Multiple Labeling and Time-Resolvable Fluorophores

Eleftherios P. Diamandis

A new time-resolved fluorescence immunoassay system involving use of the europium chelate of 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid as label is reviewed. This stable chelate by itself is not very fluorescent but, used in multiple labeling strategies, improves the achievable detection limits. By using multiple labeling, streptavidin tailing, and Eu^{3+} activation, one can create a very stable, easy-to-use reagent that is suitable for devising highly sensitive immunoassays and other biotechnological assays. This reagent, a streptavidin-based macromolecular complex, is able to detect ~300 000 molecules (~0.5 amol) of alpha-fetoprotein in a model noncompetitive immunoassay.

Additional Keyphrases: *fluoroimmunoassay · streptavidin*

Conventional fluorophores have been used for many years in various biotechnology applications, including immunoassay (*1, 2*). Currently, these fluorophores are routinely used as labels in several automated homogeneous immunoassay procedures applicable to analytes present in serum at micromolar or nanomolar concentrations. No system is commercially available that uses such fluors for immunoassay procedures having sensitivity in the picomolar or subpicomolar range, because the detection sensitivity of conventional fluors is severely compromised by background signals. Such background comes from light scatter, fluorescence of endogenous compounds in the biological sample (e.g., proteins, bilirubin, etc.), fluorescence of the cuvettes, optics, solvents, solid phases used in immunoassay, etc. The very small Stokes shifts and wide emission bands of such fluors (i.e., 28 nm Stokes shift for fluorescein) is an important limitation for several different reasons, including the following: (*a*) excitation radiation cannot be completely excluded from reaching the detector because of scattering; (*b*) in minimizing the scattering effect, narrow bandpass emission filters are used, which exclude a significant portion of the emitted radiation; and (*c*) the wide emission band is likely to overlap with emission bands of interfering compounds.

Fluorescent chelates of europium, terbium, and samarium are potential alternatives to isotopic labels, conventional fluorophores, and other nonisotopic labels for immunological and other assays. The main advantages of these fluorescent lanthanide chelates are shown in Table 1. By combining the advantages listed, background fluorescence can be virtually eliminated (Figure 1). Thus, a pulsed-light time-resolved fluorometric measurement of a europium chelate is at least 10^3-fold more sensitive than is a measurement based on conventional fluorometry.

Department of Clinical Biochemistry, Toronto Western Hospital, 399 Bathurst St., Toronto, Ontario M5T 2S8, Canada (address for correspondence), and Department of Clinical Biochemistry, University of Toronto, Toronto, Ontario M5G 1L5.

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Lanthanide Chelates for Immunological Assays

The first proposal of lanthanide chelates as labels for immunoassay applications was put forward by Wieder (*3*) and Leif et al. (*4*). A lanthanide chelate can be used as an immunological label in a simple assay configuration (Figure 2), which is identical to a configuration used for immunoradiometric assays and other nonisotopic assays. Similar immunocomplexes can be formed with competitive-type immunoassays. However, this simple assay configuration has been very difficult to use successfully, for the following reasons:

- Europium chelates absorb radiation characteristic of the ligand and emit radiation characteristic of the metal ion. The fluorescence quantum yield of the chelate depends on the efficiency of the energy transfer process from the ligand to the ion. If the energy transfer is inefficient, the chelate is not very fluorescent and sensitivity is relatively poor. The most fluorescent complexes of europium are complexes of the type $Eu(NTA)_3(TOPO)_2$, where NTA = naphthoyltrifluoroacetone and TOPO = trioctylphosphine oxide. The 1:1 complexes between Eu^{3+} and various ligands are not very fluorescent.
- Apart from the requirement that the chelate be fluorescent, it must also possess very high stability; otherwise, during the washing process necessary for heterogeneous immunoassays, Eu^{3+} would leak into the solution.

Table 1. Advantages of Lanthanide Chelates in Comparison with Conventional Fluorophores

Characteristic	Conventional fluorophores	Lanthanide chelates[a]
Stokes shift	Small (28 nm for fluorescein)	Large (290–300 nm for Eu^{3+})
Emission band	Wide	Narrow
Lifetime	Short (<100 ns)	Very long (1–1000 μs)
Overlap of excitation–emission spectra	Significant	Negligible–nonexistent
Overlap with serum autofluorescence	Significant	Negligible
Pulse excitation[a]	Difficult	Simple
Light scatter	Severe	Nonexistent
Background fluorescence[b]	Significant	Nonexistent
Quantum yield	Excellent	Good
Detection limit	~10^{-9} mol/L	~10^{-13} mol/L

[a] Used with microsecond time-resolved fluorometry.
[b] Optics, solid phases, solvents, impurities.

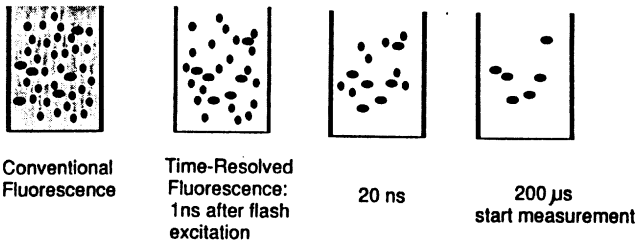

Fig. 1. Schematic diagram showing comparison of a conventional fluorometric measurement and a time-resolved fluorometric measurement

Shortly after a pulsed excitation, both short-lived and long-lived fluors emit, but at 20 ns most of the short-lived fluorescence has dissipated. At 200 μs, only the long-lived fluors emit, which can be measured under conditions of zero background with very high sensitivity. This Figure has no quantitative meaning

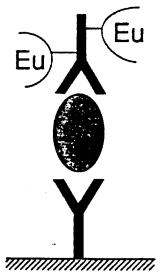

Fig. 2. "Two-site" (sandwich) assay for an analyte: one of the antibodies (capture) is immobilized on a solid support; the other (detection) is labeled with a europium chelate

The ligand binds Eu^{3+} strongly so that no leakage occurs during extensive washing. Ideally, the ligand absorbs excitation energy and transfers it to Eu^{3+}, which is, in turn, excited and subsequently emits long-lived fluorescence

Although forming very highly fluorescent complexes of Eu^{3+} with organic ligands (with long decay times, suitable for microsecond time-resolved fluorometry) is easy, these complexes are not soluble and stable enough to be linked to detection antibodies. On the other hand, the highly stable 1:1 complexes of Eu^{3+} and various aminopolycarboxylic acids can be used successfully to label antibodies with Eu^{3+} but are not very fluorescent because the energy-transfer process is not optimal with such chelates. Thus, the first time-resolved fluorometric immunoassay system described and commercialized is based on the following two steps:

- A complex (as shown in Figure 2) is formed by using detection antibodies that carry Eu^{3+} through a nonfluorescent chelate with an aminopolycarboxylic acid derivative covalently linked to the antibody.
- To measure Eu^{3+}, the derivative-antibody conjugate is released in solution at low pH and is recomplexed with appropriate organic ligands to form a highly fluorescent complex in solution.

The system, known as DELFIA™ (Pharmacia-LKB, Bromma, Sweden) has excellent sensitivity, broad dynamic range, and reagent stability. The excellent sensitivity is due to the fact that, during the immunoreaction, Eu^{3+} is carried on the antibody tightly bound to a strong chelator without any leakage. Also, during measurement, Eu^{3+} is optimally complexed by organic ligands to form a highly fluorescent complex. However, this system has been criticized for three reasons: (*a*) An extra step is needed to extract Eu^{3+} from the solid phase before measurement; (*b*) more importantly, the final measurement is based on Eu^{3+} quantification, usually at 10^{-12} to 10^{-13} mol/L, but because Eu^{3+} is a natural element present in dust, skin, etc., the possibility of contamination, especially of the reagents containing the europium chelators, presents difficulties; (*c*) because the final measurement is based on extraction, the system is not suitable for other biospecific assays, e.g., Southern, Northern, or Western blotting, DNA sequencing, or immunohistochemistry and flow cytometry.

Immunoassays with BCPDA-Labeled Detection Antibodies

About six years ago, we started research towards the synthesis of organic europium chelators that would combine good complex stability and energy transfer so that we could achieve assays based on the design of Figure 2 without the need for extraction of Eu^{3+}. Such efforts have also been undertaken by other groups; isolated reports are described by Soini et al. (*5*). The best chelator identified by our group, BCPDA, was synthesized as shown in Figure 3 (*6*).[1] BCPDA has the following structural features: a polyaromatic phenanthroline ring, which can absorb radiation subsequently used to internally excite coordinated Eu^{3+}, and a Eu^{3+}-binding site consisting of two heteroaromatic nitrogens and two carboxylic acids. Eu^{3+} can form either 1:1 or 1:2 complexes with BCPDA; the $Eu(BCPDA)_2$ complexes are more fluorescent than the 1:1 complexes (*7, 8*). BCPDA also possesses two sulfonylchloride groups, which are used to link the molecule to proteins through ε-amino groups of lysines or to other primary amine-containing compounds (*7*).

Earlier reports by our group established optimal procedures for labeling proteins with BCPDA (*7*). In general, we found it easy to derivatize all amino groups of many different proteins; however, for biologically active macromolecules, e.g., antibodies, avidin, streptavidin, ex-

[1] Nonstandard abbreviations: BCPDA, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid; SBMC, streptavidin-based macromolecular complex; SA, streptavidin; and TG, bovine thyroglobulin.

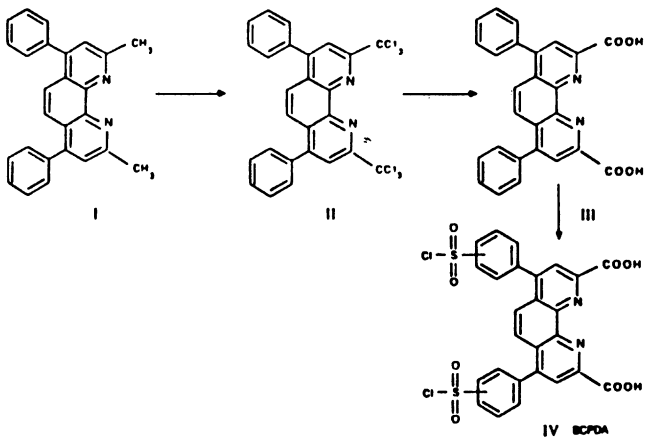

Fig. 3. Synthesis of 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) (IV) from the commercially available compound bathocuproine (I)

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tensive labeling results in partial or full loss of biological activity. Thus, optimal labeling is mandatory, not just desirable (*7*). Below, I describe how BCPDA was used as a label to devise highly sensitive time-resolved fluorescence immunoassays.

Labeling

Polyclonal and monoclonal anti-cortisol antibodies and other monoclonal detection antibodies were successfully labeled with BCPDA with a load of 12–18 BCPDA molecules per antibody molecule without significant loss of the antibody's binding activity. Additionally, second antibodies (e.g., goat anti-mouse IgG) were also labeled successfully with BCPDA at the above ratios. These antibodies were used successfully for both competitive-type and noncompetitive-type assays (e.g., in the configuration shown in Figure 2), with the fluorescent immunocomplex measured directly on the solid phase. Although it was clearly established that in such assays, with BCPDA as label, the immunocomplex does not lose Eu^{3+} during repeated washing and is fluorescent so that there was no need for Eu^{3+} extraction (unpublished data), the configuration was not highly sensitive. We attributed the low sensitivity partly to the fact that only 1:1 complexes of Eu^{3+} with BCPDA could form because of the presence of excess Eu^{3+}.

The issue of low sensitivity was addressed in a series of efforts to amplify the above system so that highly sensitive assays could be achieved.

Antibody Labeling

Antibodies possess many ϵ-amino groups (>50 per molecule) but derivatization of >18 per molecule with BCPDA inactivates the antibody. We decided to increase the antibody load by covalently linking it with a carrier protein that is already highly labeled with BCPDA. The conjugation chemistry used is diagrammatically shown in Figure 4. For the carrier protein, we used bovine serum albumin, which could carry as many as 40 BCPDA molecules (Figure 5). The antibody conjugate was used successfully in a competitive-type assay of cortisol with a signal amplification about double that of the directly labeled antibodies (*9*).

Biotin–Streptavidin System

This versatile system has recently been reviewed (*10*). In most immunoassay applications, biotin is introduced into detection antibodies at a load of 10–20 biotins per antibody, by reacting the ϵ-amino groups of lysines with an *N*-hydroxysuccinimide ester of biotin in a very simple one-step reaction. Streptavidin (SA), labeled with a reporter molecule, is added in a separate step to bind to biotin and thus reveal the presence of the immunocomplex. A final immunocomplex on the solid phase for an alpha-fetoprotein assay is shown in Figure 6 (left). The biotin–streptavidin system introduces amplification in an immunoassay because (*a*) at least 10–15 biotins could be available to bind labeled streptavidins and (*b*) streptavidin can carry as many as 14 BCPDA molecules without any loss of its biotin-binding activity (*7*). Thus,

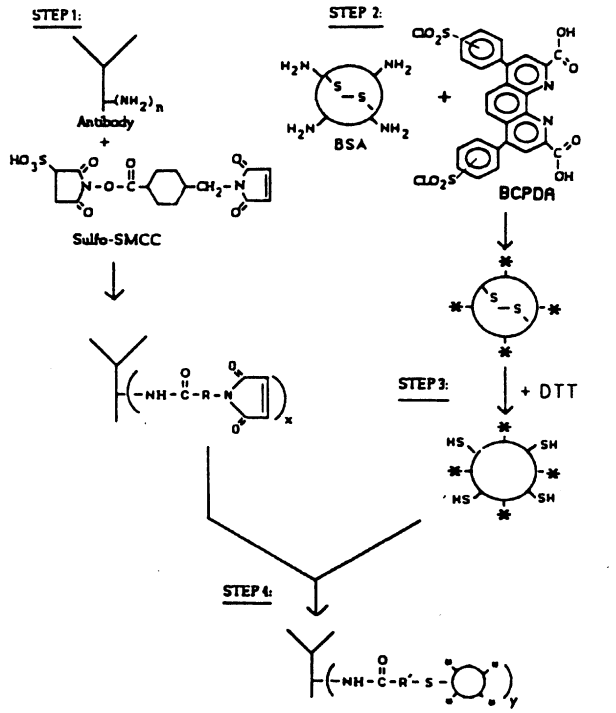


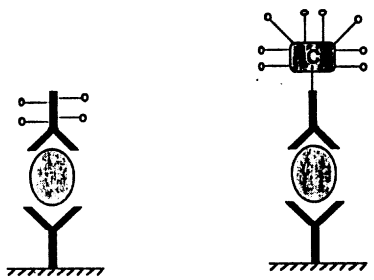
Fig. 4. Procedure for the preparation of antibody–bovine serum albumin (BSA)–BCPDA conjugates

In step 1, antibody is reacted with sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) to introduce maleimide groups. In step 2, BSA is coupled to BCPDA. In step 3, labeled BSA is reduced by dithiothreitol (DTT) to create free SH groups. In step 4, the derivatized antibody is coupled to reduced BSA. *n*, *x*, and *y* represent the number of total amino groups, derivatized amino groups, and conjugated amino groups, respectively. Reprinted from reference *9* by permission

the total amplification can be as high as 14 × 15 = 210-fold in comparison with only 15- to 18-fold achieved by using directly labeled antibodies. An extra 16-fold amplification in signal has been observed in a model alpha-fetoprotein assay when $SA(BCPDA)_{14}$ and biotinylated detection antibodies are used instead of BCPDA-labeled detection antibodies (*11*).

Streptavidin Labeling

In our previous studies of labeling various proteins with BCPDA (*7*), we noticed that we could easily incorporate into the proteins as many or, in some instances,



Signal amplification 15-18-fold

Signal amplification 40-fold

Fig. 5. Conceptual view of an assay with directly labeled BCPDA–detection antibody (*left*) and antibody covalently linked to BCPDA-labeled bovine serum albumin (*C*, right)

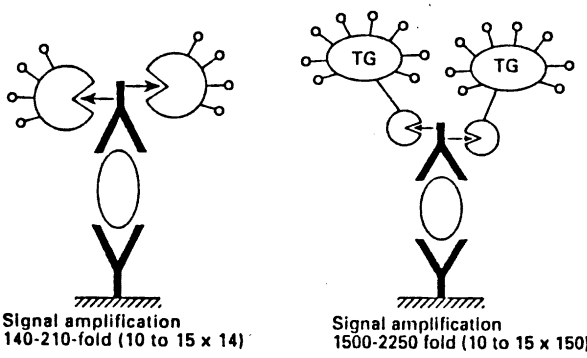


Fig. 6. Schematic of an assay involving BCPDA-labeled streptavidin [SA(BCPDA)$_{14}$] (*left*) and streptavidin covalently linked to BCPDA-labeled thyroglobulin [SA(TG)(BCPDA)$_{150}$] (*right*)

Biotin (*arrow*) is shown bound to streptavidin. —○ indicates the Eu^{3+}–BCPDA complex

more BCPDA molecules than the number of ϵ-amino groups. When we checked these proteins for fluorescence in the presence of excess Eu^{3+}, we noticed two important facts. First, no fluorescence quenching was present, although this fluorescence quenching occurs to a significant degree with polyfluoresceinated proteins or nucleic acids (*12, 13*). Fluorescence quenching with multiple fluorescence labeling occurs because of the significant overlap between excitation and emission spectra; thus, it is possible for one fluor to emit fluorescence and an adjacent fluor to absorb it. The closer the fluors, the greater the quenching. With fluorescent Eu^{3+} chelates (and probably also with fluorescent Tb^{3+} and Sm^{3+} chelates), such quenching is not expected and was not observed because there is absolutely no overlap between the excitation and emission spectra. Second, the fluorescence found was not just proportional to the degree of multiple labeling with BCPDA but was about three- to sixfold greater. This "bonus effect" was considered to be related to environmental effects of the various amino acids around the Eu^{3+} and BCPDA.

Bovine thyroglobulin (TG) is a high-M_r protein (660 000 Da) carrying ~150 ϵ-amino groups (*7*). We were able to introduce ~175 BCPDA labels per molecule of TG and obtain fluorescence equivalent to that obtained by ~900 BCPDA residues, with excess Eu^{3+}. We decided to create a new biotin-binding protein that could carry not just 14 BCPDA molecules but ~150, instead. We covalently linked streptavidin to BCPDA-labeled TG with an elegant conjugation chemistry shown in Figure 7 and further described elsewhere (*11*). The new streptavidin reagent, represented by the formula SA(TG)(BCPDA)$_{150}$, gave signal amplification and detection limit improvement by about fivefold that of SA(BCPDA)$_{14}$ (directly labeled streptavidin). Obviously, some steric hindrance effects of streptavidin binding do occur with this newer reagent, but the benefits are quite significant (Figure 6, right).

Streptavidin-Based Macromolecular Complex (SBMC)

A serendipitous observation during accelerated stability studies with the reagent SA(TG)(BCPDA)$_{150}$ led to the development of an SBMC, which now is our reagent of

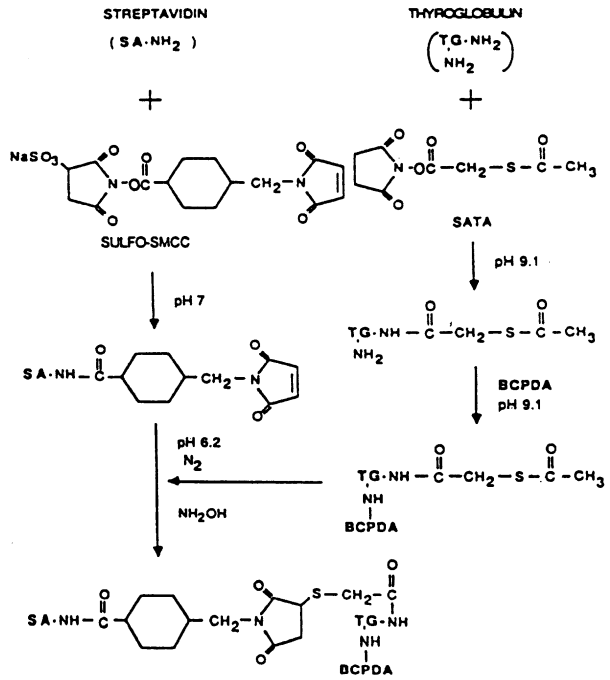


Fig. 7. Schematic representation of the conjugation reaction between streptavidin (SA) and BCPDA-labeled thyroglobulin (TG)

Streptavidin is derivatized with sulfo-SMCC (see Fig. 4) to introduce maleimide groups. TG is first thiolated with succinimidylacetylthioacetate (SATA) (~15 thiols/TG) and then extensively labeled with BCPDA (~150 BCPDA/TG). The two derivatives are then reacted under nitrogen and in the presence of hydroxylamine, which liberates free thiols. The conjugate is SA(TG)(BCPDA)$_{150}$. Reprinted from reference *11* by permission

choice for both immunological and nucleic acid hybridization assays (*14*). During the conjugation reaction shown in Figure 7, we routinely use a fivefold molar excess of thiolated-BCPDA-labeled TG over streptavidin derivatized with succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate, to promote quantitative conjugation of streptavidin. Routinely, we do not remove the excess of thiolated-BCPDA-labeled TG because we have not observed any significant increase in the background signal with its presence. However, when we added Eu^{3+} to the final reaction mixture after conjugation, which contained SA(TG)(BCPDA)$_{150}$ and TG(BCPDA)$_{150}$, and incubated at 50–55 °C for a few hours, we observed that a new, more sensitive reagent is formed under controlled reaction conditions, especially when Eu^{3+} concentration is carefully controlled. After extensive studies (*14*), we proposed that under such conditions, a SBMC is formed (Figure 8), consisting of (*a*) SA(TG)(BCPDA)$_{150}$, (*b*) TG(BCPDA)$_{150}$, and (*c*) Eu^{3+} at a molar ratio of about 1/3.3/480. We postulated that this SBMC is formed by Eu^{3+} acting as a linker between components *a* and *b*. The SBMC is very stable and does not disintegrate during the immunoassay procedure or during extensive washings. We found that no Eu^{3+} leakage occurs even if the tracer diluent does not contain Eu^{3+}. Moreover, we found that the SBMC is stable for years when kept as a stock solution containing 15 mg of streptavidin per liter. The operational structure of the SBMC is SA(TG)$_{3.3}$-(BCPDA)$_{480}$ with a M_r of ~(2.5–3) × 10^6. The theoretical amplifications with various reagents described here are shown in Table 2.

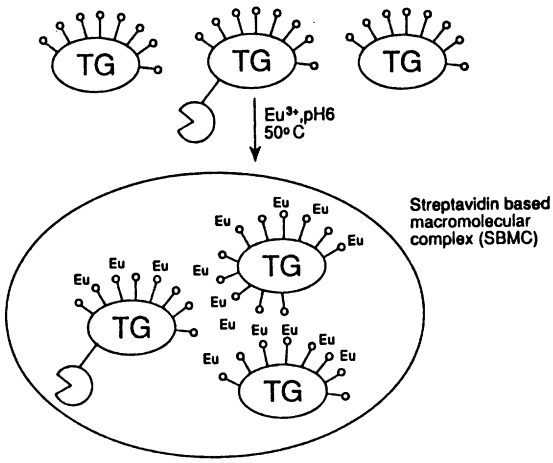


Fig. 8. Schematic representation of the proposed mechanism of formation of the streptavidin-based macromolecular complex; complexed with biotin (not shown), the system gives a signal amplification of 4500- to 6750-fold

—○ = BCPDA. The Figure has no quantitative meaning. For more details, see text and reference *14*

Table 2. Theoretical Amplifications with Various BCPDA-Labeled Reagents

Reagent	Amplification, -fold	Schematic
$Ab(BCPDA)_{15-18}$	15–18	Fig. 5
$Ab(BSA)(BCPDA)_{40}$	40	Fig. 5
$SA(BCPDA)_{14}$	140–210	Fig. 6
$SA(TG)(BCPDA)_{150}$	1500–2250	Fig. 6
SBMC	4500–6750	Fig. 9

BSA, bovine serum albumin.

Comparisons and Applications

My co-workers and I recently compared $SA(BCPDA)_{14}$, $SA(TG)(BCPDA)_{150}$, and $SA(TG)_{3.3}(BCPDA)_{480}$ in various noncompetitive immunoassay systems, using identical conditions and protocols. Results are summarized in Table 3. The last-named reagent improved both signal amplification and detection limits by eight- to 26-fold compared with $SA(TG)(BCPDA)_{150}$. Using the SBMC in an alpha-fetoprotein assay designed to maximize sensitivity, we were able to detect ~300 000 molecules (~2 amol) of alpha-fetoprotein (*15*), which is the lowest detection limit reported for any time-resolved fluorometric assay (Table 4). The SBMC can be used in any application in which a biotinylated reactant is used. Documented applications other than immunoassay include DNA hybridization[2] and Western blotting. Currently, the same reagent is being evaluated for Northern blotting, DNA sequencing, and detection of PCR products. Other possible applications include flow-cytometry and immunohistochemistry.

Further Amplification Strategies

We have also extensively investigated strategies that could increase the biotinylation load. This increased biotinylation load, in combination with the SBMC, should yield even further total amplification of the proposed detection system. Among the many possibilities tested, two produced useful results.

The principle of the first approach is shown in Figure 9. Anti-lutropin detection antibodies are labeled with digoxigenin, by use of an *N*-hydroxysuccinimide ester of digoxigenin. The immunological assay is performed as usual and the digoxigenin is further reacted with a biotinylated anti-digoxigenin monoclonal antibody. We then complete the assay by using the SBMC. A comparison of this assay with an assay involving a biotinylated anti-lutropin detection antibody showed a 2.5-fold signal amplification and improvement in detection limit with the digoxigenin-labeled anti-lutropin antibody (Diamandis and Christopoulos, unpublished results).

The other approach is based on the use of horseradish peroxidase (EC 1.11.1.7)-catalyzed deposition of biotins on the solid phase as described by Bobrow et al. (*16*). In this approach, peroxidase is the label on the streptavidin or the detection antibodies (Figure 10). After completing the immunocomplex in the conventional manner, the enzyme substrate is added, H_2O_2, and the conjugate biotin–tyramine. The peroxidase catalyzes the deposition of biotin–tyramine from solution onto the coating antibody of the solid phase, thus markedly promoting the amount of biotin deposited in a manner linearly related to the amount of peroxidase present. These biotins are then detected by the SBMC.[3] With this system, we

[2] Christopoulos TK, Diamandis EP, Wilson G. Quantification of nucleic acids on nitrocellulose membranes with time-resolved fluorometry. Manuscript submitted.

[3] Christopoulos TK, Kitching R, Diamandis EP. Time-resolved fluoroimmunoassays using enzymatic amplification. Manuscript submitted.

Table 3. Comparison of BCPDA-Labeled Streptavidin-Based Reagents in Various Immunoassays

	Detection limit		
Analyte	$SA(BCPDA)_{14}$	$SA(TG)(BCPDA)_{150}$	$SA(TG)_{3.3}(BCPDA)_{480}$
Choriogonadotropin, int. unit/L	1.00	0.32	0.03
Lutropin, int. unit/L	1.50	0.23	0.03
Prolactin, μg/L	0.61	0.15	0.01
Carcinoembryonic antigen, μg/L	0.36	0.11	0.01
Thyrotropin, milli-int. unit/L	0.45	0.09	0.01
Follitropin, int. unit/L	0.70	0.13	0.01
Somatotropin, μg/L	0.27	0.08	0.003

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Table 4. Detection Limits for Alpha-Fetoprotein (AFP) Assays in Various Protocols

Detection anti-AFP antibody	Coating anti-AFP antibody, μL	Detection limit ng/L	mol/L × 10^{-14}	Molecules/well, × 10^3
Flat-bottom wells				
Monoclonal	100	12	20.2	6067
Polyclonal	100	4.6	7.7	2309
U-bottom wells				
Polyclonal	20	4.3	7.2	863
Polyclonal	10	5.5	9.2	552
Polyclonal	5	5.7	9.5	286

Adapted from reference *15*.

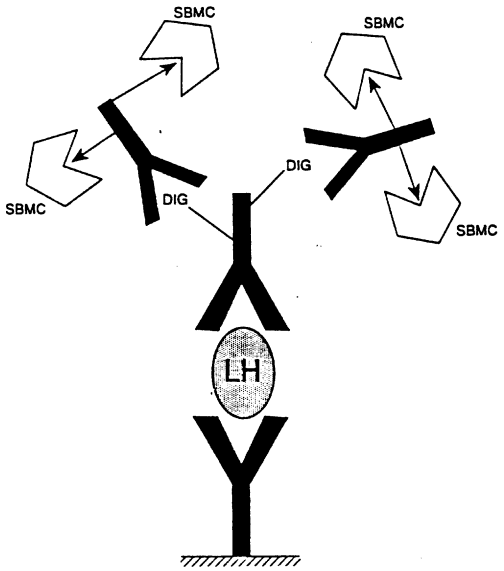

Fig. 9. Haptenized detection antibodies with digoxigenin (DIG), and linkage of the SBMC to the immunocomplex by using biotinylated anti-digoxigenin monoclonal antibodies

amplified the signal by 10- to 30-fold in comparison with biotinylated detection antibodies. However, the detection limit was improved by only about three- to sixfold because of an increase in background. In conclusion, although some of the reagents described here are complicated, and considerable organic chemistry expertise is needed to prepare and purify them, these reagents are extremely stable and easy to use. The reagents are preferable to enzyme-based systems because they do not need special conditions of temperature control, exact timing, or extra steps for substrate incubation. In addition, the system described here is completely contamination-free because the label (BCPDA) is a compound not present in nature—another advantage over both the Eu^{3+}- and the enzyme-based assays. The system I have described introduces amplification factors similar to those achieved by the use of enzymes.

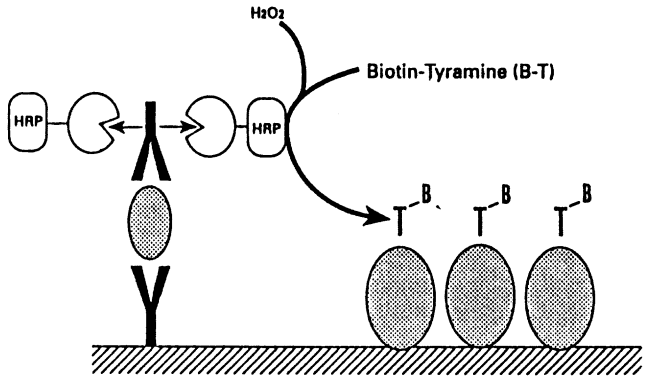

Fig. 10. Catalytic deposition of biotin–tyramine conjugate (B-T) on the proteins-coated well by the action of horseradish peroxidase (HRP), in the presence of H_2O_2

HRP is conjugated to either streptavidin or to detection antibody (this option is not shown). The immobilized B-T can be detected by use of the SBMC (not shown). For more details see text and reference *16*

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