Streptavidin-Based Macromolecular Complex Labeled with a Europium Chelator Suitable for Time-Resolved Fluorescence Immunoassay Applications

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We found that when we incubate SA-TG-(BCPDA)$_{150}$ (a conjugate of streptavidin (SA) with thyroglobulin (TG), which is labeled with the europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA)), with BCPDA-labeled thyroglobulin [TG-(BCPDA)$_{150}$], in the presence of a suitable amount of Eu^{3+}, a new macromolecular complex is formed with a molecular weight of about 3×10^6. This macromolecular complex is stable and can be used as a streptavidin-based universal detection system for devising time-resolved fluorescence immunoassays. This new reagent yields an 8–26-fold improvement in the detection limits of seven different immunofluorometric assays in comparison to the reagent SA-TG-(BCPDA)$_{150}$, which we have recently described (*Anal. Chem.* 1989, *61*, 48–53).

INTRODUCTION

We have recently described a new approach in devising time-resolved fluorescence immunoassays (*1–5*). The proposed system differs from the one previously described (Delfia, LKB Wallac) (*6–9*) with respect to the following: (a) The label is a europium chelator, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) instead of Eu^{3+}. (b) Labeled streptavidin is used as a universal detection reagent with biotinylated antibodies as complementary reagents instead of Eu^{3+}-labeled antibodies. (c) The final fluorescent immunocomplex is measured directly on the dry solid phase instead of extracting Eu^{3+} and recomplexing it in solution before the measurement is taken. The advantages and disadvantages to each assay design are outlined in detail elsewhere (*1*, *10*). In the Delfia design, the label is Eu^{3+}. This metal cation is extracted from the solid-phase immunocomplex and measured in solution after complexation with organic ligands. Thus, any external source of Eu^{3+}, e.g., from dust or skin surfaces, will contaminate the system and will cause positive errors in the measurements. One of the limitations of our system is that only a 1:1 BCPDA:Eu^{3+} complex is formed (the system works with excess Eu^{3+} to eliminate the contamination problems of the Delfia design), resulting in inferior sensitivities. This is due to the relatively low fluorescence quantum yield of the 1:1 complexes in comparison to complexes of the Eu^{3+}, $(\text{chelator})_n$ type, where n can vary between 2 and 5.

We have recently described a method (*11*) that improved the sensitivity of our assay design. The method involved labeling of a streptavidin–thyroglobulin conjugate with BCPDA, instead of directly labeling streptavidin. Thus, we were able to incorporate approximately 150 BCPDA molecules per streptavidin (*11*) as compared to 14 incorporated with direct labeling (*12*). We did not observe any quenching (inner filter) effects from multiple labeling, presumably due to the large Stokes shift of the label (BCPDA–Eu^{3+} complex) and the lack of overlap between the excitation and emission spectrum. We have shown that by using the universal detection reagent based on the streptavidin–thyroglobulin conjugate, we were able to improve the detection limit of a model α-fetoprotein assay by a factor of 25-fold or 5-fold in comparison to detection reagents based on BCPDA-labeled antibodies or streptavidin, respectively (*11*).

In this paper, we describe an activation process that involves incubation under controlled conditions of a mixture containing BCPDA-labeled streptavidin–thyroglobulin conjugate [SA-TG-(BCPDA)$_{150}$], free BCPDA-labeled thyroglobulin [TG-(BCPDA)$_{150}$], and a fixed amount of Eu^{3+}. After incubation at relatively high temperature (50 °C), a new streptavidin-based macromolecular complex is formed that can be isolated and used for immunoassays in a manner exactly the same as directly labeled streptavidin. However, an improvement in detection limit of approximately 33–90-fold and 8–26-fold can be achieved in comparison to directly labeled streptavidin and SA-TG-(BCPDA)$_{150}$. We postulate that the new macromolecular complex consists of a Eu^{3+} nucleus complexed with BCPDA present on thyroglobulin, which is either unconjugated or covalently conjugated to streptavidin.

EXPERIMENTAL SECTION

Materials. The Eu^{3+} chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) was synthesized as described previously (*13*). Affinity purified streptavidin (SA) and bovine thyroglobulin (TG) were obtained from Sigma Chemical Co., St. Louis, MO. The conjugate SA-TG-(BCPDA)$_{150}$ was synthesized and isolated from unconjugated SA by gel filtration, as described previously (*11*). White opaque 12-well microtiter strips (Immulon II type) were products of Dynatech Labs, Alexandria, VA. All other chemicals used were from Sigma except where otherwise stated. The monoclonal antibodies used for the immunoassays described are available from CyberFluor Inc. All other reagents necessary for the immunoassays were the same as described elsewhere (*14*) for a prolactin immunofluorometric assay.

Instrumentation. For measurement of solid-phase fluorescence, we used the CyberFluor 615 immunoanalyzer, a time-resolved fluorometer. Spectra were recorded on a HP Model 8450A diode array spectrophotometer (Hewlett-Packard Canada, Mississauga, Ontario). Sephacryl S-400 gel, the filtration column, the peristaltic pump, the optical unit, and fraction collector were all from Pharmacia Canada, Dorval,

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PQ. The fluorescence of solutions was measured on the Arcus time-resolved fluorometer (LKB Wallac, Turku, Finland).

Methods. Coating of the microtitration strips with monoclonal antibodies and the biotinylation procedure of the second antibodies are described in detail elsewhere (*14*). Streptavidin was directly labeled with BCPDA as described in ref 12. By this method, 14 ± 1 BCPDA molecules are conjugated to one streptavidin molecule. We have also conjugated streptavidin to BCPDA-labeled thyroglobulin as described in ref 11 to obtain the complex SA-TG-$(BCPDA)_{150}$. The SA-TG-$(BCPDA)_{150}$ complex is isolated from unconjugated SA by passage through an Ultrogel A-34 column. These BCPDA-labeled streptavidin-based compounds are diluted into a working solution containing 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. The concentration of SA in the working solutions for the directly labeled SA and the SA-TG-$(BCPDA)_{150}$ was 3 and 0.3 mg/L, respectively. The streptavidin concentration was assessed by using a small amount of radioactive streptavidin as tracer, as described in refs 11 and 12. 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}) (*12*).

General Immunofluorometric Assay Procedure. The procedure involves pipetting of 50 μL of analyte standards (analytes used are growth hormone, prolactin, carcinoembryonic antigen, thyrotropin, follitropin, lutropin, and choriogonadotropin) and 50 μL of biotinylated monoclonal antibody solution (~100–300 ng/well) in monoclonal antibody-coated microtitration wells and incubating for 3 h at room temperature with continuous shaking. The coating and biotinylated monoclonal antibodies are specific for each analyte, and they are available through CyberFluor Inc. The wells are then washed three times with the wash solution. After washing, 100 μL of either directly labeled streptavidin solution [SA-$(BCPDA)_{14}$], streptavidin–thyroglobulin conjugate solution [SA-TG-$(BCPDA)_{150}$], or streptavidin-based macromolecular complex solution (see below), diluted as described earlier or later in the text, are added, and the wells incubated for 30 min at room temperature with shaking. After an additional three washes, the wells are dried with a forced-air plate dryer and measured on the CyberFluor Model 615 immunoanalyzer.

Preparation of the Streptavidin-Based Macromolecular Complex (SBMC). The conjugate SA-TG-$(BCPDA)_{150}$ was synthesized exactly as described in ref 11. In the conjugation reaction mixture, a molar ratio of 1:5 between sulfo-SMCC-activated streptavidin and BCPDA-labeled thiolated thyroglobulin was used. The resulting conjugate was isolated by using an Ultrogel A-34 gel filtration column, which affords complete removal of unreacted streptavidin. The peak at the void volume of this column contains the conjugate SA-TG-$(BCPDA)_{150}$ and a fraction of the excess unreacted BCPDA-labeled thyroglobulin [TG-$(BCPDA)_{150}$] (see Results and Discussion). The void volume eluate (approximately 26 mL, having a streptavidin concentration of approximately 0.35 g/L) is then diluted in a 75 mmol/L 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer of pH 6.0 to obtain 600 mL of solution having a streptavidin concentration of 15 mg/L. The BCPDA concentration in this solution, determined by absorbance measurements at 325 nm, is approximately 130 μmol/L. This mixture is then titrated with Eu^{3+} to achieve a BCPDA saturation by Eu^{3+} (1:1 stoichiometry) of about 80 ± 5% (a concentrated 1 mmol/L $EuCl_3$ solution of pH 5.0 was added). The mixture was then incubated at 50 °C for 3 h and subsequently cooled to room temperature and filtered through a 0.45-μm filter.

Streptavidin-Based Macromolecular Complex Dilution and Use. The Eu^{3+}-enhanced or the nonenhanced streptavidin–thyroglobulin conjugate was diluted to 0.3 mg/L with respect to streptavidin in a solution that contained 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. This working solution (100 μL) was then added to each well of the analyte immunoassay (during the second incubation step), as described above.

Isolation and Constituent Identification of the Streptavidin-Based Macromolecular Complex. The SBMC was isolated from any free BCPDA-labeled thyroglobulin by gel filtration chromatography on a Sephacryl S-400 column (2.6 × 35 cm) as described in the legend of Figure 3. The 325-nm absorbance, the radioactivity (^{125}I-SA), and the relative fluorescence signal on the prolactin assay of the isolated SBMC were quantified. The SBMC was then broken down into its constituents (streptavidin covalently linked to BCPDA-labeled thyroglobulin; BCPDA-labeled thyroglobulin; Eu^{3+}) by the following procedure: The SBMC was incubated overnight at room temperature with ethylenediaminetetraacetic acid (EDTA) in Na_2CO_3, pH 9.1, in a molar amount that was 30 times that of the BCPDA present. The resulting mixture was applied to a Sephadex G-25 column and eluted with 0.1 M $NaHCO_3$, pH 8. The effluent absorbance was monitored at 280 nm. The amount of Eu^{3+} in each fraction collected was assessed by diluting an aliquot of each fraction 100-fold in 100 mM acetate buffer, pH 3.5 (solution A). Then we pipetted 10 μL of solution A into a clear microtitre strip to which 200 μL of an enhancement solution, containing the Eu^{3+} chelators napthoyltrifluoroacetone and trioctylphosphine oxide (*9*) (available commercially by LKB Wallac), was added. The fluorescence was then measured on the Arcus fluorometer. The Eu^{3+} concentration was quantitated by using a standard curve that was generated with an identical procedure. The protein peak eluting near the void volume of the column was pooled, and the absorbance at 325 nm, the radioactivity (^{125}I-SA), and the relative fluorescence signal on the prolactin assay were determined.

RESULTS AND DISCUSSION

We have recently described the synthesis and isolation of a streptavidin–thyroglobulin conjugate that was multiply labeled with the novel fluorescent europium chelator, BCPDA (*11*). This conjugate, SA-TG-$(BCPDA)_{150}$, was synthesized in the presence of excess TG-$(BCPDA)_{150}$ (5-fold molar excess over streptavidin). The isolated conjugate, SA-TG-$(BCPDA)_{150}$, obtained from the Ultrogel A-34 column, also contains free (unconjugated) TG-$(BCPDA)_{150}$ but in a (2.3 ± 0.2)-fold molar excess over SA-TG-$(BCPDA)_{150}$, as determined by radioactivity (^{125}I-SA) and through the quantitation of BCPDA by its specific absorbance at 325 nm. Part of the free TG-$(BCPDA)_{150}$ is removed from the mixture during the gel filtration isolation of SA-TG-$(BCPDA)_{150}$. We here describe the formation of a streptavidin-based macromolecular complex (SBMC) from the isolated conjugate mixture containing SA-TG-$(BCPDA)_{150}$ and a 2.3-fold excess of TG-$(BCPDA)_{150}$. To the isolated SA-TG-$(BCPDA)_{150}$ conjugate containing excess TG-$(BCPDA)_{150}$, increasing amounts of Eu^{3+} were added, and the resulting mixtures were incubated at 50 °C for 3 h as described in Methods. The solutions were then cooled and filtered, and the total absorbance at 325 nm (where BCPDA absorbs specifically) was determined. Each solution was also assessed as a detection reagent in conjunction with biotinylated antibodies, on a complete prolactin assay where a 25 μg/L standard prolactin solution was used. The detection reagent involved the dilution of the conjugate solutions in a Eu^{3+}-containing buffer, as described under Methods. The results are summarized in Figure 1. It can be seen that by

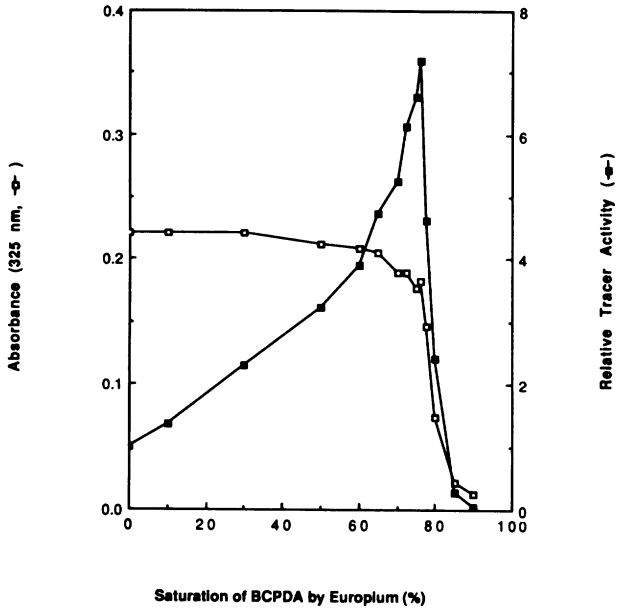

Figure 1. Assessment of the SA-TG-(BCPDA)$_{150}$ conjugate after incubation with excess TG-(BCPDA)$_{150}$ in 75 mM MES, pH 6, at 50 °C for 3 h and in the presence of Eu^{3+}. The amount of Eu^{3+} added was expressed as a molar ratio to the BCPDA molecules covalently linked to TG. The product was assessed by its absorbance at 325 nm (□) and its relative fluorescence signal (relative tracer activity) for a standard solution containing 25 μg/L prolactin in a time-resolved fluorescence immunoassay (■). For more details, definitions, and discussions, see the text.

increasing the amount of Eu^{3+} up to a certain point (65% of the required equivalent concentration for 1:1 stoichiometry in relation to BCPDA concentration), the absorbance of BCPDA remains relatively constant. After this point, increasing amounts of Eu^{3+} cause a decrease in BCPDA absorbance because of SA-TG-(BCPDA)$_{150}$ and free TG-(BCPDA)$_{150}$ precipitation (which is also identified visibly). In the same figure, the relative activity of the Eu^{3+}-enhanced conjugate is shown. The relative activity is expressed as follows: An activity of 1.00 is arbitrarily defined for the fluorescence reading observed with a 25 μg/L prolactin standard in a complete prolactin assay, when the conjugate SA-TG-(BCPDA)$_{150}$ is used without activation with Eu^{3+}. That is, the conjugate is used as described previously (*11*). After each Eu^{3+} addition, the relative activity was calculated by using the ratio of observed fluorescence with Eu^{3+} enhancement over the fluorescence observed without Eu^{3+} enhancement. It can be seen that a maximum of 7.2-fold amplification in signal can be achieved by incubating with an optimum amount of Eu^{3+}, as described in Methods. However, Eu^{3+} addition that is over the optimal results in conjugate precipitation and the subsequent loss of activity (Figure 1). The optimum Eu^{3+} concentration in the mixture which is suitable to give maximum amplification lies in a very narrow range, (Figure 1); therefore, it is preferable, for the SBMC preparation, to empirically titrate aliquots of the initial conjugate with Eu^{3+} in the range of 70–85% saturation of BCPDA and test the aliquots in a complete assay.

We have also constructed complete prolactin calibration curves in the range 0.1–25 μg/L by using (a) streptavidin directly labeled with 14 BCPDA molecules, (b) SA-TG-(BCPDA)$_{150}$ conjugate without Eu^{3+} activation, and (c) SBMC but with varying amounts of Eu^{3+}. It can be seen from Table I that maximum signal is observed with the fraction of 76% BCPDA saturation by Eu^{3+}. The observed amplification over (a) and (b) is about 35-fold and 7-fold, respectively.

Fully enhanced fractions of the SBMC were stored at 4 °C and tested frequently in complete immunoassays, to assess

Table I. Linear Regression Analysis of Prolactin Calibration Curves Using Various Streptavidin-Based Detection Reagents[a]

detect reagent	BCPDA saturation by Eu^{3+}, %	slope	y intercept	corr coeff
SA(BCPDA)$_{14}$		109	−13	0.998
SA-(TG)-(BCPDA)$_{150}$		535	−257	0.997
SBMC[b]	30	919	−371	0.998
	60	1532	−678	0.997
	70	2516	−1325	0.997
	76	2970	−1362	0.998
	80	1151	−300	0.999
	85	145	17	0.997

[a] The *y* axis is in arbitrary fluorescence units and the *x* axis is prolactin concentration (μg/L). [b] SBMC is the streptavidin-based macromolecular complex formed after the addition of Eu^{3+} to saturated BCPDA at the desired level. For other definitions and discussion see text.

their stability. It was found that the stored concentrates (concentration of 15 mg/L in terms of streptavidin) are stable for at least 1 year. These concentrates are diluted 50-fold in a suitable buffer just before use in the assay; the buffer was 50 mmol/L Tris, pH 7.20, containing 9 g of NaCl, 40 g of BSA, and 0.5 g of sodium azide per liter. This buffer must be supplemented with excess Eu^{3+} (10^{-5} mol/L) when directly labeled streptavidin or nonenhanced SA-TG-(BCPDA)$_{150}$ is used as a detection reagent. We have examined both the requirement of the maximally enhanced SBMC for Eu^{3+} in the dilution buffer and the stability of the diluted SBMC with time, in the presence or absence of Eu^{3+} in the diluent. We observed that the SBMC gives a similar signal and demonstrates much longer stability when diluted in the absence of Eu^{3+}. The diluted SBMC in the presence of excess Eu^{3+} loses its activity with time (~50% and 20% activity remains at 5 and 100 days, respectively) presumably through a slow precipitation process. The diluted SBMC in the absence of Eu^{3+} retains at least 80% of its activity 70 days after dilution.

The Eu^{3+} enhancement of the SA-TG-(BCPDA)$_{150}$ conjugate was investigated at various temperatures and pH values. A pH of 6 was found to be optimal, though conjugate enhancement would occur to a lesser extent at any pH between 5 and 8 (data not shown). The temperature of enhancement primarily altered the rate of formation of the SBMC, i.e., completion of the enhancement would occur at about 48, 16, and 3 h for the temperatures 25, 37, and 50 °C, respectively. Temperatures above 50 °C generally decreased the activity of the conjugate.

In Figure 2 we present a gel filtration chromatography experiment. The dashed line represents the elution pattern of the Ultrogel A34 isolated conjugate obtained from the conjugation reaction between streptavidin and BCPDA-labeled thyroglobulin (initial molar ratio 1:5). There is a small peak eluting around 70 mL corresponding to a molecular weight of approximately (2.5–3) × 10^6, followed by a continuous spectrum of absorbance up to 105 mL, where there is a peak corresponding to a molecular weight of approximately 0.8 × 10^6. The peak eluting around 70 mL is likely due to a conjugate of the form SA-[TG-(BCPDA)$_{150}$]$_3$ while between this volume and 105 mL another conjugate of the form SA-[TG-(BCPDA)$_{150}$]$_2$ may be eluting. The bulk of the material, however, consists of SA-TG-(BCPDA)$_{150}$ and unconjugated TG-(BCPDA)$_{150}$, both eluting around 105 mL. By monitoring the radioactivity from ^{125}I-streptavidin, incorporated as a marker in the conjugation mixture, we could estimate that approximate percentages of SA-[TG-(BCPDA)$_{150}$]$_3$:SA-[TG-(BCPDA)$_{150}$]$_2$:SA-TG-(BCPDA)$_{150}$ are 5:20:75.

Table II. Comparison of Amplification and Detection Limits with the Three Streptavidin-Based Reagents for Seven Immunofluorometric Assays

analyte	fluorescence, arbitrary units[a]		
	$SA(BCPDA)_{14}$	SA-(TG)-$(BCPDA)_{150}$	SA-$(TG)_{3.3}$-$(BCPDA)_{480}$
choriogonadotropin, U/L			
0	82 (5)	111 (6)	213 (7)
5	158 (12)	408 (13)	4376 (201)
detect limit[b]	1.00	0.32	0.028
lutropin, U/L			
0	127 (8)	163 (9)	288 (11)
2	162 (6)	401 (10)	2604 (101)
detect limit	1.50	0.23	0.029
prolactin, μg/L			
0	102 (8)	230 (19)	317 (12)
5	292 (7)	2100 (58)	16202 (691)
detect limit	0.61	0.15	0.012
carcinoembryonic antigen, μg/L			
0	53 (3)	84 (6)	206 (14)
5	187 (8)	911 (28)	20417 (653)
detect limit	0.36	0.11	0.011
thyrotropin, mU/L			
0	60 (3)	88 (3)	169 (7)
0.25	65 (3)	115 (4)	798 (50)
detect limit	0.45	0.09	0.01
follitropin, U/L			
0	57 (4)	106 (4)	180 (4)
2	93 (7)	275 (10)	3888 (128)
detect limit	0.70	0.13	0.01
growth hormone, μg/L			
0	148 (15)	219 (24)	220 (12)
0.5	233 (8)	666 (19)	6632 (172)
detect limit	0.27	0.08	0.003

[a] The standard deviation of each measurement is given in brackets (n = 12 in all cases). [b] The detection limit (DL), defined as the lowest concentration that could be distinguished from 0 with 99% confidence, was calculated from the formula DL = $(3DB)/(C - A)$, where D = concentration of second standard solution, B = standard deviation of the fluorescence signal of the first (zero) standard solution, C = the fluorescence signal of the second standard solution, and A = the fluorescence signal of the first standard solution.

After the enhancement reaction was performed, the conjugate mixture (initially corresponding to the dashed line of Figure 3) was rechromatographed on the same column generating the elution pattern represented by the solid line. Little or no unreacted TG-$(BCPDA)_{150}$ could be seen, the major product now appearing as a macromolecular complex of approximately (2.5–3.0) × 10^6 molecular weight.

Further investigation into the composition of the SBMC with respect to the ratio of streptavidin covalently linked to BCPDA-labeled thyroglobulin, BCPDA-labeled thyroglobulin, and Eu^{3+} was performed by incubating overnight at room temperature, as described in Methods, the isolated SBMC obtained from the Sephacryl S-400 column (the pooled protein peak having absorbance at 280 nm and eluting around 70 mL (Figure 2)) with 30 times excess EDTA. The resulting solution was applied and eluted from a Sephadex G-25 column. The addition of EDTA should extract and complex the Eu^{3+} present in the SBMC due to its superior affinity for Eu^{3+} (K_a = 10^{18} M^{-1}) over that of BCPDA (K_a = 10^{10} M^{-1}). We found that all of the protein eluted near the void volume of the column and the recovery was 100% as determined by radioactivity (^{125}I-SA). Furthermore, Eu^{3+} was not found to be associated with the protein but eluted in subsequent fractions closer to the bed volume of the column. The molar ratio of SA to BCPDA-labeled thyroglobulin in the pooled protein fractions was found to remain at the ratio of 1:3.3; however, its activity as a detection reagent for the prolactin assay now correlated to the original nonenhanced SA-TG-$(BCPDA)_{150}$ conjugate and not to the SBMC (data not shown). Therefore, the composition of the SBMC appears to be 1:2.3:480 with respect to SA-TG-$(BCPDA)_{150}$, TG-$(BCPDA)_{150}$, and Eu^{3+} (see also Figure 3 for further explanations).

These results prompted us to suggest the following mechanism of formation of the streptavidin-based macromolecular complex (SBMC): Upon the addition of Eu^{3+}, unconjugated (free) TG-$(BCPDA)_{150}$ molecules present in excess in the isolated mixture (Figure 3B) aggregate and combine with SA-TG-$(BCPDA)_{150}$ molecules to form the macromolecular complex (Figure 3). This complex is stable in solution if it possesses certain overall net negative charge but precipitates if the Eu^{3+} concentration exceeds a certain limit. The formation of this aggregate is relatively slow but is complete after 3 h incubation at 50 °C.

In practical terms, this new streptavidin based macromolecular complex is very useful. Its preparation, using the previously published conjugation procedure (*11*) and the enhancement procedure described here is relatively simple. The actual reagent can be used in the same manner as the directly labeled streptavidin preparation. Additionally, all the advantages of the time-resolved fluorescence immunoassay system that we have developed (*1*, *10*), are still present with the use of this reagent.

In Table II, we present comparative analytical data for seven different immunofluorometric procedures. In all assays, three different detection reagents were used: $SA(BCPDA)_{14}$, SA-TG-$(BCPDA)_{150}$, and the SBMC [represented by the formula SA-$(TG)_{3.3}$-$(BCPDA)_{480}$]. The detection limit, defined as the lowest concentration of analyte that could be distinguished from zero with 99% confidence (*15*), was calculated as shown in Table II. It can be seen that with the new reagent, the detection limit was improved by a factor of 33–90-fold and 8–26-fold in comparison to $SA(BCPDA)_{14}$ and SA-TG-$(BCPDA)_{150}$. The precision of the assays tested was similar with all reagents studied (Table II and other data at higher analyte concentrations that are not shown).

We have also examined the specificity of the assays shown in Table II by performing cross-reactivity experiments for other substances that may interfere. The percent cross-re-

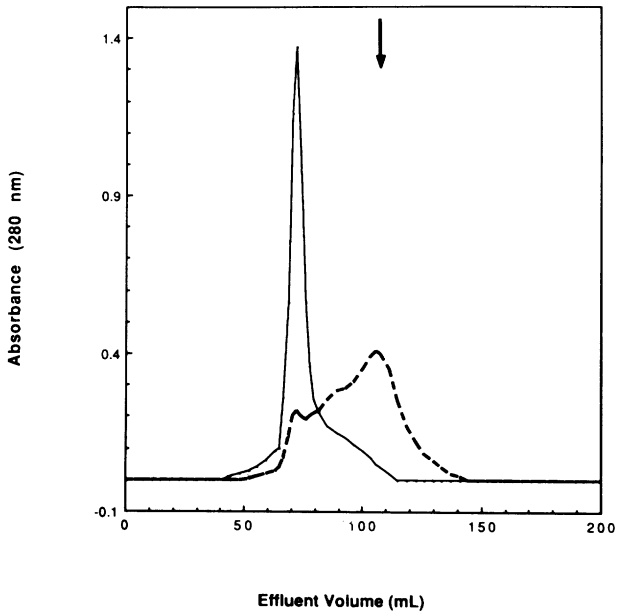

Figure 2. Elution behavior of the SA-TG-$(BCPDA)_{150}$ conjugate in the presence of excess TG-$(BCPDA)_{150}$ from a Sephacryl S-400 column (2.6 × 35 cm). These chromatograms were obtained when approximately 0.8 nmol of SA-TG-$(BCPDA)_{150}$ and 3.3 nmol of TG-$(BCPDA)_{150}$ were applied to the column that was equilibrated and eluted with 50 mM MES, pH 6, at a flow rate of 0.4 mL/min before (---) and after (—) a 3-h incubation with Eu^{3+} (76% of the BCPDA present) in 75 mM MES, pH 6 at 50 °C. The arrow denotes the position at which TG-$(BCPDA)_{150}$ elutes from this column under identical conditions. For discussion, see text.

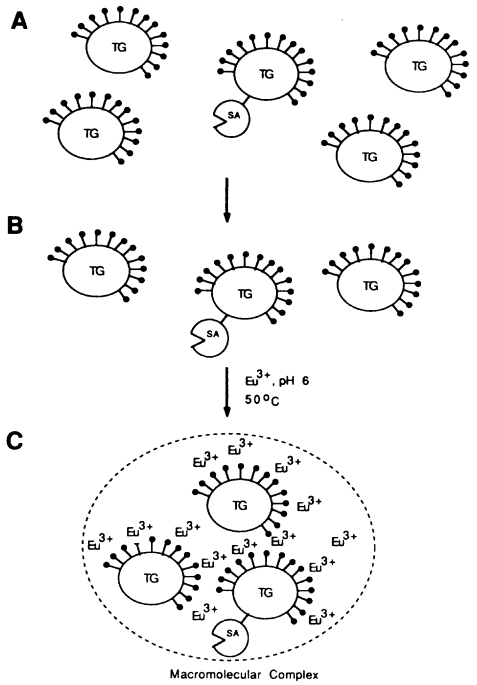

Figure 3. Schematic representation of the proposed mechanism of formation of the macromolecular complex, where -● = BCPDA, SA = streptavidin, and TG = thyroglobulin. This scheme illustrates the components in the conjugate solution after the conjugation reaction (A), after isolation from the Ultrogel A34 column (B), and after incubation with Eu^{3+} at 50 °C (C). The figure has no quantitative meaning. For quantitative aspects, see text.

activity is calculated here as the ratio of the analyte-equivalent concentration resulting from the cross-reacting substance over the concentration of the cross-reacting substance that produced the cross-reactivity, expressed as a percentage. In the choriogonadotropin assay, only lutropin showed a slight cross-reactivity (2%) at a level of 500 U/L. Thyrotropin (up to 200 mU/L) and follitropin (up to 200 U/L) sowed negligible cross-reactivity (<0.1%). In the lutropin assay choriogonadotropin showed a 5% cross-reactivity at a level of 500 U/L. Follitropin (up to 200 U/L) and thyrotropin (up to 200 mU/L) showed negligible cross-reactivity (<0.1%). In the prolactin assay, the cross-reactivity of the following hormones was negligible (<0.1%): choriogonadotropin (up to 50 000 U/L); lutropin (up to 200 U/L); growth hormone (up to 125 μg/L); follitropin (up to 200 U/L); and thyrotropin (up to 100 U/L). In the thyrotropin assay, the cross-reactivity of the following hormones was negligible (<0.1%): lutropin (up to 2500 U/L); follitropin (up to 2500 U/L); and choriogonadotropin (up to 50 000 U/L). In the follitropin assay, the cross-reactivity of the following hormones was negligible (<0.1%): lutropin (up to 1000 U/L) and thyrotropin (up to 1000 mU/L). Choriogonadotropin showed a slight interference (2%) up to a level of 1000 U/L. In the growth hormone assay, the following hormones showed negligible cross-reactivity (<0.1%): prolactin (up to 400 μg/L); follitropin (up to 300 U/L); thyrotropin (up to 1000 U/L); and lutropin (up to 250 U/L).

In conclusion, we here present a novel universal detection reagent that is based on labeled streptavidin. This reagent is suitable for time-resolved fluorometric applications using biotinylated reactants as complementary reagents. Significant improvements in detection limits were obtained in comparison to previous reagents described by our group. We anticipate that the new reagent will find applications as an ultrasensitive detection system in areas other than immunoassay, e.g., in immunohistochemistry and DNA probing.

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