Intensely Luminescent Immunoreactive Conjugates of Proteins and Dipicolinate-Based Polymeric Tb(III) Chelates

Jagannath B. Lamture[†] and Theodore G. Wensel*

Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030. Received June 27, 1994[⊗]

Partial alkylation of polylysine with 4-(iodoacetamido)-2,6-dimethylpyridine dicarboxylate (IADP), followed by exhaustive reaction with succinic anhydride, yielded polymers (PLDS, polymer of lysine, dipicolinate, and succinate) containing large numbers (50−100) of 4-substituted dipicolinic acid moieties per molecule, with the remaining lysyl side chains succinylated. Competition experiments showed that PLDS binds Tb(III) ions with much higher affinity than does EDTA and strongly enhances the visible luminescence they emit when excited with ultraviolet light. Carbodiimide-mediated coupling to proteins, including bovine serum albumin, ovalbumin, and protein A, yielded PLDS−protein conjugates whose Tb(III) chelates displayed intense green luminescence and millisecond excited state lifetimes. These conjugates retained sufficient immunoreactivity to allow their use in sensitive luminescence-based immunodetection schemes for proteins immobilized on nitrocellulose. The presence of 10 ng of ovalbumin could be easily visualized by eye when probed with rabbit anti-ovalbumin and PLDS−protein A−Tb(III). The ease of preparation of PLDS−protein−Tb(III) conjugates, and their favorable luminescence properties, make them promising reagents for use in time-resolved luminescence immunoassays and other ultrasensitive detection schemes for macromolecules.

INTRODUCTION

The use of time-gated measurements of millisecond emission from lanthanide ions to enhance background rejection and sensitivity in luminescence-based immunoassays is well established (Soini and Lovgren, 1987). The EDTA- or DTPA-based bifunctional chelating agents used to couple lanthanides such as Eu(III) to proteins, in general, do not give rise to strong sensitized emission from the bound lanthanide ions. For this reason, commercially available systems have used a multistep protocol involving dissociation of the bound ion and treatment with an enhancing reagent that does give rise to strong sensitized emission (Soini and Lovgren, 1987). In contrast to their complexes with EDTA-based chelating agents, complexes of Tb(III) or Eu(III) with chelators whose coordinating atoms are involved in conjugated π electron systems can display very strong sensitized emission. For Tb(III), one of the most efficient sensitizing chelators (Barela and Sherry, 1976) is dipicolinic acid (DPA[1]). Until recently, bifunctional reagents for coupling DPA groups to macromolecules have not been available. Recently, however, reactive 4-substituted DPA analogues have been described (Mukkala *et al.*, 1992; Lamture and Wensel, 1993), including a 4-(iodoacetamido) derivative (IADP) that readily alkylates proteins and whose synthesis is quite simple. The main drawback to this reagent is that the stability of its Tb(III) complexes is expected to be rather low (dissociation constants in the micromolar range), as observed for DPA itself (Grenthe, 1961), and because of the ability of each Tb(III) to coordinate three dipcolinates, protein conjugates derived from this reagent appear to be crosslinked by Tb(III).

In order to obtain a reagent with high affinity for Tb(III) and the ability to supply sufficient ligands within a single molecule to occupy terbium's nine coordination sites, we have developed a polymeric conjugate of IADP and polylysine that can be coupled to proteins, that binds Tb(III) ions with very high affinity, and that very efficiently enhances their luminescence. It has the added advantage that multiple luminescent Tb(III)−DPA complexes are present in each labeled protein, even if only one site on the protein is modified with the polymer, so that the molar luminescence intensity is brighter than that of conventional monomeric fluorophores.

EXPERIMENTAL PROCEDURES

Materials. Reagents for preparation of IADP were as described (Lamture and Wensel, 1993). Other reagents were obtained commercially and used without further purification: EDC and $Tb(NO_3)_3$ atomic absorption standard solution from Aldrich, *N*-hydroxysulfosuccinimide from Pierce, polylysine, proteins, antisera, and Sephadex G-25 from Sigma. All reactions with polylysine and its derivatives were carried out in siliconized microfuge tubes. Buffers used were as follows: TBS, 154 mM NaCl, 10 mM Tris·HCl, pH 7.4; borate buffer, 0.1 M potassium borate, pH 9; MOPS/citrate, 0.1 M MOPS, 0.1 M sodium citrate, adjusted to pH 7 with NaOH; bicarbonate buffer, 0.1 M $NaHCO_3$, pH 9; BLOTTO, TBS with 5% (w/v) dry milk, 0.5% (v/v) Nonidet P-40 detergent, 3 mM NaN_3.

Instrumentation. UV spectra were measured on a Hewlett-Packard HP 8452 diode array spectrophotometer. Fluorescence measurements were made on an Aminco-Bowman spectrofluorometer modified as described (Ramdas *et al.*, 1990). Lifetime measurements were carried out using an instrument based on a UV-optimized argon ion laser (to be described elsewhere (Lamture *et al.* submitted)) and similar in design to one described previously (Wensel and Meares, 1983).

* To whom correspondence should be addressed. E-mail: twensel@bcm.tmc.edu. Tel. (713) 798-6994. Fax: (713) 796-9438.

[†] Current address: Center for Biotechnology, Baylor College of Medicine, Woodlands, TX 77381.

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1994.

[1] Abbreviations: M_r, average relative molecular weight for polylysine preparation; DPA, dipicolinic acid or the dipicolinate moiety within the polymers; IADP, 4-(iodoacetamido)-2,6-dimethylpyridine dicarboxylate, PLD, product of reaction between polylysine and IADP; PLDS, succinylated PLD; MOPS, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin; EDC, 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride.

1043-1802/95/2906-0088$09.00/0 © 1995 American Chemical Society

Preparation of 4-(Iodoacetamido)-2,6-dimethylpyridine Dicarboxylate (IADP). IADP was synthesized as described previously (Lamture and Wensel 1993). Briefly, 4-amino 2,6-dimethylpyridine dicarboxylate (0.071 mmol) was mixed with iodoacetic anhydride (0.22 mmol) and a drop of concentrated H_2SO_4 at 60 °C for 30 min. The reaction mixture was then cooled in ice–water, and methanol (1 mL) was added to it slowly. After solvent removal, the crude reaction mixture was chromatographed over silica gel (1 × 20 cm), using hexane:ethyl acetate (1:1) as an eluant. After the faster moving impurities were removed the product was eluted and recovered after solvent removal in 67% yield as a white powder, which was characterized by TLC, ^1H and ^{13}C NMR, and GC–MS analysis as described (Lamture and Wensel, 1993).

Labeling of Polylysine with IADP (Synthesis of PLD/PLDS). A mixture of poly-L-lysine hydrobromide (Sigma, nominal average M_r = 26 000 or 10 000, 2–4 mg, 9.5–19 μmol, of lysyl residues) and IADP (4–12.5 mg, 10.6–33 μmol) in 0.1 M $NaHCO_3$ (1.6 mL) was stirred at 55 °C overnight to yield dipicolinic acid-modified polylysine (PLD). Modification of amino groups was monitored by the TNBS color test (Means and Feeney, 1971). To this reaction mixture was added succinic anhydride (20 mg, 200 μmol), and the mixture was stirred at room temperature for 8 h. The pH of the reaction was constantly maintained at 8.5 by 2 N NaOH throughout the course of the reaction. The product was separated from low molecular weight reactants and side products by centrifugation through Sephadex G-25 equilibrated in bicarbonate buffer. When the product was filtered through nitrocellulose using a Slot Blot apparatus, treated with $TbCl_3$, and examined under ultraviolet light, the product, but not IADP, polylysine, or succinylated polylysine, displayed intense green emission. On the basis of the TNBS test before and after modification of polylysine, it was found that more than 90% of the original amino groups were modified to yield PLDS (succinylated PLD). SDS polyacrylamide gel electrophoresis, followed by staining with $TbCl_3$ and UV illumination, resulted in a broad band of green luminescence centered at a position corresponding to an apparent M_r of about 29 000 (for reactions starting with 10 000 M_r polylysine and ~0.5 DPA groups per lysyl residue). Lanes loaded with polylysine or IADP did not display this luminescent band. The concentration of dipicolinate groups was estimated from the absorbance at 254 nm, using the molar extinction coefficient of IADP of 9629 M^{-1}·cm^{-1} at 254 nm (determined by spectrophotometry of a highly purified sample of IADP dimethyl ester after mild alkaline hydrolysis). In general, estimated yields of DPA group incorporation into PLDS derived from UV absorbance measurements agreed well with those derived from TNBS tests, so the TNBS test was used routinely to determine the average number of DPA groups per polymer, before succinylation, and UV absorbance was used to determine the concentration of polymer and DPA groups thereafter. In four different preparations tested, succinylation yields were indistinguishable from complete modification.

Competition between EDTA and PLDS or DPA. Samples for competition experiments all contained 1 μM $Tb(NO_3)_3$ and MOPS/citrate buffer, and emission was monitored at 546 nm with 278 nm excitation. To measure the exchange of Tb(III) from PLDS or DPA to EDTA, PLDS or DPA was added first at a final concentration of 2 μM DPA groups (20 nM PLDS for the preparation used, which was prepared from M_r 26 000 polylysine) and the emission measured before and after addition of 1 mM EDTA. Samples were monitored immediately after EDTA addition and several more times over a 2–3 day period, while the samples were kept at room temperature. To measure exchange of Tb(III) *from* EDTA to PLDS or DPA , EDTA was added first at various concentrations (10 μm to 1.0 mM) and the emission measured before and at various times after addition of PLDS or DPA to a final concentration of 2 μM DPA groups.

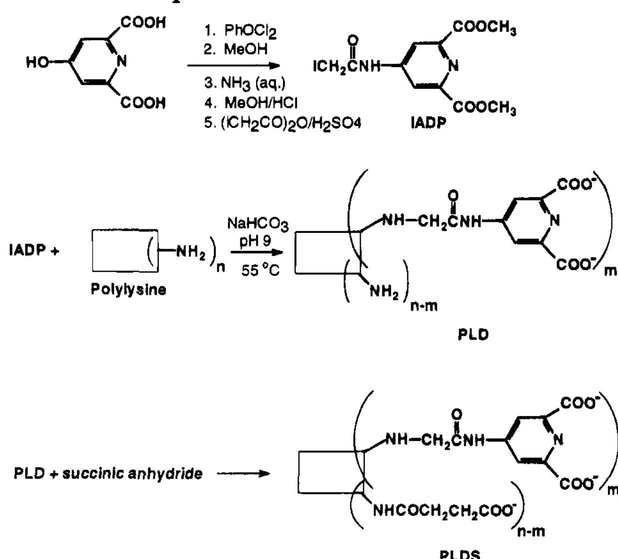
Conjugation of Proteins with PLDS. Typical reaction conditions are illustrated by the conditions for labeling bovine serum albumin (BSA): To an aqueous solution of PLDS (from M_r 10 000 polylysine, ~0.5 DPA/lysyl) in water (~4.9 nmol) was added EDC (500 μg) and *N*-hydroxysulfosuccinimide (500 μg) in a final volume of 160 μL, pH 4. After 3 min at room temperature to allow activation of PLDS, 30 μL of potassium borate buffer containing 151 μM BSA (4.5 nmol) was added and the pH was adjusted to 8.5 with NaOH. After 2 h at room temperature, the sample was analyzed by SDS polyacrylamide gel electrophoresis (Laemmli, 1970) and by immobilization on a nitrocellulose slot blot, followed by staining with $TbCl_3$. To test the stability of the Tb(III) complex, after initial visualization under UV illumination, the blots were incubated with 0.1 M EDTA, pH 8, and examined 48 h and 46 days later. Similar conditions were used to conjugate PLDS with ovalbumin, protein A, and avidin, using PLDS from M_r 26 000 polylysine, ~0.9 DPA/lysyl, and PLDS/protein molar ratios of 0.58 (ovalbumin), 1.32 (avidin), or 0.65 (protein A).

Immunodetection of proteins on blots using specific antisera and Tb–PLDS–protein conjugates. Aliquots of BSA (0.5–20 μg) were blotted onto a nitrocellulose membrane using a BioRad Bio-Dot slot-blot apparatus and blocked by a 2 h incubation in gelatin suspension. The membrane strips were washed in 2 × TBS and separately incubated overnight with rabbit BSA-specific antiserum or normal rabbit serum (both from Sigma) at a dilution of 1:80. The membranes were washed with 2 × TBS (4 × 25 mL) and incubated with a 2 × TBS solution of PLDS–BSA (350 nM, from M_r 26 000 polylysine, ~0.9 DPA/lysyl) for 1 h. Finally, the membranes were soaked in $TbCl_3$ (47 μM) for 15 min and then washed with 2 mM EDTA and visualized/photographed under UV (254 nm) light. For immunodetection of ovalbumin on blots using ovalbumin antiserum and PLDS–protein A, aliquots of ovalbumin (0.01–3 μg) were blotted onto nitrocellulose and blocked with BLOTTO (5% w/v dry milk in TBS, 0.5% NP-40, 3 mM NaN_3). They were then washed with water and 2 × TBS, followed by 4 h incubation in ovalbumin antiserum (rabbit, Sigma) at a dilution of 1:130 in 2 × TBS. The strips were washed with 2 × TBS, and incubated in a 2 × TBS solution of PLDS–protein A (380 nM, from M_r 26 000 polylysine, ~0.9 DPA/lysyl) for 30 min before addition of $TbCl_3$ (100 μM) and UV illumination.

RESULTS AND DISCUSSION

Preparation of polylysine derivatized with dipicolinate and succinate (PLDS). Reaction between IADP and polylysine (Scheme 1) was used to prepare polylysine derivatives that had multiple dipicolinic acid moieties, which were then treated with succinic anhydride to succinylate any remaining unmodified primary amine groups. The labeling yield was dependent on the molar ratios of the reactants. With a 50% molar excess of IADP over lysyl side chains, 80%–90% labeling efficiency was achieved (starting with polylysine of nominal M_r 26 000), while at a 0.5:1 ratio of IADP to lysyl side chains virtually all of the IADP reacted with the polymer

Scheme 1. Preparation of PLDS



to give a labeling efficiency of approximately 50% (starting with polylysine of nominal M_r 26 000 or 10 000). Labeling efficiency of remaining amine groups on the polymer by succinylation was generally > 90%, regardless of the stoichiometry of the IADP labeling step. The succinylation step, which was designed to increase the number of carboxylates available for subsequent coupling to proteins, appeared to improve the solubility of the polymer and also minimized problems with formation of PLDS–PLDS crosslinks during subsequent protein coupling reactions. While our initial work has used lower molecular weight polylysines to minimize problems with solubility and surface adsorbtion, preliminary experiments (Lamture and Wensel, unpublished results) suggest that higher polymers (M_r 100 000–150 000) may also be useful for preparing derivatives with higher numbers of chelating groups per molecule.

Luminescence Properties of Tb–PLDS. Addition of $TbCl_3$ to PLDS resulted in strong sensitized Tb(III) emission, with an excitation spectrum (Figure 1A) similar to that of complexes of Tb(III) with 4-acetamidodipicolinate (Lamture et al., 1995), and a typical Tb(III) emission spectrum. The excited state lifetime of Tb–PLDS was found to be approximately 1.4 ms, somewhat longer than that observed for a 1:3 complex of Tb(III) with IADP (1.25 ms) and comparable to that of a 1:3 complex of Tb(III) with 4-acetamidodipicolinate (1.4 ms; Lamture et al., 1995), suggesting that the average number of polymer atoms coordinated to each bound ion is close to 9. Although lifetime heterogeneity is to be expected as a result of heterogeneous environments for bound metal ions, the decay curve was fit reasonably well by a single exponential function, suggesting that the various Tb(III) binding sites are not terribly dissimilar. Alternatively, some sites may have much shorter lifetimes but not be observable because of their small contribution to the total emission.

Stability of Tb-PLDS. Figure 2 shows the results of experiments comparing the stability of Tb–PLDS relative to Tb complexes with EDTA and dipicolinic acid (DPA). Because the ligand exchange reactions are quite slow, the equilibria were approached from either side. First, a solution containing 2 μM DPA groups, in monomeric (DPA) or polymeric (20 nM PLDS) form, was mixed with 1 μM $Tb(NO_3)_3$ to ensure quantitative complexation of Tb. Then these complexes were challenged with 1 mM EDTA. Emission intensity at 546 nm was used to follow ligand exchange because luminescence from TbEDTA is

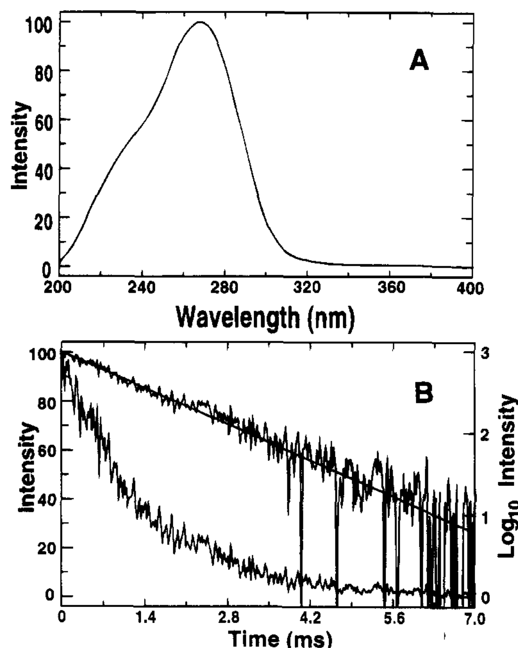


Figure 1. Luminescence of Tb–PLDS. A. Excitation spectrum (emission at 546 nm). B. Emission spectrum (excitation at 285 nm). C. Emission decay kinetics. A solution containing 50 nM Tb–PLDS was excited with ~20 ms pulses of 275 nm light from an argon ion laser and the luminescence at 546 nm monitored as a function of time after the end of the excitation pulse. Both linear and semilogarithmic plots of the same data are shown, and the theoretical curve drawn on the semilogarithmic plot corresponds to a best-fit lifetime of 1.37 ms. The polylysine used for this PLDS preparation had a nominal M_r of 10 000 and a DPA/lysyl substitution ratio of approximately 0.4.

much weaker than that from Tb–PLDS or Tb–DPA complexes; with the instrumental settings used, the Tb–EDTA signal was only a few percent above the background signal detected from buffer alone, while the Tb–PLDS and Tb–DPA signals were more than 10-fold, and more than 23-fold higher than the buffer signal, respectively (Figure 2). In the case of PLDS, the signal declined by less than 5% after 24 h (Figure 2A) or 3 days (data not shown) challenge with EDTA. In contrast, 1 mM EDTA decreased the signal from Tb–DPA to a level near background almost immediately (Figure 2B). When chelators were added to 1 μM Tb in the reverse order (i.e., EDTA before DPA or PLDS), DPA and PLDS again showed strikingly different behavior. Addition of 2 μM DPA to 10 μM EDTA and 1 μM Tb resulted in almost no increase in emission intensity (Figure 2B), even when monitored for 2 days (data not shown). When 20 nM PLDS was added to a mixture of 10 μM EDTA and 1 μM Tb, there was a slow increase in luminescence, which reached a final value nearly equal to that of the Tb–PLDS solution with no EDTA, with a half-time of about 13 min (Figure 2A). When the same experiment was carried out using 1 mM EDTA and the same final concentrations of Tb and PLDS, the luminescence increased much more slowly (half-time of 4.4 h) and reached a final value (measured 3 days later) that was 64% of that measured for Tb–PLDS without EDTA (data not shown). These results suggest that Tb–PLDS complexes are approximately 50 000 times more stable than Tb–EDTA, whose stability constant (for the fully deprotonated form of EDTA) is on the order of 10^{18} M^{-1} (Martell and Smith, 1974). In addition, the dissociation of Tb(III) from PLDS, once bound, is so slow as to be practically unmeasurable using the methods desribed here. We have observed by visual inspection under UV

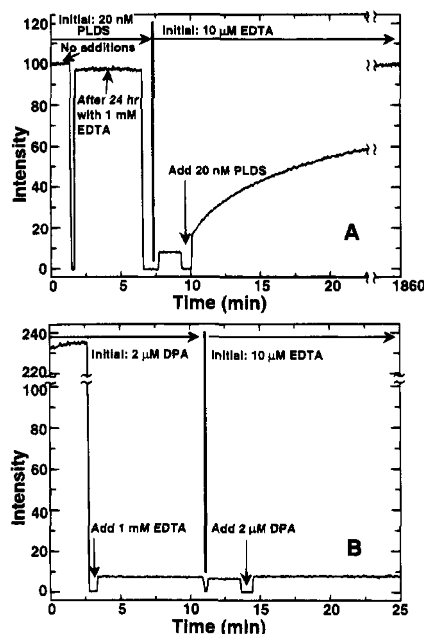


Figure 2. Competition between EDTA and PLDS or DPA. The 546 nm emission from samples excited at 278 nm was monitored continuously in samples containing 1.0 μM $Tb(NO_3)_3$, MOPS/citrate buffer, and one or both of the following added at the indicated concentrations: PLDS (20 nM, from M_r 26 000 polylysine, 2 μM DPA groups), DPA (2 μM), or EDTA (10 μm or 1.0 mM) added in the indicated order. Deflections of the traces to zero resulted from closing of the emission shutter as samples were changed or chelators added. A. Comparison of PLDS and EDTA. Emission first was collected from a sample containing 1 μM Tb and 20 nM PLDS and then from a sample that was identical, except that 1 mM EDTA had been added 24 h before the measurement shown, but after Tb and PLDS. The emission from this sample had not changed substantially when checked again 2 days later (not shown). Then a sample containing 10 μM EDTA and 1 μM Tb was monitored for a few minutes before addition of 20 nM PLDS. The increase in emission over the first 13 min after PLDS addition is shown, as well as the final value measured 2 days later. B. Comparison of DPA and PLDS. To a sample containing initially 2 μM DPA and t μM Tb was added EDTA to 1 mM and emission monitored before and after EDTA addition. Then a sample containing 10 μM EDTA and 1 μM Tb was monitored briefly before addition of DPA to 2 μM. When checked again 2 days later, the emission from this sample had not changed significantly (not shown).

illumination that Tb remains complexed with PLDS bound to nitrocellulose membranes after soaking for 11 days in 0.1 M EDTA.

Stoichiometry of Tb(III) Binding by PLDS. A typical result from titration of PLDS with standard $Tb(NO_3)_3$ is shown in Figure 3. This titration indicates a binding stoichiometry of approximately one Tb(III) per seven DPA groups on PLDS or roughly 14 Tb(III) bound, on average, to each PLDS polymer containing, in this preparation, ~100 DPA groups per polymer. Similar titrations with different preparations of PLDS gave similar results, with the number of DPA groups per bound Tb(III) varying from 3 to 7. Lower levels of DPA substitution on polylysine tended to give higher ratios of Tb:DPA groups. These results suggest that at higher levels of DPA substitution (e.g., 80%−90%) as many as half of the DPA groups attached to PLDS may not be able to bind Tb(III), perhaps as a result of steric constraints. Although precautions were taken to minimize contamination with adventitious metal ions, we cannot rule out that some chelating sites were occupied by such contaminants. Therefore, the apparent stoichiometries observed must be considered as lower limits on the number of potential Tb(III) binding sites.

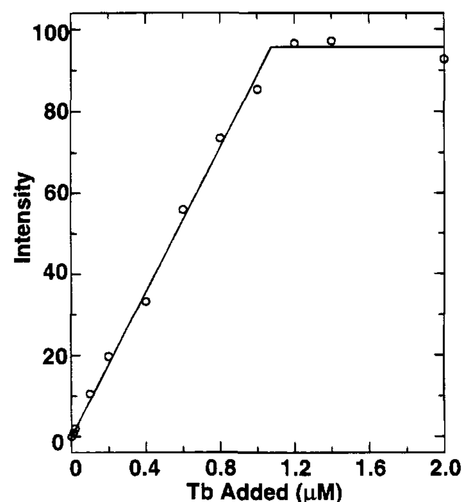


Figure 3. Titration of PLDS with $Tb(NO_3)_3$ Luminescence intensities were measured at 546 nm (excitation at 285 nm) for samples containing 75 nM PLDS (7.5 μM DPA groups, prepared from polylysine of nominal average M_r 26 000) and the indicated concentrations of $Tb(NO_3)_3$ prepared by dilution of a commercial atomic absorption standard solution.

Protein Conjugates of PLDS. Covalent coupling of PLDS to protein was achieved for bovine serum albumin (BSA), ovalbumin, protein A, avidin, and IgG. In each case, SDS−PAGE showed new bands appearing above the bands of the unlabeled proteins, and well above the PLDS bands, that showed intense green luminescence when soaked in $TbCl_3$ and illuminated with UV light. For BSA (Figure 4) and protein A, Coomassie staining showed that the majority of the protein had been converted to PLDS conjugates (and possibly some comigrating homo-oligomeric conjugates) while for avidin and ovalbumin, the yield of conjugation was relatively low (<30%). No obvious solubility problems were encountered for the BSA and protein A conjugates, but there were minor higher molecular weight species that failed to enter the stacking gel or were trapped at the top of the resolving gel. These species were positive for both sensitized Tb(III) emission and for Coomassie blue staining.

Stability of Tb(III) Complexes with PLDS−BSA. To assess the stability of Tb−PLDS−BSA, the nitrocellulose sheets with immobilized Tb−PLDS−BSA were soaked in 0.1 M EDTA. After 46 days, the luminescence from the immobilized complex was still clearly visible under UV illumination.

Immunodetection on Nitrocellulose Using Tb−PLDS−Protein Conjugates. When BSA was immobilized on nitrocellulose and probed successively with BSA antiserum, PLDS−BSA, and $TbCl_3$, bright green luminescence was observed under UV illumination (Figure 5). This detection procedure is obviously suboptimal in terms of sensitivity because of quenching by nitrocellulose, the ability of only a fraction of the bound antibodies to bind the conjugate, and the lack of time-gating. Nonetheless, this result demonstrates the feasibility of immunodetection using PLDS-labeled proteins. By using Tb−PLDS−protein A to detect antibodies in ovalbumin antiserum bound to slot-blotted ovalbumin, we were able to detect by eye as little as 10 ng of ovalbumin, a sensitivity comparable to that routinely achieved by enzyme-linked immunodetection schemes. Somewhat poorer results (~100 ng detection limit) were observed when avidin was labeled with PLDS and used in a similar assay to detect biotin-labeled goat-anti-rabbit antibodies bound to ovalbumin−antibody complexes on nitrocellulose. It will likely be possible to obtain better results

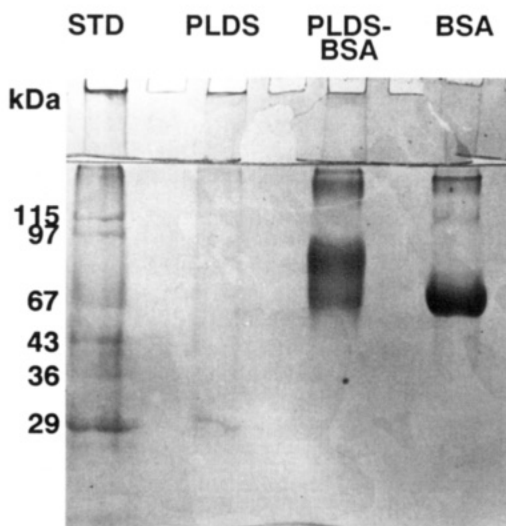


Figure 4. SDS−PAGE analysis of PLDS−BSA. Photograph of a Coomassie-Blue stained polyacrylamide (12% acrylamide) SDS gel loaded with 10 μg of BSA, before and after reaction with activated PLDS, as described in the text. Molecular weight markers are carbonic anhydrase (29 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), chicken ovalbumin (43 kDa), BSA (67 kDa), glycogen phosphorylase (97 kDa), and β-galactosidase (115 kDa). Eleven μg of PLDS was loaded in the indicated lane, but the diffuse band is only faintly visualized by the stain. The PLDS was prepared from polylysine of nominal average M_r 10 000 and contained approximately 0.5 DPA/lysyl groups.

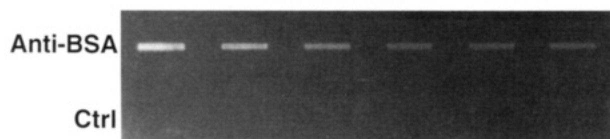


Figure 5. Detection of BSA in immunoblots using Tb−PLDS−BSA. BSA was serially diluted, and amounts ranging from 0.5 to 20 μg were blotted onto nitrocellulose under vacuum and then incubated successively with rabbit serum (either BSA antiserum, anti-BSA, or normal rabbit serum, Ctrl, as indicated), PLDS BSA (preparation of Figure 4), and $TbCl_3$ as described in the text. The nitrocellulose strips were photographed through green filters (546 nm band pass and 540 nm high pass dielectric filters) while illuminated from below by a transilluminator (305 nm).

with avidin or streptavidin by increasing reactant concentrations sufficiently to improve the labeling yield and by protecting lysines essential for biotin binding during the labeling reaction.

SUMMARY

The results reported here demonstrate that Tb(III) complexes with PLDS can be readily prepared and covalently coupled to proteins in ways that preserve binding activities essential for immunochemistry. Because these conjugates display emission intensities comparable to those of commonly used fluorophores, and offer the added advantage of background rejection through time-gating, they represent a class of reagents with great promise for ultrasensitive immunoassays and other techniques for detection of specific macromolecules.

ACKNOWLEDGMENT

This work was supported by the Welch Foundation, the Whitaker Foundation, and the Texas Advanced Technology Program.

LITERATURE CITED

Barela, T. D.; Sherry, A. D. (1976) A simple, one-step fluorometric method for determination of nanomolar concentrations of terbium. *Anal. Biochem. 76*, 351−357.

Grenthe, I. (1961) Stability relationships among the rare earth dipicolinates. *J. Am. Chem. Soc. 83*, 360−364.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680−685.

Lamture, J. B.; Wensel, T. G. (1993) A novel reagent for labeling macromolecules with intensely luminscent lanthanide complexes. *Tetrahedron Lett. 34*, 4141−4144.

Lamture, J. B., Zhou, Z., Suresh Kumar, A., and Wensel, T. G. (1995). Luminescence properties of Tb(III) complexes with 4-substituted dipicolinic acid analogues. *Inorg. Chem.*, in press.

Martell, A. E., and Smith, R. M. (1974) *Critical Stability Constants*, Vol. 1, p 205, Plenum Press, New York.

Means, G. E., and Feeney, R. E. (1971) *Chemical Modification of Proteins*, p 217, Holden-Day, Inc., San Francisco.

Mukkala, V.-M.; Sund, C. Kwiatkowski, N.; Pasanen, P.; Hogberg, M.; Kankare, J. (1992) New heteroaromatic complexing agents and luminescence of their Europium(III) and Terbium(III) chelates. *Helv. Chim. Acta 75*, 1621−1632.

Ramdas, L.; Disher, R. M.; Wensel, T. G. (1991) Nucleotide exchange and cGMP phosphodiesterase activation by pertussis toxin-inactivated transducin. *Biochemistry 30*, 11637−11645.

Soini, E.; Lovgren, T. (1987) Time-resolved fluorescence of lanthanide probes and applications in biotechnology. *CRC Crit. Rev. Anal. Chem. 18*, 105−154.

Wensel, T. G.; Meares, C. F. (1983) Electrostatic properties of myoglobin probed by diffusion-enhanced energy transfer. *Biochemistry 22*, 6247−6284.

BC940091N