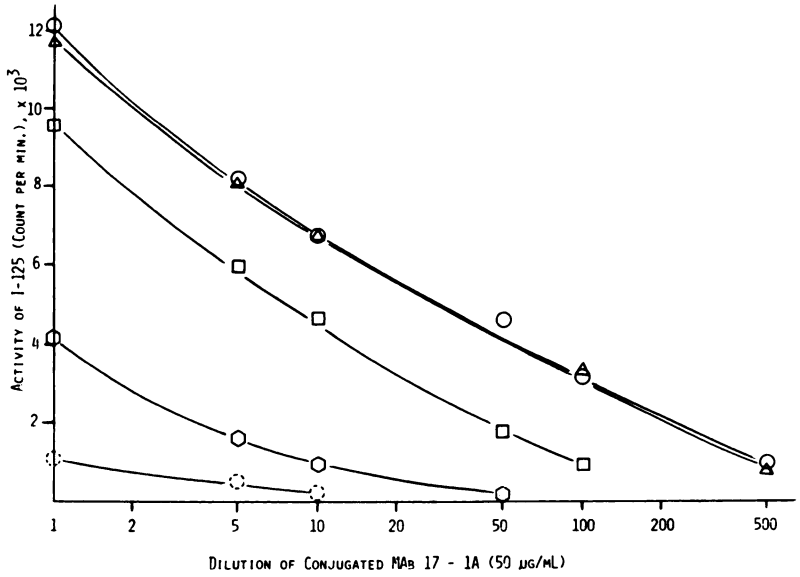

FIG. 2. Binding assay of DTPA-conjugated MAb-17-1A with colon cancer cells. (○) = MAb-17-1A stock solution, (△) = MAb-17-1A containing 1.3 DTPA, (□) = MAb-17-1A containing 6.5 DTPA, (○) = MAb-17-1A containing 11.2 DTPA, (◌) = MAb-17-1A containing 30 DTPA.

TABLE 2. DTPA CONJUGATION TO MAb 17-1A CYCLIC DTPA ANHYDRIDE METHOD*

cDTPAA: MAb ratio	Buffer solution	pH		Total Ab	
		Initial	Final	% Active	#In/Ab
5000	0.1*M* Bicarbonate	8.2	7.3	<5	28.9(28.1-29.8)
2000	0.1*M* Bicarbonate	8.2	7.5	<5	28.4
1000	0.1*M* Bicarbonate	8.2	8.2	<5	31.2(28.6-33.3)
500	0.1*M* Bicarbonate	8.2	8.2	12(10-15)	11.0(9.6-12.0)
100	0.1*M* Bicarbonate	8.2	8.2	60(40-55)	3.6(2.4-4.3)
50	0.1*M* Bicarbonate	8.2	8.2	93(90-95)	1.3(0.6-2.1)

* The concentration of antibody was 300 μg/ml.
The data are average numbers of triplicate experiments.

(25,700), β-lactoglobulin (18,400), and cytochrome C (12,300). After the completion of electrophoresis, gels were stained with 0.02% Coomassie brilliant blue R 250 in 25% methanol/10% acetic acid. Subsequently gels were destained by equilibrating with 5% acetic acid/2% glycerol.

RESULTS

DTPA conjugation. Comparing the reactions at the cDTPAA-to-Ab ratio of 5000 in four different buffer solutions, the reaction in 0.1*M* Hepes buffer at pH 7 incorporated seven indium atoms per Ab molecule with 49% retention of Ab binding activity. The reaction reduced the pH of the buffer solution to 4.3 due to hydrolysis of the anhydride, which produces four acetic acid molecules. With 0.1*M* phosphate buffer, the determination of indium-atom incorporation was not successful, due to the formation of a white precipitate upon the addition of $InCl_3$ containing a tracer amount of In-111. With 0.1*M* borate buffer at pH 8.6, 12 indium atoms were incorporated per Ab molecule, with 46% retention of the binding activity. The pH of the solution at the end of the conjugation reaction was 4.8. With 0.1*M* bicarbonate buffer at pH 8.2, 11 indium atoms were obtained per Ab molecule with 50% retention of the activity. The reaction reduced the pH of the solution to 7.5. Since the bicarbonate buffer was the most stable buffer and the conjugation reaction is more efficient at a higher pH, this buffer was chosen for further studies on DTPA conjugation to Ab and MAb-17-1A.

The reaction between cDTPAA and Ab at molar ratios of 2000, 1000, 500, and 100 in 0.1*M* bicarbonate buffer (pH 8.2) gave rise to 11, 9, 8, and 2 indium atoms incorporated per Ab molecule, with retentions of 47%, 55%, 59%, and 77% binding activity (Table 1). The pH of the solution at the end of the reaction was 7.5 for the molar ratio of 2000. For the lower molar ratios, the pH remained constant at 8.2. For the conjugation reaction with MAb-17-1A, 29, 28, 31, 11, 4, and 1 indium atoms were incorporated, with retentions of <5%, <5%, <5%, 12%, 60%, and 93% activity, respectively, when the molar ratios were 5000, 2000, 1000, 500, 100, and 50 (Table 2). The trend for the pH drop was the same as that for Ab.

Electrophoresis. Electrophoresis of MAb-17-1A and of DTPA conjugated MAb-17-1A was done with SDS polyacrylamide gel (10%), before and after the antibodies were reduced with 5% β-mercaptoethanol. The electrophoresis of unreduced antibodies showed only one band close to the top of the gel (Fig. 3). The reduced antibody, containing less than 6.5 DTPA groups per antibody molecule, showed mainly two bands of 25,000 and 50,000 molecular weight representing IgG light and heavy chains. When the conjugated antibody containing 9.6 to 30 DTPA groups was analyzed (Fig. 4), the intensities of bands corresponding to mol wts ~75,000 and ~150,000 became stronger at the expense of the bands for mol wts 25,000 and 50,000.

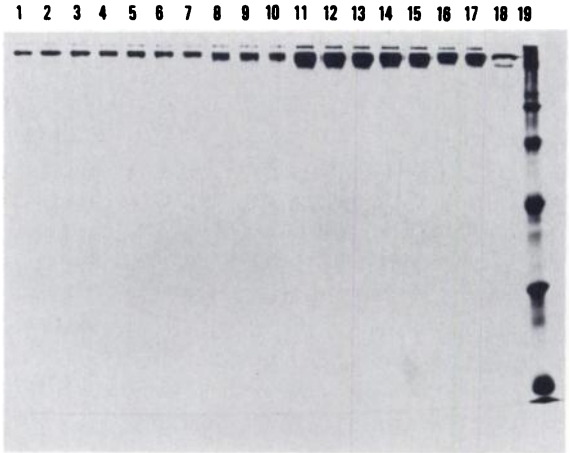

FIG. 3. SDS polyacrylamide gel containing DTPA-conjugated MAb-17-1A, stained with Coomassie blue. Electrophoresis was run in absence of β-mercaptoethanol. Lanes 1 and 18 are for MAb-17-1A. Lanes 2 to 10 are for conjugaged MAb-17-1A containing 0.6, 1.3, 2.1, 3.1, 4.2, 6.5, 9.6, 11.2, and 12 DTPA per MAb-17-1A, respectively. Lanes 11 to 17 are for DTPA-conjugated MAb-17-1A containing 30 DTPA. Lane 19 is for mol wt markers.

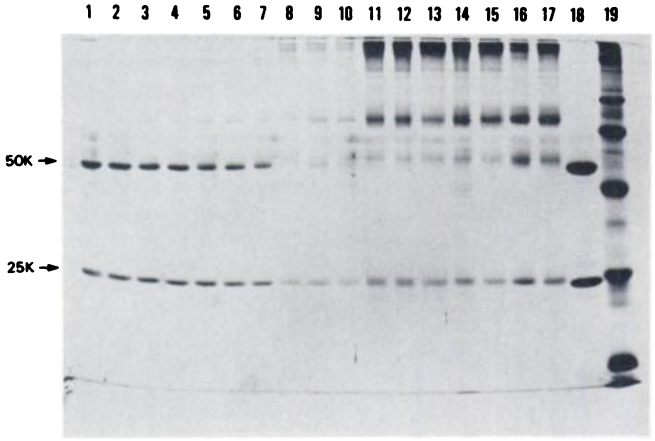

FIG. 4. SDS polyacrylamide gel containing DTPA-conjugated MAb-17-1A, stained with Coomassie blue. Electrophoresis was run in presence of β-mercaptoethanol. Lanes as in Fig. 3.

DISCUSSION

Acylation of proteins using carboxylic acid anhydrides was reported to take place at the amino group of lysine, the hydroxyl group of tyrosine, the sulfhydryl group of cysteine, and the imidazole group of histidine. Among the acylated products, the *N*-acylated product at the ϵ-amino group of lysine is very stable. Acylated products at the sulfhydryl group and at the imidazole group are very unstable and revert spontaneously to the starting amino acids. The *O*-acylated product at the hydroxyl group of tyrosine also hydrolyses readily to form the starting tyrosine. These side reactions and the hydrolysis of the anhydride with water consume the major part of the anhydride, causing a low acylation yield at the amino group of lysine, especially when the concentration of the amino group is very low.

Since the acylation reaction is pH-dependent, we have attempted to optimize the conjugation by varying the pH of the reaction media. The conjugation reaction was more efficient in a buffer with a higher pH (Table 1). This indicates that neutral ϵ-amino groups of lysine residues are the reactive species responsible for DTPA conjugation to antibody. Among the buffer solutions we have studied, 0.1*M* bicarbonate buffer at pH 8.2 was the best, giving a stable pH and also a higher DTPA conjugation. The number of DTPA conjugated per antibody molecules from the reaction in the bicarbonate buffer increased proportionately as the cDTPAA-to-antibody molar ratio was increased. On the other hand, the antibody-binding activity of the conjugated antibody was inversely proportional to the number of DTPA molecules conjugated per antibody molecule. Thus one can optimize the balance between the number of DTPA molecules conjugated per antibody molecule and the reduced antibody-binding activity by varying the cDTPAA-to-antibody ratio or by varying the pH of the medium.

Comparing Ab and MAb-17-1A, the latter was more susceptible to deactivation. The percentage binding activities for the conjugated MAb-17-1A were 93%, 60%, 12%, and <5% of unconjugated MAb-17-1A when the numbers of indium atoms incorporated per antibody molecule were 1, 4, 11, and 31, respectively. For the conjugated Ab, the percentage binding activity changed from 91% for unconjugated Ab to 77%, 59%, 55%, and 47% for Ab containing 2, 8, 9, and 11 indium atoms.

SDS polyacrylamide gel electrophoresis of the conjugated MAb-17-1A in a reducing condition with β-mercaptoethanol showed high-molecular-weight bands corresponding to mol wt 75,000 and of >150,000. The high-molecular-weight bands seem to be caused by interchain cross linkages between light and heavy chains. When the conjugated antibody containing more than 9.6 DTPA molecules per antibody was analyzed, the intensity of these bands became much stronger compared with the bands for 25,000 and 50,000 mol wt. Therefore, the interchain cross linkage seems to be responsible, to a great extent, for deactivation of the antibody-binding activity.

In conclusion, the DTPA conjugation reaction is more efficient at a higher pH. Among buffer systems we studied, 0.1*M* bicarbonate buffer at pH 8.2 was the best, giving a stable pH and a higher DTPA conjugation. Antibody-binding activity decreases as the molecular ratio of DTPA to antibody increases.

FOOTNOTES

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