Multiple Fluorescence Labeling with Europium Chelators. Application to Time-Resolved Fluoroimmunoassays

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Multiple fluorescence labeling with conventional probes like fluorescein, to improve the detection limit of labeled reactants, is not usually successful because of fluorescence quenching. In contrast, we found that the europium chelator 4,7-bis-(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) can be incorporated into proteins at very high molar ratios. Working with thyroglobulin as a model protein, we found that when 160 BCPDA molecules are incorporated into one thyroglobulin molecule, the fluorescence emitted by the labeled protein in the presence of excess Eu^{3+}, is equivalent to that emitted by approximately 900 molecules of unconjugated BCPDA:Eu^{3+} complexes. We took advantage of the lack of any quenching effects and of the enhancement observed with the multiply labeled protein, to develop a universal reagent system consisting of (a) streptavidin covalently coupled to BCPDA labeled thyroglobulin and (b) excess Eu^{3+}. With this approach, streptavidin is heavily labeled through thyroglobulin and retains its full biotin binding activity. We used the reagent to develop a highly sensitive time-resolved heterogeneous immunofluorometric assay of α-fetoprotein (AFP) in serum, using monoclonal antibodies. One antibody is immobilized in white microtitration wells (solid-phase) and the other is biotinylated. We demonstrate that this assay, using the newly developed reagent, is 25-fold more sensitive than the one using directly BCPDA labeled antibody and 5-fold more sensitive than an assay that uses BCPDA-labeled streptavidin. The detection limit of the assay with the new reagent was down to 60 amol of AFP per well. We conclude that multiple fluorescence labeling with europium chelators is an effective method of extending the sensitivity of currently used fluorescence immunoassay procedures.

INTRODUCTION

Fluorescence labeling with conventional probes like fluorescein, rhodamine, dansyl chloride, and fluorescamine and the newer labels Texas Red and phycobiliproteins is widely used for analytical and nonanalytical applications (*1–6*). Fluorescent labels are now used extensively in the field of fluorescence immunoassay as alternatives to the radioactive labels. However, the limit of detection is restricted to the micromolar and nanomolar range mainly because of the high background fluorescence readings encountered in the measurements and the limited number of labels incorporated into the immunoreactants.

In principle, the sensitivity of the fluoroimmunoassay could be improved by attaching many fluorescent groups to the labeled reagent. This could be achieved directly (for macromolecular reagents) or indirectly through conjugation of the reagent to a carrier macromolecule labeled with multiple fluorescent molecules. Unfortunately, these approaches do not work since the increased tagging level soon produces concentration quenching which reduces first the quantum efficiency and then the total emission (*7–9*). For example, when a macromolecule was labeled with 32 fluorescein molecules, an emission intensity equivalent to only two free fluorescein molecules was obtained (*9*). More recently, a bulking agent that is covalently bound to the antigen has been used to disperse the fluorescein labels and minimize concentration quenching. With this technique, a maximum of 8.8 fluorescein molecules have been incorporated, yielding a fluorescence intensity equivalent to that of 7.7 free fluorescein molecules (*10*). Alternatively, multiple labeling can be used with fluorophores which are not subject to concentration quenching. Multi-umbelliferone-substituted polylysine labels have been used successfully in steroid fluoroimmunoassays (*11*). The umbelliferone fluorophore showed no concentration quenching at the level of 1 fluor/10 lysine residues.

Hirschfeld (*9*) proposed an elegant method to overcome the problem of concentration quenching of fluorescein. He demonstrated that when the sample is illuminated to complete photochemical bleaching using a powerful laser, each fluorophore emits the same maximum number of photons. He thus achieved a linear increase of the signal with the number of labels used, regardless of concentration quenching. Using this technique, which is nevertheless not practical because of the complex and costly instrumentation, he was able to detect single antibody molecules labeled with multi-fluorescein-substituted polyethylenimine (*12*).

In this paper, we propose for the first time that the fluorescent europium chelates can be used for multiple fluorescence labeling without any fluorescence quenching. We found that when the europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) was introduced into proteins at a molar ratio as high as 160, then the 1:1 complex which formed with Eu^{3+}, fluoresced with an intensity greater than that of 160 1:1 complexes of unconjugated BCPDA:Eu^{3+} in solution. We found no quenching effects from multiple labeling. In fact, we observed an enhancement in the expected fluorescence as a result of multiple labeling; the enhancement factor ranged from 3- to 6-fold and was protein dependent. We took advantage of the lack of quenching effects after multiple labeling and created a new universal immunological reagent that can be used for highly sensitive time-resolved fluorescence immunoassays. The reagent consists of streptavidin conjugated covalently to thyroglobulin which itself carries about 160 BCPDA residues. The reagent also contains excess Eu^{3+} which saturates BCPDA and forms the fluorescent complex. When this reagent is used for devising time-resolved fluorescence immunoassays with biotinylated antibodies as complementary reagents, a theoretical amplification factor of about 10^4 can be introduced [10-fold from biotin bound to antibody, 160-fold from BCPDA bound to thyroglobulin, and 6-fold from the enhancement effect]. The practical amplification observed is lower. Our results suggest that multiple fluorescence labeling is a powerful method of increasing the sensitivity of analytical methodologies provided that the labels used do not suffer from quenching effects. We here demonstrate for the first time that

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the fluorescent europium chelates are suitable labels for such applications.

EXPERIMENTAL SECTION

Materials. 4,7-Bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) was synthesized as described previously (*13*). Sulfosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC), was purchased from Pierce Chemical Co., Rockford, IL. *N*-Succinimidyl *S*-acetylthioacetate (SATA) was obtained from Calbiochem Behring Diagnostics, La Jolla, CA.

Ethylmaleimide was from Eastman Kodak Co., Rochester NY. Protein concentration was carried out by using Centriprep and Centricon concentrators from Amicon Canada, Ltd., Oakville, ON, Canada.

Fluoroimmunoassay microtitration plates, Microfluor W, white opaque 96-well plates were purchased from Dynatech Labs, Alexandria, VA.

Affinity purified streptavidin and bovine thyroglobulin were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals used were also from Sigma except where otherwise stated.

The monoclonal antibodies used for the α-fetoprotein immunoassay are available from CyberFluor Inc.

Instrumentation. For measurement of solid-phase fluorescence we used the CyberFluor 615 Immunoanalyzer, a time-resolved fluorometer. The fluorescence of solutions was measured on the Arcus time-resolved fluorometer (LKB Wallac, Turku, Finland). Spectra were recorded on a HP Model 8450A diode array spectrophotometer (Hewlett-Packard Canada, Mississauga, ON). Sephadex G-25 gel filtration columns, the peristaltic pump, the optical unit, and fraction collector were all from Pharmacia Canada, Dorval, PQ. Ultrogel A34 was purchased from Fisher Scientific, Toronto, Ontario. The Speed Vac Concentrator was obtained from Emerston Instruments, Scarborough, ON.

Methods. The α-fetoprotein monoclonal detection antibody was labeled with BCPDA, isolated, characterized, and diluted to 5 mg/L as described in ref 16. Coating of the microtitration strips with the α-fetoprotein monoclonal antibody and the biotinylation procedure of the second antibody were as described elsewhere (*14*, *15*).

Streptavidin was labeled with BCPDA as described in ref 15. By this method, 14 ± 1 BCPDA molecules are conjugated to one streptavidin molecule. The labeled streptavidin working solution contained 3 mg/L streptavidin and 4×10^{-5} mol/L Eu^{3+} in a 50 mM Tris buffer at pH 7.20 containing 9 g of NaCl, 40 g of BSA, and 0.5 g of sodium azide per liter. In all cases, the extent of BCPDA labeling was assessed by absorbance measurements at 325 nm, where only BCPDA absorbs (molar extinction coefficient of 1.5×10^4 M^{-1} cm^{-1}) (*16*). Characterization of the labeled protein by fluorescence was performed as follows: BCPDA was hydrolyzed by incubating a weighed amount in 0.1 M carbonate buffer of pH 9.50, at 40 °C, for 2 h. This stock solution (10^{-4} M) was diluted appropriately to cover the range between 10^{-7} and 3×10^{-12} M. The dilution buffer was 10 mM Tris buffer of pH 7.80, containing 10^{-6} M Eu^{3+}. A standard curve was then constructed by plotting the fluorescence reading of each solution (Arcus fluorometer, 200 μL/well) versus the concentration of the standard BCPDA solution. BCPDA labeled protein was then diluted in the same buffer and the fluorescence measured as above. The BCPDA concentration could then be determined from the calibration curve.

Solid-Phase Binding Assay. White opaque microtiter wells were coated with 0, 25, 50, 100, 150, and 200 ng of biotinylated goat anti-human IgM antibody (from Tago, Inc., Burlingame, CA) by adding 100 μL per well in a 0.1 M bicarbonate solution and incubating overnight at 4 °C. The wells were then washed with a solution containing 9 g of NaCl and 0.5 g of Tween 20 per liter and blocked for 4 h at room temperature by adding 100 μL per well of a 0.1 M bicarbonate solution containing 10 g/L bovine serum albumin (BSA). The wells were then rewashed and shaken dry. One hundred microliters of BCPDA-labeled streptavidin or BCPDA-labeled streptavidin–thyroglobulin conjugate were then added at different dilutions and incubated as described in the legend of Figure 3.

Derivatization of Thyroglobulin with SATA and Labeling with BCPDA. Fifty milligrams of bovine thyroglobulin [TG; mol. wt. 670 000 with 150 amino groups per molecule (*17*)] were dissolved in 12 mL of a 0.1 M carbonate buffer of pH 9.1. TG was then initially derivatized with 1.6 mg of *N*-succinimidyl *S*-acetylthioacetate (SATA) dissolved in 80 μL of dimethylformamide; the SATA solution (~0.5 equivalent per amino group) was added dropwise in four portions to the stirring TG solution. The reaction was left to proceed for 1 h at room temperature. The protein was then extensively labeled with BCPDA by adding 40 mg of BCPDA dissolved in 500 μL of anhydrous ethanol (in five portions, dropwise) under continuous stirring. The mixture was stirred for 1 h at room temperature. The SATA derivatized, BCPDA labeled TG was isolated from unreacted SATA and BCPDA by extensive dialysis (molecular weight cutoff 12–14000) against a 0.1 M phosphate buffer of pH 6.2. The final product was then concentrated to approximately 0.3 mL by using the Amicon concentrator.

Streptavidin Activation with Sulfo-SMCC. One milligram of streptavidin [SA; mol wt 60 000 with 20 amino groups per molecule (*18*)] was dissolved in 200 μL of a 50 mM phosphate buffer of pH 7.0. To this solution 50 000 counts/min of ^{125}I-labeled streptavidin (obtained from Amersham Corp., Oakville, ON) was added to serve as a tracer for the accurate quantitation of the conjugation reaction. Streptavidin was then activated by the addition of 0.22 mg of sulfo-SMCC (~1.5 equiv per amino group) dissolved in 50 μL of a 0.1 M phosphate buffer of pH 7.0 to the continuously stirred streptavidin solution. The reaction was allowed to proceed for 1 h at room temperature. The derivatized protein was isolated on a preequilibrated Sephadex G-25 column (1 × 15 cm) that was eluted with a 0.1 M phosphate buffer of pH 6.2. The protein peak eluting near the void volume of the column was pooled and concentrated to approximately 0.1 mL with the Amicon Centricon concentrator.

Conjugation of Activated Streptavidin to Labeled Thyroglobulin. The derivatized TG and streptavidin solutions were mixed in a glass Pyrex screw-cap test tube. The approximate molar ratio of TG:SA was about 5. The volume was then reduced down to 250–300 μL by repeating the following cycle three times: The solution was purged with N_2 gas for 30 s followed by centrifugation for 3 min in the Speed-Vac concentrator under reduced pressure. Vacuum was released under nitrogen. The conjugation reaction was then initiated by the addition of 50 μL of a 0.5 M NH_2OH solution of pH 7.0 that had been purged with N_2 for at least 15 min. The reaction mixture is then incubated for 2 h at 37 °C under N_2. After incubation, 10 μL of a 0.12 M β-mercaptoethanol solution was added and the mixture incubated for 15 min at room temperature. Subsequently, 20 μL of a 0.24 M *N*-ethylmaleimide solution was added and the mixture incubated for another 15 min. The conjugate can then be isolated immediately or the next day after storage at 4 °C, as described below.

Conjugate Isolation. The reaction mixture is diluted with a 0.1 M bicarbonate solution of pH 8.0 to a final volume of 2 mL and then applied to a preequilibrated Ultrogel A34 column (3 × 42 cm) that was eluted with a 0.1 M bicarbonate solution. Five milliliter fractions were collected and counted on a γ counter (from LKB Wallac). The fractions obtained near the void volume of the column were also assessed for their binding activity on microtiter wells coated with biotinylated antibody (solid-phase binding assay) and in a complete α-fetoprotein assay. For these assays, an aliquot of the fractions was preliminarily diluted to approximately 0.15 μg/mL in terms of streptavidin concentration using the radioactivity counts as the point of reference. The diluent was a 50 mM Tris buffer of pH 7.80 containing Eu^{3+} (4×10^{-5} M), BSA (40 g/L) and sodium azide (0.5 g/L). The active fractions (in terms of binding to the biotinylated antibody) were pooled and diluted to 15 μg/mL in terms of streptavidin concentration using radioactivity as reference, in a 50 mM acetate buffer of pH 6.0. This solution was stored at 4 °C and diluted 100-fold in the europium-containing diluent when it was used for an assay. From the procedure described above approximately 50 mL of concentrate can be obtained (sufficient for about 50 000 assays).

α-Fetoprotein Assay. (a) With Directly Labeled Antibody. Twenty-microliter portions of α-fetoprotein standards were pipetted into the AFP antibody coated microtitration wells. One hundred microliters of the directly labeled antibody solution (5 μg/mL in antibody, containing 10^{-5} M Eu^{3+}) was added and

incubated for 90 min at 37 °C. The wells were then washed three times with the wash solution and dried for 3 min with cool air and the fluorescence was measured on the CyberFluor Model 615 Immunoanalyzer.

(b) With Biotinylated Antibody. The procedure is as above but utilizes the biotinylated second antibody instead of the directly labeled antibody. After washing, 100 μL of either directly labeled streptavidin solution (3 μg/mL) or the streptavidin thyroglobulin conjugate solution containing 40 μM Eu^{3+}, diluted as described above, was added to the wells and incubated for 30 min at 37 °C. After an additional three washes, the wells were dried and measured on the CyberFluor Model 615 Immunoanalyzer, as described above.

RESULTS AND DISCUSSION

The attractive properties of the fluorescent europium chelates and their application as immunological labels for time-resolved fluoroimmunoassays have been recently reviewed (*6, 19*). Soini and Kojola (*20*) calculated the theoretical detection limits in a hypothetical immunoassay of insulin by using either Eu^{3+} or ^{125}I as the label and concluded that Eu^{3+} is a more sensitive label. Ekins suggested that the potential sensitivity of a noncompetitive immunoassay system is better when alternative labels other than ^{125}I are used because much higher specific activities can be achieved (*21*). There are now several examples in the literature in which the sensitivities of immunoassays based on alternative labels are better than those obtained with radioimmunoassays and immunoradiometric assays.

When the fluorescent europium chelates are used as immunological labels, there are two general immunoassay configurations:

(1) Use Eu^{3+} as the label, bound to antibody molecules via a bridge such as an ethylenediaminetetraacetic acid (EDTA) derivative (*22*). After the immunological reaction is complete, Eu^{3+} is dissociated from EDTA by lowering the pH; then it is complexed with appropriate ligands (such as naphthoyltrifluoroacetone (NTA) and trioctylphosphine oxide (TOPO)) and is measured in a time-resolved mode, in solution. This two-step procedure is necessary since the EDTA–Eu^{3+} complex is not fluorescent. This configuration suffers from a number of disadvantages, the most prominent of which is its vulnerability to exogenous Eu^{3+} contamination (*19*). However, due to the optimized complexation of Eu^{3+} and the formation of the Eu^{3+}–$(NTA)_3(TOPO)_2$ complex, the detection limit is very low ($\sim 10^{-13}$ mol/L of Eu^{3+}) (*23*).

2. Use a europium chelator as the label and introduce excess Eu^{3+} to form the fluorescent complex (*24*). A suitable chelator for this purpose has been synthesized (4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid) (BCPDA) (*13*) and applied to the immunoassay of a number of biological compounds (*19*). In principle, this assay configuration is advantageous because Eu^{3+} contamination problems are eliminated and fluorescence can be measured directly on the solid phase. This approach suffers from one major disadvantage; because Eu^{3+} is used in excess, only the 1:1 BCPDA:Eu^{3+} complex is formed and it has a lower fluorescence yield than complexes of the form Eu^{3+}–$(BCPDA)_n$, where $n = 2$ or 3 (*13, 16*). Thus, BCPDA can be detected down to approximately 10^{-11} mol/L (*13*), which is 2 orders of magnitude higher than the detection limit of measuring Eu^{3+} with excess chelators.

To solve this problem, we decided to introduce amplification to the system so that sensitivity is improved without compromising the advantages of the assay design or the simplicity of performance. We chose to use the biotin–streptavidin system and multiple fluorescence labeling. Contrary to observations with conventional fluorescence probes, multiple labeling with BCPDA resulted in conjugates free from quenching effects. This is presumably due to the exceptional

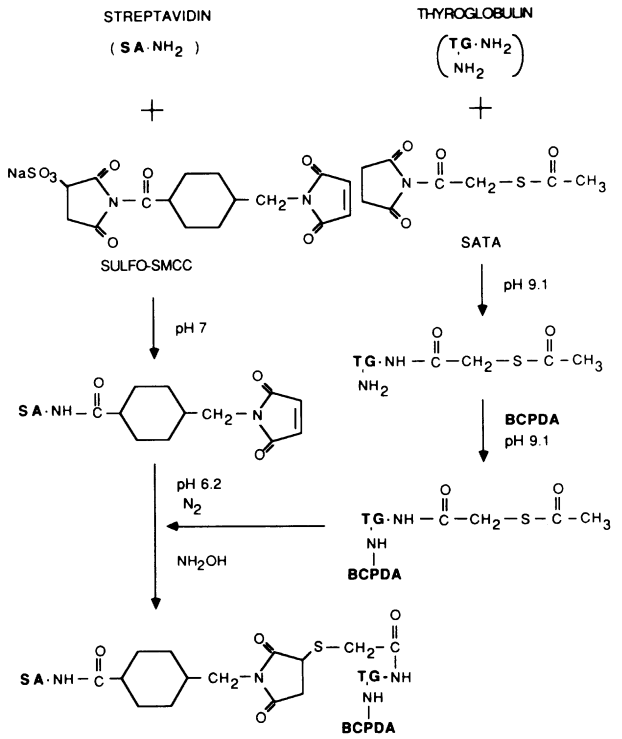

Figure 1. Schematic representation of the conjugation reaction between streptavidin and BCPDA labeled thyroglobulin. Details are given in the text.

Stokes shift observed with such complexes (excitation at 325 nm with a sharp emission band at 615 nm) such that there is essentially no overlap between the excitation and the emission spectra. Additionally, we noticed a fluorescence "bonus" effect after multiple labeling, as described in detail elsewhere (*16*).

We initially used antibodies directly labeled with BCPDA or antibodies covalently bound to labeled bovine serum albumin to devise immunoassays for cortisol (*16, 25*). With such methods about 10–15 and 40 BCPDA molecules, respectively, can be incorporated per antibody molecule. In a more sensitive assay design, we labeled streptavidin with about 14 BCPDA molecules and used biotinylated antibodies as complementary reagents (*14, 15*). The resulting total amplification is approximately 140-fold assuming a load of ~10 biotin molecules/antibody, without considering a bonus effect from labeled streptavidin (which does exist and is about 3-fold in terms of enhancement).

In this paper, streptavidin was conjugated to the carrier protein, bovine thyroglobulin (TG), which carries 160 BCPDA residues. The resulting SA–TG conjugate retains the ability to bind to biotinylated antibodies and it can be used for highly sensitive time-resolved immunoassays. The theoretical amplification factor introduced is about 10^4-fold (10 biotins × 160 BCPDA × 6 = 9600-fold) when considering the 6-fold bonus effect from the multiple labeling (*16*).

In Figure 1 the individual steps of the conjugation reaction are illustrated in an attempt to show the chemistry involved. The reaction is similar to that proposed by Katz (*26*) with respect to the incorporation of a maleimide group onto one protein and a thiol ester onto another. The initiation of the conjugation reaction occurs upon the addition of hydroxylamine which hydrolyzes the thiol ester and generates a thiol group which then reacts with the maleimide on the streptavidin forming the protein conjugate. The difference in our reaction lies in the activating and the thiolating reagents which are sulfo-SMCC (*27*) and SATA (*28*), respectively.

This reaction scheme is the preferred method by which this conjugation can be performed using sulfo-SMCC and SATA,

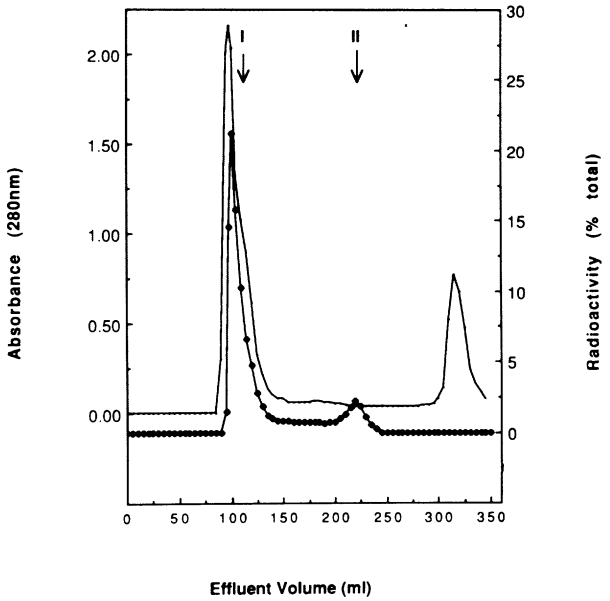

Figure 2. Elution behavior of the BCPDA–TG–SA conjugate from an Ultrogel A34 column (3 × 42 cm). This chromatogram was obtained when a reaction mixture containing 200 μg of SA and 11 mg of BCPDA labeled TG was applied to an Ultrogel A34 column that was equilibrated and eluted with a 0.1 M $NaHCO_3$ solution, pH 8, at a flow rate of 1 mL/min. The optical density of the effluent was monitored at 280 nm. The volume of each fraction was 5 mL. The symbols I and II denote the position at which BCPDA labeled TG and unconjugated SA, respectively, elute from this column under identical conditions.

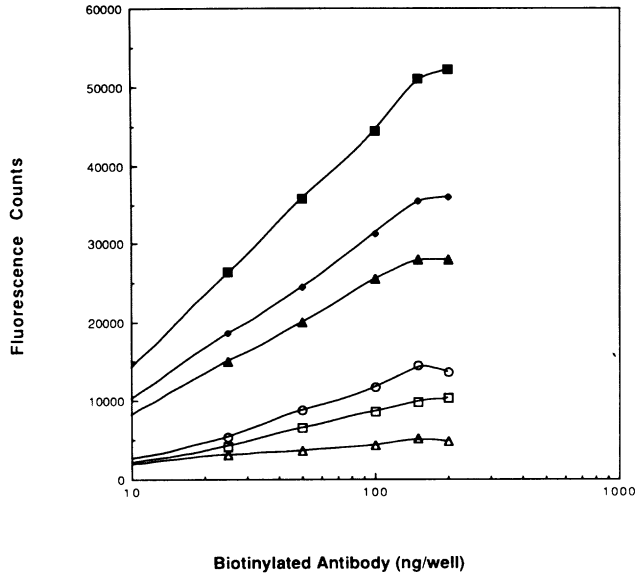

Figure 3. Fluorescence quantitation of the binding of streptavidin to biotinylated antibody coated microtitration plates. Plates were coated with varying amounts of biotinylated antibody against human IgM as outlined in Materials and Methods. BCPDA labeled SA or BCPDA–TG–SA conjugate was diluted in 50 mM Tris, pH 7.2, containing 4% BSA, 0.9% NaCl, 0.05% NaN_3, and 40 μM Eu^{3+}, to yield different concentrations (in terms of streptavidin) as follows: 0.33 μg/mL (triangles), 0.5 (diamonds), 1.0 (squares), or 3.0 (circles). One hundred microliters of each solution of BCPDA–SA (open symbols) or BCPDA–TG–SA (closed symbols) was applied in duplicate to each of the microtitre wells containing varying amounts of the biotinylated antibody and incubated for 1 h at 37 °C. The plates were then washed and dried and the fluorescence counts measured with the CyberFluor 615 fluorometer. The fluorescence counts were plotted against the amount of biotinylated antibody immobilized on the well.

for the following reasons: (1) The BCPDA labeling reaction is optimal at pH 9.1 (*16*). (2) Malimides are most stable below pH 7. The TG cannot first be activated by the conversion of some of its amino groups into malimide derivatives prior to extensive labeling at pH 9.1 because the malimide groups will undergo hydrolysis. Thus, SATA is initially used to convert approximately 5% of the TG amino groups into thiol esters. The degree of thiolation was assessed by the assay of Grassetti and Murray (*29*) by using 2,2′-dithiodipyridine. The subsequent reaction with BCPDA results in the incorporation of about 160 fluors, as determined by its absorbance at 325 nm.

The SATA derivative of the BCPDA-labeled TG should be used within 48 h to ensure that the incorporated thiols remain protected. Hydrolysis of the acetyl derivative with time could result in homopolymerization of TG through disulfide bond formation.

The activation conditions for SA with 1.5 equiv of sulfo-SMCC per amino group results in the incorporation of three to four maleimide groups per SA molecule, as determined by the 2,2′-dithiodipyridine method (*29*). The resulting derivative of SA is relatively stable in phosphate buffer at pH 6.2. However, immediate usage ensures the integrity of the maleimide groups.

The most critical step in this conjugation reaction appears to be the complete removal of oxygen from the solution. The reaction must proceed under reducing conditions in order to allow the free thiols to react with the maleimide groups and minimize the formation of disulfide bonds. The addition of mercaptoethanol after 2 h of conjugation results in blocking any remaining maleimide on streptavidin as well as reducing any disulfide bonds that may have formed between thyroglobulin molecules. The addition of *N*-ethylmaleimide results in the blocking of all the free thiols in the reaction mixture.

The isolation of the BCPDA–TG–SA conjugate employs the molecular sieving properties of the Ultrogel A34 column which is capable of fractionating proteins having molecular weights from 1×10^4 to 7.5×10^5 (Figure 2). This column affords a large separation between the conjugated and the unconjugated SA; BCPDA–TG–SA would have a molecular weight that exceeds 8×10^5 as compared to 6×10^4 for SA. This separation was monitored by the radioactivity that was associated only with SA. The presence of ^{125}I radioactivity near the void volume of this column proves that SA has been conjugated to the BCPDA–TG. SA contains no free thiol group and, therefore, the activated SA could not form a homopolymer of high molecular weight.

This column does not separate the unconjugated BCPDA–TG from that covalently bound to SA, as indicated in Figure 2. However, elaborate methods of purification of BCPDA–TG–SA from BCPDA–TG, such as with an iminobiotin column, were not necessary. We found that the excess BCPDA–TG does not contribute significantly to the overall background fluorescence observed in an α-fetoprotein assay which was used as the working model (data not shown).

Figure 3 illustrates the improved sensitivity obtained with the BCPDA–TG–SA conjugate in comparison to directly labeled SA in studies of binding to microtiter wells that are coated with biotinylated antibodies. The fluorescence readings observed with the conjugate are at least 5 times higher than those obtained with BCPDA labeled streptavidin at the same concentration.

During the developmental stage of our investigation, we tried a number of other chemical conjugation procedures. We have used the periodate conjugation method (*30*) after activation of BCPDA labeled TG with $NaIO_4$ to generate aldehyde groups on the sugar moieties of TG. The aldehyde groups were then reacted with the amino groups of SA and the Schiff base stabilized by reduction with sodium borohydride. We have also used the well-known homobifunctional reagent benzoquinone and the newer reagent *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) according to the procedures

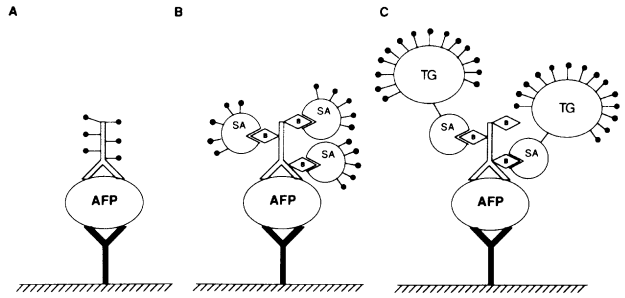

Figure 4. Schematic representation of the BCPDA labeled reagents and their utilization in the AFP assay. The figure has no quantitative meaning. Solid phase is indicated by a horizontal line with hatched lines. The solid ball and stick represents BCPDA. AFP = α-fetoprotein. B = biotin. SA = streptavidin. TG = thyroglobulin. For quantitative aspects see text.

described in ref 30. In all cases active conjugates were produced. However, the yields were lower than those obtained with the sulfo-SMCC–SATA procedure.

We have also investigated a number of proteins other than TG to serve as carriers of BCPDA. These proteins include BSA, hemocyanin, myosin, ferritin, catalase, and a series of polylysine copolymers having different ratios of lysine/amino acid and different molecular weights. These carrier proteins were selected because they have a large number of amino groups so as to carry as many BCPDA molecules as possible. We found that of the proteins tested, TG conjugates gave the best signals in the solid-phase binding assay and the AFP sandwich assay (see below) as compared to conjugates with BSA, hemocyanin, and ferritin (data not shown). Myosin and polylysine copolymers polymerized and precipitated upon conjugation to BCPDA and were not studied further.

The stability of the BCPDA–TG–SA conjugate was assessed at 4 and 37 °C. We found the stability to be best when the conjugate is stored as a relatively concentrated solution (15 μg/mL in terms of streptavidin) in a 0.05 M acetate buffer at pH 6.0. This solution is usually diluted 50–100-fold in a 50 mM Tris buffer of pH 7.20, containing 40 g of BSA per liter and Eu^{3+} (4×10^{-5} M) just before an assay. Stored as a concentrate, the conjugate was found to be stable for at least 6 months at 4 °C. Accelerated stability studies showed that the conjugate was stable for at least 2 weeks at 37 °C. According to the literature, this finding suggests that the BCPDA–TG–SA conjugate is stable for at least 2 years at 4 °C (*31*). After dilution, the reagent is stable for 1 day at room temperature, making it convenient for routine use.

We developed an immunoassay procedure for α-fetoprotein in serum using two monoclonal antibodies. One antibody is immobilized in white opaque microtitration wells to form the solid phase, as described in ref 15. The second antibody (detection antibody) is either directly labeled with BCPDA as described in ref 16 for a cortisol monoclonal antibody or biotinylated as described in ref 15. The molar ratio of BCPDA to the antibody as determined by absorbance measurements at 325 nm was 10.

We used three different methods of performing the AFP assay, as illustrated in Figure 4, in order to demonstrate the amplification of each system: (a) directly labeled detection antibody, (b) biotinylated detection antibody in conjunction with directly labeled streptavidin, (c) biotinylated detection antibody in conjunction with streptavidin conjugated to labeled thyroglobulin, as described above. The calibration curves obtained are shown in Figure 5. We calculated the detection limit in each case [defined as the concentration of AFP which can be distinguished from zero with 95% confidence] and found it to be 5, 1, and 0.2 ng/mL, respectively. The improvement in signal of system b over system a is approximately 15-fold. The detection limit improves only approximately 5-fold because the background signal (obtained with the zero standard) due to nonspecific adsorption to the wells of the reagents used was higher in system b than it was in system a [around 200 and 40 arbitrary fluorescence units, respectively]. The improvement in signal of system c over system b is about 5-fold and so is the improvement in the detection limit. Background signals in systems b and c were similar.

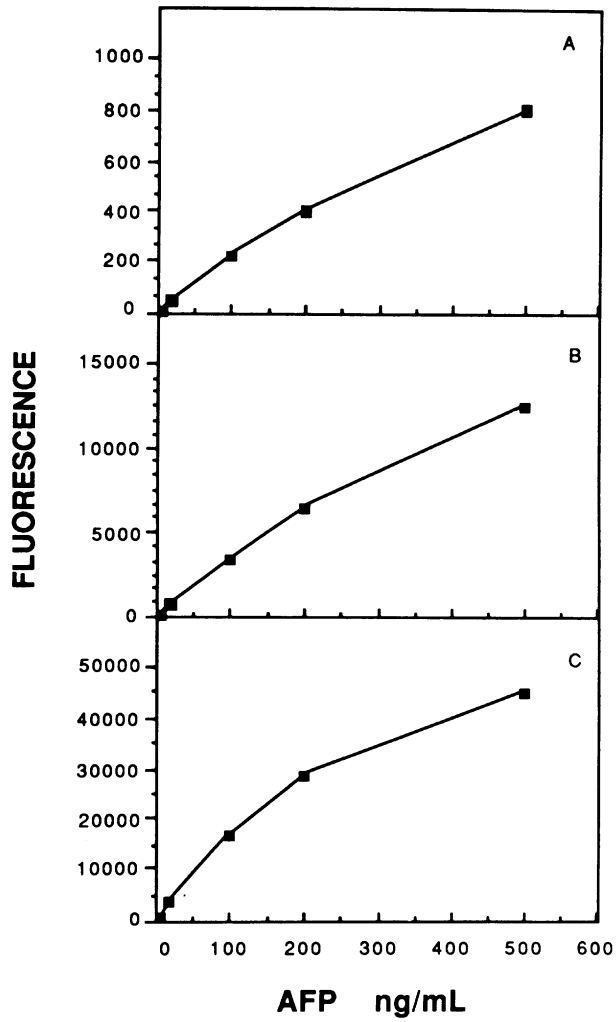

Figure 5. Calibration curves for the α-fetoprotein assay with directly labeled antibody (A) or biotinylated antibody and directly labeled streptavidin (B) or streptavidin conjugated to labeled thyroglobulin (C). More details are given under Methods. The final product in each case is shown in Figure 4.

On the basis of the BCPDA load of system c over system b, we would expect an improvement in signal of system c of at least 10-fold and more likely 20- to 30-fold because of the higher "bonus effect" described earlier for TG over streptavidin (*16*). The 5-fold increase observed in both the solid-phase binding assay and the AFP assay may be due to the fact that since the SA–TG conjugate is bulky, not as many molecules can bind to antibody conjugated biotin moieties (for steric reasons) as directly labeled streptavidin.

In conclusion, we describe methods for multiple labeling of analytical reagents that can be used successfully for devising highly sensitive immunoassays. We have demonstrated that by using europium chelators as fluorescent labels, the well-known quenching effects of multilabeled reagents are not observed. Using multiple labeling, we were able to improve the detection limit of an AFP immunoassay by a factor of up to 25-fold. The same improvements can be obtained for other analytes provided that specific biotinylated antibodies are used as complementary reagents.

LITERATURE CITED

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