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Spectrophotometric Determination and Removal of Unchelated Europium Ions from Solutions Containing Eu-Diethylenetriaminepentaacetic Acid Chelate-Peptide Conjugates¹

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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 upon attempted purification. We compared the use of EmporeTM 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, EmporeTM chelating disks were found to give much higher recoveries of the probes under the conditions utilized. 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.

Keywords

Europium; Chelate; Xylenol orange; EmporeTM; Chelex[®]

Introductory Statement

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

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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 (DTPA²) chelated probe Eu-DTPA-PEGO-MSH(7)-NH₂ 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]. However, the presence of unchelated Eu ions in formulations of probes such as Eu-DTPA-PEGO-MSH(7)-NH₂ 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 Figure 1).

Probes like Eu-DTPA-PEGO-MSH(7)-NH₂ 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 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 TFA during the cleavage step. Hence, the desired metal is usually introduced after cleavage of the peptide from the resin and purification, utilizing an excess of a metal halide (1.2–3 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 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 non-peptide 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. Since it is very common to deal with milligram quantities of probes such as Eu-DTPA-PEGO-MSH(7)-NH₂, efficiency

²Abbreviations used: CCK2R, Cholecystokinin receptor subtype 2; CCK(4), Trp-Nle-Asp-Phe; Cl-HOBt, 6-chloro-1-hydroxybenzotriazole; DCM, Dichloromethane; DIC, Diisopropyl carbodiimide; DMF, N,N-dimethylformamide; DMSO, Dimethylsulfoxide; DOTA, 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; ESI, Electrospray ionization; Fmoc, 9-fluorenylmethoxycarbonyl; FT-ICR, Fourier transform ion cyclotron resonance; HEK, Human embryonic kidney; hMC4R, Human melanocortin 4 receptor; HOBt, 1-hydroxybenzotriazole; HPLC, High performance liquid chromatography; HRMS, High resolution mass spectrometry; LRMS, Low resolution mass spectrometry; MSH(7), Ser-Nle-Glu-His-DPhe-Arg-Trp; PEGO, 19-amino-5-oxo-3,10,13,16-tetraoxa-6-azanonadecan-1-oic acid; THF, Tetrahydrofuran; TFA, Trifluoroacetic acid.

(i.e. the 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 herein 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. Dimethylsulfoxide (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 HPLC was performed using a 19 × 250 mm Waters XBridge™ 10 µm OBD™ C₁₈ preparative HPLC column. A linear gradient of mobile phase was used over 45 min from 0–90% acetonitrile/water containing 0.1% TFA. The flow rate was 10 mL/min, and a dual channel 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 C₁₈ analytical HPLC column. A linear gradient of mobile phase was used over 30 min from 10–90% 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 company) 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 Corporation). 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 1–30 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)-NH₂

DTPA-PEGO-MSH(7)-NH₂ 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 shaken for 2 min. This solution was removed, 20% piperidine in DMF (15 mL) was

again added, and the mixture shaken for another 18 min. After the removal of the solution, the resin was washed sequentially with DMF (3 × 15 mL), DCM (3 × 15 mL), DMF (3 × 15 mL), 0.5 M 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 DIC (512 mg, 4.08 mmol) over two minutes. This solution was then added to the resin, and the syringe shaken for 1 h. The coupling solution was removed and the resin was washed with DMF (3 × 15 mL), DCM (3 × 15 mL), and DMF (3 × 15 mL). Free amine groups were capped by shaking the resin with acetic anhydride/pyridine (6 mL, 1:1) for 20 min. The resin was washed again with DMF (3 × 15 mL), DCM (3 × 15 mL), and DMF (3 × 15 mL). The coupling cycle was then repeated for each of the remaining amino acids in the sequence. The Kaiser test was used to determine coupling completion at each attachment step. The PEGO linker [10] was then attached following the same coupling cycle, with the activation of Fmoc-PEGO-OH (2.04 mmol in 4.1 mL of DCM) with Cl-HOBt (345 mg, 2.04 mmol) and DIC (512 mg, 4.08 mmol) in DMF (10 mL).

One fourth of the resin made above (0.17 mmol) was placed in a syringe reactor, and 20% piperidine in DMF (4 mL) was added. The syringe was shaken for 2 min, and the solution removed. Further 20% piperidine in DMF (4 mL) was added, and the syringe shaken for 18 min. The resin was then washed thoroughly with dry DMSO (6 × 4 mL). DTPA dianhydride (620 mg, 1.7 mmol, 10 eq) and HOBt monohydrate (520 mg, 3.4 mmol, 20 eq) were placed in a capped vial with dry DMSO (5 mL). This suspension was heated at 70 °C for 5 min. The suspension cleared, and the solution was stirred for a further 15 min at rt. This solution was then taken into the syringe reactor and shaken for 1 h. The resin was then washed (5 min shaking for each wash) with DMSO (2 × 4 mL), THF (2 × 4 mL), 20% aqueous THF (4 mL), 5% diisopropylethylamine in THF (4 mL), THF (3 × 4 mL), and DCM (4 mL) in that order.

A cleavage cocktail (3 mL) consisting of TFA, thioanisole, triisopropylsilane, and H₂O (9:1:0.3:0.3) was injected into the syringe reactor, which was shaken for 4 h at rt. The solution was then collected into a 15 mL centrifuge tube and the resin washed for 2 min with further aliquots of TFA (2 × 2 mL). The combined TFA solutions were concentrated in the centrifuge tube under a stream of argon, and the product precipitated by the addition of cold ether (8 mL). The tube was centrifuged and the supernatant removed. The pellet was washed with cold ether (3 × 6 mL), air dried, dissolved in 1 M acetic acid, and lyophilized. The resultant solid was subjected to reversed-phase HPLC, product-containing fractions combined, and lyophilized to give 174.6 mg (62% yield) of the product DTPA-PEGO-MSH(7)-NH₂ as a fluffy white solid. LRMS (ESI) m/z calculated for C₇₄H₁₁₂N₁₉O₂₅ [M + H]⁺ 1666.8, observed 1666.5; calculated for C₇₄H₁₁₃N₁₉O₂₅ [M + 2H]²⁺ 833.9, observed 833.9.

Synthesis of Eu-DTPA-PEGO-MSH(7)-NH₂

The metal-free precursor DTPA-PEGO-MSH(7)-NH₂ (11.0 mg, 6.60 μmol) was dissolved in 0.1 M ammonium acetate solution at a concentration of 1 mg/mL. The pH was then adjusted

to 7–8 with aqueous 0.1 M NH_4OH , and $\text{EuCl}_3 \cdot 6 \text{H}_2\text{O}$ in water (353.6 μL of a 0.022 M solution, 7.92 μmol , 1.2 eq) was added. The reaction mixture was stirred at rt overnight. Salts were removed using a Sep-Pak[®] C_{18} reverse-phase (500 mg) column as previously reported [3, 6]. The product-containing fractions were concentrated and lyophilized to obtain the product Eu-DTPA-PEGO-MSH(7)- NH_2 as a fluffy white solid (9.1 mg) in 76% yield. HRMS (FT-ICR) m/z calculated for $\text{C}_{74}\text{H}_{110}\text{N}_{19}\text{O}_{25}^{153}\text{Eu} [\text{M}+2\text{H}]^{2+}$ 908.8567, observed 908.8503; analytical HPLC, t_R = 12.2 min.

Preparation of acetate buffer (pH = 5.81)

To a beaker of distilled water (800 mL) was added concentrated acetic acid (2.87 mL). The pH was adjusted to 5.81 with a solution of 1 M sodium hydroxide. The volume was then brought up to 1 L using distilled water.

Preparation of xylenol orange solution

The tetrasodium salt of xylenol orange (8 mg, 10.5 μmol) was dissolved in acetate buffer (250 mL) to yield an orange-colored solution. The UV/visible spectrum (350 – 700 nm) was recorded for this solution for comparison purposes. The solution was then frozen in 20 mL aliquots and stored at -20°C until needed. This solution was stable for at least three months, as previously reported [11].

Preparation of the standard curve for the Eu-xylenol orange complex

A series of Eu ion solutions (range 50–400 μM) were prepared using an atomic absorption standard solution of europium(III) nitrate (970 ppm) diluted into acetate buffer. Xylenol orange solution (950 μL) was added to a semi-micro cuvette, followed by 50 μL of a Eu ion solution. The mixture was shaken vigorously, and the absorbance of the solution was read. A calibration curve was plotted using the absorbance at 573 nm (corrected by subtracting the absorbance of an acetate buffer blank) versus Eu ion concentration.

Spectrophotometric determination of unchelated europium ion concentration

The Eu-DTPA-PEGO-peptide conjugates analyzed in this work were first dissolved in DMSO and/or water at a peptide concentration of 1–2 mM. To quantify the unbound Eu ion content of a given solution, 50 μL of the solution was mixed with the xylenol orange solution (950 μL) in a cuvette and the absorbance at 573 nm read. The standard curve was then used to calculate the concentration of unchelated Eu ion in the solution.

Use of Empore[™] chelating disks for removal of unchelated europium ions

An Empore[™] chelating disk (47 mm) was placed on a sintered glass filter holder (47 mm) base fitted to a 1 L filter flask. The disk was wetted with 2–5 mL of distilled water, as per the manufacturer's instructions. This resulted in swelling of the disk. The solvent reservoir was then clamped onto the base, completing the apparatus. Nitric acid (3 M, 20 mL) was added, the disk allowed to soak in this solution for 1 min, then a vacuum was applied, drawing the acid solution through the filter. The disk was washed on the filter under vacuum with water. The disk was allowed to go dry between each wash ($2 \times 50 \text{ mL}$). To put the disk in its most active ammonium form, 100 mL of 0.1 M ammonium acetate buffer (pH 5.3,

made up in HPLC grade water) was added to the solvent reservoir and the disk allowed to soak for 1 min. The solution was then drawn through the disk by applying a vacuum, and the disk left to dry for 5–20 min before sample loading. During this time, the apparatus was disassembled, cleaned thoroughly with distilled water followed by HPLC grade water, and the apparatus reassembled for use.

The peptide sample (10–14 mg) in water (3 mL/mg, pH 5 to 7) was passed through the disk at a flow rate of 30–40 mL/min to allow maximum binding interactions. The disk was then washed with water (50 mL) and acetonitrile/water (20:80, 25 mL). The combined filtrates were transferred to a round bottom flask and lyophilized to give the purified product in quantitative recovery. The product was analyzed by HRMS and analytical HPLC. The success of unchelated Eu ion removal was assessed by comparison of the results of xylenol orange assays before and after the passage of the sample through the disk.

Use of Chelex® 100 resin for removal of unchelated europium ions

Chelex® 100 resin (analytical grade, 2 g) was placed in a polypropylene syringe equipped with a polypropylene frit. The resin was washed with 1 M hydrochloric acid (15 mL, syringe shaken for 5 min), followed by water (2 × 15 mL, syringe shaken for 5 min). To put the resin in its most active ammonium form, 15 mL of 0.1 M ammonium acetate buffer (pH 5.3) was added and the syringe was shaken for 20 min. After removal of the ammonium acetate solution, the peptide sample (4–6 mg) in water (3 mL/mg, pH 5 to 7) was then loaded into the syringe and the syringe was shaken for 1 h. The solution was then collected in a round bottom flask and the resin washed with 20/80 acetonitrile/water (5 mL, syringe shaken for 5 min), and water (5 mL, syringe shaken for 5 min). The solution and washings were combined, concentrated, and lyophilized, giving the purified product in <30% recovery. The product was analyzed by HRMS and analytical HPLC. The success of unchelated Eu ion removal was assessed using xylenol orange assays as previously described.

Results and Discussion

Given the low yield (9%) previously reported [3] for the synthesis of Eu-DTPA-PEGO-MSH(7)-NH₂, an approach leading to a higher synthetic efficiency was devised, utilizing a different resin for solid-phase peptide synthesis (see Scheme 1). The metal-free peptide precursor DTPA-PEGO-MSH(7)-NH₂ was obtained in 64% yield following HPLC purification, and was characterized by mass spectrometry. Europium(III) was then introduced using the previously published procedures, affording Eu-DTPA-PEGO-MSH(7)-NH₂ in good yield (76%) and purity based upon mass spectral and analytical HPLC analyses. However, unchelated Eu ion is not detectable by either of these characterization methods, so an assay based on xylenol orange chelation was developed and employed.

Xylenol orange has long been the indicator of choice for detection of polyvalent metal ions [12,13]. The fact that metal complexes with xylenol orange have stability constants (log K ~ 5) far lower than DTPA-metal complexes (log K > 20) ensures that unchelated metal ions can be detected selectively in the presence of a DTPA-metal chelate [14,15]. Xylenol orange has two absorption maxima in its UV-visible spectrum, at 433 and 573 nm (Figure 2). In the presence of lanthanide ions, the xylenol orange solution undergoes a color change from

orange to violet that can be visually detected at concentrations higher than 50 μM . Based on this knowledge, Barge et al. published a method for quantification of unchelated Gd ions in solution with Gd chelates. The ratio of the Gd-xyleneol orange complex absorbances at 573 and 433 nm was used to construct a standard curve [11]. However, in adapting this method for use with Eu-DTPA chelates, we observed that a plot of absorbance at 573 nm versus metal ion concentration afforded better linearity over the concentration range 50–400 μM than the reported ratiometric method (see Figure 3).

Prior to evaluating possible purification methods, it was necessary to gauge the concentrations of unchelated Eu ions present in test samples from independent syntheses of Eu-DTPA-PEGO-MSH(7)-NH₂ and Eu-DTPA-PEGO-CCK(4)-NH₂ [Elshan and Mash, unpublished synthesis]. A previously reported Gd-DOTA chelate that had been purified by normal phase column chromatography [16] was used as a negative control to validate our assay protocol. Using the xylenol orange spectrophotometric assay for determination of unchelated Eu ion concentration described in the Experimental Section, the control Gd-DOTA chelate did not exhibit a detectable level of unchelated Gd ions (i.e., no increase in absorbance at 573 compared to the reagent blank).

Using the xylenol orange assay, simple visual observation confirmed the presence of concentrations of unchelated Eu ions higher than 50 μM in all the test samples. The unchelated Eu ion concentrations were quantitatively determined using standard curve A,

and the percentages of unchelated Eu ion (out of the total Eu content) were calculated (Table 1).

Having recognized unchelated Eu ion contamination as a problem during the synthesis of probe Eu-DTPA-PEGO-MSH(7)-NH₂, we experimented with the use of different amounts of EuCl₃ in the chelation reaction. Decreasing the amount of EuCl₃ from 3 equivalents to 1.2 equivalents did not significantly decrease the amount of free Eu after purification of the probe as described [3] by passage through a Sep-Pak® C₁₈ column. Increasing the number of water washes during the purification step on the Sep-Pak® C₁₈ column and/or preparative reversed-phase HPLC under buffered conditions (pH 6) to prevent detachment of the DTPA-chelated Eu failed to satisfactorily remove the unchelated Eu ions. We presume that, in addition to DTPA chelation, Eu ions must also associate with PEGO and/or the peptide components of the probe. Given these results, a purification technique that actively removes unchelated Eu ions without disrupting the Eu-DTPA chelate was required.

Two methods for removal of unchelated Eu ions were evaluated: (a) filtration through Empore™ chelating disks, and (b) shaking with Chelex® 100 resin. Both of these commercially available products employ iminodiacetate functionalities on a solid support and are capable of actively binding divalent and trivalent metal cations. A comparative study of the thermodynamic and kinetic properties of metal sorption onto these materials was recently reported by Alberti et al. [17]. While the thermodynamic properties of metal sorption were not significantly different for the metals studied in their work, some metals were found to have slower kinetics of sorption to the chelating disks. Lanthanides were not included in this study, making predictions about Eu ion sorption difficult.

In the event, purification of both Eu-DTPA-PEGO-MSH(7)-NH₂ and Eu-DTPA-PEGO-CCK(4)-NH₂ probes using filtration through Empore™ chelating disks yielded quantitative recoveries with no apparent extrusion of the metal chelated to DTPA (as evidenced by the absence of metal-free DTPA-peptide in the mass spectra). Analysis of the purified conjugates using the xylenol orange assay confirmed, to the limit of detection (ca 10 µM), the removal of unchelated Eu ions. Shaking of contaminated samples of Eu-DTPA-PEGO-MSH(7)-NH₂ with Chelex® 100 resin also removed unchelated Eu ions, but gave a much lower recovery of the probe (<30%). We operated well below the manufacturer-specified capacities for the chelation disks (breakthrough capacity per disk = 0.30 mmol Cu²⁺), and the resin (wet capacity = 0.26 meq/g) to ensure complete removal of unchelated Eu ions.

In conclusion, the preparation of probe Eu-DTPA-PEGO-MSH(7)-NH₂ by the methods described herein gave an overall yield of 47%, a significant improvement over the 9% yield achieved previously [3]. Use of Empore™ chelating disks provides an efficient method for the selective removal of unchelated Eu ions, which may be detected using a modified xylenol orange-based spectrophotometric assay. These methods should find general use in the preparation of water-soluble metal chelate-peptide conjugates.

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Figure 1.

Saturation binding curves for Eu-DTPA-PEGO-MSH(7)-NH₂ using HEK293 cells expressing hMC4R/CCK2R. Total binding (●). Non-specific binding (■). (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 equivalents of unchelated Eu ion showing fluorescence intensity that is one order of magnitude higher.

Figure 2. Absorption spectrum for xylenol orange (2 μ M in acetate buffer, pH 5.81). λ_{max} 433 and 573 nm.

Figure 3. Calibration curves for the Eu-xylene orange complex. (A) Using the absorbance at 573 nm ($R^2 = 0.9998$); (B) Using the absorbance ratio 573 nm/433 nm ($R^2 = 0.9794$).

Scheme 1.

Synthesis of DTPA-PEGO-MSH(7)-NH₂ using an N^α-Fmoc solid-phase peptide synthesis strategy.

Table 1
Determination of unchelated europium ion concentrations in Eu-DTPA-PEGO-peptide conjugate preparations.

Chelate-Peptide Conjugate		Unchelated Europium		
Structure ^{a,b}	Sample	Concentration ^c (μM)	Absorbance (573 nm)	% of Total Eu
Eu-DTPA-PEGO-MSH(7)-NH ₂	1	1100	0.56	24
	2	1300	1.16	36
	3	1300	0.62 ^e	37
Eu-DTPA-PEGO-CCK(4)-NH ₂	4	1700	0.99 ^e	42
	5	1700	0.50 ^f	42

^aThe peptide MSH(7) is Ser-Nle-Glu-His-DPhe-Arg-Trp. The peptide CCK(4) is Trp-Met-Asp-Phe.
^bChelates prepared using 2.0 equivalents of EuCl₃.
^cConcentrations of the chelate-peptide conjugates were determined using analytical HPLC by comparison with a co-injected standard (0.50 mM) solution of D-tryptophan.
^dCalibration curve A was used in these determinations.
^eChelate-peptide conjugate solution diluted by a factor of two for analysis.
^fChelate-peptide conjugate solution diluted by a factor of four for analysis.