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Spectrophotometric determination and removal of unchelated europium ions from solutions containing Eu-diethylenetriaminepentaacetic acid chelate–peptide conjugates

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ABSTRACT

Europium chelates conjugated with peptide ligands are routinely used as probes for conducting in vitro binding experiments. The presence of unchelated Eu ions in these formulations gives high background luminescence and can lead to poor results in binding assays. In our experience, the reported methods for purification of these probes do not achieve adequate removal of unchelated metal ions in a reliable manner. In this work, a xylenol orange-based assay for the quantification of unchelated metal ions was streamlined and used to determine levels of metal ion contamination as well as the success of metal ion removal on attempted purification. We compared the use of Empore chelating disks and Chelex 100 resin for the selective removal of unchelated Eu ions from several Eu-diethylenetriaminepentaacetic acid chelate–peptide conjugates. Both purification methods gave complete and selective removal of the contaminant metal ions. However, Empore chelating disks were found to give much higher recoveries of the probes under the conditions used. Related to the issue of probe recovery, we also describe a significantly more efficient method for the synthesis of one such probe using Rink amide AM resin in place of Tentagel S resin.

Interactions between lanthanides and various multidentate chelates have been studied extensively because of the utility of these complexes in biology and medicine [1–5]. Due to their unique properties [5], luminescent lanthanide chelates are versatile nonradiolabeled alternatives to organic fluorophores, particularly in applications where background autofluorescence is a significant problem [6]. For example, the europium diethylenetriaminepentaacetic acid (DTPA)[1] chelated probe Eu-DTPA-PEGO-MSH(7)-NH_2 and similar compounds have been routinely used to conduct in vitro binding experiments, where the time-resolved fluorescence from the lanthanide provides the readout in the assay [3].

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[1] *Abbreviations used:* DTPA, diethylenetriaminepentaacetic acid; PEGO, 19-amino-5-oxo-3,10,13,16-tetraoxa-6-azanonadecan-1-oic acid; MSH(7), Ser-Nle-Glu-His-DPhe-Arg-Trp; TFA, trifluoroacetic acid; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DCM, dichloromethane; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; DMF, *N,N*-dimethylformamide; HPLC, high-performance liquid chromatography; UV, ultraviolet; ESI, electrospray ionization; FT–ICR, Fourier transform ion cyclotron resonance; Fmoc, 9-fluorenylmethyoxycarbonyl; HOBt, 1-hydroxybenzotriazole; DIC, diisopropyl carbodiimide; RT, room temperature; LRMS, low-resolution mass spectrometry; HRMS, high-resolution mass spectrometry; CCK(4), Trp-Nle-Asp-Phe.

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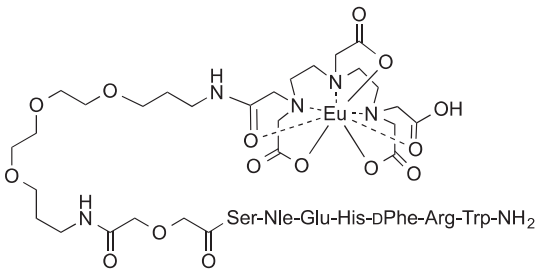
However, the presence of unchelated Eu ions in formulations of probes such as Eu-DTPA-PEGO-MSH(7)-NH_2 can result in high background fluorescence and can lead to poor results in binding assays by overshadowing specific binding of the probe to targeted receptors (see Fig. 1).



Eu-DTPA-PEGO-MSH(7)-NH_2

Probes such as Eu-DTPA-PEGO-MSH(7)-NH_2 are generally assembled using solid-phase peptide synthesis to first make the metal-free peptide bearing an N-terminal DTPA unit. Although it would greatly simplify the purification process, the lanthanide

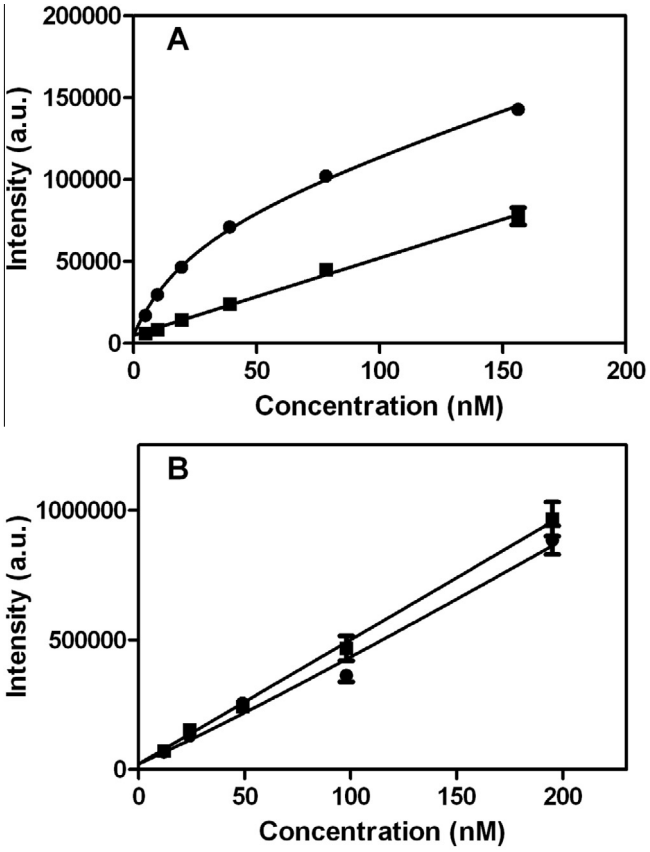


Fig.1. Saturation binding curves for Eu-DTPA-PEGO-MSH(7)-NH_2 using human embryonic kidney 293 cells expressing human melanocortin 4 receptor/cholecystokinin receptor subtype 2 (hMC4R/CCK2R). Total binding (●) and nonspecific binding (■) are shown. (A) Previously reported [3] binding data using a formulation of this probe with an undetectable level of unchelated Eu ion contamination. (B) Binding data observed for a formulation of this probe contaminated with 0.32 eq of unchelated Eu ion showing fluorescence intensity that is one order of magnitude higher.

cannot be introduced while the DTPA unit is on the solid support because Ln-DTPA chelates are acid labile and will not survive exposure to trifluoroacetic acid (TFA) during the cleavage step. Hence, the desired metal is usually introduced after cleavage of the peptide from the resin and purification, using an excess of a metal halide (1.2–3.0 eq). In our hands, purification using the commonly employed technique of reversed-phase chromatography [3,6] did not achieve adequate removal of unchelated metal ion contaminants. Recent literature describes the removal of excess metal ions from DTPA and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelates by means of "passive" removal techniques such as size-exclusion chromatography and dialysis [7,8]. Alternatively, "active" removal of excess metal ions has recently been demonstrated using Chelex 100 resin for a nonpeptide compound that contained a DTPA chelate [9]. However, this method has not been validated with metal chelates linked to peptides. We are unaware of any other well-detailed published methods with the potential to remove unchelated Eu ions in a reliable manner while leaving the Eu-DTPA chelate unaffected. Because it is very common to deal with milligram quantities of probes such as Eu-DTPA-PEGO-MSH(7)-NH_2, efficiency (i.e., percentage recovery) of the method is a major concern. Recovery can greatly diminish if multiple purification and/or transfer steps are necessary. We have developed and report here an active method that removes unchelated lanthanide ion contamination quickly, completely, and conveniently.

Materials and methods

Dichloromethane (DCM) and tetrahydrofuran (THF) were dried by passage through activated alumina. Dimethyl sulfoxide (DMSO) and *N,N*-dimethylformamide (DMF) were dried by contact with activated 4-Å molecular sieves, followed by distillation under reduced pressure. All other reagents were used as supplied. Preparative scale reversed-phase high-performance liquid chromatography (HPLC) was performed using a 19 × 250-mm Waters XBridge 10-μm OBD C_{18} preparative HPLC column. A linear gradient of mobile phase was used over 45 min from 0 to 90% acetonitrile/water containing 0.1% TFA. The flow rate was 10 ml/min, and a dual channel ultraviolet (UV) detector was used at 230 and 280 nm. Analytical HPLC for the Eu-labeled samples was performed on a 3.0 × 150-mm Waters XBridge 3.5-μm C_{18} analytical HPLC column. A linear gradient of mobile phase was used over 30 min from 10 to 90% acetonitrile/triethylammonium acetate buffer (pH 6.0). The flow rate was 0.3 ml/min, and a dual channel UV detector was used at 220 and 280 nm. Empore chelating disks (47 mm, 3M) or Chelex 100 resin (analytical grade, Bio-Rad Laboratories) were used in the Eu ion removal process. A Beckman Φ 350 pH/temperature/mV meter equipped with a FUTURA refillable combination pH electrode was used for pH measurements. UV–visible spectra were recorded on a UV-2401PC UV–visible spectrophotometer (Shimadzu). Electrospray ionization (ESI) mass measurements were performed on a Bruker 9.4 T Apex-Qh hybrid Fourier transform ion cyclotron resonance (FT–ICR) instrument using standard ESI conditions. The samples were dissolved in acetonitrile/water (1:1) containing 0.1% formic acid in a concentration range of 1 to 30 μM. Time-resolved fluorescence was measured using a VICTOR X4 2030 Multilabel Reader (PerkinElmer) employing the standard Eu time-resolved fluorescence measurement settings (340 nm excitation, 400 μs delay, and emission collection for 400 μs at 615 nm).

Data analysis

Data analysis was done using GraphPad Prism software (version 5.04) with linear regression analysis to construct the calibration curves. Saturation binding data were analyzed using nonlinear regression analysis and fitted to classic one-site total binding and nonspecific binding equations.

Synthesis of DTPA-PEGO-MSH(7)-NH_2

DTPA-PEGO-MSH(7)-NH_2 was synthesized manually via an N^{α}-Fmoc solid-phase peptide synthesis strategy (Scheme 1) using a modified version of the previously published procedure [3]. Instead of Tentagel S resin (0.24 mmol/g loading) used in the previous work, Rink amide AM resin (200–400 mesh, 0.68 mmol/g loading) was used. Resin (1 g) was allowed to swell in THF for 1 h in a polypropylene syringe equipped with a polypropylene frit. THF was removed, a solution of 20% piperidine in DMF (15 ml) was added, and the tube was shaken for 2 min. This solution was removed, 20% piperidine in DMF (15 ml) was again added, and the mixture was shaken for another 18 min. After the removal of the solution, the resin was washed with DMF (3 × 15 ml), DCM (3 × 15 ml), DMF (3 × 15 ml), 0.5 M 1-hydroxybenzotriazole (HOBt) in DMF (15 ml), 0.5 M HOBt in DMF + a drop of 0.01 M bromophenol blue solution in DMF (15 ml), DMF (2 × 15 ml), and DCM (15 ml) in that order. The amino acid (3 eq, 2.04 mmol) to be coupled was activated by reaction in DMF (15 ml) in a glass vial with Cl–HOBt (345 mg, 2.04 mmol) and diisopropyl carbodiimide (DIC, 512 mg, 4.08 mmol) over 2 min. This solution was then added to the resin, and the syringe was shaken for 1 h. The coupling solution was removed, and the resin was washed with DMF (3 × 15 ml), DCM