A Fluorescence Quenching Method for Estimating Chelating Groups in Chelate-Conjugated Macromolecules

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Received May 19, 1992; accepted July 23, 1992

A terbium–dipicolinic acid (Tb–DPA) fluorescence quenching method for estimating free chelating groups conjugated to protein molecules was developed. This method was based on competitive displacement of DPA from binding to terbium by stronger chelating groups such as diethylenetriaminepentaacetic acid (DTPA), EDTA, nitrilotriacetic acid (NTA), DTPA-conjugated bovine serum albumin (BSA-DTPA), or DTPA-conjugated immunoglobulin G (IgG-DTPA), resulting in a significant reduction in terbium fluorescence. The chelating ability of the tested reagent, from high to low, was in the following order: BSA-DTPA > DTPA > IgG-DTPA > EDTA, NTA. At low terbium concentrations, the reduction was linear for DTPA. This fluorescence quenching method was not only rapid, simple, and as accurate as conventional radioisotopic or chromatographic methods, but also sensitive and reproducible. The detection limit was 10 n*M* for DTPA. The interrun coefficient of variation was at most 8%. The advantage of this method over other indirect methods is that it reveals the actual chelating ability of the tested macromolecule, unencumbered by complicating factors such as trace metal contamination and dimer/polymer formation during conjugation.

KEY WORDS: chelates; fluorescence quenching.

INTRODUCTION

Labeling monoclonal antibodies with radioactive iodine is a popular method for monitoring their distribution *in vivo* (1–10). The drawback with radioactive iodine is rapid deiodination (11). But it may be overcome by labeling the antibodies through chelation with a variety of radionuclides, including ^{99}Tc, ^{67}Ga, and ^{111}In. Since few biological macromolecules form stable chelates with radionuclides, the possibility of covalently introducing chelating groups to the macromolecules may be considered. Such an approach may offer the advantage that the coupled protein can be stored and labeled with a variety of metallic radionuclides when needed.

Many transition metals possess spectroscopic properties which may be useful in biochemical applications. Hnatowich *et al.* (4,8) described methods of labeling antibodies and other proteins using the bifunctional chelation approach, in which the strong chelating DTPA[4] was covalently attached to the antibody for binding with ^{111}In(III). The terbium–dipicolinic acid (Tb–DPA) fluorescent method reported here was first developed by Wilschut *et al.* (12) to monitor the mixing of liposomal aqueous contents during fusion. This method was based on the interaction between Tb(III) ions in one liposomal population and DPA in another population, rapidly forming the strong fluorescent Tb–DPA complex upon fusion. Since fluorescence is generated through an internal energy transfer from the ligand to the metal ion, the complex is excited at a wavelength close to the maximum absorption of DPA, while the fluorescence spectrum is characteristic for Tb(III). The fluorescence intensity of Tb(III) on its own is very low but is enhanced 10^4-fold by its interaction with DPA. Being a relatively weak chelate compared with the bifunctional chelate often used to carry radionuclides [such as DTPA (diethylenetriaminepentaacetic acid) and EDTA] in monoclonal antibody systems, DPA can be displaced from binding by stronger chelating groups, including DTPA (Scheme I). The implicit assumption is that displacement of DPA by stronger chelating groups is quantitative, so that the possibility of mixed ligand complex formation is low. Since the resulting Tb–DTPA complex is nonfluorescent, the terbium fluorescence is significantly reduced. Provided that the reduction in fluorescence is linear with the concentration of DTPA or other chelate-coupled macromolecules, it will be possible to measure the unknown concentration of available chelating groups on macromolecular quenchers.

MATERIALS AND METHODS

Materials

Terbium chloride hexahydrate, 1-ethyl-1,3-(3-dimethylaminopropyl)carbodiimide (EDC), DTPA, and nitrilotriacetic acid (NTA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dipicolinic acid (pyridine-2,6-dicarboxylic acid), Folin reagent, fluorescamine, and bovine IgG were purchased from Sigma (St. Louis, MO). *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) and L-histidine were purchased from Research Organics Inc. (Cleveland, OH). Cupric sulfate · $5H_2O$ was purchased from J. T. Baker (Phillipsburg, NJ). Bovine albumin fraction V was purchased from USB (Cleveland, OH), Sephadex G-50 was purchased from Pharmacia (Piscataway, NJ), and AG1-X8 resin was purchased from Bio-Rad (Richmond, CA). The dialysis tubing was purchased from Spectrum Medical Industries (Los Angeles, CA). ^{111}In-Indium chloride was purchased from NEN Dupont (Boston, MA).

Synthesis and Purification of BSA-DTPA and IgG-DTPA

Eighty milligrams of bovine serum albumin (BSA) was

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[4] Abbreviations used: BSA, bovine serum albumin; DTPA, diethylenetriaminepentaacetic acid; DPA, dipicolinic acid; EDC, 1-ethyl-1-3-(3-dimethylaminopropyl)carbodiimide; His, L-histidine; NTA, nitrilotriacetic acid; Tb, terbium; TES, *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid.

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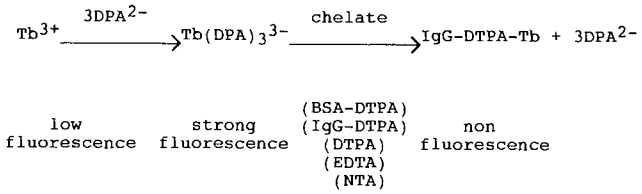

Scheme I. Principle of the $Tb(DPA)_3$ fluorescence quenching method for titrating the available chelate groups on macromolecules.

dissolved in 3.2 mL of Millipore-grade water. The chelating agent DTPA was neutralized to pH 5.0 before use by the addition of 1 *N* NaOH. Six hundred microliters of 200 m*M* DTPA was then added to the BSA solution with stirring, while maintaining the pH at 3.80. EDC (0.52 g) was dissolved in 0.8 mL of Millipore-grade water and added dropwise to the BSA-DTPA solution, while carefully controlling the pH between 2.70 and 2.85 using 0.1 *N* NaOH or HCl. The coupling reaction was allowed to proceed for approximately 20 hr at room temperature. IgG-DTPA was synthesized under the same conditions.

The BSA-DTPA reaction solution was transferred to a dialysis bag (MW cutoff 12,000–14,000) and dialyzed against 4 L of 0.9% NaCl at 4°C with four changes of solution every 24 hr. BSA-DTPA was then passed through a Sephadex G-50 column (0.8 × 31 cm) and eluted with 0.1 *N* acetic acid to remove trace amounts of free DTPA. The macromolecular fraction was collected for the $^{111}In(III)$ binding study and checked for purity. IgG-DTPA was purified by the same procedure. It, however, was passed through a Sephadex G-50 column twice to remove residual DTPA.

The total protein concentration of purified BSA-DTPA and IgG-DTPA was determined by Lowry assay (13) in a Hitachi U-1100 Spectrophotometer, using 10 mg/mL BSA or 100 μg/mL IgG as a standard stock solution. The unreacted protein concentration was measured using the fluorescamine assay in a Perkin-Elmer 650-10S fluorescence spectrophotometer. The degree of conjugation was calculated by the following equation:

$$\% \text{ conjugation} = (C_L - C_F)/C_F * 100\%$$

where C_L and C_F indicate the protein concentration determined by the Lowry assay and the fluorescamine assay, respectively. According to this procedure, the percentage conjugation of DTPA to BSA was 43.9 and 34.5% on two separate occasions.

Purification of $^{111}In(III)$ and Binding of $^{111}In(III)$ to BSA-DTPA or IgG-DTPA

$^{111}In(III)$ was purified using ion-exchange chromatography on a AG1-X8 (chloride form) column, as described by Hwang (14). The purified $InCl_3$ was incubated with, first, 11.6 m*M* citrate for 10 min and, then, 100 μL of BSA-DTPA or IgG-DTPA (in 0.1 *N* acetic acid), purified by Sephadex G-50 chromatography, for 1 hr. Two hundred microliters of the resulting BSA-DTPA-In(III) or IgG-DTPA-In(III) were applied to the same Sephadex G-50 column and eluted with 0.1 *N* acetic acid. Thirty tubes (1 mL/tube) were collected, and the radioactivity of each fraction was monitored with a Packard Auto-Gamma scintillation spectrometer (Model 5002). The percentage of In(III)-bound macromolecule was determined by quantitating the radioactivity associated with BSA-DTPA (or IgG-DTPA) and with unbound $^{111}In(III)$.

Fluorescence Spectrum of $Tb(DPA)_3$

Five micromoles of $Tb(DPA)_3$ complex in 0.9% NaCl and 2 m*M* TES/His (pH 7.4) buffer was excited at 276 nm, and the fluorescence intensity was measured from 250 to 600 nm. Two other samples of 100 μg/mL BSA-DTPA and 42 μg/mL IgG-DTPA in 0.9% NaCl and 2 m*M* TES/His (pH 7.4) buffer were also measured to check for possible interference.

Standard Curve of $Tb(DPA)_3$

The standard curve of $Tb(DPA)_3$ complex was obtained using Tb(III) concentrations ranging from 0 to 1.2 μ*M* at a DPA/Tb(III) ratio of 5. An excess of DPA was used to ensure that all of Tb(III) was chelated. Fluorescence was measured in a Perkin–Elmer 650-10S fluorescence spectrophotometer with an excitation wavelength of 276 nm and an emission wavelength of 491 nm. Each concentration was measured in triplicate.

Fluorescence Quenching Measurements

Two millimolar TES/His buffers in volumes ranging from 0–800 μL were added to 100 μL each of 50 μ*M* DPA and 10 μ*M* $TbCl_3$, vortexed, and incubated for 2 min to form a strong and stable fluorescent $Tb(DPA)_3$ complex. To quench the fluorescence of $Tb(DPA)_3$, 50 μ*M* NTA, or 20 μ*M* DPA, EDTA, BSA-DTPA, and IgG-DTPA, in volumes ranging from 0 to 800 μL, were then added to the above $Tb(DPA)_3$ complex to a final volume of 1 mL. The concentrations of the quenching reagents were chosen according to their chelating abilities to generate a quenching curve within the linear range. After incubating the sample for 5 min, the fluorescence was measured. Incubation was conducted in triplicate.

The reproducibility of the fluorescence-quenching method was tested by repeating the sample preparation and fluorescence-quenching measurement described above five times for each quenching reagent. The linear regression equation and standard deviation of the slope for each reagent were calculated.

RESULTS

Purity Check of BSA-DTPA and IgG-DTPA by $^{111}In(III)$ Binding

In studying the quenching effects of chelate-conjugated macromolecules, it is imperative that unreacted DTPA in the conjugation procedure be eliminated. A trace amount of free DTPA contamination can cause a false quenching effect. Extensive dialysis and gel filtration chromatography were capable of removing all free DTPA from the BSA-DTPA-In(III) and IgG-DTPA-In(III) conjugates. For chelate-conjugated macromolecules, 100% radioactivity was eluted at the macromolecular fraction, while the free DTPA-In(III) (control) was eluted at bed volume (data not shown).

Fluorescence Emission Spectrum and Calibration Curves of $Tb(DPA)_3$ Complex

The fluorescence emission spectrum of $Tb(DPA)_3$ is displayed in Fig. 1. The first peak at 491 nm is the emission peak, while the second peak at 551 nm is the second overtone of the Rayleigh scatter of the exciting light at 276 nm. In the BSA-DTPA and IgG-DTPA controls, there existed an interference emission peak at 551 nm. Since the second peak was related to the light-scattering nature of colloidal particles, the shorter wavelength of 491 nm was chosen as the wavelength for fluorescence measurement.

Figure 2 shows the calibration curves of the $Tb(DPA)_3$ complex obtained 7 days apart using the same samples stored at 4°C. The fluorescence intensity was found to be linear with a $Tb(DPA)_3$ concentration from 0 to 1.2 μM. There was no appreciable difference in the slopes of the two standard curves.

Quenching of $Tb(DPA)_3$ by DTPA, EDTA, NTA, BSA-DTPA, and IgG-DTPA

Figure 3 shows the quenching curves of $Tb(DPA)_3$ by DTPA (20 μM), EDTA (20 μM), and NTA (50 μM) solutions. The order of quenching efficiency was DTPA, EDTA, and NTA. Figure 3 also shows the quenching curves of $Tb(DPA)_3$ by BSA-DTPA and IgG-DTPA. BSA-DTPA was more effective than IgG-DTPA.

Table I shows the linear regression equation, correlation coefficient, and number of molecules of different quenching reagents equivalent to 1 molecule of DTPA. The latter was calculated as the ratio of $\text{slope}_{DTPA}/\text{slope}_X$ using DTPA as a standard quenching reagent. This ranged from 0.46 for BSA-DTPA and 12.39 for NTA. The interrun variation of the assay ranged from 2.39% (BSA-DTPA) to 8.6% (IgG-DTPA) (data not shown).

DISCUSSION

Several methods are available to measure chelate-to-macromolecule ratios. Sundberg *et al.* (15) used ^{14}C-labeled azo-EDTA to determine the incorporated EDTA group. Meares *et al.* (16) measured the number of protein-bound chelating groups by titration with a ^{57}Co standard. Oser *et al.* (17) used a time-resolved fluorometry method to quantitate

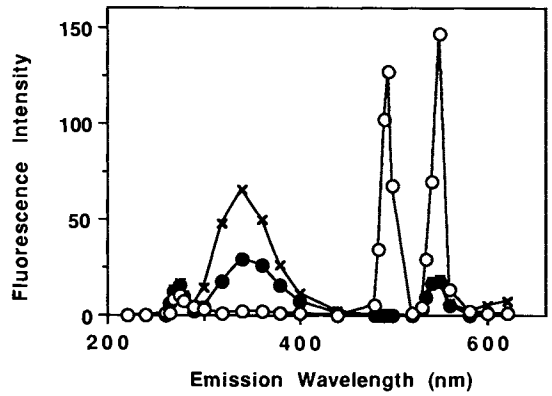

Fig. 1. Fluorescence emission spectra of $Tb(DPA)_3$ complex in solution (○), BSA-DTPA (●), and IgG-DTPA (+).

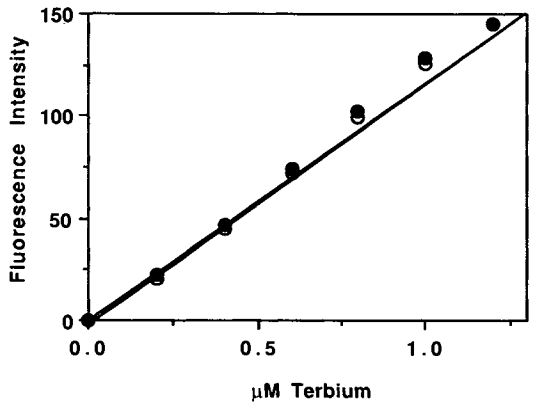

Fig. 2. Calibration curves of $Tb(DPA)_3$ complex obtained 7 days apart.

the DNA-bound DTPA group using europium ions. Other methods to measure the degree of conjugation include (a) measuring DTPA anhydride before and after conjugation by the CO_2 method (18); (b) measuring the disappearance of certain amino groups per milligram or mole of protein, before and after conjugation, using the fluorescamine method (19); and (c) measuring the concentration of free chelating groups, before and after conjugation, by HPLC, TLC, or dialysis followed by measuring UV absorbance at 254 nm. All of the preceding methods reveal only the percentage of conjugation, not the actual number of chelating groups on the chelate-conjugated proteins available for binding. There are four major reasons the percentage of conjugation may not reflect the actual binding ability of the chelate-coupled protein: (a) the possibility that the actual available number of chelate groups per macromolecule is affected by factors such as trace metal contamination during the reaction or purification process, (b) steric hindrance of the macromolecules, (c) the formation of dimer or polymer during conjugation, and (d) the possibility of intra- or intermolecular reaction.

In this study, a fluorescence quenching method to determine the actual number of chelate groups per protein molecule, using DTPA as a standard, was developed. This method is sensitive and reproducible. In addition, it is simple

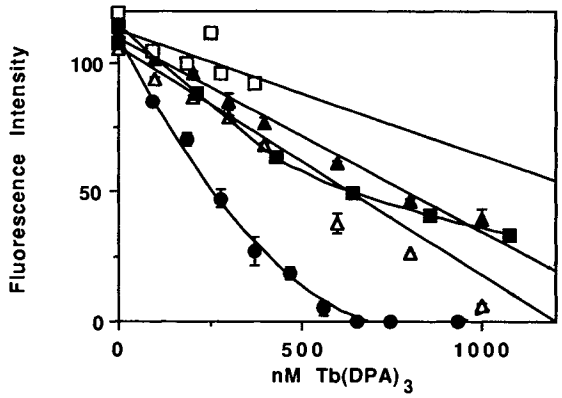

Fig. 3. Quenching of fluorescence due to $Tb(DPA)_3$ by 20 μM DTPA (△), 20 μM EDTA (▲), 50 μM NTA (□), and 20 μM BSA-DTPA (●) and IgG-DTPA (■). The error bar represents the standard deviation for $n = 3$. Where not shown, it is smaller than the size of the symbol.

Table I. Regression Equations, Correlation Coefficients, and Number of DTPA Equivalents of Various Quenching Agents

Quenching reagent	Regression equation[a]		Molar ratio, X^b/DTPA
DTPA	$F = 105.75 - 0.1008C$	0.996	1
EDTA	$F = 109.50 - 0.075C$	0.984	
NTA	$F = 116.32 - 0.0081C$	0.966	12.39
BSA-DTPA			
Trial 1	$F = 111.87 - 0.2207C$	0.988	0.46
Trial 2	$F = 103.5 - 0.185C$	0.993	0.55
IgG-DTPA	$F = 104.91 - 0.075C$	0.966	1.34

[a] F, fluorescence intensity; C, concentration.
[b] X stands for DTPA, EDTA, NTA, BSA-DTPA, or IgG-DTPA.

and rapid, involving neither radionuclide nor a time-consuming separation process. The radioactive ^{111}In(III) was used here merely to confirm the purity of DTPA-conjugated protein as well as its direct involvement in fluorescence quenching. Fluorescence detection can be reduced to minutes instead of hours, compared to conventional radioisotopic or chromatographic methods.

A limitation of the method reported here is that it does not reveal the absolute number of DTPA per macromolecule; it gives only the number of DTPA-conjugated protein equivalent to one molecule of free DTPA. As shown in Table I, 1.34 molecules of EDTA and 12.39 molecules of NTA are equivalent to 1 molecule of DTPA in chelating ability, against DTPA as a standard. The weaker chelating ability of NTA and EDTA is to be expected from the fewer number of carboxyl groups available for chelation. Compared with the five carboxyl groups for DTPA, NTA has only three and EDTA has four. The weaker chelating ability of IgG-DTPA than DTPA is due to either the low degree of conjugation, the steric effect of protein molecules, or the unavailability of at least one functional group on DTPA due to participation in conjugation. In contrast, BSA-DTPA is a stronger chelate than DTPA; therefore, it is likely that 1 molecule of BSA-DTPA conjugate possesses more than one DTPA group. Because different chelates have different binding constants, the number of DTPA-conjugated proteins equivalent to 1 molecule of free DTPA is not the absolute number of DTPA group per protein. Only when the binding constant of each chelate is known will it be possible to calculate the absolute number of DTPA per macromolecule. The deviations from linearity observed for BSA-DTPA and IgG-DTPA at high Tb(III) concentrations (Fig. 3) are probably due to binding of Tb(III) to non-DTPA groups on BSA and IgG.

In conclusion, the fluorescence quenching method presented here is extremely rapid, simple, and sensitive to 10 nM. It requires neither special apparatus nor radionuclide. The accuracy of this method is comparable to those based on ^{14}C, ^{111}In, and ^{57}Co. This method, therefore, can find useful applications in immunodiagnostics, drug targeting, and gene or immunotherapy.

ACKNOWLEDGMENTS

This work was supported in part by Grants DK34013 and CA37528 from the National Institutes of Health, Bethesda, Maryland, and by the Gavin S. Herbert Professorship.

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