Protocol

Journal of PeptideScience

Received: 15 April 2010 Revised: 27 August 2010 Accepted: 27 August 2010 Published online in Wiley Online Library: 11 January 2011

(wileyonlinelibrary.com) DOI 10.1002/psc.1307

General method for selective labelling of double-chain cysteine-rich peptides with a lanthanide chelate via solid-phase synthesis

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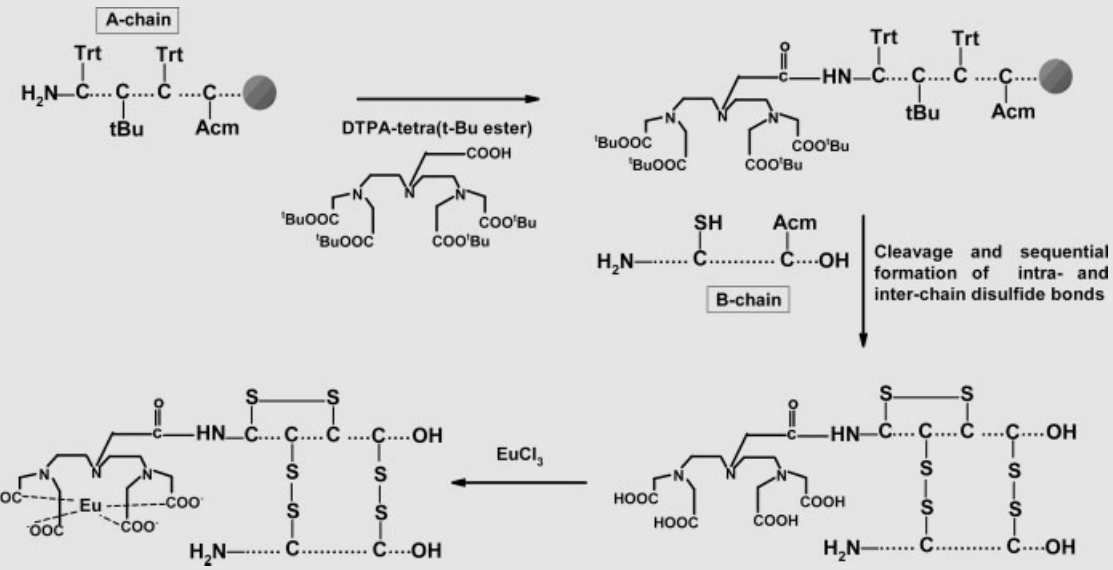
The use of lanthanides in preference to radioisotopes as probes for various biological assays has gained enormous popularity. The introduction of lanthanide chelates to peptides/proteins can be carried out either in solution using a commercially available labelling kit or by solid-phase peptide synthesis using an appropriate lanthanide chelate. Herein, a detailed protocol for the latter is provided for the labelling of peptides or small proteins with diethylenetriamine-*N*, *N*, *N*′′, *N*′′-tetra-tert-butyl acetate-*N*′-acetic acid (DTPA) chelate or other similar chelates on a solid support using a chimeric insulin-like peptide composed of human insulin-like peptide 5 (INSL5) A-chain and relaxin-3 B-chain as a model peptide. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: lanthanide; chelate; time-resolved fluorescent; solid phase

REACTION SCHEME



GENERAL OPTIMIZED PROCEDURE

N-terminal conjugation of DTPA chelate on solid support. The diethylenetriamine-*N*,*N*,*N*",*N*"-tetra-tert-butyl acetate-*N*'-acetic acid (DTPA) chelate (3 eq) was coupled to the *N*-terminus of resin-bound relaxin-3 B-chain using HBTU (3 eq) and DIEA (3.5 eq) at room temperature for 30 min. The completeness of coupling was confirmed using the trinitrobenzenesulfonic acid (TNBSA) test. After cleavage of peptide from the solid support, it was combined with INSL5 A-chain by sequential regioselective formation of the three disulfide bonds in solution. DTPA-coupled relaxin-3B/INSL5A was dissolved in H_2O before adding $EuCl_3$ (2 eq). The final product was purified using TEA-based buffers (pH 6.5).

Scope and Comments

A range of biological molecules have been labelled with either one or two chelates of specific trivalent lanthanide ions (Eu^{3+}, Sm^{3+}, and Tb^{3+}) for use as a sensitive detection probe in various assay systems such as dissociation-enhanced lanthanide

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fluoroimmunoassay (DELFIA) [1–5], time-resolved amplified cryptate emission (TRACE) [6–8], and enzymatically amplified time-resolved fluorescence immunoassay [9].

Although the luminescence properties of lanthanide chelate complexes were discovered in 1942 [10], it was another 40 years before their application was first reported [11]. However, over the last decade, the use of luminescence lanthanide chelates as a sensitive probe has gained enormous attention and many researchers now use them in place of radionuclei labels and other organic fluorescence dyes.

Lanthanides on their own emit only a very weak fluorescence but this can be enhanced by chelating the ions with an appropriate organic ligand via what is known as an antenna effect. The advantage of luminescence lanthanide complexes over other organic fluorescence dyes is the characteristics of the emitted fluorescent light by lanthanide. These include (i) the long decay time of the emitted fluorescence which lasts up to milliseconds and allows the decay of short-lived background noise before the measurement of the specific fluorescent signal, (ii) the large Stoke's shift, and (iii) sharp emission profile [12]. These characteristics contribute to the high sensitivity and low background signal and hence make lanthanide an ideal choice for use as a probe in the development of time-resolved fluorescence bioassays.

Lanthanide (III) chelates are commonly conjugated to free amino groups of peptides or proteins in solution using the DELFIA labelling kit (Perkin Elmer, Melbourne, Australia). The reaction is usually performed with an excess of the succinimide-activated chelate. However and not unexpectedly, in peptides or proteins bearing more than one free amino group, this approach of labelling often leads to a mixture of mono- and multi-labelled biomolecules, which makes subsequent purification potentially very laborious. The isolation and characterisation of the active species of the mono-labelled biomolecule from the complex mixture is difficult and often impossible. In order to circumvent these problems, site-specific incorporation of the chelate into solid phase-assembled and bound biomolecules can be successfully undertaken. Solid-phase synthesis has been reported for labelling oligonucleotides for use in DNA studies [13] as well as for the preparation of small, labelled peptides [14,15]. Recently, we have reported a highly efficient solid-phase synthesis methodology for site-specifically incorporating a single lanthanide chelate into two-chain peptides having multiple disulfide bonds [16].

In this report, we describe a protocol which includes a stepwise solid-phase synthesis of each of human relaxin-3 B-chain and INSL5 A-chain and the *N*-terminal coupling of the lanthanide (III) chelate on solid support. Following cleavage of the chains, the sequential regioselective formation of the three disulfide bonds between them is outlined in detail. The choice of buffer for RP-HPLC as well as the appropriate choice of matrix and its preparation for MALDI-TOF/TOF MS characterisation are also explained. The general procedure can be adapted to any synthetic peptide.

Experimental Procedure

Synthesis of linear peptides

The linear INSL5 A- and relaxin-3 B-chains were synthesised as *C*-terminal amides using Fmoc-PAL-PEG-PS resin with substitution of *ca* 0.20 mmol/g (Applied Biosystems Inc., Melbourne, Australia). The side-chain protecting groups of the amino acids were all TFA-labile except for cysteines in positions A24 and B22 which were acetamidomethyl (Acm)-protected and cysteine in position A11 that was protected with *tert*-butyl (tBu). Both chains were assembled on a CEM Liberty™ microwave peptide synthesizer (AI Scientific, Scarborough, QLD, Australia) on a 0.1 mmol scale using a fivefold molar excess of Fmoc-protected amino acids (0.5 mmol) that were activated by using fivefold excess of HBTU in the presence of DIEA (tenfold excess). The N^{α}-Fmoc protecting groups were removed using piperidine (20% v/v) in DMF. The couplings were carried out at 75 °C using 25 W microwave power in except for the cysteines and histidine which were coupled at 30 °C for 30 min to minimise racemisation. The DTPA (3 eq, 0.3 mmol) obtained from Macrocyclics (Dallas, TX, USA, http://www.macrocyclics.com/) was coupled manually to the *N*-terminus of solid-phase bound peptide using HBTU (0.3 mmol) and an excess of DIEA (0.35 mmol). The success of the DTPA coupling was monitored using 2,4,6-TNBSA test [17], which was negative and confirmed the absence of a free amino group at the *N*-terminus. The linear polypeptide chains were cleaved from solid support by stirring the resins in 20 ml of TFA : 3,6-dioxa-1,8-octanedithiol (DODT) : H_2O : TIPS (94 : 2.5 : 2.5 : 1%) for 90 min at room temperature. The resin was filtered and the cleaved peptides were precipitated in ice-cold diethyl ether and collected by centrifugation (3000 rpm, 3 min). The pellet was washed in ice-cold diethyl ether for at least three times (**i**).

Sequential formation of the regioselective disulfide bonds

The intra-A-chain disulfide bond was formed between the cysteines at position A10 and A15 under oxidative conditions. First, the peptide was dissolved in NH_4HCO_3 (1 mg/3 ml) and a 1 mM solution of 2,2′-dipyridyl disulfide (DPDS) (Fluka, Bucha, Switzerland) in methanol (0.2 ml/1 mg peptide) was added while stirring [18]. The progress of the reaction was monitored by RP-HPLC at 30-min intervals on a Waters XBridge™ column (4.6 × 250 mm, C18, 5 μm). The oxidised A-chain was purified on a Waters XBridge™ preparative column (19 × 150 mm, C18, 5 μm) using 0.1% TFA in water (solvent A) and 0.1% TFA in 100% acetonitrile (solvent B) running from 20 to 50% B at a flow rate of 10 ml/min over 30 min (**ii**).

To enable the formation of the first interchain disulfide bond, the *tert*-butyl protecting group on cysteine at position A11 needs to be converted to a 2-pyridylsulfenyl derivative so it can react with the free thiol group of the cysteine on the B-chain. This was done by first dissolving the peptide in a mixture of TFA : thioanisole (9 : 1 v/v) (50 μg/μl) and adding DPDS (4 eq). The mixture was chilled on iced to about 0 °C before adding equivalent volume of TFMSA in TFA (1 : 4 v/v) [19]. The reaction was continued for 45 min after which the reaction mixture was precipitated with cold diethyl ether, pellet collected by centrifugation and washed at least four times to remove excess DPDS (**iii**).

Before combining the A-chain with the B-chain to form the first interchain disulfide bond, the A-chain was first dissolved in NH_4HCO_3, pH 8.5, and then a twofold excess of europium chloride solution in water was added which formed a solid precipitate. The addition of excess water solubilised the precipitate and the resulting mixture was stirred until the solution was clear. The formation of Eu-DTPA-A-chain (**iv**) was monitored by MALDI-TOF mass spectroscopy. The formation of the Eu and DTPA complex *before* chain combination is to protect the DTPA chelate from any modification which may occur during this step. An equimolar amount of the B-chain was dissolved in H_2O and it was added to the stirred solution of the A-chain. The mixture was stirred for 30 min and the combined A–B intermediate (**v**) was then purified by RP-HPLC using 0.1% TFA-based buffer which liberates the europium ion from chelation with DTPA.

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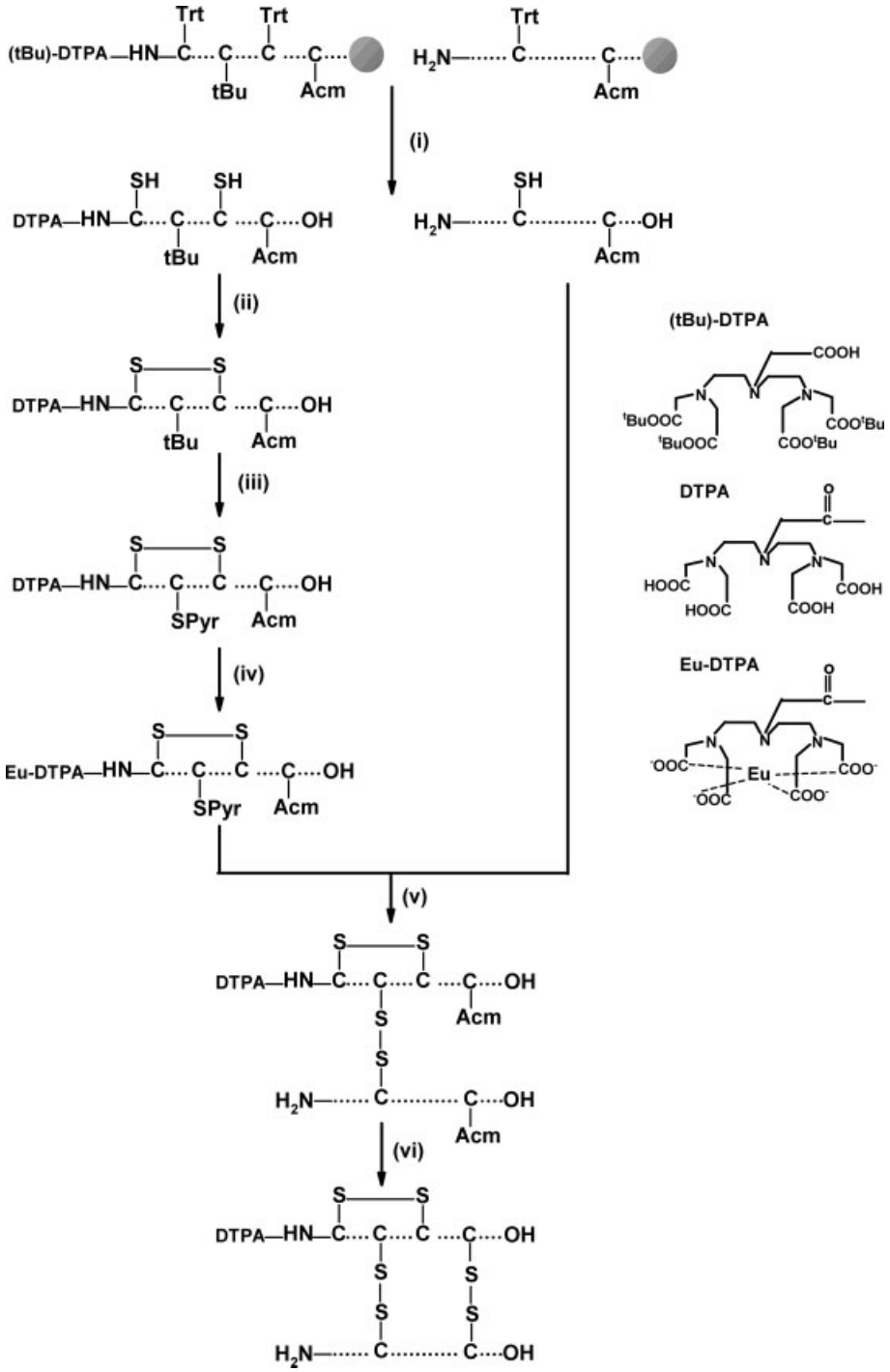
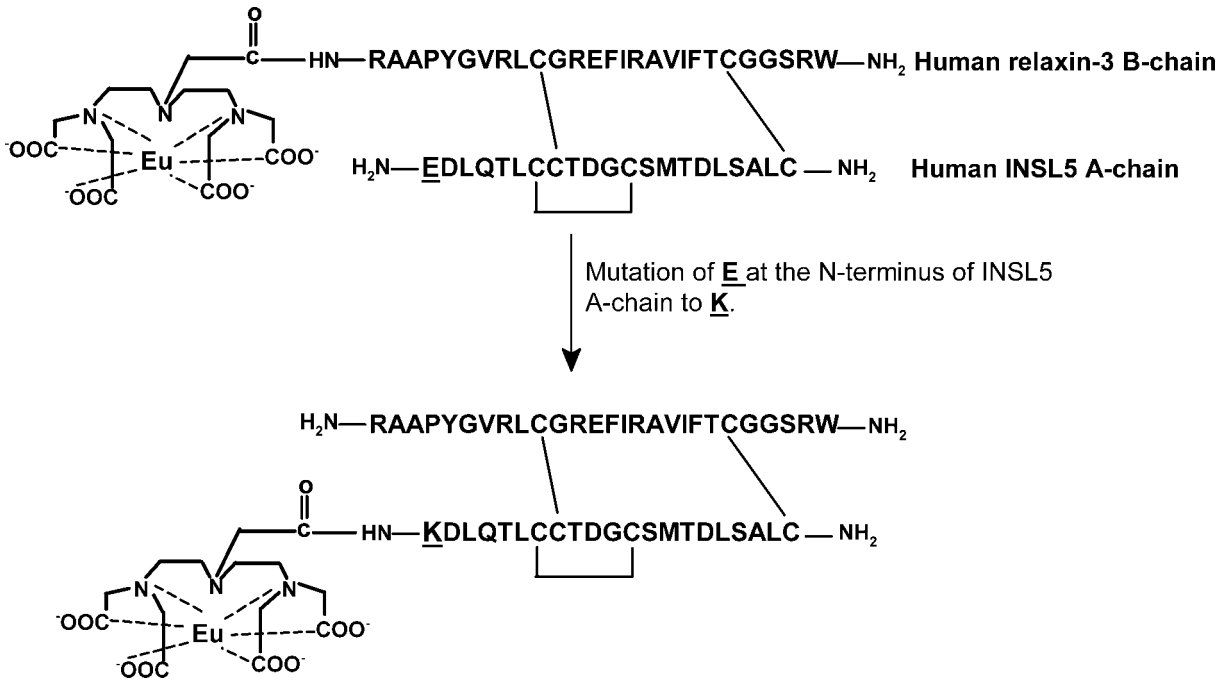


Scheme 1. Solid-phase synthesis of human INSL3 and incorporation of DTPA chelate at the *N*-terminus of the A-chain and sequential formation of the regioselective disulfide bonds.

Formation of the second interchain disulfide bond was carried out by oxidative removal of the acetamidomethyl groups in positions A24 and B22. The intermediate peptide (**v**) (6.5 mg) was dissolved in acetic acid (2 mg/ml) and 60 mM HCl (5% v/v) was added to the mixture followed by addition of 20 mM iodine (23 eq/Acm). The mixture was stirred in the dark for 1 h and the progression of the reaction was monitored by analytical RP-HPLC at 30-min intervals. Upon completion, the mixture was poured onto chilled diethyl ether to precipitate the peptide which was collected by centrifugation. The peptide was dissolved in water and a few drops of 20 mM solution of ascorbic acid were added to quench the remaining traces of iodine. The peptide was then purified by preparative RP-HPLC using 20 mM TEA, pH 6.5 (solvent A) and 20 mM TEA in 90% acetonitrile (solvent B) at a flow rate of 10 ml/min with gradient of 20–50% B in 30 min. The removal of the solvent left a yellow oily peptide which was re-lyophilised in 50% acetonitrile in water to give a white powder (**vi**) (0.5 mg, 9.3% yield).



Scheme 2. Due to peptide insolubility in water, E at the *N*-terminus of INSL5 A-chain was mutated to K followed by coupling of the DTPA chelator at the *N*-terminus of INSL5 A-chain. The resulting labelled peptide had an overall charge of +2 and it was water soluble.

The final step was the re-addition of europium to form a complex with the DTPA chelate at the *N*-terminus of the A-chain. This was achieved by dissolving the peptide (**vi**) in water and adding 2 equivalents of europium chloride. The labelled chimeric peptide was then purified by RP-HPLC using 20 mM TEA-based buffer and it was re-lyophilised in 50% acetonitrile in water to give a white powder (**vii**) (0.3 mg, 60%). The excess of europium used to recharge the DTPA chelate at the final step should be calculated carefully as too much europium can non-specifically bind to the peptide, especially those rich in negatively charged side-chains (Scheme 1).

Unfortunately, after successful synthesis and characterisation of the chimeric DTPA-relaxin-3 B-chain and INSL5 A-chain, the peptide had poor water solubility possibly due to the net zero charge of the peptide and addition of the large water-insoluble DTPA chelate. In order to check the receptor-binding affinity of this labelled peptide, it was dissolved in either 50% acetonitrile or DMSO and then diluted in the receptor-binding buffer before using it in the whole cell receptor-binding assay. The labelled peptide had a poor receptor-binding affinity with the dissociation constant (K_d) of 15.1 nM which was thought to be likely due to its poor solubility or the toxicity of the solvent. In order to address this problem, the chimeric peptide which had an overall net charge of zero was modified to give it an overall charge of +2. This was achieved by replacing Glu at the *N*-terminus of INSL5 A-chain with Lys and also coupling DTPA to the *N*-terminus of INSL5 A-chain instead of relaxin-3 B-chain. This modification not only improved the water solubility of the labelled peptide but also its receptor-binding affinity with dissociation constant (K_d) of 9.1 nM (Scheme 2).

Peptide characterisation

The peptides at each intermediate step were characterised using MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). The matrix used was either sinapinic acid or α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics) which were made up in 50% acetonitrile containing either 0.05% TFA or 0.05% formic acid. The intermediates at step (**iv**) and the final product were characterised by sinapinic acid made up in 0.05% formic acid which does not dissociate europium from the DTPA chelate and the mass obtained is the mass of the Eu-DTPA-relaxin-3B/INSL5A, whereas a TFA-based matrix causes the dissociation of the europium and only the mass of peptide plus DTPA is observed.

Limitations and notes

1. The site of incorporation of the DTPA chelate into peptide and protein obviously needs to be carefully selected so that the bulky size of the DTPA chelate does not interfere with the pharmacophore of the biomolecule. This example uses the *N*-terminus of the resin-bound peptide but placement elsewhere in the peptide can be achieved by use of, for example, Lys(Dde) followed by selective removal of the N^{ε}-protecting group and subsequent incorporation of the DTPA.
2. HPLC purification needs to be carried out at near-neutral pH due to the loss of the europium from the complex in acidic solution.
3. Labelling of peptides with DTPA may make them less water soluble which, in turn, may affect the bioassays. One possible solution is to dissolve the labelled peptide in DMSO and slowly dilute this with receptor-binding buffer before use. It is not recommended that peptides are stored in DMSO for extended periods as the solvent may oxidise methionine and tryptophan residues in the peptide (if present). Before labelling the peptide with any chelate, the overall charge of the peptide should also be taken into consideration. If the peptide contains net zero charge, then incorporation of lysine or arginine residues may also help to prevent any solubility issues with the final labelled peptide.

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4. Detailed protocols for the use of europium-labelled peptides and proteins in whole cell receptor binding assays are given in [16,20].

Acknowledgement

Fazel Shabanpoor is a recipient of David Hay Postgraduate Write-up award from the University of Melbourne. This work was supported by NHMRC Project grants (#350245 and 509048) to JDW.

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