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Solid-Phase Synthesis of Europium-Labeled Human INSL3 as a Novel Probe for the Study of Ligand−Receptor Interactions

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An efficient solid-phase synthesis protocol has been developed which, together with regioselective sequential formation of the three disulfide bonds, enabled the preparation of specifically monolanthanide (europium)-labeled human insulin-like peptide 3 (INSL3) for the study of its interaction with its G-protein-coupled receptor, RXFP2, via time-resolved fluorometry. A commercially available chelator, diethylene triamine pentaacetic acid (DTPA), was coupled to the N-terminus of the INSL3 A-chain on the solid phase, and then a coordination complex between europium ion and DTPA was formed using $EuCl_3$ to protect the chelator from production of an unidentified adduct during subsequent combination of the A- and B-chains. The labeled peptide was purified in high yield using high-performance liquid chromatography with nearly neutral pH buffers to prevent the liberation of Eu^{3+} from the chelator. Using time-resolved fluorometry, saturation binding assays were undertaken to determine the binding affinity (pK_d) of labeled INSL3 for RXFP2 in HEK-293T cells stably expressing RXFP2. The dissociation constant of DTPA-labeled INSL3 (9.05 ± 0.03, $n = 3$) that was obtained from saturation binding experiments was comparable to that of ^{125}I-labeled INSL3 (9.59 ± 0.09, $n = 3$). The receptor binding affinity (pK_i) of human INSL3 was determined to be 9.27 ± 0.06, $n = 3$, using Eu-DTPA-INSL3 as a labeled ligand, which again is similar to that obtained when ^{125}I-INSL3 was used as labeled ligand (9.34 ± 0.02, $n = 4$). This novel lanthanide-coordinated, DTPA-labeled INSL3 has excellent sensitivity, stability, and high specific activity, properties that will be particularly beneficial in high-throughput screening of INSL3 analogues in structure−activity studies.

Insulin-like peptide 3 (INSL3), also referred to as Leydig-insulin-like (Ley-I-L) and relaxin-like factor (RLF) (*1*) is a circulating peptide hormone belonging to the relaxin-insulin superfamily of peptides. The heterodimeric peptide hormone INSL3 consists of two chains (A and B) connected via three disulfide bonds (*1–3*). INSL3 and its G-protein coupled receptor (GPCR), RXFP2 (also known as LGR8), have been shown to play important roles in the reproductive system including testicular descent during fetal development as a result of INSL3-mediated gubernacular growth (*4–6*) and meiotic progression of arrested oocytes in the female and suppression of male germ cell apoptosis (*7*). Additionally, recent studies have shown the pathological involvement of INSL3 in certain cancers, as its expression is up-regulated in human thyroid carcinoma, neoplastic Leydig cells, and mammary epithelial cells (*8*).

Recent structure−activity relationship studies have provided considerable insight into the molecular interaction of INSL3 with its receptor associated with reproductive processes. The rapidly emerging physiological and pathological importance of INSL3 has led to efforts to develop antagonists which prevent binding and signaling of INSL3. The structure-based development and discovery of such antagonists requires the design and synthesis of various analogues. In order to test these analogues for their receptor binding affinity, radioactive ^{125}I-labeled INSL3 has typically been employed. However, the use of this peptide has drawbacks including its short half-life, high cost, and the need for its preparation immediately prior to use. Additionally, there are safety concerns associated with the use of radionuclides. To overcome these problems, we undertook the development of efficient methods for the preparation of an INSL3 labeled with a lanthanide for evaluation and use in a time-resolved fluorescence receptor binding assay.

The development of such lanthanide-based assays to study ligand−receptor interactions provides an attractive alternative to the traditional radionuclide-based assays. Due to their unique fluorescent properties, chelates of specific trivalent lanthanide ions (Eu^{3+}, Tb^{3+}, and Sm^{3+}) have been coupled to molecules of biological interest and the resultant labeled molecules used as probes in various biological assays such as kinase assays (*9*), immunofluorometric detection assays (*10*), and ligand−receptor interaction assays (*11*). Normally, aqueous solutions of Eu^{3+}, Tb^{3+}, or Sm^{3+} produce such a weak fluorescence that it cannot be detected by a regular fluorometer (*12*). However, these rare earths form stable coordination complexes with appropriate signal-enhancing ligands and, following irradiation with near-ultraviolet light, the ligand is excited (*13*). Then by intersystem crossing (known as an antenna effect), energy is transferred from the signal-enhancing ligand to the central lanthanide ion, which becomes excited (*13*). When the excited lanthanide ion returns to the ground state, it emits a strong fluorescence. The signal-enhancing ligands enhance the fluorescent emission of lanthanide ions by shielding them from the quenching effects of water (*14*).

The emitted fluorescence has four attractive characteristics: (1) the emission wavelength is barely affected by the structure of the ligand. (2) It has a long fluorescence lifetime (∼ms) which

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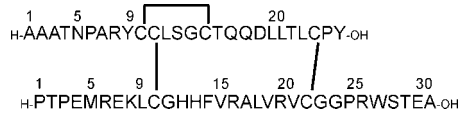


Figure 1. The primary structure of human INSL3. Of the three free amino groups, there is one each at the N-terminus of the A- and B-chains and one at the side-chain of lysine 8 on the B-chain.

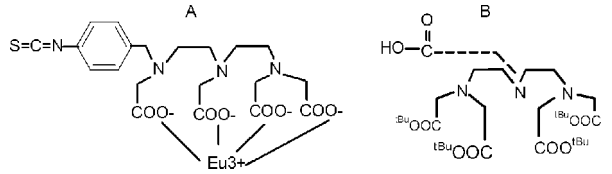


Figure 2. (A) Chemical structure of an activated Eu-DTTA chelate for postsynthesis acylation of free amino groups. (B) Chemical structure of tBu-protected DTPA for direct acylation of the N-terminal amino group in solid-phase peptide synthesis.

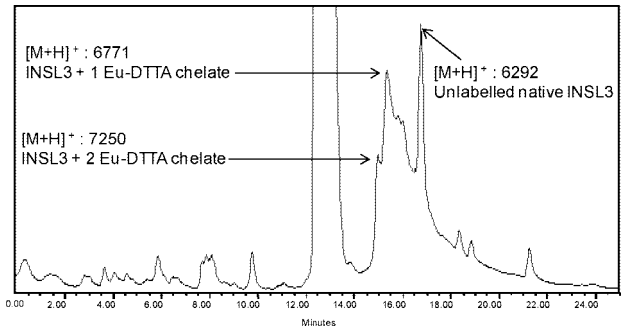


Figure 3. -HPLC chromatogram of human INSL3 following postsynthesis labeling with Eu-DTTA complex. A complex mixture of mono- and dilabeled peptides was obtained which could not be satisfactorily resolved.

allows measurement of fluorescence signal to be made after the decay of short-lived (micro- to picoseconds) background fluorescence from plates, cells, and reagents that are often a source of background in many fluorescence measurements (*10, 15, 16*). (3) It is characterized by a large Stokes shift (difference between the excitation maximum and emission maximum) compared to the short Stokes shift of organic fluorescent dyes in which the excitation and emission spectrum often overlap. (4) It exhibits a sharp emission peak which causes a large emission intensity that is easily detectable. These factors contribute to the low background and high sensitivity of lanthanide time-resolved fluorescence, making it comparable to that of radionuclide detection.

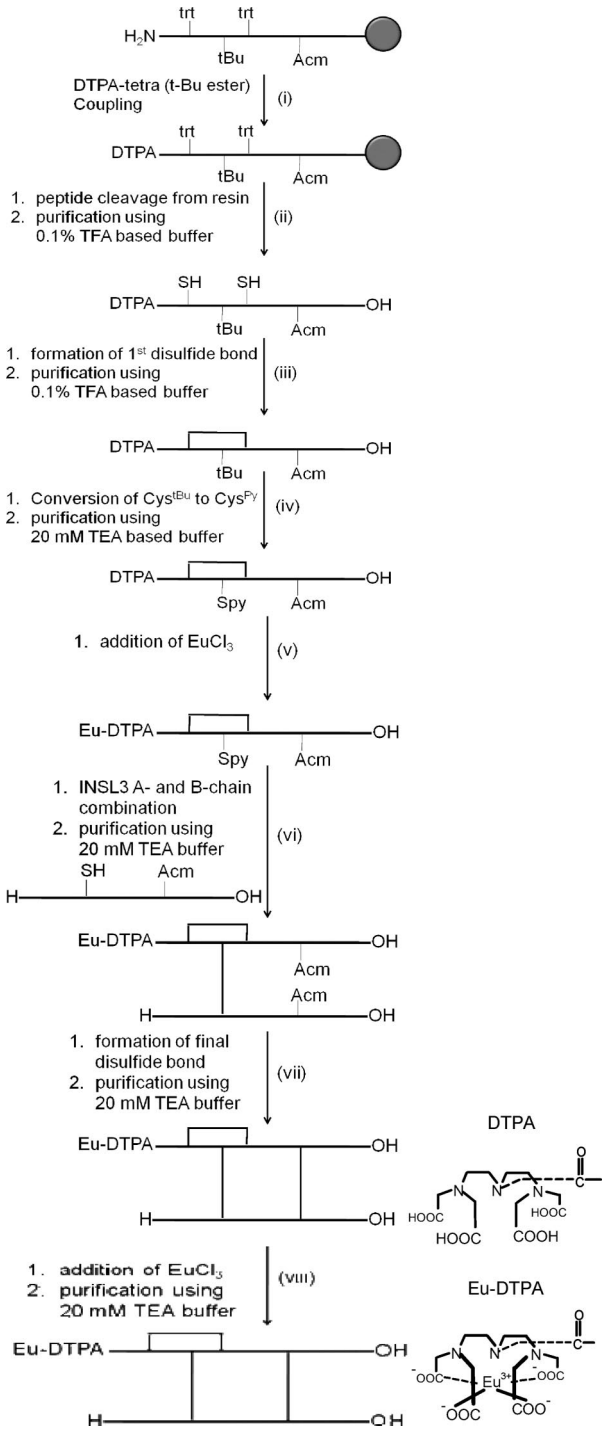
In this study, we have developed an efficient solid-phase synthesis technique to prepare specifically monolabeled peptide, which was subsequently shown to possess biophysical properties that render it suitable for receptor binding studies. INSL3 was labeled specifically at the *N*-terminus of the synthetic A-chain with the chelator, diethylenetriamine-*N*,*N*,*N*′′*N*′′-tetra-*tert*-butyl acetate-*N*′-acetic acid (DTPA). The labeled peptide was subsequently combined with the B-chain via regioselective disulfide bond formation. After chemical characterization of labeled INSL3, we performed saturation binding assays to determine maximum specific binding (B_{max}) and the receptor binding affinity of Eu-DTPA-labeled INSL3 (pK_d). We then performed competition binding assays to determine the binding affinity (pK_i) of the native INSL3 for RXFP2 in HEK 293 cell line using Eu-DTPA-INSL3 as labeled ligand.

EXPERIMENTAL PROCEDURES

Materials. 9-Fluorenylmethoxycarbonyl (Fmoc) protected L-α-amino acids; 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate (HBTU), *N*,*N*-dimethylformamide (DMF), piperidine, and trifluoroacetic acid (TFA) were obtained from Auspep (West Melbourne, Australia). Fmoc-L-Tyr(tBu)-PEG-PS and Fmoc-L-Ala-PEG-PS resins with substitution of 0.20 mmol/g were purchased from Applied Biosystems (Melbourne, Australia). Methanol, diethyl ether, dichloromethane, Merck (Kilsyth, Melbourne, Australia), 3,6-dioxa-1,8-octanedithiol (DODT), triisopropylsilane (TIPS), diisopropylethylamine (DIPEA), 1,2,4,5-benzene-tetracarboxylic dianhydride (Sigma-Aldrich, Sydney, Australia), 2,2′-dipyridyl disulfide (DPDS), (Fluka-Switzerland); diethylenetriamine-*N*,*N*,*N*′′,*N*′′-tetra-*tert*-butyl acetate-*N*′-acetic acid (DTPA) (Macrocyclic, USA). N^1-(*p*-isothiocyanatobenzyl)-diethylenetriamine-*N*, N^2, *N*, N^3-tetraacetic acid (Eu-DTTA) (Perkin-Elmer, Australia). Acetonitrile and NH_4HCO_3, $(NH_4)_2CO_3$: BDH laboratory supplies, (Poole, UK) and trifluoromethanesulfonic acid (TFMSA) (MP Biomedicals, Seven Hills NSW Australia). All other reagents: Sigma Castle Hill, NSW. Dulbecco's modified Eagles' medium (DMEM), RPMI 1640 medium, 2 mM L-glutamine, fetal calf serum, and penicillin/streptomycin were all obtained from Trace Biosciences (Castle Hill, New South Wales). All other reagents were obtained from Sigma-Aldrich (New South Wales, Australia).

Peptide Synthesis. All linear precursor peptides were solid-phase synthesized (*17*) as C-terminal acids on PAC-PEG-PS resin (0.2 mmol/g loading) using Fmoc chemistry. They were assembled on a CEM Liberty microwave peptide synthesizer (Ai Scientific, Queensland, and Australia). The side chain protecting groups of trifunctional amino acids were trifluoroacetic acid labile, except for acetamidomethyl (Acm)-protected cysteines in positions A24 and B22, and *tert*-butyl (tBu)-protected cysteine in position A11. All peptides were synthesized on a 0.1 mmol scale using a 5-fold molar excess of Fmoc-protected amino acids (0.5 mmol) that were activated by using 5-fold excess of HBTU in the presence of DIEA (*18, 19*). N^{α}-Fmoc protecting groups were removed by treating the resin-attached peptide with piperidine (20% v/v) in DMF. The coupling and deprotection were carried out at 75 °C using 25 W microwave power for 5 min and 60 W microwave power for 3 min, respectively. The chelator, DTPA-tetra(tBu ester) (3 equiv, 0.3 mmol), was coupled to the N-terminus of the solid-phase bound A-chain. The 2,4,6-trinitrobenzenesulphonic acid (TNBSA) test (*20*) was carried out to determine the success of coupling the DTPA. Each resin-bound polypeptide chain was cleaved from the solid support by treatment with a cocktail of trifluoroacetic acid (TFA)/3,6-dioxa-1,8-octanedithiol (DODT)/H_2O/triisopropylsilane (TIPS) (94%/2.5%/2.5%/1%, 20 mL) for 90 min. The cleaved peptide was precipitated in ice-cold diethyl ether and centrifuged at 3000 rpm for 3 min. The pellet was washed by resuspending it in ice-cold diethyl ether and centrifuging it again. This washing process was repeated at least three times. The stepwise synthesis of disulfide bonds was carried out by first forming the intra-A-chain disulfide bond between cysteines at positions A10 and A15. The cysteines were oxidized by first dissolving the linear A-chain in 0.1 M NH_4HCO_3 (1 mg/3 mL) to which a 1 mM solution of 2,2′-dispyridyl disulfide (DPDS) (*21*) in methanol (0.2 mL/ 1 mg peptide) was added while stirring. An aliquot of the mixture was analyzed by RP-HPLC to monitor the oxidation process. The oxidized INSL3 A-chains were analyzed and purified by preparative RP-HPLC on Waters XBridge columns (4.6 × 250 mm, C18, 5 μm) and (19 × 150 mm, C18, 5 μm), respectively, using two different buffer systems. INSL3 A-chain was purified using 0.1% trifluoroacetic acid in water (solvent A) and 0.1%

Scheme 1. Schematic Diagram of Synthesis of Eu-DTPA-INSL3 and Its Reagents and Conditions[a]



[a] (i) DTPA (3 equiv), HBTU (3 equiv), DIEA (3.5 equiv); (ii) TFA:DoDt:H_2O:TIPS (94:2.5:2.5:1); (iii) 1 mg peptide/0.2 mL of 1 mM DPDS; (iv) peptide:DPDS (1:4 μmol) dissolved in TFA: thioanisole (9:1) in 50 μg/μL, chilled on ice to 0 °C and then similar volume of TFA:TFMSA (4:1) was added; stirred for 1 hr at 0 °C and then purificaion of DTPA-INSL3 A-chain using 20 mM TEA buffer, pH 6.5, (v) DTPA-INSL3 A-chain and $EuCl_3$ (2 equiv) were dissolved in H_2O and stirred at room temperature for 30 min; (vi) Eu-DTPA-INSL3 A-chain dissolved in NH_4CO_3 mixed with INSL3 B-chain in water (1:1 mol) and stirred for 30 min; (vii) 60 mM HCl, acetic acid (2 mg/ mL), 20 mM I_2 (23 equiv/Acm), 1 h at rt, peptide was purified using 20 mM TEA based buffer, (viii) Eu-DTPA-INSL3 and $EuCl_3$ (2 equiv) were dissolved in H_2O and stirred at room temperature for 30 min and then final product was purified using 20 mM TEA based buffer to prevent the liberation of Eu^{3+} from the chelation with DTPA chelator.

trifluoroacetic acid in 100% acetonitrile (solvent B) running from 20% to 50% B at a flow rate of 10 mL/min over 30 min. In order to form the first interchain disulfide bond, the *tert*-butyl protecting group on cysteine in position A11 was converted to 2-pyridylsulfenyl derivative by dissolving the A-chain and DPDS (1:4 μmol) in TFA and thioanisole (9:1 v/v) (50 μg/ μL). The mixture was chilled on ice after which a similar volume of TFMSA in TFA (1:4 v/v) was added and the reaction continued for 45 min at 0 °C (*22*). The A-chain was precipitated with cold diethyl ether, and the pellet collected by centrifugation and washed 4 times to remove excess DPDS. The INSL3 A-chain derivative was purified using 0.1% TFA based buffers whereas the INSL3 A-chain with DTPA attached was purified using 20 mM triethylammonium acetate (TEA), pH 6.5, (solvent A) and 20 mM TEA in 90% acetonitrile (solvent B) with gradient of 20−50% B in 30 min. The first interchain disulfide bond was formed by reacting equimolar amounts of purified INSL3 B-chain with thiol-activated A-chain. The A-chain was dissolved in NH_4HCO_3, pH 8.5 and the B-chain, dissolved in water, was then added with stirring. The progress of the reaction was monitored using RP-HPLC. The reaction was complete after 30 min and the combined A- and B-chains were then purified by RP-HPLC using 0.1% TFA based buffers. In the case of INSL3 A-chain with the DTPA chelator at the N-terminus, the A-chain was first dissolved in NH_4HCO_3, pH 8.5, and then a 2-fold excess of europium chloride ($EuCl_3$) solution in water was added and the reaction was stirred for 10 min to form the complex of Eu-DTPA-A-chain before adding the equimolar amounts of INSL3 B-chain. The combined INSL3 Eu-DTPA-A-chain and B-chain was purified using 20 mM TEA based buffers. The formation of the last disulfide bond was achieved by oxidative removal of the acetamidomethyl groups in positions A24 and B22 by dissolving the peptide in 60 mM HCL (5% v/v) and acetic acid (2 mg/mL) followed by addition of 20 mM iodine (23 equiv/Acm). The reaction was stirred at room temperature for 1 h during which the progress was assessed by analytical RP-HPLC. After the completion of reaction, the mixture was poured onto chilled diethyl ether to precipitate the peptide, which was collected by centrifugation. The peptide was dissolved in water and 20 mM ascorbic acid to quench the remaining traces of iodine and then purified by preparative RP-HPLC on a Waters XBridge column (19 × 150 mm, C18, 5 μm) using 20 mM TEA-based buffers as described above. The solvent was removed by lyophilization which left a yellow oily peptide that was relyophilized in 50% acetonitrile in water to give a white powder. After purification, the labeled INSL3 containing the DTPA chelator was redissolved in H_2O and then 2 equiv of europium chloride was added and stirred for an hour. The formation of complex between europium and DTPA chelator was monitored by RP-HPLC and MALDI-TOF/TOF mass spectroscopy. The labeled INSL3 was then purified by RP-HPLC using 20 mM TEA-based buffer.

Postsynthesis Labeling of INSL3. The postsynthesis labeling of INSL3 with Eu-DTTA complex was attempted using a 10-fold excess of Eu-DTTA over peptide at three different reaction conditions: (1) 50 mM $NaHCO_3$ + 150 mM NaCl, pH 8.5 (*23*), (2) 100 mM Na_2CO_3, pH 9.2 (according to the DELFIA labeling kit form PerkinElmer), (3) 100 mM Na_2CO_3, pH 6.5. The reactions were carried out at 4 °C overnight.

Peptide Characterization. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS, Bruker Daltonics, Germany) was used to characterize the peptides at each intermediate step using sinapinic acid, α-cyano-4-hydroxycinnamic acid, and 2,5-dihydroxy benzoic acid (Bruker Daltonics, Germany) based on the molecular size of the peptide. The matrices were made up in 50% acetonitrile containing either 0.05% TFA or 0.05% formic acid. The peptide

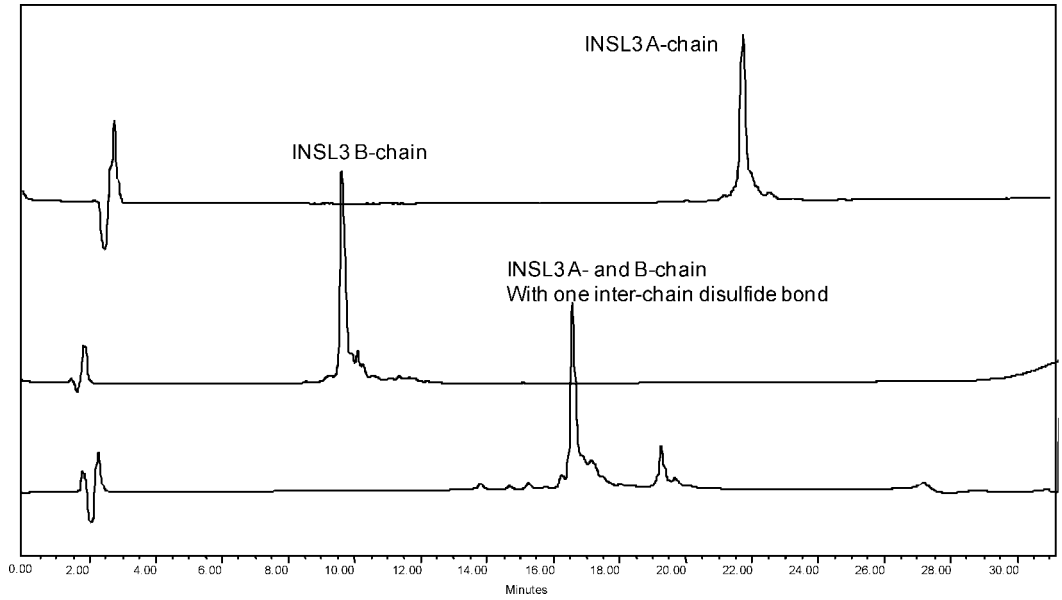


Figure 4. RP-HPLC monitoring of DTPA-INSL3 A-chain combination with INSL3 B-chain. The single-chain peptides each have a correct molecular mass as determined by MALDI-TOF/TOF. However, the combined A- and B-chain product had a *m/z* 55.2 higher than the expected monoisotopic mass (see Figure 5).

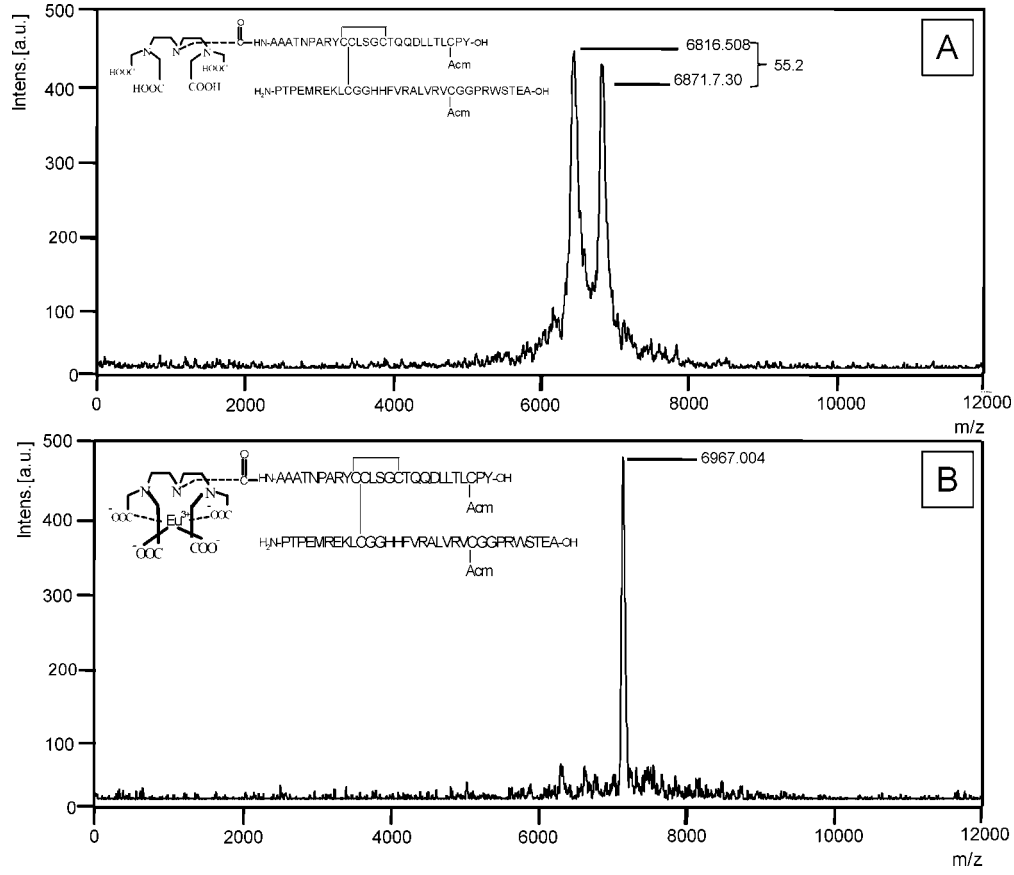


Figure 5. (A) Mass spectrum of DTPA-labeled INSL3 after the formation of first interchain disulfide bond which showed a *m/z* of 55.2 Da higher ($[M + H]^+ = 6724.244$) than the expected monoisotopic mass ($[M + H]^+ = 6668.989$). (B) Protection of the DTPA on the A-chain with Eu^{3+} prior to combination with B-chain gave a labeled INSL3 with the correct monoisotopic mass (expected $[M + H]^+ = 6966.85$).

content was determined using vapor-phase acid hydrolysis in 6 M HCL containing 2% phenol at 110 °C for 24 h. The individual amino acids were converted to stable, fluorescent derivatives using a Waters AccQ.Tag kit (Waters, Sydney, Australia). The derivatized amino acids were separated using a Shim-Pak XR-ODS (3 × 75 mm, 2.2 μm) column on Shimadzu RP-HPLC system (Shimadzu, Victoria, Australia).

Circular Dichroism Spectroscopy. The peptides were made up to a concentration of 0.1 μM in phosphate buffered saline (PBS: 10 mM potassium phosphate buffer containing 137 mM NaCl pH 7.4). The far UV circular dichroism (CD) spectra of peptides were acquired using a Jasco J815 spectropolarimeter between the wavelengths of 195−250 nm at room temperature with a resolution of 0.1 nm, bandwidth of 0.1 nm, and a cell of

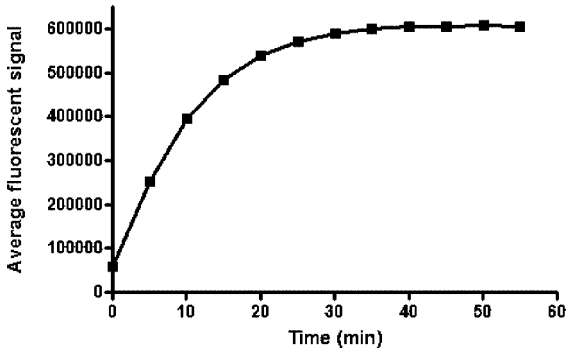


Figure 6. Measurement of Eu release from DTPA chelate. 10 μL of 10 nM Eu-DTPA-INSL3 peptide was mixed with enhancement solution in triplicate and the fluorescence was measured at 5 min interval for 60 min. The signal reached its maximum at ~600 000 average fluorescent units (AFU) at time 40 min.

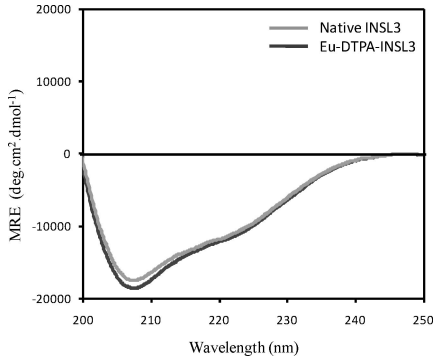


Figure 7. CD spectra of human INSL3 (solid gray line) and Eu-DTPA-INSL3 (solid black line) measured at a concentration of 0.1 μM in PBS (pH 7.4) at room temperature. The two peptides showed similar spectra and a calculated helicity of approximately 33%.

0.1 cm path length. The recorded spectra in millidegrees of ellipticity (θ) were converted to mean residue ellipticity (MRE) in deg.cm^2 dmol^{-1}.

Cell Culture and Ligand Binding Assays. Subcultures of human embryonic kidney cells (HEK 293T) stably transfected with RXFPR2 (LGR8) were plated out at a density of 80 000 cells per 200 μL per well in a 96 well Isoplate with white wall and clear bottom precoated with poly(L-lysine). The binding assay was carried out using a modification of a method previously described by us (*24*). Briefly, at the start of each experiment, cell culture medium was removed and the cells were washed with phosphate buffer saline (PBS) (250 μL per well). 1% BSA in receptor binding buffer (250 μL) was then added to each well and allowed to stand at room temperature for 1 h. The appropriate concentration of INSL3 and Eu-labeled INSL3, in 1% BSA in receptor binding buffer, was added to each well in triplicate and incubated at room temperature for 1 h. The cells were washed with PBS (250 μL) once, and then 100 μL of enhancement solution (purchased from Perkin-Elmer, composed of β-NTA, TOPO and 0.1% (w/v) Triton X-100 in 0.1 M acetic acid adjusted to pH 3.2) was added to each well, and after 45 min, the fluorescent measurement was carried out on a Victor^3 Plate Reader (Perkin-Elmer, USA) at excitation of 340 nm and emission of 614 nm with the delay time and counting time of 400 μs each.

Data Analysis. The CD spectra data were first transformed from machine unit θ to $\Delta\varepsilon$ using Graphpad *PRISM 4* (Graphpad Ins., San Diego, USA) with user-defined formula ($\varepsilon = \theta \times (0.1 \times \text{MRW})/(\text{P} \times \text{C}) \times 3298$). The converted values were then submitted to the DichroWeb server (*25–28*) for calculation of secondary structure using the CDSSTR (*29*) and K2D (*30*) analysis algorithms. Graphpad *PRISM 4* was used to analyze the receptor-binding assays data, which were expressed as mean ± SEM of percentage specific binding of Eu-chelate-labeled INSL3 from triplicate treatments from at least three independent experiments. The value for the dissociation constant (k_d) was determined by fitting saturation binding data to a one-site hyperbolic model. The inhibition constants were determined from IC_{50} values using the Cheng-Prusoff equation (*31*).

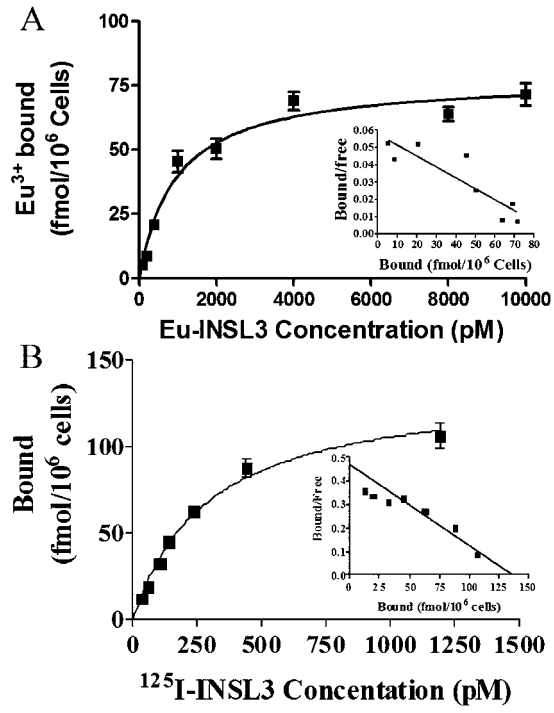


Figure 8. Saturation binding curves of Eu-DTPA-INSL3 and ^{125}I-INSL3 in HEK293T cells stably transfected to express RXFP2. Different concentrations of labeled peptides were added to cells and incubated at room temperature for 60 min. Nonspecific binding was determined in the presence of 500 nM INSL3. The pK_d and B_{max} of the labeled peptides were determined to be 9.05 ± 0.03 (n = 3); 78074 (receptors/cell) ± 15834 SEM (n = 3); and 9.59 ± 0.09; 83518 (receptors/cell) ± 13061 SEM, respectively. GraphPad *Prism* software was used to fit the data using the nonlinear regression and one site binding model.

RESULTS AND DISCUSSION

Molecular Design and Postsynthesis Labeling. Human INSL3 has two chains (a 26 residue A-chain and a 31 residue B-chain) each containing a free terminal α-amino group. There is also an ε-amino group on the side chain of lysine B8 (Figure 1). We initially undertook postsynthesis labeling of synthetic native human INSL3 with commercially available activated Eu-DTTA chelate, N^1-(*p*-isothiocyanato benzyl)-diethylenetriamine-N,N^2,N,N^3-tetraacetic acid (Figure 2A). First, we synthesized human INSL3 by Fmoc chemistry using standard solid-phase techniques that have been optimized in our laboratory (*32*), employing regioselectively S-protecting groups for the sequential formation of each of the three disulfide bonds. The postsynthesis labeling of INSL3 was then carried out using different reaction conditions, each of which yielded a mixture of mono- and multilabeled peptides, as well as unlabeled INSL3 (Figure 3). Although it was possible to identify some of the species present in the complex mixture obtained, they were poorly resolved making their complete deconvolution and purification exceptionally difficult. As the determination of precise binding parameters requires knowledge of the true specific activity of a ligand, we thus sought an alternative approach to develop a molecularly defined Eu-labeled INSL3.

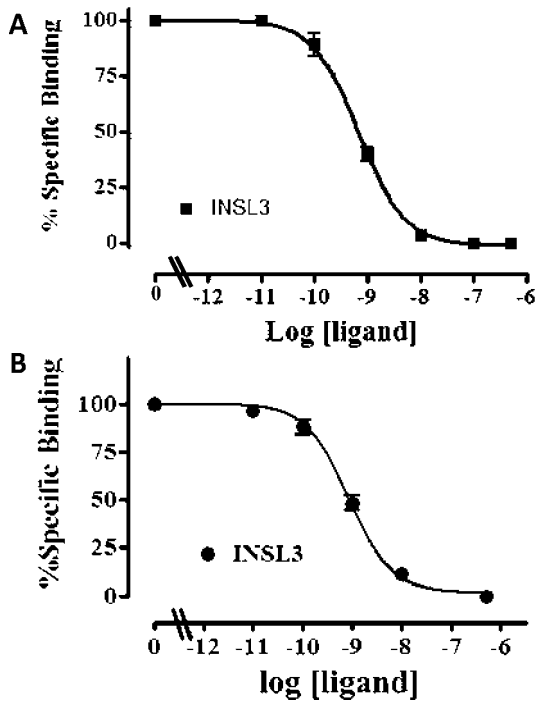


Figure 9. Competition binding curves of INSL3 using Eu-DTPA-INSL3 (A) and ^{125}I-INSL3 (B) as labeled ligand in HEK293T cells stably transfected to express RXFP2. Different concentrations of INSL3 were added to cells and incubated at room temperature for 60 min. INSL3 was competed off the receptor with 0.5 nM of Eu-DTPA-INSL3. The pK_i of INSL3 was determined to be 9.27 ± 0.06 (n = 3) using Eu-DTPA-INSL3 as labeled ligand and 9.34 ± 0.02 (n = 4) when ^{125}I-INSL3 used as labeled ligand. GraphPad *Prism* software was used to fit the data using the nonlinear regression and one site competition model.

Synthesis of INSL3 and Incorporation of DTPA Chelator on Solid Phase. Structure−activity relationship studies with INSL3, relaxin, and other members of this superfamily of peptide hormones which each have a similar core tertiary structure have shown that the N-terminus of the A-chain is tolerant to a range of chemical modifications (*33*). We thus chose to prepare a Eu-chelate labeled INSL3 in which the chelator was incorporated specifically and solely at the N-terminus of the A-chain. To do this, we first synthesized human INSL3 A-chain on the solid phase and concluded this by coupling the empty chelator DTPA (Figure 2B) (Scheme 1). Our plan was that this would allow us to use standard TFA-based HPLC buffers—these are not compatible with Eu-loaded chelates, as the chelates are unstable at low pH—rather than the less well-resolving TEA acetate buffers. Following the removal of the chelator-labeled A-chain from the solid support, the intrachain disulfide bond was formed via 2-pyridyldisulfide-mediated oxidation (*21*). The resulting modified peptide, which possessed a *tert*-butyl protecting group on the thiol of Cys 11 and a Acm group on the thiol of Cys 24, was then subjected to treatment with 2-pyridyldisulfide in TFMSA/TFA to convert the tert-butyl functionality to an S-pyridinyl group. In subsequent preliminary experiments, despite the apparent successful combination of the A- and B-chains via thiolysis of the S-pyridinyl by the free thiol of Cys 10 of the B-chain (Figure 4), MALDI-TOF MS showed the resulting product to have a molecular mass that was 55.2 higher than the expected monoisotopic mass ($[M + H]^+$= 6668.98) (Figure 5A) group. Although this modification did not hinder formation of the final disulfide bond (via iodine oxidation of the Acm groups on Cys A24 and B22), the resulting DTPA-peptide failed to form a complex with the Eu^{3+} when treated with an excess of $EuCl_3$. This suggested that the modification was of the DTPA moiety itself. In order to overcome this, we modified the protocol by loading the DTPA on the A-chain with Eu^{3+} prior to combining the two chains. This strategy effectively protected the four free carboxylic groups on the DTPA chelator during chain combination, and the resulting chain-combined peptide could thus be obtained with the correct monoisotopic mass (Figure 5B). The final stage of the synthesis (the formation of second interchain disulfide bond using iodine in acetic acid) is carried out at low pH, conditions which might be expected to lead to the loss of Eu^{3+} from the DTPA. Although MS analysis indicated that only a small proportion of Eu^{3+} was lost during the final disulfide bond formation, we nevertheless chose to reload the chelate with Eu^{3+} after this step to ensure that the final product was completely Eu-chelated. The formation of the complex between europium and DTPA-labeled INSL3 was monitored by RP-HPLC and MALDI-TOF/TOF MS. The final desired Eu-chelate labeled INSL3 was then purified by RP-HPLC buffer using TEA acetate buffer at near-neutral pH (6.5) to prevent the liberation of europium from the DTPA.

The peptide purity was shown by analytical RP-HPLC to be greater than 95%, and to have a peptide content of 64% as assessed by amino acid analysis. A monoisotopic m/z of 6821 corresponding to the singly Eu-coordinated DTPA-labeled INSL3 was obtained using MALDI-TOF MS, which contrasts to previous studies on synthetic lanthanide complexes of synthetic peptides, in which multiple isomers were obtained (*34*). The overall yield of synthesis was 7.7%, which compares favorably to that obtained for native human INSL3 (10%) obtained using a similar synthesis protocol (*35*).

In order to monitor the time that it takes for the fluorescent signal of Eu-coordinated DTPA INSL3 (as a result of the liberation of Eu^{3+} from chelation with DTPA) to reach its maximum, the release of Eu^{3+} from DTPA chelate was monitored by measuring the fluorescence of 10 μL of 10 nM Eu-DTPA-INSL3 in 100 μL of enhancement solution in triplicate at 5 min intervals for 60 min (Figure 6). The signal reached its maximum at ∼600 000 average fluorescence units (AFU) at time 40 min. This time was used in the subsequent assays as the end point for liberation of europium before measurement of fluorescent signal.

Circular Dichroism Spectroscopy. The CD spectra of synthetic native human INSL3 and DTPA-labeled INSL3 were measured in PBS (pH 7.4) (Figure 7). The two peptides showed a similar CD spectroscopic profile with calculated helicity of approximately 33%. This indicated that the coupling of DTPA to the N-terminus of the INSL3 A-chain had no apparent influence on the secondary structure of INSL3 and is therefore unlikely to have perturbed its tertiary structure.

Whole Cell Receptor Binding Assay. Eu-chelate-labeled and ^{125}I-labeled INSL3 were compared under identical saturation binding conditions using HEK293T cells stably transfected to express the INSL3 receptor RXFP2. To determine the total binding, cells were incubated with different concentrations of Eu-DTPA-INSL3 and ^{125}I-INSL3 in a 96 well plate for 60 min at room temperature to reach equilibrium. To measure the nonspecific binding, 500 nM unlabeled INSL3 was used to competitively dissociate Eu-chelate-labeled INSL3. The nonspecific binding was found to be less than 15% of total binding at any concentration of Eu-DTPA-INSL3 used. Like ^{125}I-INSL3, Eu-DTPA-INSL3 binds in a specific and saturable manner and it was best fitted to a one-site binding model (Figure 8). The calculated dissociation constant (pK_d) for Eu-DTPA-INSL3 was 9.05 ± 0.03 (n = 3) and maximal binding B_{max} was 78074 ± 15834 receptors/cell (n = 3). The pK_d and B_{max} determined for Eu-DTPA-INSL3 agreed with the pK_d (9.59 ± 0.09, n = 3) and B_{max} (83518 ± 13061 receptors/cell) values for ^{125}I-INSL3. There was a slight decrease in pK_d of Eu-DTPA-INSL3

compared to ^{125}I-INSL3 that is statistically significant ($p < 0.005$). This decline in affinity of Eu-DTPA-INSL3 for its receptor RXFP2 is possibly due to the relatively large size of the DTPA moiety compared to the smaller ^{125}I atom. This phenomenon of a slight loss in ligand−receptor affinity has also been reported in other cases where DTPA labeling of bioactive peptides has been described (*36*–*38*). Assays using the Eu-chelate-labeled INSL3 were highly reproducible, showed excellent signal-to-noise ratios, and were faster than radioisotopic-based binding assays, as the measurement can be made directly in the same plate as the assay (in the case of radionuclei-based assays, the cells need to be transferred for signal measurement).

Once the Eu-DTPA-INSL3 was characterized by saturation binding experiments, it was then used as a labeled ligand in competition binding assays (Figure 9) to determine the receptor binding affinity (pK_i) of native human INSL3 for RXFP2. INSL3 displaced Eu-DTPA-INSL3 (0.5 nM) with pK_i value of 9.27 ± 0.06 ($n = 3$), which was similar to those previously obtained using ^{125}I-INSL3 (9.34 ± 0.02, $n = 4$) (*3*).

CONCLUSION

The use of lanthanide-labeled ligands to study ligand−receptor interactions has become increasingly popular over the past decade. Such peptides have a longer shelf life compared to radiolabeled peptides, offering greater reproducibility of results, since the same stock of labeled ligand can be used over a long period of time. In this study, we have demonstrated a novel and efficient methodology for effective preparation of Eu-coordinating, DTPA-lableled INSL3 via solid-phase synthesis. The site-specific incorporation of DTPA chelator on the solid phase gives a monolabeled peptide with high yield and purity compared to the postsynthesis labeling using DTTA chelator. Eu-DTPA-labeled INSL3 showed binding properties that are comparable to that of ^{125}I-INSL3. Lanthanide detection methods are presently being applied to other members of the relaxin-insulin superfamily of peptide hormones.

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