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A Europium Chelate for Quantitative Point-of-Care Immunoassays Using Direct Surface Measurement

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New labels and assay techniques are needed to improve the sensitivity and quantitativeness of point-of-care immunotesting while sustaining the rapidity and ease of use of the assays. We synthesized a novel, intrinsically fluorescent nonadentate europium chelate with two chromophores and hydrophilic α-galactose side groups. The chelate is highly fluorescent, soluble in water, and provides effective shielding of Eu from water. The performance of the nonadentate chelate was compared with a heptadentate chelate in a dry reagent immunoassay for human chorionic gonadotropin (hCG). After 15-min incubation and washing, time-resolved fluorescence was measured directly from a wet or dried well surface. Contrary to the heptadentate label, the effect of aqueous quenching on the nonadentate label was found to be insignificant, with calculated analytical detection limits (background + 3 SD) of 0.9 and 0.7 IU/L hCG for wet and dry measurements, respectively, and a linear range up to 5000 IU/L. The CVs for the new label were <8% at the cutoff of 25 IU/L and above in both whole blood and plasma. The novel nonadentate label facilitates short turnaround times and simple instrumentation due to the absence of all signal development steps, at the same time retaining an excellent immunoassay performance.

In recent years, an ever-increasing number of new point-of-care (POC) tests have been introduced to the market, most based on simple manual assay devices using the lateral flow immunochromatographic assay principle. The analytical performances of most of these assays, however, are not comparable with those of the complex and sophisticated assay techniques employed in routine testing. While the most sensitive and reproducible immunoassays used in clinical laboratories are based on heterogeneous, noncompetitive assay principles,[1−3] the design of fully quantitative, wide-range POC assays with low detection limits obviously presents great challenges due to the additional demands for short turnaround times, small size, and ease of use of the instrumentation, as well as the capability of using whole blood as sample material.

Currently, many research groups concentrate on the development of miniaturized, rapid assay formats based on optical immunosensors[4−8] and microparticles.[9,10] These types of assays facilitate smaller sample volumes and reduced consumption of reagents and, in some cases, also real-time or multianalyte measurements. The major challenge especially for sensor systems intended for clinical use is, however, the discrimination between specific and nonspecific signals in complex biological matrixes such as whole blood. Accordingly, only one commercial immunosensor application[11] has been introduced for point-of-care testing (POCT) thus far.

Recently, however, several commercial POC immunoanalyzers have been brought to the market that are based on assay and detection technologies very similar to those used in advanced analytical systems.[12−14] Although the detection limits are not as low as those obtained in centralized testing, these pioneering assays have already shown significantly improved precision of results when compared to immunochromatographic assay formats, as well as linear assay ranges exceeding 3 orders of magnitude. The turnaround times of 15−20 min and the somewhat downsized instrumentation have been accomplished by reducing the sample capacity of the analyzers as well as the number and duration of assay steps. Further simplification of the assay procedures and the analyzers is, however, still feasible as the current assays require either on-board centrifugation of whole blood samples,[12] separate steps for enzymatic signal enhancement,[12,13] or at least drying of the solid phase prior to direct surface measurement.[14] All these extra steps, in addition to being possible sources of error,

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tend to increase the assay times as well as the complexity and, therefore, the cost and the difficulty of maintenance of the instrumentation.

In the search for sensitive, fully quantitative yet robust assay formats for POCT, selection of the label and the detection method is obviously in a key position. To obtain high sensitivity and assay precision, a label with a high specific activity and low nonspecific binding characteristics is required. Time-resolved fluorometry,[15−18] regarded as one of the most sensitive detection methods that currently exist, is one of the very few technologies that can offer these characteristics and at the same time omit separate signal enhancement or amplification steps through direct surface measurement of the label. Accordingly, a number of intrinsically fluorescent lanthanide chelates have been developed and used in modern bioanalytical applications.[14,19−21] A common drawback affecting the measurement is, however, quenching by water since particularly europium has a strong tendency to fill its first coordination sphere with water molecules up to the coordination number nine. Drying the surface prior to measurement prevents the nonradiative energy transfer from the europium ions to the quencher molecules but again results in an increased number of steps in the assay procedure.[14,20,21] The environmental sensitivity of the fluorophores can, however, be decreased by structural modifications that exclude water molecules from the coordination sphere although only few bioanalytical applications using these kinds of low molecular weight lanthanide chelates[22] and cryptates[23] have been described.

In this study, we have developed a new intrinsically fluorescent europium chelate, {2,2′,2″,2‴-{[2-(4-isothiocyanatophenyl)ethylimino]-bis(methylene)bis{4-{[4-(α-galactopyranoxy)phenyl]ethynyl}-pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetato)}europium-(III) (**1** in Figure 1), here referred to as the nonadentate label, that forms a nine-coordinate complex with the metal ion, thus providing high thermodynamic stability and minimal fluorescence quenching by water. Due to the two sugar side groups incorporated with the chelate structure, the complex is soluble in water which, for its part, facilitates a gentle and efficient coupling to antibodies via the aromatic isothiocyanato group of the chelate and decreases the tendency of nonspecific binding.

We have employed the new label in an all-in-one time-resolved fluorometric POC assay for human chorionic gonadotropin (hCG),[24] a glycoprotein hormone normally produced by the trophoblast cells of placenta and used for the early detection of pregnancy. The performance characteristics of the new label were compared with those of a well-characterized heptadentate label, {2,2′,2″,2‴-{[4-[(4-isothiocyanatophenyl)ethynyl]pyridine-2,6-diyl]-bis(methylenenitrilo)}tetrakis(acetato)}europium(III)[25] (**2** in Figure 1), the direct measurement of which has successfully been employed in immunohistochemistry,[20,26] in situ hybridization,[26] and in vitro diagnostics using either microparticles as solid-phase[9,10] or a well-based assay format similar to the one described here.[14,27,28] We laid especially high emphasis on studying the fluorescence characteristics of the chelates in different moisture conditions, as the aim of the study was to develop a sensitive, fully quantitative POC assay format with an absolute minimum number of assay steps, including the direct use of whole blood samples and no drying of the solid phase prior to measurement.

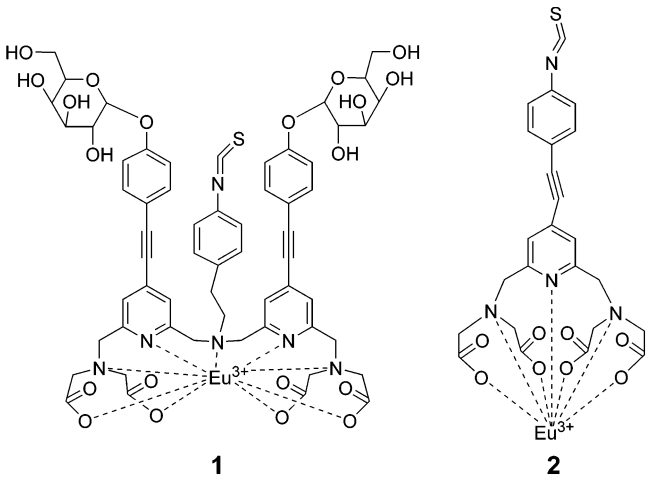

Figure 1. Structures of the nonadentate (**1**) and heptadentate (**2**) europium chelates: {2,2′,2″,2‴-{[2-(4-Isothiocyanatophenyl)ethylimino]-bis(methylene)bis{4-{[4-(α-galactopyranoxy)phenyl]ethynyl}-pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetato)}europium(III) (**1**); {2,2′,2″,2‴-{[4-[(4-Isothiocyanatophenyl)ethynyl]pyridine-2,6-diyl]-bis(methylenenitrilo)}tetrakis(acetato)}europium(III) (**2**).

EXPERIMENTAL SECTION

Materials. The low-fluorescence Maxisorp single wells made of irradiated polystyrene were purchased from Nunc. The DELFIA reagents, the heptadentate europium chelate **2**,[25] and the biotinylation reagent biotin isothiocyanate (BITC) were obtained from PerkinElmer Life Sciences/Wallac Oy. The monoclonal anti-hCGβ capture and detection antibodies were acquired from Medix Biochemica and OEM, respectively, and the streptavidin was from BioSpa.

Except for the tetra(*tert*-butyl)2,2′,2″,2‴-{[2-(4-aminophenyl)-ethylimino]bis(methylene)bis(4-bromopyridine-6,2-diyl)bis(methylenenitrilo)tetrakis(acetate) (**6**), which was synthesized as described elsewhere,[29] all reagents and solvents used in the organic syntheses were commercially available and at least of reagent grade.

HCG Calibrators and Clinical Samples. The hCG calibration solutions were prepared by diluting the Fourth International

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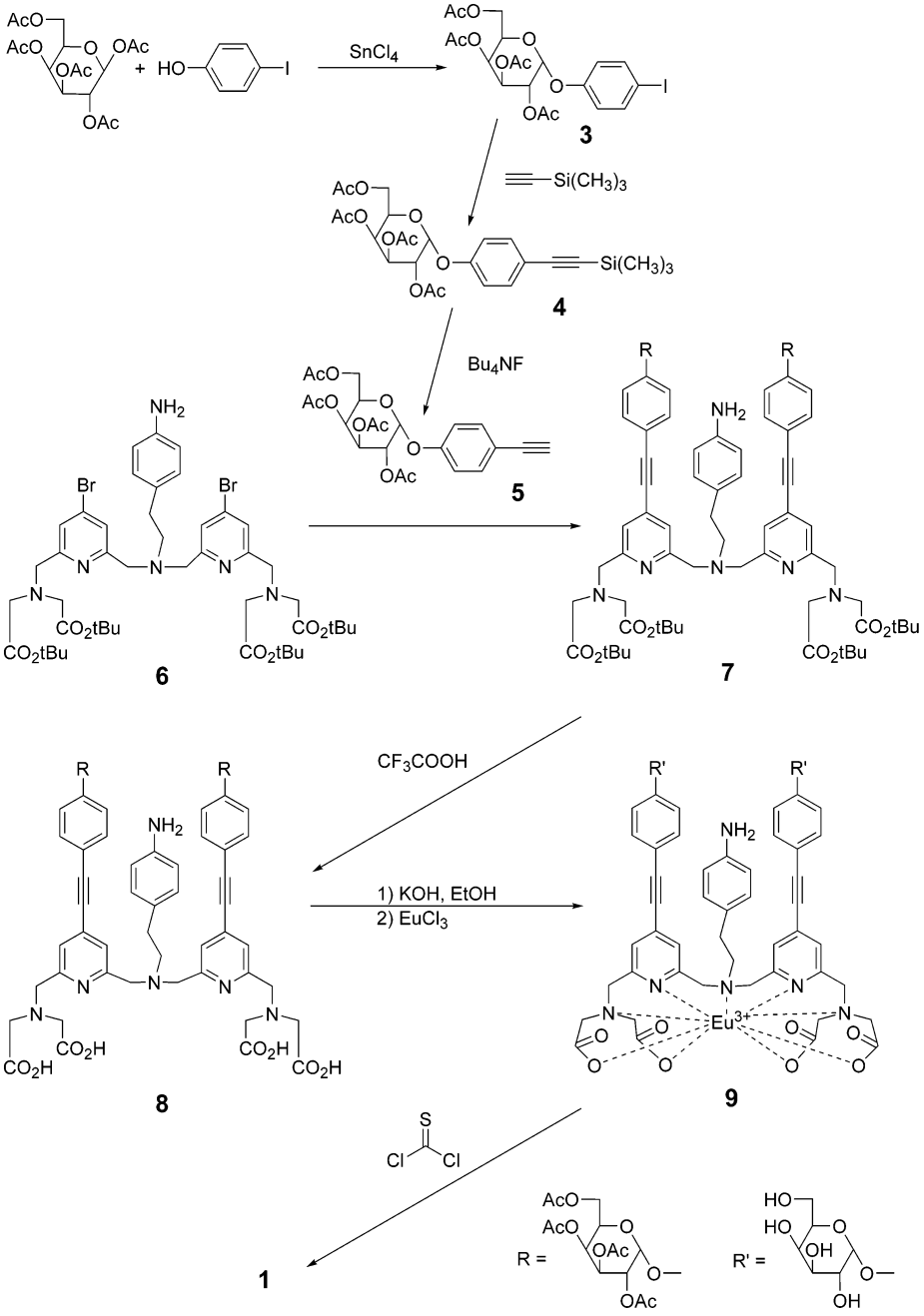
Scheme 1. Synthesis of the Nonadentate Europium Chelate



Standard for Chorionic Gonadotropin (75/589), purchased from the National Institute for Biological Standards and Control (United Kingdom), in a pool of normal human male serum. All blood samples used in this study were obtained from volunteers of the staff, and the procedures followed were in compliance with the Helsinki Declaration of 1975 as revised in 1996. For precision studies, pooled heparin plasma samples were spiked with the hCG standard material and frozen in aliquots at −70 °C until use. The heparin whole blood samples were spiked similarly but analyzed fresh.

Safety Considerations. All reagents of the organic syntheses should be handled with caution. Thiophosgene and tin tetrachloride are toxic. Both of these reagents are toxic by inhalation, and mechanical ventilation should be used. Tin tetrachloride is toxic also in contact with skin and if swallowed; thiophosgene is harmful in corresponding conditions. Personal protection should be used when handling these reagents, and special care should be taken when pouring the reaction mixture into the aqueous sodium hydrogen carbonate because the reaction is vigorous. Appropriate safety measures should also be followed when using the potassium hydroxide and trifluoroacetic acids due to their corrosive nature. Skin and eye contact and inhalation should be avoided with the rest of the reagents as well.

Synthesis of {2,2′,2″,2‴-{[2-(4-Isothiocyanatophenyl)-ethylimino]bis(methylene)bis{4-{[4-(α-galactopyranoxy)-phenyl]ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}-tetrakis(acetato)}europium(III) (1). The novel nonadentate label was prepared according to the steps shown in Scheme 1, as described below. In flash chromatography (FC) we used Kieselgel 60 (0.040−0.063 or 0.063−0.200) silica gels (Merck).

(a) 4-Iodophenyl 2,3,4,6-Tetra-*O*-acetyl-α-galactopyranoside (3). 1,2,3,4,6-Penta-*O*-acetyl-β-D-galactopyranose (12.0 g, 30.7 mmol) and 4-iodophenol (9.5 g, 43.2 mmol) were dissolved in dry ethanol-free chloroform (60 mL). Tin tetrachloride (10.8 mL, 92.5 mmol) was added, and the reaction was stirred at 62−64 °C for 7 h. Subsequently, the solution was poured into ice-cold aqueous sodium hydrogen carbonate (600 mL) and diluted with dichloromethane (600 mL). The solution was then filtered through Celite (Aldrich), and the precipitate washed with additional 150 mL of dichloromethane. The organic phase was rapidly washed with ice−water (400 mL), dried (Na_2SO_4), and evaporated to give a brown syrup. The product was purified by FC (Kieselgel 0.040−0.063) using AcOEt/petroleum (1:3) to give **3** (yield 10.95 g, 65%).

(b) 4-[(Trimethylsilyl)ethynyl]phenoxy 2,3,4,6-Tetra-*O*-acetyl-α-galactopyranoside (4). Compound **3** (10.9 g, 19.9 mmol), bis(triphenylphosphine)palladium(II) chloride (280 mg, 0.40 mmol), and CuI (152 mg, 0.80 mmol) were dissolved in a mixture of dry Et_3N (68 mL) and THF (78 mL), and the solution was deaerated with N_2. (Trimethylsilyl)acetylene (3.5 mL, 24.8 mmol) was added, and the reaction stirred for 1.5 h at room temperature. The mixture was then filtered and the filtrate evaporated. The residue was dissolved in $CHCl_3$ (150 mL) and washed with H_2O (2 × 60 mL), dried (Na_2SO_4), and evaporated. The product was purified by FC (Kieselgel 0.063−0.200, AcOEt in petroleum ether, 10 → 20%). The yield was 7.43 g (66%).

(c) 4-Ethynylphenoxy 2,3,4,6-Tetra-*O*-acetyl-α-galactopyranoside (5). Compound **4** (7.4 g, 14.2 mmol) was dissolved in dry dichloromethane (110 mL) and deaerated with N_2. Tetrabutylammonium fluoride (4.45 g, 17.02 mmol) was added to the mixture and stirred for 30 min at room temperature, after which the mixture was washed with 10% citric acid in H_2O (70 mL) and H_2O (4 × 110 mL), dried (Na_2SO_4), and evaporated. The product was purified by FC (Kieselgel 0.063−0.200, AcOEt in petroleum ether, 20 → 40%) with a yield of 4.3 g (68%).

(d) Tetra(*tert*-butyl) 2,2′,2″,2‴-{[2-(4-Aminophenyl)ethylimino]bis(methylene)bis{4-{[4-(2,3,4,6-tetra-*O*-acetyl-α-galactopyranoxy)phenyl]ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetate) (7). Compound **6** was synthesized as earlier described,[29] dissolved (1.0 g, 1.0 mmol) together with bis(triphenylphosphine)palladium(II) chloride (28 mg, 0.040 mmol) and CuI (15 mg, 0.078 mmol) in a mixture of dry Et_3N (10 mL) and THF (10 mL), and then deaerated with N_2. Compound **5** (1.08 g, 2.4 mmol) was added to the mixture, and the reaction was stirred overnight at 53 °C, after which it was filtered and the filtrate evaporated. The residue was dissolved in $CHCl_3$ (80 mL), washed with H_2O (2 × 35 mL), dried (Na_2SO_4), and then evaporated. The product was purified by FC (Kieselgel 0.063−0.200, MeOH in dichloromethane, first 0.5 → 2% and finally 10%). The yield was 1.34 g (77%).

(e) 2,2′,2″,2‴-{[2-(4-Aminophenyl)ethylimino]bis(methylene)bis{4-{[4-(2,3,4,6-tetra-*O*-acetyl-α-galactopyranoxy)phenyl]ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetic acid) (8). A solution of **7** (1.3 g, 0.753 mmol) in CF_3COOH (13 mL) was stirred for 2 h at room temperature. After evaporation without heating, the mixture was triturated with Et_2O (50 mL) and filtered. The yield was 1.3 g.

(f) {2,2′,2″,2‴-{[2-(4-Aminophenyl)ethylimino]bis(methylene)bis{4-{[4-(α-galactopyranoxy)phenyl]ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetato)}europium(III) (9). A mixture of **8** (1.0 g, 0.544 mmol), 0.5 M KOH in EtOH (55 mL), and H_2O (25 mL) was stirred for 2 h at room temperature. After evaporation, the residue was dissolved in H_2O (19 mL) and the pH adjusted to 6.5 with 6 M HCl. $EuCl_3$ (237 mg, 0.647 mmol) in H_2O (6.6 mL) was added within 15 min and the pH maintained at 6.5 with solid $NaCO_3$. After stirring the reaction for 30 min, the pH was raised to 8.5 with 1 M NaOH and the precipitate removed by centrifugation. The filtrate was then treated with acetone and the precipitate collected by centrifugation. The product was washed with acetone and used in the next step without further purification. The yield was 2.43 g.

(g) {2,2′,2″,2‴-{[2-(4-Isothiocyanatophenyl)ethylimino]bis(methylene)bis{4-{[4-(α-galactopyranoxy)phenyl]ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetato)}europium(III) (1). An aqueous solution of **9** (2.3 g, 1.75 mmol) was added slowly (within 25 min) to a mixture of thiophosgene (530 μL, 7.0 mmol), $NaHCO_3$ (736 mg, 8.8 mmol), and $CHCl_3$ (27 mL). After stirring for 1 h, the H_2O phase was washed with $CHCl_3$ (3 × 50 mL). The pH of the aqueous solution was then adjusted to 7.0 with 1 M CH_3COOH, and acetone was added. The product was collected by centrifugation and washed with acetone to give **1** (2.26 g).

Labeling of Antibodies. The labeling of the detection antibody was performed in 10 mmol/L borate buffer, pH 8.6−9.0, using 50- and 150-fold molar excesses of the hepta- and nonadentate chelates, respectively. The reactions were carried out overnight at +4 °C for the heptadentate and at room temperature for the nonadentate labels. Labeled antibodies were separated from excess free chelate on Superdex 200 HR 10/30 or Superdex 200 HiLoad 26/60 gel filtration columns (Pharmacia Biotech) using Tris−saline−azide (6.1 g/L Tris, 9.0 g/L NaCl, and 0.5 g/L NaN_3), pH 7.75, as elution buffer. The fractions containing the antibody were pooled and the europium concentrations measured against a europium calibrator. The labeling degrees obtained were 3.6 and 3.5 Eu/IgG for the hepta- and nonadentate labels, respectively.

Biotinylation of the capture antibody was performed in 50 mmol/L $NaHCO_3$, pH 9.8, using a 30-fold molar excess of the biotinylation reagent (BITC) that was first dissolved in a small volume of dimethylformamide. The reaction was carried out for 4 h at room temperature and the excess free reagent removed using NAP-5 and NAP-10 (Amersham Pharmacia) or Superdex 200 HR 10/30 (Pharmacia Biotech) gel filtration columns, and the Tris−saline−azide buffer, pH 7.75, for elution.

Finally, bovine serum albumin was added to a concentration of 1 g/L to the solutions containing the europium-labeled and the biotinylated antibodies. The antibodies were stored at 4 °C.

Fluorescence Properties of the Antibody Conjugates. The fluorescence properties of the antibody-coupled europium chelates were measured in the combined assay/wash buffer used in the hCG immunoassays, containing 5 mmol/L HEPES, 2.1 g/L NaCl, 0.1 mmol/L EDTA, 0.055 g/L Tween 20, and 1 g/L Germall II, pH 7.75. The excitation and emission spectra, fluorescence intensities, and decay times were determined using a LS55 luminescence spectrometer (PerkinElmer Instruments), while the molar extinction coefficients (absorbance) were determined with

a UV-2100 spectrophotometer (Shimadzu). Additionally, decay times were also measured from antibody conjugates bound to solid phase, either covered with 30 μL of the combined assay/wash buffer or after aspirating and drying of the wells. The solid-phase determinations were performed using a Cary Eclipse fluorescence spectrophotometer (Varian).

Preparation of the All-in-One Dry Reagent Wells. The streptavidin coating of the single wells was performed through physical adsorption. A 750-ng sample of streptavidin was added per well in 150 μL of coating buffer, containing 100 mmol/L NaH_2PO_4 and 50 mmol/L citric acid, pH 5.0, and incubated overnight at room temperature. The coated wells were washed twice (5 mmol/L Tris, 154 mmol/L NaCl, 5 g/L Tween 20, and 1 g/L Germall II, pH 7.75) and saturated overnight at room temperature with 300 μL/well of a solution containing 26.6 g/L bovine serum albumin, 50 mmol/L Tris, 154 mmol/L NaCl, 330 mmol/L D-sorbitol, and 0.5 g/L NaN_3, pH 7.0. After saturation, the wells were aspirated and dried for 3 h in a dry-condition cabinet (35 °C, relative air humidity 5%).

For preparing the assay-specific dry reagent wells, 400 ng of biotinylated capture antibody was added in the wells in 50 μL of coating buffer containing 50 mmol/L Tris, 154 mmol/L NaCl, 20 μmol/L DTPA, 0.1 g/L Tween 40, 5 g/L BSA, 0.5 g/L bovine γ-globulin, 0.5 g/L NaN_3, and 0.02 g/L cherry red, pH 7.75, and incubated overnight at room temperature. The wells were again washed and the antibodies covered with a protective solution, 40 μL/well, containing 37.5 mmol/L Tris, 120 mmol/L NaCl, 0.375 g/L NaN_3, 0.6 g/L bovine γ-globulin, 25 g/L bovine serum albumin, 50 g/L D-trehalose, 0.1 g/L native mouse IgG, 0.05 g/L denatured mouse IgG, 2 g/L casein, and 10 g/L PVA 6000, pH 7.75. Drying of the wells was performed overnight in a dry-condition cabinet at 35 °C with 5% relative air humidity. The labeled detection antibody (200 ng/well) was applied on top of the protective layer in a 1-μL drop of a buffer containing 50 mmol/L Tris, 154 mmol/L NaCl, 0.5 g/L NaN_3, 125 g/L D-trehalose, and 5 g/L bovine serum albumin, pH 7.75, and immediately dried with a steam of warm air. The dry reagent wells were stored protected from humidity at room temperature.

Immunoassay for hCG. The noncompetitive, one-step immunoassays for hCG were performed on a fully automated Aio immunoanalyzer (Innotrac Diagnostics) that allows time-resolved fluorescence measured directly from a solid phase. In the automated assay procedure, 10 μL of sample (whole blood or plasma) and 20 μL of the combined assay/wash buffer were dispensed in the dry reagent well and incubated for 15 min (i.e., until equilibrium) with slow shaking at 36 °C. The wells were washed and aspirated a total of 6 times, with the last aspiration adjusted so that the wells were either aspirated dry or a well-defined amount of the assay/wash buffer (from 10 ± 1 to 150 ± 10 μL) was left in the well. For dry measurements, a steam of hot (95 °C) air was blown to the assay cups for 40 s prior to measurement to ensure dryness of the wells, while in the wet measurement mode, this step was omitted. The measurement of europium fluorescence from the well surface was carried out using the default measurement settings of the immunoanalyzer: excitation wavelength, 340 nm; emission wavelength, 615 nm; delay time, 400 μs; window time, 400 μs, cycling time, 1 ms; measurement time, 1 s (i.e., counts resulting from 1000 sequential excitations were integrated for each measurement).

Table 1. Fluorescence Properties of the Antibody-Coupled Chelates

	λ_{exc} (nm)	λ_{em} (nm)	$\tau_{liquid\ phase}$ (μs)	$\tau_{solid\ phase}$ (μs) wet[a]	dry	$\epsilon\Phi$	Φ
9-dentate	325	615	1000	1000	1150	4787	0.127
7-dentate	329	615	390	490	990	1337	0.042

[a] 30 μL of the combined assay/wash buffer per well.

To compare the sensitivity of solid-phase and dissociation-enhanced measurements of the novel nonadentate label, some of the assay cups first measured dry in the Aio immunoanalyzer were collected and remeasured using the commercial dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) principle (PerkinElmer Life Sciences/Wallac Oy).[30] In DELFIA, the europium ions are dissociated from the original, nonfluorescent chelates at low pH using a special enhancement solution. The enhancement solution also contains β-diketone, detergent, and chelator that interact to form a strongly fluorescent, micellar complex with the dissociated lanthanide ions. For this purpose, 200 μL of the enhancement solution (DELFIA reagent) was dispensed in each well and incubated for 60 min at room temperature with slow shaking (>90% dissociation obtained for the nonadentate chelate). The fluorescence counts were measured using a Victor 1420 multilabel counter (PerkinElmer Life Sciences/Wallac Oy) with the default settings for europium measurement.

RESULTS

Syntheses. The novel nonadentate europium(III) chelate **1** was prepared according to the route shown in Scheme 1. The organometallic coupling of aryl bromide **6**[29] with the terminal acetylene (4-ethynylphenoxy)-2,3,4,6-tetra-*O*-acetyl-α-galactopyranoside (**5**) gave compound **7**. Compound **5** was prepared starting from 4-iodophenol and 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose. The reaction of 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose and 4-iodophenol with tin tetrachloride in $CHCl_3$ gave a good yield of α-anomer 4-iodophenyl 2,3,4,6-tetra-*O*-acetyl-α-galactopyranoside (**3**).[31] The 4-iodo atom of **3** reacted with (trimethylsilyl)acetylene in the presence of a palladium catalyst and copper(I) iodide. The silyl protection was easily removed by tetrabutylammonium fluoride to give **5**. The ester groups of **7** were hydrolyzed with trifluoroacetic acid. The acetyl groups of **8** were saponified, and the europium(III) chelate was prepared by stirring the tetrakis-(acetic acid) with $EuCl_3$ in slightly acidic conditions. Finally, the amino group of **9** was activated with thiophosgene.

Fluorescence Properties of the Antibody Conjugates. The excitation (λ_{exc}) and emission (λ_{em}) maximums, fluorescence yields ($\epsilon\Phi$), and quantum yields (Φ) of the nona- and heptadentate europium chelates were determined using the combined assay/wash buffer for dilution (Table 1). Both chelates had an excitation

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maximum in the range of 320−340 nm and a sharp emission at 615 nm, facilitating the use of the default settings for europium measurement in the Aio immunoanalyzer and Victor 1420 multilabel counter. The fluorescence and quantum yields were, however, more than 3-fold higher for the nonadentate chelate compared to the heptadentate one.

The fluorescence decay times (τ) of the label−antibody conjugates were measured both free in liquid phase and bound to the surface of an assay well, the latter either wet (30 μL of buffer/well) or after aspirating and drying of the wells (Table 1). Unlike the heptadentate label, the fluorescence lifetime of which significantly increased after drying, the nonadentate europium chelate demonstrated rather uniform and long standing (in the range of ∼1 ms) decay times in all the different measurement conditions, thus indicating good tolerance against aqueous quenching.

Measurement of the decay times also facilitated estimation of the number of water molecules, q, in the first coordination sphere of the europium ions using the equation derived by Horrocks and Sudnick[32] and later specified by Beeby et al.:[33]

$$q = A(\tau^{-1}_{H_2O} - \tau^{-1}_{D_2O} - 0.25)$$

where A is a proportionality constant suggested to be 1.2 waters·ms for europium[33] and τ is the experimental fluorescence lifetime in milliseconds. Although the exact value for q can be calculated only based on parallel measurements in both ordinary and deuterated water, good estimates can be obtained using an average decay constant of 0.5 ms^{-1} (corresponding to a decay time of 2 ms) for a europium(III) chelate in D_2O.[34,35] By this adaptation, q computes to be ∼0.3 for the antibody-coupled nonadentate chelate and ∼2.2 for the corresponding heptadentate one, both measured free in the aqueous solution. In the case of the heptadentate label, however, the binding of the label-antibody conjugate onto the well surface resulted in a slight increase in the label's decay time, thus decreasing the calculated q to ∼1.5. Such a change may imply, for example, some kind of a structural change in the label−antibody conjugate that leads to the exclusion of some of the coordinated water molecules or, alternatively, protection of the chelate by the proximity of the surface.

Moisture Tolerance of the Europium Chelates in hCG Immunoassay. To assess the moisture tolerance of the two chelates in a noncompetitive assay for hCG, we performed the immunoassays as usual and then measured the fluorescence intensities from the assay wells in different moisture conditions. Accordingly, fluorescence was measured directly from a set of aspirated but still moist wells, from wells containing different volumes (10−150 μL) of the combined assay/wash buffer, and from aspirated wells that were further dried with hot air.

As expected, the signal intensity of the heptadentate chelate was already decreased by 50% when measured from an aspirated instead of a dry surface, and only ∼20% of the maximal fluorescence level could be recovered from the wells containing liquid (Figure 2). For the nonadentate chelate, however, no decrease in fluorescence was observed when the wells were measured after aspiration, and ∼60% of maximal signal level could be obtained also from wells containing buffer. Furthermore, the intensity of fluorescence and the corresponding signal/noise ratios were independent of the amount of buffer per well for both chelates in the wet measurement mode. Therefore, we selected a residual buffer volume of 30 μL for subsequent experiments.

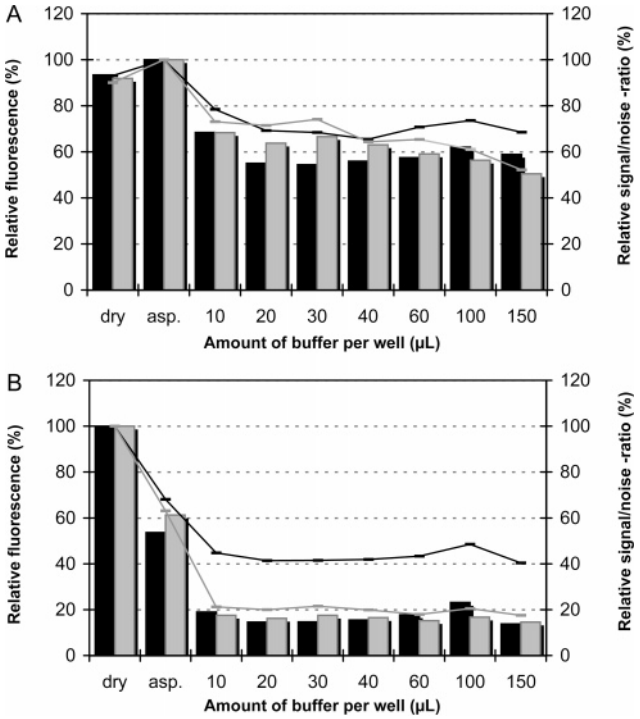

Figure 2. Moisture tolerance of the nonadentate (A) and heptadentate (B) chelates in hCG assay. The one-step assays were performed using 25 (black box) or 1000 (gray box) IU/L hCG. Fluorescence was measured either directly from aspirated (moist) well surface, from wells containing 10−150 μL of the combined wash/assay buffer, or from aspirated wells dried with hot air. The relative fluorescence signals are indicated with columns and the relative signal-to-noise ratios with lines. The calculations are based on four replicate measurements.

Calibration Curves. The calibration curves and analytical detection limits (calculated as the mean of background + 3 SD) obtained with the dry, moist (aspirated wells), and wet (30 μL of buffer/well) measurements using either the nona- or heptadentate europium chelates as labels are shown in Figure 3. Consistent with the fluorescence intensity measurements, the analytical detection limits obtained in the different moisture conditions were significantly divergent for the assays using the heptadentate label (1.7, 2.4, and 5.1 IU/L hCG for the dry, moist, and wet measurements, respectively) compared with the rather consistent detection limits calculated for the ones using the nonadentate label (0.7, 0.8, and 0.9 IU/L hCG, in the same order as above). The differences in the detection limits were, however, generally smaller than could have been predicted from the fluorescence intensity studies, mainly because the background fluorescence of the assays followed the change in the specific signal.

For comparison, a calibration curve was run for the assay employing the nonadentate label also using the DELFIA principle

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Table 2. Assay Variability

		heparin whole blood	heparin plasma	
		within-run CV%[a] 25/500/5000 IU/L hCG	within-run CV%[a] 25/500/5000 IU/L hCG	between-run CV%[b] 25/500/5000 IU/L hCG
9-dentate	dry	8.0/5.3/3.5	4.6/4.0/1.5	4.0/4.2/4.7
	wet	4.7/6.2/4.0	3.5/4.7/2.8	7.9/4.9/4.3
	DELFIA[c]	8.1/3.7/2.1	8.9/2.7/2.4	
7-dentate	dry	7.0/8.3/8.3	7.2/4.8/2.4	
	wet	15/6.9/3.8	14/4.9/2.4	

[a] $n = 12$. [b] Duplicate samples run for 12 days; 2 runs per day. [c] Four wells from the total of 72 were excluded from calculations due to falsely increased signal intensities.

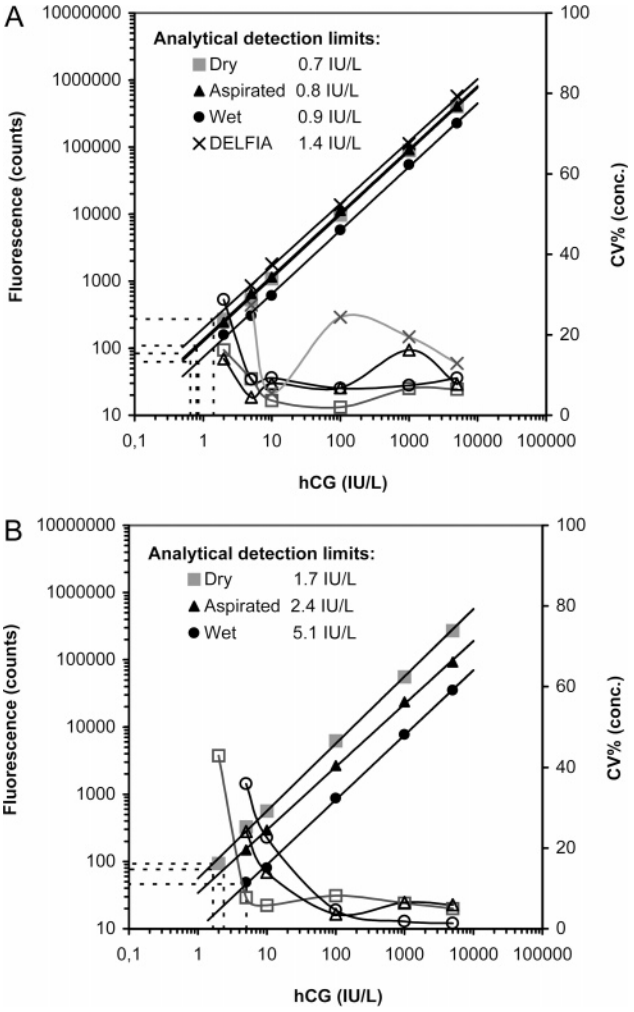

Figure 3. Calibration curves (solid symbols) and precision profiles (open symbols) using the nonadentate (A) and heptadentate (B) chelates as labels in the hCG assay. Fluorescence was measured either from aspirated (moist) wells, from wells containing 30 μL of the combined wash/assay buffer, or from aspirated wells dried with hot air. The values shown are the mean of four replicates. The analytical sensitivities of the assays were calculated as the mean of background (12 replicates) + 3 SD and are indicated with dotted lines.

for detection (Figure 3A). In the current assay format, however, the use of this measurement principle led to an increase in the background level as well as an increased well-to-well variation, and the analytical detection limit of 1.4 IU/L hCG obtained using the DELFIA method did not exceed those of the direct surface measurements.

Furthermore, although very good results were obtained by measuring the hCG assay wells immediately after washing and aspiration (without drying), this measurement mode was, in our opinion, potentially too susceptible to environmental influences and disturbances resulting, for example, from variation in air humidity, temperature, or aspiration efficiency. Since these kinds of changes can affect the moisture level in the cups and, therefore, compromise the accuracy of the fluorescence detection, we decided to omit this mode of measurement in the subsequent experiments and concentrate on the more robust wet and dry measurements.

Assay Precision and Recovery. The variability and recovery of the hCG assay were studied by adding known amounts of purified hCG to hCG-free human heparinized whole blood and plasma samples (Table 2). The highest within-assay variation, 14−15% at the hCG concentration of 25 IU/L, was seen for the wet measurements of the heptadentate label, being a consequence of the decreased signal level in these measurements due to aqueous quenching. For the nonadentate label, the within-assay CVs were in the range of 1.5−8.0% for the wet and dry measurements and 2.1−8.9% for the DELFIA measurements, although in the latter case 4 wells (so-called "flyers") from a total of 72 were excluded from the calculations due to significantly (falsely) increased signal intensities. The between-assay variability was tested for the nonadentate chelate only, and it was found to be <8% for both wet and dry measurements.

The analytical recovery of the rapid hCG immunoassay is shown in Table 3. Recoveries were in the same range for whole blood and plasma, being 92−114 and 86−122% for the assays using the nona- and heptadentate labels, respectively, indicating the accuracy of the method and an absence of interference from whole blood. In all, no differences were detected between whole blood, plasma, and serum samples in any of the measurements. The average background fluorescence counts using any of these matrixes remained below 700 using the heptadentate label and below 600 using the nonadentate label, of which ~150 fluorescence counts originated from the polystyrene cups and the instrument itself.

DISCUSSION

New labels and assay techniques are needed to keep up with the expectations set for the analytical performance of future POCT. Time-resolved fluorometry, which has for long been applied in both clinical routine and research applications (for review, see

Table 3. Analytical Recovery of HCG (n = 12)

				heparin plasma		heparin whole blood	
		original hCG (IU/L)	added hCG (IU/L)	measured (IU/L)	recovery (%)	measured (IU/L)	recovery (%)
9-dentate	dry	0	25/500/5000	27/566/5549	109/113/111	27/572/5249	107/114/105
	wet	0	25/500/5000	24/546/5485	97/109/110	23/534/5024	92/107/100
7-dentate	dry	0	25/500/5000	27/503/4464	107/101/89	31/538/4321	122/108/86
	wet	0	25/500/5000	24/463/4733	97/93/95	23/469/4705	93/94/94

refs 36 and 37), provides an excellent way of creating highly sensitive yet robust assay formats suitable for quantitative and rapid testing. The measurement ranges and sensitivities obtainable with the time-resolved fluorescence technology are generally orders of magnitude higher than for conventional fluorescence, deriving from the unique long-lived emission properties of the lanthanide ions and the effective elimination of the rapidly decaying background fluorescence originating from serum proteins, plastic ware, and light scattering.[15−18]

In the current study, we focused on time-resolved surface measurement of two intrinsically fluorescent europium chelates, a previously produced heptadentate and a novel nonadentate, and the applicability of them in a single-well-based POC assay platform. Although successfully used in several different bioaffinity applications,[9,10,14,20,26−28] the heptadentate label was, however, known to be sensitive to aqueous quenching due to the molecular structure that allows water molecules in the first coordination sphere of the lanthanide ion. In the nonadentate label, however, all the coordination sites of the europium ion are occupied by carboxylate and amine moieties, providing a very high thermodynamic and kinetic stability as well as minimal nonradiative energy transfer to the quencher molecules. The measured fluorescence decay times of the two chelates were consistent with these structural details. While significant quenching was observed for the antibody-conjugated heptadentate label in the presence of water molecules, the fluorescence lifetime of the nonadentate label was only slightly decreased and remained in the range of ∼1 ms. Correspondingly, a significantly higher quantum yield (Φ), i.e., the portion of excited label molecules that emit light, was measured for the antibody-conjugated nonadentate label (12.7%) free in an aqueous solvent compared to the heptadentate one (4.2%).

Furthermore, two separate chromophores were incorporated in the nonadentate label to facilitate more efficient absorption of excitation light. Combined with the effective shielding from water molecules, almost a 4 times higher total fluorescence yield was measured for the antibody-conjugated nonadentate label compared to the heptadentate one. As expected, the difference in the fluorescence yields was directly reflected in the signal-to-noise ratios obtained in the wet measurements and, therefore, in the detection limits of the hCG assay, although the degree of background discrimination was slightly enhanced by the concurrent decrease of background fluorescence as well. Taken together, a 3-fold deterioration of the analytical detection limit was observed for the heptadentate label when measured in the wet instead of the dry mode (from 1.7 to 5.1 IU/L hCG), while the corresponding loss was <30% for the nonadentate label with the analytical detection limit remaining below 1 IU/L hCG.

The nonadentate chelate was also measured using the commercial DELFIA measurement principle, in which a separate step for fluorescence enhancement is employed.[30] However, this method of detection was not fully compatible with the current assay format and resulted in an increased background level and assay variation. Mostly this was due to the gentle washing procedure that was optimized for the direct surface detection at the bottom of the well. In the DELFIA measurements, however, the fraction of label that was nonspecifically bound to the sidewalls of the wells could be dissociated and detected, occasionally also resulting in significant aberrations (so-called "flyer" wells). However, the reproducibility and sensitivity of the DELFIA measurements could most likely have been improved using more efficient washings, although they were not applied in this study.

Although increasing the number of chromophores generally tends to increase the hydrophobicity of the chelate and, therefore, the extent of nonspecific binding, no such increase in background was observed for the novel chelate when compared with the heptadentate chelate in direct surface measurements. Most likely this was due to the balancing effect of the two hydrophilic α-galactose side groups, which also rendered the chelate soluble in aqueous solvents. Most importantly, the binding activity of the detection antibody was preserved after conjugation with the new label, even though it must be noted that since the ultimate sensitivity of the hCG assay was not a subject of investigation for this study, the labeling degree of the detection antibody was kept relatively low (3.6 and 3.5 europiums/IgG molecule using the hepta- and nonadentate chelates, respectively). Owing to the gentle coupling via the chelate's isothiocyanato groups and the antibody's primary amino groups, however, it is possible that the labeling degree of the antibody could be multiplied without a significant change in its binding characteristics, as shown by earlier studies using corresponding low molecular weight lanthanide chelates.[18,30] Even so, the assay sensitivities readily obtained using the nonadentate label were more than sufficient for the intended use for detection of pregnancy, with the within- and between-assay CVs below 8% at the common hCG cutoff concentration of 25 IU/L[38] in both wet and dry measurements. Furthermore, the assays were linear at least up to 5000 IU/L hCG, thus covering almost 4 orders of magnitude.

Even though a number of intrinsically fluorescent lanthanide chelates have been developed and applied in different bioaffinity applications during the past years, the current nonadentate chelate

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is one of the few that can be measured wet without significantly compromising the fluorescence yield and that fulfills all the strict requirements set for use in bioaffinity applications. However, in addition to increasing the number of lanthanide-coordinating groups in the ligand structure, other alternatives exist to similarly avoid aqueous quenching of the fluorescence. One of the commonest approaches is to incorporate the fluorescent chelates into polymeric latex or polystyrene particles and thus physically protect them from environmental effects. A major advantage of this approach is that large numbers of label molecules can be included in the particles, thus also offering a way of improving assay sensitivity by signal amplification.[39,40] Correspondingly, several other types of novel particulate labels have been applied in ultrasensitive bioassays, including quantum dots,[41,42] down-[43] and upconverting[44] phosphors, and plasmon-resonant particles.[45] In general, however, the particulate labels suffer from several limitations that also have obstructed their use in routine applications. Due to their relatively large size (inner diameters usually 100 nm or higher), the particles diffuse very slowly and have an increased tendency of aggregation and nonspecific binding, resulting in slow kinetics and assay variation. In some cases, the particles may also cause sterical hindrance to the binding of biomolecules. The assay procedures using these labels are usually complicated and require, in addition to the prolonged incubation times, separate steps for incubating the antigen and label with the solid-phase antibody.

Due to the reasons described above, heterogeneous assays utilizing the particulate labels are not easily automated and are therefore presently mostly used for research purposes. However, the high-sensitivity particulate labels do have potential use in simple immunochromatographic assay formats where the diffusion distances are short and the assay kinetics little affected by the slow diffusion properties of the particles. For example, preliminary results describing the use of upconverting phosphors in lateral flow assay systems have recently been reported,[46,47] with an analytical detection limit (background + 2 SD) of 10 pg/mL hCG in a Hepes-based buffer, equal to ~0.9 IU/L hCG.[46] However, due to the limitations inherent to the labels and the assay format, the reproducibility and linearity of this and most of the current immunochromatographic assays do not yet correspond to those of the more advanced immunoassay formats.

Sufficient analytical quality of results is, however, extremely important regardless of whether the assays are performed in central laboratories or in POC conditions, although this is often overlooked in the latter case. With novel labels and assay techniques, analytical information can, however, be rapidly obtained in POC situations also without sacrificing the sensitivity and quantitativeness of the immunoassays, as shown in this study. By using the novel intrinsically fluorescent nonadentate europium chelate and the all-in-one dry reagent chemistry, we were able to diminish the number of assay steps to three, comprising the one-step incubation, washing, and direct measurement of time-resolved fluorescence from a wet well surface. Combined with the independency of the signal level both on the sample type (i.e., unprocessed whole blood versus plasma and serum) and on the amount of residual buffer per well (10−150 μL), the current assay format provides an extremely robust and rapid, yet sensitive and fully quantitative immunoassay procedure that can easily be automated. Although the assays were now run with the midsized Aio immunoanalyzer, a simpler and smaller instrument, targeted for use in rapid diagnostics for example at the physician's office, is currently under development. Further improvements of the assay platform will also include, in addition to applying it for other analytes, a further reduction in the total assay time. Although we used an incubation time of 15 min in the current study, the small reaction volumes, effective shaking, increased reaction temperature (36 °C), carefully selected antibodies, and high solid-phase binding capacity facilitated more than 80% of equilibrium to be reached in only 5 min. Therefore, the use of a 5-min incubation time in the current assay format should not significantly affect the assay performance while, however, remarkably shortening the time to results.

In conclusion, the newly synthesized 9-dentate label provided several advantages for the development of a robust and sensitive POC immunoassay format with direct surface measurement of time-resolved fluorescence. The novel label also has a high potential for adaptation to many different applications requiring spatial resolution, including immunohistochemistry, in situ hybridization, and microarray analysis.

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SUPPORTING INFORMATION AVAILABLE

The NMR and mass spectra of compounds **3**−**8**, and the UV and mass spectra of compounds **9** and **1.** This material is available free of charge via the Internet at http://pubs.acs.org.

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