Anal. Chem. **2002,** *74,* 5845−5850

Performance Evaluation of the Phosphorescent Porphyrin Label: Solid-Phase Immunoassay of α-Fetoprotein

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Phosphorescent conjugates of antibodies, neutravidin, and biotin (pentylamine derivative) were synthesized using previously described monofunctional labeling reagent of platinum(II) coproporphyrin-I with isothiocyanate reactive group (PtCP-NCS). These conjugates, which can be considered as standard reagents for a range of bioanalytical applications, were evaluated in solid-phase immunoassay schemes with the clinical analyte α-fetoprotein (AFP). A custom-designed time-resolved phosphorescence plate reader based on a compact and low-cost 532-nm laser and optimized for measurement of porphyrin labels was used. Using optimized tracers, instrumentation and assay protocols, subpicomolar detection limits were obtained both for PtCP label in solution and for AFP in solid-phase immunoassay. This sensitivity is comparable with standard time-resolved fluorescence immunoassays with lanthanide labels. The performance of metalloporphyrin labels, instrumentation, and solid-phase immunoassays as an alternative to the established detection platforms is discussed.

Since their introduction more than twenty years ago, time-resolved fluoroimmunoassays (TR-FIA) have found widespread application as sensitive and versatile nonisotopic detection methods in bioanalytics.[1−8] These have been based almost exclusively on lanthanide chelates or cryptates where the long-lived emission of the europium ion or otherwise allows for temporal resolution and elimination of fluorescence background, thus dramatically increasing sensitivity.[2,5] Although many heterogeneous immunoassay kits, using lanthanide-labeled antibodies or avidin, have been commercialized by various groups, e.g., PerkinElmer Life Sciences (DELFIA), Cyberfluor (FIAgen), CIS Bio International (TRACE), the DELFIA (Perkin-Elmer Wallac) remains the leading and most sensitive method for TR-FIA.[3,8] The probe and assay technology has been accompanied by the development of dedicated time-resolved fluorescence readers such as the Victor series (PerkinElmer Life Sciences), which facilitate multilabel measurement[9] attaining high sensitivity and sample throughput.

We have previously outlined the potential of hydrophilic phosphorescent metalloporphyrin labels as alternatives to lanthanides for assays requiring high sensitivity.[10−12] Briefly, metalloporphyrins display high quantum yields (10−40%), intense absorption bands in the 360−400- and 500−550-nm region accompanied by red emission (600−750 nm), with Stokes' shifts of greater than 100 nm, and long phosphorescent lifetimes (10−1000 μs). To date, the limitations associated with metalloporphyrins for these applications has been mainly due to the lack of functional labeling reagents to allow nondeliterious labeling of biomolecules.[12,13] Thus, coupling agents such as carbodiimides were employed[13−18] to bind tetracarboxyporphyrins to target molecules. This coupling chemistry resulted in aggregated bioconjugates with compromised activity and performance.[15] This was amplified by the lack of instrumentation dedicated specifically to measurement of time-resolved metalloporphyrin phosphorescence,[12] despite the fact that these labels have favorable photophysical properties and are compatible with 532-nm laser excitation. Recently, the syn-

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(1) Hemmila, I. In *Fluorescence Spectroscopy, New Methods and Applications*; Wolfbeis, O. S., Ed.; Springer-Verlag: Berlin, 1993; Chapter 18, pp 259−266.
(2) Gudgin Dickson, E. P.; Pollak, A.; Diamandis, E. P. *J. Photochem. Photobiol. B* **1995**, *27*, 3−19.
(3) Hemmila, I.; Webb, S. *Drug Discovery Technol.* **1997**, *2* (9), 373−381.
(4) Hemmila, I. In *Applications of Fluorescence in Immunoassays*; Winefordner, J. D., Kolthoff, I. M., Eds.; Wiley & Sons: New York, 1991.
(5) Soini, E.; Hemmila, I. *Clin. Chem.* **1979**, *25*, 353−361.
(6) Soini, E.; Kojola, H. *Clin. Chem.* **1983**, *29*, 65−68.
(7) Hemmila, I. *Clin. Chem.* **1985**, *31*, 359−370.
(8) Hemmila, I. *J. Biomol. Screening* **1999**, *4*(6), 303−307.
(9) Xu, Y.-Y.; Hemmila, I.; Lovgren, T. *Analyst* **1992**, *117*, 1061−1069.
(10) Savitsky, A. P.; Papkovsky, D. P.; Ponomarev, G. V.; Berezin, I. V. *Dokl. Akad. Nauk SSSR* **1989**, *304*, 1005.
(11) Papkovsky, D. P. *Appl. Fluoresc. Technol.* **1991**, *3*, 16−24.
(12) O'Riordan, T. C.; Soini, A. E.; Papkovsky, D. P. *Anal. Biochem.* **2001**, *290*, 366−375.
(13) Martsev, S. P.; Preygerzon, V. A.; Mel'nikova, Y. I.; Kravchuk, Z. I.; Ponomarev, G. V.; Lunev, V. E.; Savitsky, A. P. *J. Immunol. Methods* **1995**, *186*, 293−304.
(14) Bedel-Cloutour, C. H.; Maneta-Peyret, L.; Pereyre, M.; Bezian, J.-H. *J. Immunol. Methods* **1991**, *144*, 35−41.
(15) Savitsky, A. P.; Papkovsky, D. P.; Berezin, I. V. *Dokl. Akad. Nauk SSSR* **1987**, *293*, 744.
(16) de Haas, R.; van Gijlswijk, R. P. M.; van der Tol, E. B.; Zijlmans, H. J.; Bakker-Schut, T.; Bonnet, J.; Verwoerd, N. P.; Tanke, H. J. *J. Histochem. Cytochem.* **1997**, *45* (9), 1279−1292.
(17) de Haas, R.; van Gijlswijk, R. P. M.; van der Tol, E. B.; Veuskens, J.; van Gijssel, H. E.; Tijdens, R. B.; Bonnet, J.; Verwoerd, N. P.; Tanke, H. J. *J. Histochem. Cytochem.* **1999**, *47* (2), 183−196.
(18) Tanke, H. J.; de Haas, R.; Sagner, G.; Ganser, M.; van Gijlswijk, R. P. M. *Cytom.* **1998**, *33*, 453−459.

10.1021/ac020346n CCC: $22.00
Published on Web 10/11/2002

thesis of a monofunctional labeling reagent of platinum(II) coproporphyrin-I with isothiocyanate reactive group (PtCP-NCS) was described.[19] This reagent allowed facile labeling of proteins and overcame the problems described above. Furthermore, these conjugates were applied to model binding assays and displayed good sensitivity using nondedicated instrumentation.[12] Additional developments have also been reported with these labels such as applications of PtCP bioconjugates in time-resolved microscopy[20] and further development of labeling chemistry to offset the effects of bioconjugate inactivation.[21]

In this paper, we present the application of phosphorescent bioconjugates of the PtCP-NCS label to the measurement of the clinically important analyte α-fetoprotein (AFP). Sensitive detection of AFP is essential in clinical laboratories as increased levels are associated with a number of conditions including primary hepatocellular carcinoma, nonseminomatous testicular carcinomas, ovarian carcinomas, and other epithelial tumors. Increased AFP levels are also associated with nonmalignant hepatic diseases such as viral hepatitis and active cirrhosis.[22] Monoclonal antibodies, neutravidin, and biotin were labeled with PtCP-NCS and each was used as the tracer in an optimized solid-phase immunoassay. Phosphorescence measurements were made on a custom-designed instrument (plate reader), which employs a low-cost compact 532-nm laser, to which the PtCP label is particularly suited. We demonstrate the performance of this instrument and the newly synthesized metalloporphyrin bioconjugates in a time-resolved phosphorescence immunoassay (TR-PIA) and compare with both clinical requirements and literature data on TR-FIA-based assays, which use either DELFIA[23] or FIAgen[24] formats. Finally, we discuss the overall performance and bioanalytical potential of the phosphorescent metalloporphyrin labels and the instrument and potential applications in comparison with established labels and techniques.

EXPERIMENTAL SECTION

Materials. The labeling reagent PtCP-NCS was synthesized according to the described method.[19] The monoclonal antihuman AFP antibodies (clones 5107 and 5108) were obtained from Medix Biochemica (Kauniainen, Finland). AFP standard (X0900) and polyclonal rabbit antihuman AFP antibodies (A0008) were obtained from Dako (Cambridge, U.K.). EZ-link 5-(biotinamido)-pentylamine and NeutravidinTM were obtained from Pierce). NAP 5 desalting columns were obtained from Amersham Pharmacia Biotech. Maxisorp 96-well black microtiter plates were obtained from Nunc. Triethylammonium acetate buffer and cetyltrimethylammonium bromide (CTAB) were obtained from Alkem. 5,10,-15,20-Tetraphenylporphyrin (TPP), Biotin-NHS, dimethyl sulfoxide (DMSO), acetonitrile, Tween-20, casein, and all other reagents and solvents were obtained from Sigma-Aldrich. All buffers were made using Millipore grade water.

Labeling of Proteins with PtCP-NCS. Labeling of proteins was carried out by standard procedures described elsewhere.[12] Briefly, the reaction was carried out with the protein (typically 1 mg/mL) equilibrated in 0.1 M carbonate buffer, pH 9.7. A small quantity of PtCP-NCS was weighed and diluted to a concentration of 3 mg/mL (3×10^{-3} M) in DMSO. An aliquot was added to the protein solution to give a molar excess of dye of 20, followed by gentle vortexing and incubation for 4 h at room temperature. The conjugate fraction was isolated from free dye by gel filtration on a NAP 5 column equilibrated with 0.1 M potassium phosphate containing 0.3 M NaCl, pH 7.4. Fractions were analyzed spectrophotometrically on a HP 8453 diode array spectrophotometer with determinations of conjugate concentration and dye/protein ratio carried out using equations described previously.[12]

Labeling of Biotin. For labeling of biotin with PtCP-NCS, a 1 mg/mL quantity of 5-(biotinamido)pentylamine was dissolved in 0.1 M sodium borate, pH 9.7, followed by addition of an appropriate volume of a 3 mg/mL concentration of metalloporphyrin in DMSO to ensure a 50 molar excess of biotin. The reaction mixture was incubated for 30 min at 37 °C and then purified by semi-preparative HPLC. An Agilent 1100 series HPLC system consisted of a quaternary pump, UV−visible diode array detector, and an autosampler, and a Hypersil ODS C-18 reversed-phase column (5 μm, 250 mm × 4.6 mm, Supelco) was used. The 100-μL aliquots of the reaction mixture were injected in 0.1 M triethylammonium acetate, pH 6.5, and eluted with an ascending gradient of acetonitrile. The conjugate peak was collected, concentrated, and analyzed spectrophotometrically as noted above.

Biotinylation of Proteins. A quantity of biotin-NHS, typically 0.2 mg, was weighed and dissolved in DMSO to a concentration of 10 mg/mL. This solution was added to the protein solution (1 mg/mL in 0.1 M carbonate buffer, pH 9.0), at a ratio of 100 μg (50 molar excess for antibodies) of biotin-NHS/mg of protein. The reaction mixture was incubated for 3 h at room temperature followed by the addition of 20 μL of 1 M NH_4Cl/mg of protein. Following a subsequent 10-min incubation at room temperature, the biotinylated protein was separated from uncoupled biotin on a NAP 5 column, as described above.[25]

Spectral Measurements. Absorption spectra (range 250−600 nm) were recorded on a HP-8453 diode array spectrophotometer (Agilent) using standard 1-cm quartz cells. Time-resolved phosphorescence spectra (range 600−800 nm) and lifetimes were measured on an LS-50B luminescence spectrometer (Perkin-Elmer) equipped with an R928 red-sensitive PMT (Hamamatsu). Phosphorescence emission efficiency of the conjugates were measured in 3 mM CTAB with respect to the free PtCP, whose quantum yield was itself measured against a standard of known emission yield: ϕ(TPP in benzene) = 0.11.[26] Interference by molecular oxygen and other quenchers of the phosphorescence of porphyrin labels was eliminated by the addition of Na_2SO_3 (chemical deoxygenator, see below). The phosphorescent lifetime of the dye and bioconjugates was measured under the same conditions, using Fldm software (Perkin-Elmer) with the decay analyzed by single-exponential fitting using Microsoft Excel.

(19) Soini, A. E.; Yashunsky, D. V.; Meltola, N. J.; Ponomarev, G. V. *J. Porphyrins Phthalocyanines* **2001**, *5*, 735−741.
(20) Soini, A. E.; Seveus, L.; Meltola, N. J.; Papkovsky, D. B.; Soini, E. *Microsc. Res. Tech.* **2002**, *58* (2), 125−131.
(21) Soini, A. E.; Yashunsky, D. V.; Meltola, N. J.; Ponomarev, G. V. *Bioconjugate Chem.*, submitted.
(22) Chan, D. W.; Sell, S. In *Teitz textbook of clinical chemistry*, 3rd ed.; Burtis, C. A.. Ashwood, E. R., Eds.; W. B. Saunders Co.: Philadelphia, 1999; pp 722−749.
(23) Suonpaa, M. U.; Lavi, J. T.; Hemmila, I. A.; Lovgren, T. N. *Clin. Chim. Acta* **1985**, *145*, 341−348.
(24) Christopoulos, T. K.; Lianidou, E. S.; Diamandis, E. P. *Clin. Chem.* **1990**, *36*(8), 1497−1502.
(25) Bayer, E. A.; Wilchek, M. *Methods Biochem. Anal.* **1980**, *26*, 1−45.
(26) Atwater, B. W. *J. Fluoresc.* **1992**, *2* (4), 237−246.

Immunoassay Schemes and Procedures. Microtiter plates were passively coated with 100 μL of a 2 μg/mL concentration of monoclonal antihuman AFP antibody (clone 5107) by dilution in 0.1 M carbonate buffer, pH 9.5, and incubation overnight at 4 °C. The plates were washed with 0.1 M tris-buffered saline (TBS, 0.1 M tris and 0.15 M NaCl, pH 7.7,), containing 0.1% (v/v) Tween-20 and 5 mg/mL casein (washing, dilution buffer). The wells were refilled with 200 μL of TBS-casein and blocked for 1 h at 37 °C, followed by aspiration. Antigen standard curves were made by dilution of the AFP standard in the above buffer (given concentrations assessed against the WHO international standard preparation 72/225) and addition of 100 μL of each concentration followed by incubation for 1 h at 37 °C.

The sandwich assay was completed by addition of 100 μL of a 20 nM concentration of secondary antibody PtCP conjugate, monoclonal antihuman AFP antibody (clone 5108), incubated for 1 h at 37 °C and washed extensively prior to measurement. In immunoassay schemes where either the neutravidin-PtCP or biotin-PtCP conjugates were used as tracers, 100 μL of a 20 nM concentration of biotinylated secondary antibody (clone 5108) in dilution buffer was added and incubated for 1 h at 37 °C. This was followed by addition of 100 μL of a 50 nM concentration of either labeled or unlabeled neutravidin followed by incubation for 1 h at 37 °C and extensive washing. In the bridged assay, 100 μL of a 10 nM concentration of biotin-PtCP was added and incubated for 1 h at 37 °C followed by washing. Bound conjugate was then desorbed into solution by addition of 200 μL of 3 mM CTAB, pH 11.5, and incubation on a plate shaker for 20 min at room temperature, followed by the addition of 20 μL of phosphate−sulfite solution (PSS: 50 mg/mL KH_2PO_4 and 50 mg/mL Na_2SO_3) to each sample well, to remove oxygen and adjust pH. The plate was then measured on the time-resolved phosphorescence plate reader.

Estimation of Limits of Detection and Data Fitting. The detection limit was taken as the analyte concentration that gives a signal equal to the sum of the average signal given by a zero dose (i.e., background signal) and twice the standard deviation of this signal. The calculations were made according to a slightly altered equation described previously[24] where the limit of detection (LOD) = $3SD_o/S$, where SD_o is the standard deviation of the signal given where no analyte was added and S is the slope of the calibration between the points of earliest increase of signal. This was calculated as $\delta F/\delta C$, where δF was taken as the difference in signal (counts/s) between the mean background and the counts displaying the first increase in signal and δC is taken as the difference in concentration between the zero dose and the concentration showing first increase in signal, i.e., equal to the first standard concentration showing the first increase in signal. Four-parameter log−logistic (4 PL) fits, used for both calibrations in solution and immunoassay calibrations, were carried out using Mathcad software (Mathsoft).

Plate Fluorometer. The optical unit for time-resolved phosphorescence measurements is incorporated into a standard Fluoroscan Ascent microplate fluorometer (Thermo Labsystems Oy, Helsinki, Finland), which controls the XY movement of 96- and 384-well microtiter plates (SSI standard). A 10-mW frequency-doubled diode-pumped solid-state Nd:YVO_4 laser (532 nm, average cw power 10 mW, type Gl10dT, BremLas Lasertechnik Bremen GmbH, Bremen, Germany) was used for excitation. This laser is functioning in cw mode, but it can be modulated by an external CMOS-level logic signal to produce pulses of variable duration from 1 μs upward and up to 10 kHz repetition rate.

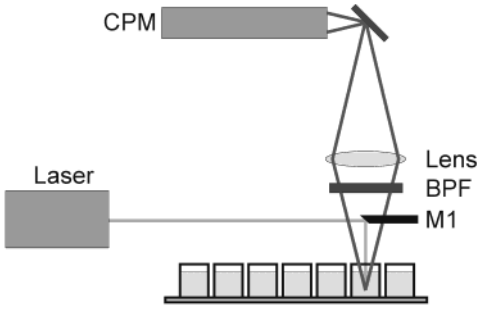

Figure 1. Optical setup of the custom-designed plate reader based on 532-nm laser, 645-nm band-pass filter, focusing lens, and photomultiplier tube.

An epifluorescence optical setup, as shown in Figure 1, is used for sample illumination and luminescence detection. The naturally diverging laser beam is reflected into the sample by an aluminum-coated mirror positioned at a 45° angle with respect to the incident beam. The reflecting mirror has an ellipsoidal shape of semiaxes 3 and 5 mm, respectively. Sample emission that passes around this mirror is then filtered with a band-pass filter (D645, Chroma Technology Corp., Brattleboro, VT). The collected photons are focused by a plano-convex lens 25 × 50 FL (32478, Edmund Industrial Optics, Barrington, NJ) onto the multialkali photocathode of the channel photomultiplier tube C952-P (PerkinElmer Optoelectronics Heimann, Wiesbaden, Germany). The single photon current pulses generated by the photomultiplier tube are converted to TTL/CMOS level logic pulses and counted by multichannel scaler circuitry.

The dwell time, i.e., the duration of each consequent counting interval, can be chosen from the software from 0.1 μs upward. The collected data are transferred to a PC via serial RS-232 data communication. In addition to the measurement of time-resolved luminescence, the multichannel scaler and instrument software allow for the measurement of the luminescence decay curve and calculation of emission lifetime (within the same measurement cycle).

RESULTS AND DISCUSSION

Conjugation Procedure. The scheme of conjugation and kinetics of protein labeling with PtCP-NCS has been described elsewhere.[12] The reaction of PtCP-NCS with primary amino groups of biomolecules takes place under mild conditions (0.1 M carbonate buffer, pH 9.7) and results in nondeliterious labeling of the latter. Typically, to obtain acceptable labeling (2−6 dye molecules/protein molecule), incubation times of 6 h or greater are required at room temperature using a 20 molar excess. However, labeling of monoclonal anti-AFP-antibodies (clone 5108) was found to proceed much faster. Conjugates with desired levels of substitution were formed within 60−90 min. Longer incubation resulted in conjugates with 15−20 labels/protein molecule, which displayed compromised performance in the immunoassay (see below). The conjugation of PtCP-NCS to the pentylamine derivative of biotin proceeded even faster, due to the excess of biotin. After 30 min of incubation, HPLC analysis (see Figure 2) showed

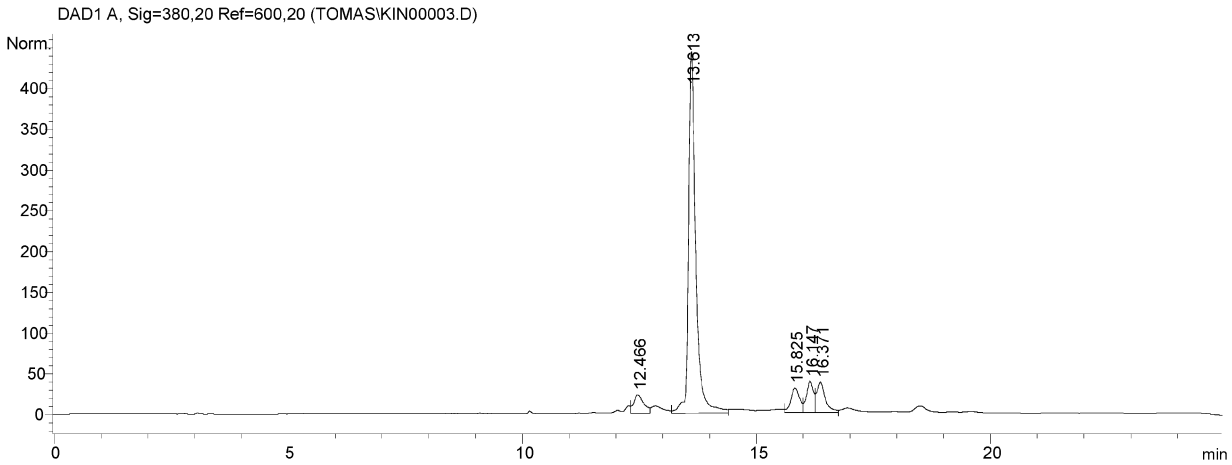

Figure 2. Elution profiles of the biotin-PtCP conjugated after 30-min incubation at room temperature, monitoring at 380 nm.

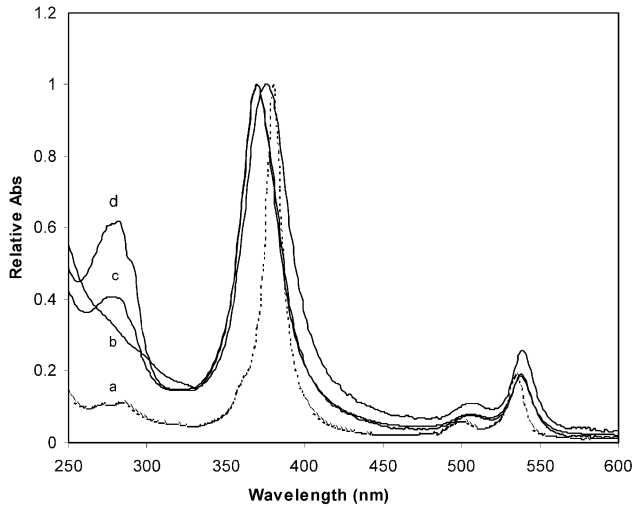

Figure 3. Absorption spectra (normalized) of free PtCP-NCS in 3 mM CTAB (a, dashed line); biotin-PtCP (b), anti-AFP−PtCP (clone 5108) (c), and neutravidin-PtCP (d) conjugates in 0.1 M phosphate buffer, pH 7.4.

traces of free PtCP-NCS (peak at 18 min), while the conjugate gives the principal peak at 13.6 min. Pure biotin-PtCP conjugate was obtained by collecting fractions of this peak eluting from the column. In these conditions, up to 100 μg of PtCP-biotin can be purified on a 4.5-mm analytical column in one run.

Photophysical Properties of Conjugates. The absorption spectra of the conjugates are shown in Figure 3. The spectra of protein conjugates, particularly the Soret band (360−400 nm), are slightly blue shifted and broadened compared to that of the free PtCP and PtCP-biotin. For calculation of conjugate concentration and degree of substitution, these changes in the PtCP label absorption caused by protein carrier were taken into account, as described previously.[12] All the conjugates retained high absorptivity characteristic to the PtCP label. Both the Soret band (360−400 nm) and the Q-band (530−535 nm) with molar extinction coefficients of 2×10^5 and 3.6×10^4 M^{-1} cm^{-1}, respectively, are suitable for excitation. The Q-band falls specifically on the emission maximum of the 532-nm laser, a factor that has led to the development of a dedicated fluorometer for the phosphorescent porphyrin labels.

Table 1. Phosphorescent Properties of PtCP Bioconjugates Used in TR-PIA Measured in Chemically Deoxygenated 3 mM CTAB

dye/conjugate	labeling ratio	absolute emission yield[a]	lifetime (μs)
PtCP-NCS	n/a[b]	0.17	60
PtCP-mAb	3.3	0.13	56
PtCP−neutravidin	2.7	0.16	61
PtCP−biotin	1	0.21	60

[a] Measured with room-temperature PtCP-1 in CTAB ($\phi = 0.28$). n/a, not applicable.

The emission properties of the conjugates and free dye are summarized in Table 1. The emission yield values and the lifetimes further augment the high sensitivity of PtCP label. The relative phosphorescence ($\epsilon\phi$) obtained from the PtCP label is ~34 000 when exciting at the Soret maximum or 6100 when exciting at the Q maximum. These values compare well those of the standard dissociation-based chelate or fluorescent chelates with relative fluorescence values of 24 500[27] and 10 000[28] reported, respectively. The microsecond lifetime range, 532-nm laser compatibility, and large Stokes' shift (emission maximum at 650 nm) facilitate efficient elimination of background fluorescence and scattering and high signal-to noise ratio.[5−7] Therefore, time-resolved detection of these conjugates can be performed with high sensitivity and low optical background using relatively simple instrumentation (see below).

Evaluation of Instrument Performance. Counting parameters of custom-made time-resolved phosphorescence plate reader were optimized for measurement of the PtCP label. The following temporal parameters gave the highest signal/noise ratio and thus were chosen as the standard parameters for TR measurement: excitation with a 1−50-μs square pulse, a 50-μs delay time, 100-μs gate time, and 1-s integration time (5000 cycles). A dilution curve of a biotin-PtCP conjugate in deoxygenated CTAB (Figure 4) displays linearity of ~4 orders of concentration with a dynamic

(27) Hemmila, I. In *Applications of fluorescence in immunoassays*; Winefordner, J. D., Kolthoff, I. M., Eds.; Wiley & Sons: New York, 1991; Vol. 7, pp 107−162.
(28) Hemmila, I.; Mukkala, V.-M.; Takalo, H. *J. Alloys Compd.* **1997**, *249*, 158−162.

Table 2. Results of the Solid-Phase Time-Resolved Phosphorescence Immunoassay of AFP Including Curve-Fit Data Using Four-Parameter Log-Logistic (4 PL) Calibration Functions and δ (% Error) Function

[AFP] (M)	[AFP] (ng/mL)	mean intensity ± standard deviation (counts/s) sandwich ($n = 4$)	amplified sandwich ($n = 4$)	bridged sandwich ($n = 4$)
1.29×10^{-8}	908.9	31266 ± 1727	50522 ± 3080	29357 ± 2228
1.29×10^{-9}	90.89	15231 ± 1576	42177 ± 3438	8229 ± 368
1.29×10^{-10}	9.09	2157 ± 96	5192 ± 437	387 ± 16
1.29×10^{-11}	9.1×10^{-1}	405 ± 29	615 ± 79	155 ± 4
1.29×10^{-12}	9.1×10^{-2}	220 ± 24	230 ± 19	132 ± 18
1.29×10^{-13}	9.1×10^{-3}	210 ± 16	191 ± 38	121 ± 2
1.29×10^{-14}	9.1×10^{-4}	208 ± 20	180 ± 23	123 ± 8
1.29×10^{-15}	9.1×10^{-5}	207 ± 17	193 ± 21	133 ± 8
4 PL calibration functions				
A		3.54×10^4	5.64×10^4	3.15×10^4
B		207.62	190.77	129.72
C		21.47	24.78	31.90
D		1.07	1.18	1.61
% error, δ ($A-D$)		2.108	8.01	5.45

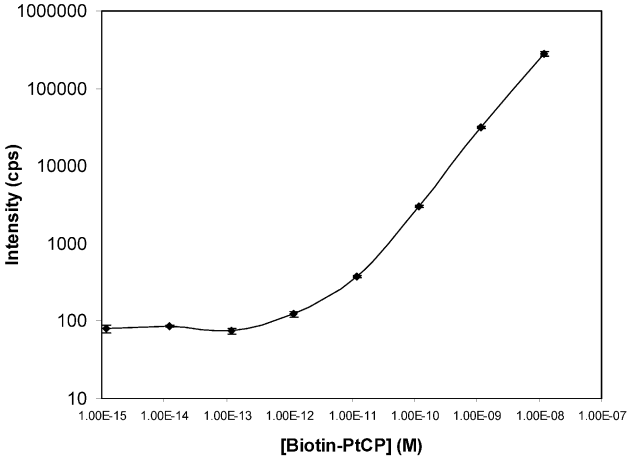

Figure 4. Dilution curve of a biotin-PtCP in solution: samples ($N = 3$) measured in deoxygenated 3 mM CTAB using temporal resolution of 50-μs delay and 100-μs gate. 4 PL calibration functions: $A = 4.19 \times 106$, $B = 79.33$, $C = 15.62$, $D = 0.99$, and δ (% error) = 6.35.

range of greater than 5 orders from subpicomolar levels and good reproducibility between replicate measurements.

Solid-Phase Immunoassay for AFP. For AFP detection, three immunoassay schemes were employed, i.e., (a) a sandwich assay using a PtCP-labeled secondary antibody, (b) an amplified sandwich assay using a biotinylated secondary antibody and PtCP-labeled neutravidin, and (c) an amplified sandwich assay using a biotinylated secondary antibody, a neutravidin bridge, and PtCP-labeled biotin as the tracer (results in Tables 2 and 3 and Figure 5).

In the optimized schemes, monoclonal antibodies tailored to two different epitopes on the AFP protein from the same commercial supplier were preferred to different commercial combinations of coating polyclonal or secondary monoclonal antibodies. In each case, parameters were optimized with respect to sensitivity and degree of nonspecific binding (NSB). In the sandwich assay, as alluded to previously,[12] the degree of PtCP substitution was critical with labeling ratios of 2−6 PtCP molecules/protein molecule found to be optimal. Increasing the degree of substitution had a detrimental effect on sensitivity by an approximate 5-fold

Table 3. Results Showing Estimated Limits of Detection for PtCP Measured in Solution and AFP Measured in Different Immunoassay Schemes

analyte	LOD pM	LOD ng/L
PtCP in solution	0.37	0.37
AFP-sandwich	1.14	80.3
AFP-amplified sandwich	0.67	47.1
AFP-bridged sandwich	8.48	597.2

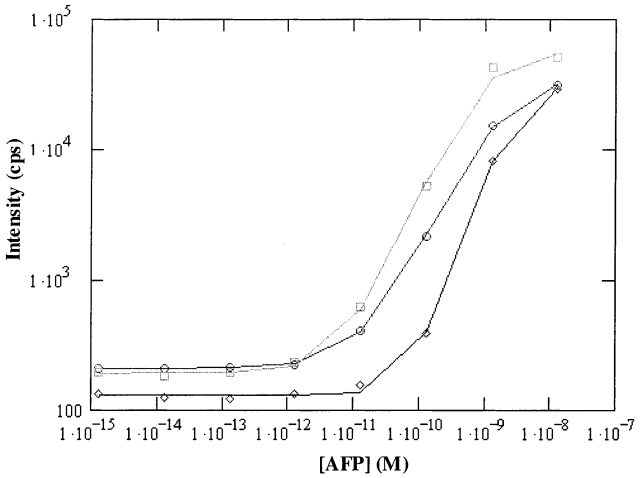

Figure 5. Four-parameter log-logistic (4 PL) fits of sandwich (○), amplified sandwich (□), and bridged sandwich (◇) optimized immunoassay schemes for AFP. Measurement conditions described above.

increase in nonspecific phosphorescence using antibodies with a substitution degree higher than 10 (results not shown). The amplified sandwich using labeled neutravidin displayed a 2-fold increase in sensitivity compared to the sandwich scheme due to slightly lower levels of nonspecific binding of the conjugate and slightly increased specific counts. The bridged system displayed significantly lower sensitivity than either the sandwich or amplified sandwich system (Table 3). This is possibly due to a significantly lower affinity constant of labeled biotin. In all cases, a high degree of intra-assay reproducibility was observed with error for the sandwich, amplified sandwich, and bridged sandwich of 2.1, 8.01, and 5.45%, respectively.

The sensitivity and dynamic range of these immunoassays for AFP obtained with the phosphorescent PtCP-based tracers and the new instrument fall well within the clinical range. Standard levels of AFP in humans are in the range of 0−15 ng/mL, while elevated levels greater than 200 ng/mL may be an indicator of clinical significance.[22] Furthermore, the assay performance and sensitivity compare well with those obtained using lanthanide labels in either DELFIA[23] or FIAgen[24] formats. Thus, the DELFIA system, where secondary antibodies were labeled with europium which was measured following dissociation into solution,[23] or variations of this assay,[29] displayed detection limits of 100 ng/l. For the amplified FIAgen system using a streptavidin−thyroglobulin complex labeled with the BCPDA reagent, detection limits of 12 ng/L were reported.[24] The limits of detection for AFP in time-resolved phosphorescence immunoassay, i.e., 47.1 ng/L for the amplified system and 80.3 ng/L for the sandwich assay, all calculated as described earlier, are very comparable with the lanthanide-based assays.

In addition to the high sensitivity, relatively straightforward labeling chemistry, and detection procedure, the new system incorporates a number of advantages compared to the established ones, particularly lanthanide-based. The long-wavelength excitation of porphyrin labels with a 532-nm laser results in low blank counts (compared to the UV excitation) and provides good spatial resolution suitable for high-density plates and 2D scanning. The system is suitable for TR luminescence measurements from solid surfaces, where a range of solid substrates commonly used in bioaffinity assays can be used, including the ones with high optical background, such as glass, membrane filters, etc. As was shown earlier,[30] the label desorption step can be eliminated, though with some decrease in sensitivity. Assay multiplexing, i.e., simultaneous detection of PtCP and PdCP labels (time resolution), as well as short-lived fluorophores (spectrally resolved) is possible. Some of these applications are now under development by our team.

CONCLUSIONS

Recently developed monofunctional reagent PtCP-NCS facilitates phosphorescent labeling of biomolecules and overcomes the previous drawbacks of metalloporphyrin conjugation chemistries where compromised bioconjugate activity was commonplace. In this study, the phosphorescent bioconjugates of antibodies, neutravidin, and biotin are described, which can be considered as standard reagents for a range of bioanalytical applications. In parallel, a specialized laser-based plate reader dedicated for time-resolved detection of metalloporphyrin labels is described. Using a clinically important analyte AFP and several assay schemes, these bioconjugates and instrumentation were evaluated in solid-phase immunoassays, where they demonstrated subpicomolar sensitivity for AFP, well comparable with lanthanide-based TR-FIA. The combination of improved tracers and dedicated measurement device provides a sensitive and versatile detection platform, which is applicable to various analytes, immunoassay schemes, and related applications.

ACKNOWLEDGMENT

Financial support of this work by the Irish Higher Educational Authority and helpful discussion of results by Dr. Vladimir Ogurtsov are gratefully acknowledged.

Received for review May 24, 2002. Accepted August 22, 2002.

AC020346N

(29) Lovgren, T.; Merio, L.; Mitrunen, K.; Makinen, M.-L.; Makela, M.; Blomberg, K.; Palenius, T.; Pettersson, K. *Clin. Chem.* **1996**, *42* (8), 1198−1201.

(30) Papkovsky, D. B.; O'Riordan, T.; Soini, A. *Biochem. Soc. Trans.* **2000**, *28* (2), 74−77.