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Streptavidin-Polyvinylamine Conjugates Labeled with a Europium Chelate: Applications in Immunoassay, Immunohistochemistry, and Microarrays

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Background: **The favorable properties of lanthanide chelates compared with conventional fluorescent probes have attracted considerable interest. A Eu^{3+} chelator, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA), has been synthesized previously.**

Methods: **We here describe immunoassay, immunohistochemistry, and microarray applications of a new streptavidin-based universal polyvinylamine (PVA) detection reagent that is multiply labeled with the europium chelate of BCPDA. Solid-phase time-resolved immunofluorometric assays for biotinylated mouse IgG and prostate-specific antigen (PSA) were developed using the new conjugate as a detection reagent. The new conjugate was also used for the immunohistochemical localization of PSA expression in paraffin-embedded prostatic tissues. A model microarray with spotted biotinylated antibody as target was also performed.**

Results: **Approximately 50–100 BCPDA moieties were covalently bound to PVA, which was then linked to streptavidin via biotin interaction. The macromolecular complex successfully recognized and bound biotinylated detection reagents, e.g., antibodies. The new reagent enabled measurement of solid phase-immobilized biotinylated mouse IgG with a detection limit of ~1 pg/assay and demonstrated excellent linearity. In an ELISA-type sandwich PSA assay that included two PSA monoclonal antibodies using the new conjugate as detection reagent, we detected 0.001 μg/L PSA (~100 fg or ~3 amol/assay). Serum samples analyzed for PSA by this method and a commercial assay gave highly correlated results. The new reagent enabled excellent immunohistochemical localization of PSA expression in prostate tissues. Using the new reagent in a model microarray experiment with biotinylated mouse IgG as target, we demonstrated excellent spatial resolution of 5- to 10-nL microspots.**

Conclusions: **The new detection reagent may find important applications in biotechnology.**

Time-resolved fluoroimmunoassays using Eu^{3+} and other lanthanide chelates as fluorescent labels have been reported frequently *(1–13)*. A Eu^{3+} chelator, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA),[4] has already been described *(14)*. This chelator forms stable fluorescent complexes with Eu^{3+}. BCPDA contains two chlorosulfophenyl groups and reacts covalently with proteins and other macromolecules via amino groups to form soluble conjugates in aqueous solutions. Another Eu^{3+} chelator, 4,4′-bis (1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedion-6″-yl) chlorosulfo-*o*-terphenyl, was reported recently *(7)*.

Fluorescent europium chelates exhibit large Stokes shifts (~290 nm) with no overlap between the excitation and emission spectra. These chelates have a very narrow

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[4] Nonstandard abbreviations: BCPDA, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic-acid; SA, streptavidin; PVA, polyvinylamine; and PSA, prostate-specific antigen.

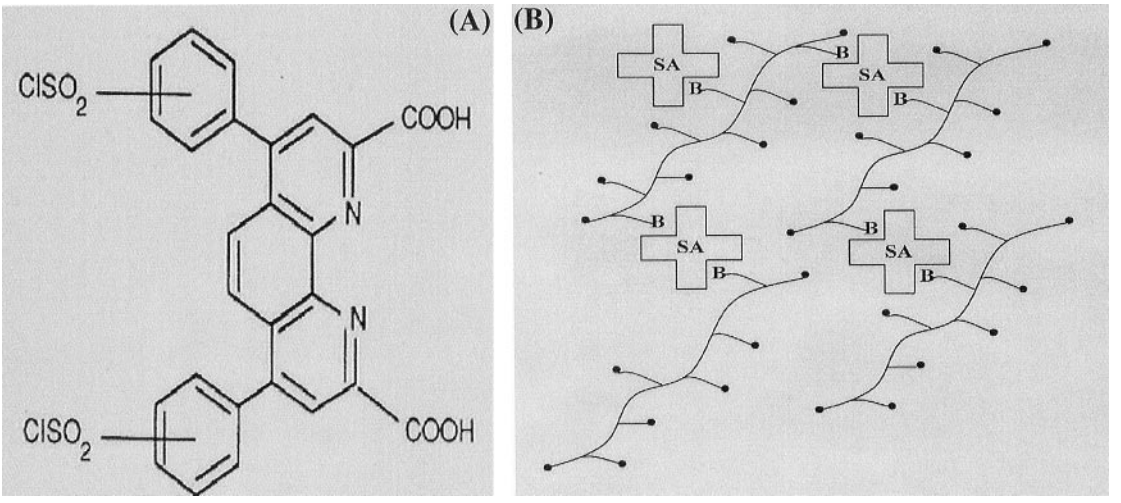

Fig. 1. BCPDA structure (*A*) and schematic representation of the macromolecular nature of the new reagent (*B*).

This figure has no quantitative meaning. *B*, biotin; *SA*, streptavidin. (*B*), the *solid ball and stick* represents BCPDA. The *curved lines* represent PVA. Each PVA molecule carries ~50–100 BCPDA-Eu^{3+} complexes and ~5–10 biotin molecules.

(10-nm bandwidth) emission spectrum at 615 nm that differs from the fluorescence spectrum of serum native. In addition, their long fluorescence lifetimes (600–1000 μs) allow use of microsecond time-resolved fluorescence measurements, which further reduces the observed background signals *(1–6, 15)*.

In principle, the sensitivity of fluorometric analysis could be improved by attaching many fluorescent groups to the labeled reagents. This approach does not always work because the increased tagging soon produces concentration quenching, which first reduces the quantum efficiency and then the total emission *(15, 16)*.

In this report, we describe preliminary immunoassay, immunohistochemistry, and microarray applications of a new streptavidin (SA)-based universal detection reagent labeled with the chelate of Eu^{3+} with BCPDA via the macromolecular carrier polyvinylamine (PVA). Our aim was to develop a reagent that, through multiple labeling, would provide signal amplification while allowing measurement of fluorescence directly from the solid phase.

Materials and Methods

PREPARATION OF THE MACROMOLECULAR COMPLEX

The detailed preparation of this reagent has been described elsewhere *(17)*. Briefly, we biotinylated PVA and then labeled it with BCPDA to produce the complex, $(biotin)_x$-PVA-$(BCPDA)_y$, where x is ~5–10 and y is ~50–100 *(17)*. We then mixed the purified complex with a carefully controlled amount of SA and Eu^{3+} to form the complex $(SA)_z$-$(biotin)_x$-PVA-$(BCPDA\text{-}Eu^{3+})_y$. The optimal amounts of the two reagents can be found by careful titration with different ratios and then testing the resulting reagent with a model solid-phase immunoassay using biotinylated mouse IgG immobilized in microtiter wells. This reagent is stable at 4 °C for at least 1 year. The molecular weight of this complex was determined by gel filtration chromatography, as described below. The SA content of the optimal reagent concentrate (×10) is 100 mg/L.

SOLID PHASE-DETECTION OF IMMOBILIZED BIOTINYLATED MOUSE IgG

Microtiter plates were coated overnight with 500 ng of sheep anti-mouse IgG in 100 μL of 0.05 mol/L Tris (pH 7.80) per well. After washing the plates, we added 100 μL of biotinylated mouse IgG, diluted in 60 g/L bovine serum albumin, and incubated the plates for 2 h at room temperature with shaking. The amount of biotinylated mouse IgG varied from 0.5 pg/well to 50 000 pg/well. After washing the plates, we added 100 μL of the SA

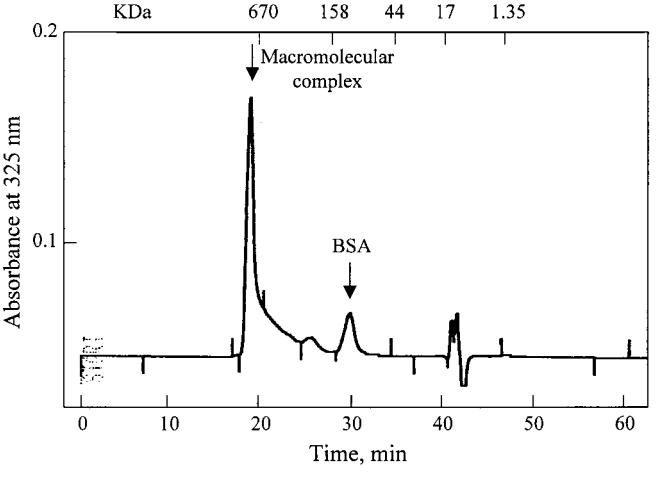

Fig. 2. Assessment of the molecular mass of the macromolecular complex by gel filtration HPLC.

The elution times of the molecular mass calibrators are shown at the *top*. The complex elutes at the void volume of the column, indicating molecular mass ≥10^6 Da. *BSA*, bovine serum albumin, a component of the mixture.

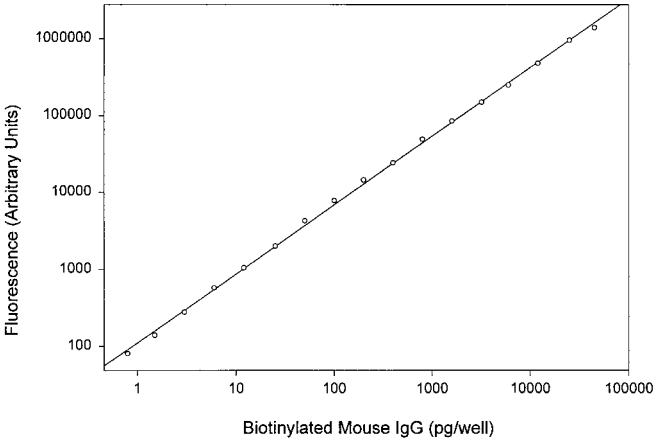

Fig. 3. Calibration curve for quantifying immobilized biotinylated mouse IgG.

The background signal obtained with the zero calibrator (no IgG added) was ~80 arbitrary fluorescence units, subtracted from all measurements.

conjugate, diluted 10-fold (diluent, 60 g/L bovine serum albumin solution in 0.1 mol/L Tris buffer, pH 7.80; ~1 μg of SA per well). After incubation for 25 min, the plates were washed and dried in a forced air microplate dryer, and the solid-phase fluorescence was measured with a CyberFluor 615 Time-Resolved Fluorometer (MDS Nordion).

GEL FILTRATION CHROMATOGRAPHY

The molecular mass of the macromolecular complex was assessed by gel filtration chromatography on a 600 × 7.5 mm Bio-Sil SEC-250 column (Bio-Rad Laboratories). The column was calibrated with a calibration solution from Bio-Rad. The mobile phase was 0.1 mol/L sodium phosphate, 0.1 mol/L sodium sulfate (pH 6.50), and the flow rate was 0.5 mL/min; absorbance was monitored at 280 nm for the protein calibrators and at 325 nm for the macromolecular complex (absorbance maximum of

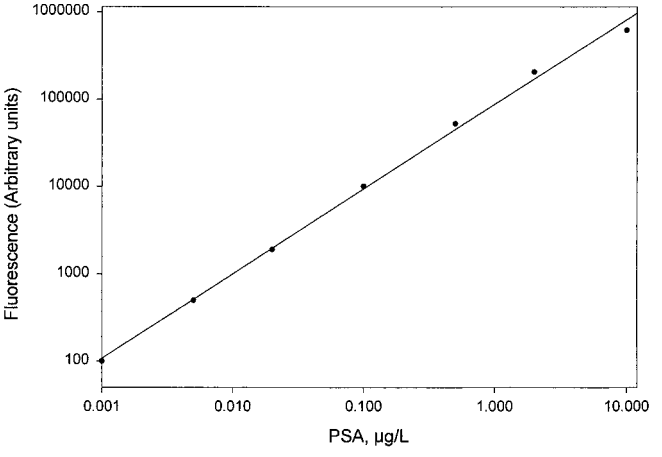

Fig. 4. Calibration curve for the PSA immunoassay with the new conjugate as detection reagent.

Background signal was ~100 arbitrary units, subtracted from all measurements. For details regarding this assay, see Ref. *(18)*.

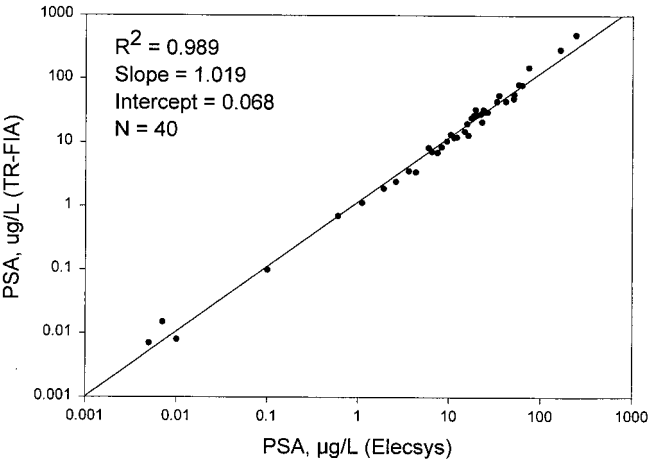

Fig. 5. Correlation between the time-resolved fluorometric immunoassay for PSA, using the newly developed reagent (*TR-FIA*) and the Roche Elecsys automated PSA immunoassay, for 40 serum samples.

Samples were diluted, as necessary, to bring them within the measuring range of the newly developed assay (0.001–10 μg/L).

BCPDA) *(14)*. The exclusion limit for this column is ~10^6 Da.

IMMUNOASSAY FOR PROSTATE-SPECIFIC ANTIGEN

The general principles of this assay have been described previously *(18)*. Briefly, the assay uses two monoclonal antibodies, one of which is coated on a microtiter plate, whereas the other one is biotinylated. Sample volume is 100 μL. After sandwich formation in the first step [capture antibody, prostate-specific antigen (PSA)-biotinylated antibody], the complex is reacted with SA-alkaline phosphatase conjugate. Enzyme activity is then measured by time-resolved fluorometry as described *(18)*. The detection limit of this assay is 0.001 μg/L, and the measuring range extends to 10 μg/L, but the dose–response curve is not linear at 1–10 μg/L *(18)*. Here, we detected the final immunocomplex, comprising the capture antibody, PSA, and biotinylated detection antibody, with our SA-based macromolecular complex. The final measurement was performed on the dry solid phase as described previously *(16, 17)*.

IMMUNOHISTOCHEMISTRY

Paraffin-embedded prostatic tissue sections (5 μm in thickness) were deparaffinized and rehydrated. A 5% universal tissue conditioner (Biomeda) was applied at room temperature for 10 min to block any nonspecific binding. At 37 °C, sections were incubated with biotinylated monoclonal mouse anti-human PSA antibody (~2 mg/L; coded 8301; Diagnostic Systems Laboratories) for 1 h. The section was then stained with $(SA)_z$-$(biotin)_x$-PVA-$(BCPDA)_y$-Eu^{3+} for another 25 min. After each step, each section was wash briefly with a 0.5 mL/L Tween 20 solution. Finally, the slides were dried with a stream of cold air and the resulting fluorescence was observed with

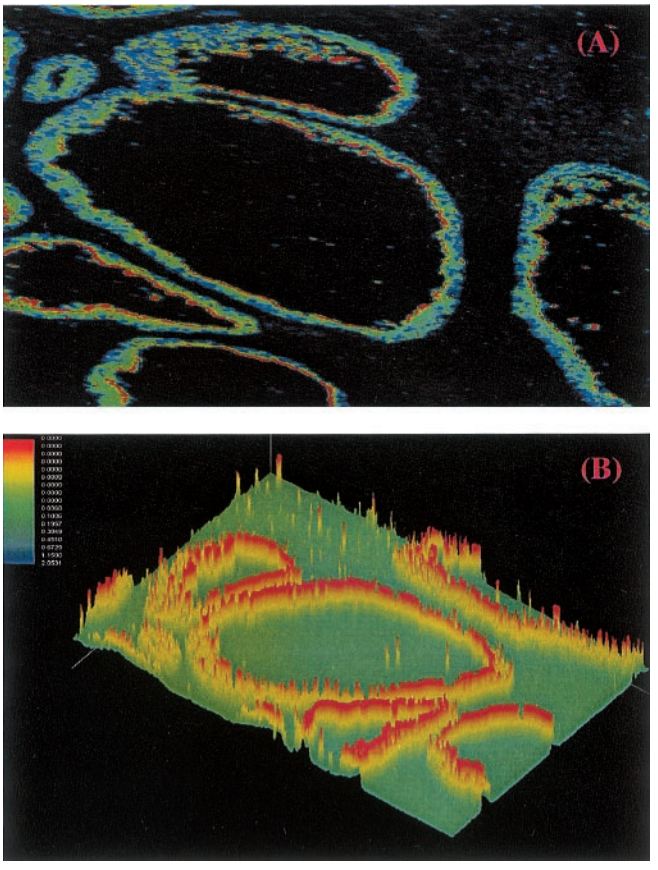

Fig. 6. Immunohistochemical localization of PSA in paraffin-embedded prostatic tissue using the new conjugate as a detection system and a biotinylated monoclonal anti-PSA antibody.

Time-resolved fluorescence images were produced using an epi-fluorescence microscope equipped for TRFI (Signifer 1432 MicroImager; Perkin-Elmer Life Sciences; Wallac Oy). The specimens were focused in bright-field mode with a halogen light source, and images were obtained with a ×10 objective during 30 s with repeated cycles of excitation by a xenon flash lamp. Images were captured using the camera software PixCel, Ver. 2.7 (Perkin-Elmer) and pseudocolored. Two- (*A*) and three-dimensional (*B*) pictures were created in Image Pro, Ver.4.0 (Media Cybernetics). The PSA immunoreactivity is seen only in the epithelial cells; the stroma is negative.

a time-resolved fluorescence microscope (Signifer 1432; MicroImager).

MICROARRAY ANALYSIS

Spots of biotinylated mouse IgG (5 nL) were immobilized on a glass slide coated with a proprietary membrane of high protein capacity (Fast™ slides, cat. no. 10484182; Schleicher & Schuell). The microspots were stained with the SA-based reagent, washed, dried, and examined with the time-resolved fluorescence microscope.

Results

We describe the synthesis and model applications of a conjugate of SA indirectly labeled with the europium chelate of BCPDA through PVA polymer. The BCPDA structure and a schematic depiction of the novel conjugate are shown in Fig. 1. The labeling reaction of BCPDA with PVA involves covalent bond formation between the $-SO_2Cl$ group of BCPDA and $-NH_2$ groups of PVA, producing $-SO_2-NH$ groups on the labeled PVA. The complex, $(SA)_z$-$(biotin)_x$-PVA-$(BCPDA)_y$-Eu^{3+}, where x is ~5–10 and y is ~50–100 *(17)*, successfully recognizes and binds other biotinylated antibodies and is very stable at 4 °C (at least 1 year). When we assessed the molecular mass of the macromolecular complex by HPLC (Fig. 2), we found that it elutes at the void volume, suggesting a molecular mass $\geq 10^6$ Da. This is consistent with cross-linking of at least eight PVA-$(BCPDA)_y$ complexes. This cross-linking is facilitated by SA, through its multiple binding sites *(19)*.

In Fig. 3, we show the sensitivity and linearity of measuring solid phase-immobilized biotinylated mouse IgG. At 10 pg/assay (~0.7 fmol), the signal-to-background ratio of fluorescence is ~10-fold. The detection limit is ~ 1 pg/assay and linearity is excellent.

As shown in Fig. 4, we could detect ~0.001 μg/L PSA (100 fg/assay; equivalent to 3×10^{-18} moles of PSA). Measurements were linear at PSA concentrations of 0.001–1 μg/L, and the measuring range was useful to 10 μg/L. Imprecision (CV) was <10% at 0.005–10 μg/L PSA and was 20% at 0.001 μg/L PSA. When we compared this assay with assays using either enzymatically amplified time-resolved fluorometry *(9)* or enzymatically triggered chemiluminescence with dioxetane substrates *(20, 21)* (same antibodies, assay conjugation, and conditions), we obtained comparable signal-to-noise ratios and detection limits, but linearity was superior with the present method at PSA concentrations of 1–10 μg/L. When we analyzed 40 serum samples from males by this method, the results were in excellent agreement with those obtained with a commercially available PSA immunoassay (Elecsys; Roche Diagnostics, Indianapolis; Fig. 5).

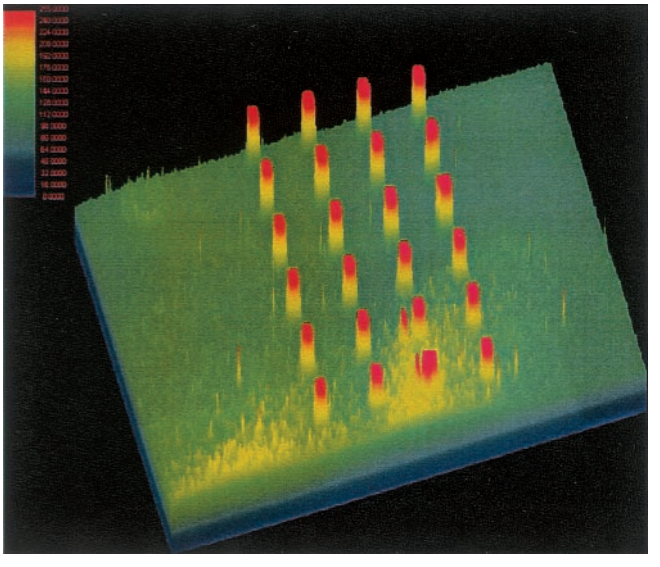

Fig. 7. Model microarray experiment using biotinylated antibody spots (~5 nL) as targets.

The same amount of biotinylated antibody (~ 250 pg/spot) was immobilized 24 times. Photographs were generated as described in the legend for Fig. 6.

We used the new reagent for the detection of PSA in prostate tissues by immunohistochemistry. The results are shown in Fig. 6. PSA is present in epithelial cells and the lumen; the stroma is negative.

In Fig. 7, we show multiple microspots (~5 nL each) of a 50 μg/L solution of biotinylated mouse IgG (~250 pg/spot). In this microarray experiment, we demonstrated the excellent spatial resolution of this technique. Microspotting of the same reagent at different dilutions indicated achievable detection limits of ~0.25–2.5 pg/spot.

Discussion

The numerous advantages of the fluorescent lanthanide chelates over conventional fluorescent probes have been summarized elsewhere *(1–6, 15)*. Lanthanide chelates can provide 100- to 1000-fold better sensitivities comparison with conventional fluorophores because of efficient background fluorescence rejection. Background rejection is achieved through the large Stokes shifts and the narrow emission bands of fluorescent lanthanide chelates as well as application of time-resolved fluorometric measurements *(1, 2, 15)*.

Fluorescent lanthanide chelates are free of quenching when multiple labeling is used *(16)*. We have taken advantage of this principle to devise macromolecular complexes, multiply labeled with lanthanide chelates, for time-resolved immunofluorometric analysis. Although we were able to achieve ng/L detection limits in model immunological assays *(16)*, the major disadvantages with the previously described macromolecular complexes were their difficult preparation, their relative instabilities, and their very large molecular masses (bovine thyroglobulin was used as a carrier in these applications). The new complexes described here have the advantage of an approximately threefold higher sensitivity and inexpensive and easy preparation by means of a simple reagent-mixing step. The preformed complexes are stable over time and are suitable for immunological assays using biotinylated antibodies as auxiliary reagents. We anticipate that the same complexes may have applications in nucleic acid hybridization assays, but we did not test these assays.

We previously described highly sensitive detection technologies based on enzymatically amplified time-resolved fluorescence using terbium chelates as labels *(9)*. Furthermore, enzymatically triggered chemiluminescence is among the most sensitive detection techniques *(20, 21)*. When we compared our new reagents with these competing technologies in the same immunological assay for PSA, we found that all three methods have similar detection limits (~3 amol/assay).

Recently, other investigators succeeded in developing Eu^{3+} fluorescent chelators that, like BCPDA, are suitable for solid-phase time-resolved immunofluorometry *(7, 22–24)*. Others have devised successful homogeneous time-resolved fluorometric immunoassays suitable for clinical applications *(25, 26)*.

The major advantage with the newly developed reagent is the ability to measure fluorescence directly from the solid phase. We describe here successful model immunoassay, immunohistochemical, and microarray applications. Others have also used time-resolved fluorometric analysis for immunohistochemistry of PSA with different Eu^{3+} chelates *(11)*. We anticipate that this new reagent may be used in diverse biotechnology applications and especially in protein microarray and high-throughput screening assays.

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References

1. Soini E, Kojola H. Time-resolved fluorometer for lanthanide chelates—a new generation of monisotopic immunoassays. Clin Chem 1983;29:65–8.
2. Soini E, Hemmila I, Dahlen P. Time-resolved fluorescence in biospecific assays [Review]. Ann Biol Clin 1990;48:567–71.
3. Diamandis EP, Christopoulos TK. Europium chelate labels in time-resolved fluorescence immunoassays and DNA hybridization assays [Review]. Anal Chem 1990;62:1149–57.
4. Hemmila I. Fluoroimmunoassays and immunofluorometric assays [Review]. Clin Chem 1985;31:359–70.
5. Diamandis EP. Time-resolved fluorometry in nucleic acid hybridization and Western blotting techniques [Review]. Electrophoresis 1993;14:866–75.
6. Hemmila I. Lanthanides as probes for time-resolved fluorometric immunoassays [Review]. Scand J Clin Lab Investig 1998;48:389–99.
7. Yuan J, Matsumoto K, Kimura H. A new tetradentate β-diketonate-europium chelate that can be covalently bound to proteins for time-resolved fluoroimmunoassay. Anal Chem 1998;70:596–601.
8. Leif RC, Thomas RA, Yopp TA, Watson BD, Guarino VR, Hindman DH, et al. Development of instrumentation and fluorochromes for automated multiparameter analysis of cells. Clin Chem 1977;23:1492–8.
9. Christopoulos TK, Diamandis EP. Enzymatically amplified time-resolved fluorescence immunoassay with terbium chelates. Anal Chem 1992;64:342–6.
10. Eriksson S, Vehniainen M, Jansen T, Meretoja V, Saviranta P, Pettersson K, Lovgren T. Dual-label time-resolved immunofluorometric assay of free and total prostate-specific antigen based on recombinant Fab fragments. Clin Chem 2000;46:658–66.
11. Bjartell A, Laine S, Pettersson K, Nilsson E, Lovgren T, Lilja H. Time-resolved fluorescence in immunocytochemical detection of prostate-specific antigen in prostatic tissue sections. Histochem J 1999;31:45–52.
12. Lovgren T, Heinonen P, Lehtinen P, Hakala H, Heinola J, Harju R, et al. Sensitive bioaffinity assays with individual microparticles and time-resolved fluorometry. Clin Chem 1997;43:1937–43.
13. Merio L, Pettersson K, Lovgren T. Monoclonal antibody-based dual-label time-resolved fluorometric assays in a simplified one-step format. Clin Chem 1996;42:1513–7.

14. Evangelista RA, Pollak A, Allore B, Templeton EF, Morton RC, Diamandis EP. A new europium chelate for protein labeling and time-resolved fluorometric applications. Clin Biochem 1988;21: 173–8.
15. Diamandis EP. Immunoassays with time-resolved fluorescence spectroscopy: principles and applications [Review]. Clin Biochem 1988;21:139–50.
16. Morton RC, Diamandis EP. Streptavidin-based macromolecular complex labelled with a europium chelator suitable for time-resolved fluorescence immunoassay applications. Anal Chem 1990;62:1841–5.
17. Scorilas A, Diamandis EP. Polyvinylamine-streptavidin complexes labeled with a europium chelator—a universal detection reagent for solid-phase time resolved fluorometric applications. Clin Biochem 2000;in press.
18. Ferguson RA, Yu H, Kalyvas M, Zammit S, Diamandis EP. Ultrasensitive detection of prostate specific antigen by a time-resolved immunofluorometric assay and the Immulite® immunochemiluminescent third generation assay: potential applications in prostate and breast cancers. Clin Chem 1996;42:675–84.
19. Diamandis EP, Christopoulos TK. The biotin-(strept)avidin system: principles and applications in biotechnology [Review]. Clin Chem 1991;37:625–36.
20. Bronstein I, Voyta JC, Vant Erve Y, Kricka LJ. Advances in ultrasensitive detection of proteins and nucleic acids with chemiluminescence: novel derivatized 1,2-dioxetane enzyme substrates [Review]. Clin Chem 1991;37:1526–7.
21. Bronstein I, Fortin J, Stanley PE, Stewart GS, Kricka LJ. Chemiluminescent and bioluminescent reporter gene assays [Review]. Anal Biochem 1994;219:169–81.
22. Yuan J, Wang G. Kimura H, Matsumoto K. Highly sensitive time-resolved fluoroimmunoassay of human immunoglobulin E by using a new europium fluorescent chelate as a label. Anal Biochem 1997;254:283–7.
23. Yuan J, Matsumoto K. Synthesis of a new tetradentate β-diketonate-europium chelate and its application for time-resolved fluorimetry of albumin. J Pharm Biomed Anal 1997;15:1397–403.
24. Kimura H, Yuan J, Wang G, Matsumoto K, Mukaida M. Highly sensitive quantitation of methamphetamine by time-resolved fluoroimmunoassay using a new europium chelate as a label. J Anal Toxicol 1999;23:11–6.
25. Mathis G. Probing molecular interactions with homogeneous techniques based on rare earth cryptates and fluorescence energy transfer [Review]. Clin Chem 1995;41:1391–7.
26. Mathis G, Socquet F, Viguier M, Darbouret B. Homogeneous immunoassays using rare earth cryptates and time resolved fluorescence: principles and specific advantages for tumor markers [Review]. Anticancer Res 1997;17:3011–4.