

## Development of a high throughput receptor binding assay using time-resolved fluorescent europium chelate

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### Introduction

The structure-activity study of insulin-like peptide 3 (INSL3) requires the design and synthesis of various analogues. In order to test the designed analogues of INSL3 for their receptor binding affinity, previously, we have been using either radioactive-labelled <sup>125</sup>I-INSL3 or <sup>33</sup>P-relaxin with pK<sub>i</sub> values of 9.68 ± 0.09 and 9.34 ± 0.24 respectively. The use of radio labelled peptides has drawbacks including their short half life, high cost, and the need for prior preparation and their toxicity. To overcome these problems, we have decided to develop a high throughput receptor binding assay using time-resolved fluorescent lanthanide europium chelate. This has been used as probe in various biological assays such as kinase assays [1], immuno-fluorometric detection assays [2] and ligand-receptor interaction assays [3]. The emitted fluorescent light from this lanthanide probe has unique characteristics such as a very long luminescence half-time, large Stoke's shift and sharp emission peak which allows measurement of signal to be made after the decay of short-lived background fluorescence [4]. These factors contribute to the low background and high sensitivity of this fluorescent probe which is becoming an increasingly common useful tool in clinical chemistry and molecular biology.

### Results and Discussion

*N*<sup>1</sup>-(*p*-isothiocyanatobenzyl)-diethylenetriamine-*N*<sup>2</sup>, *N*<sup>3</sup>, *N*<sup>3</sup>-tetraacetic acid (Eu<sup>3+</sup> DTTA) (Fig.1) was used to label INSL3. The Eu<sup>3+</sup> DTTA reacts with free amino groups on proteins and peptides.

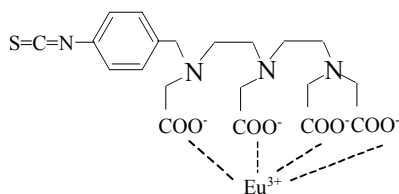


Fig. 1. Structure of Eu<sup>3+</sup> DTTA with isothiocyanate group which reacts with free amino groups at alkaline pH.

INSL3 contains two free N-termini and a lysine, therefore there is a possibility of multi-labeling. In order to prevent this, the lysine residue was mutated to arginine and the proline at the N-terminus of the B-chain was mutated to pyroglutamic acid. The modified INSL3 was then labeled with Eu<sup>3+</sup> DTTA (10 eq, 47.4 nmol) in 50 mM

NaHCO<sub>3</sub>, 150 mM NaCl, pH 8.5, at 4 °C overnight. The reaction was monitored using HPLC (A = 20mM triethylammonium acetate (TEA), pH 7.2; B = 20 mM TEA in 90% CH<sub>3</sub>CN). This condition failed to yield the desired labeled peptide. In the next step, the labeling buffer was changed to 100 mM Na<sub>2</sub>CO<sub>3</sub>, pH to 9.2 and this also didn't give the desired product. In the final attempt, lowering the pH to 6.5 also failed and the Eu<sup>3+</sup> DTTA didn't react with the N-terminus amino group of the modified INSL3 A-chain.

After failing to label the modified INSL3 using various labeling reaction conditions, we decided to label native INSL3. The labeling of native INSL3 was carried using 5 fold excess of the Eu<sup>3+</sup> DTTA in 100 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.2 at 4 °C overnight. This condition gave the desired labeled INSL3 and as expected there was mono- and di-labeled INSL3 confirmed using MALDI-TOF.

After chemical characterization of labeled INSL3, we then performed competition binding assays (Fig.3) to determine its affinity (pK<sub>i</sub>) for RXFP2 in HEK 293 cell line expressing RXFP2. The pIC<sub>50</sub> of the INSL3 for RXFP2 using Eu-INSL3 was determined to be 9.2 ± 0.06, n=6. This value is similar to that of radioactively labeled INSL3. Using Eu-INSL3, we have also tested one of the analogues of INSL3 which was previously tested with <sup>33</sup>P-relaxin and the affinity of the analogue was found to be similar in both cases (pIC<sub>50</sub> 6.35 ± 0.08, n=3 and pK<sub>i</sub> 6.65 ± 0.08).

Due to the problem of obtaining multi-labeled peptide and the difficulty of quantitating the amount of labelled peptide, we then specifically incorporated a different europium chelate DTPA-tetra(tBu ester) (Fig. 2) at the N-terminus of A-chain on solid phase. This method of labeling yielded a mono-labeled peptide which is currently being chemically and pharmacologically characterized.

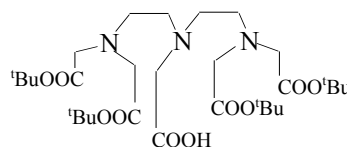


Fig. 2. Structure of DTPA with a free carboxylic group that can be coupled to a free amino group of peptides on solid phase.

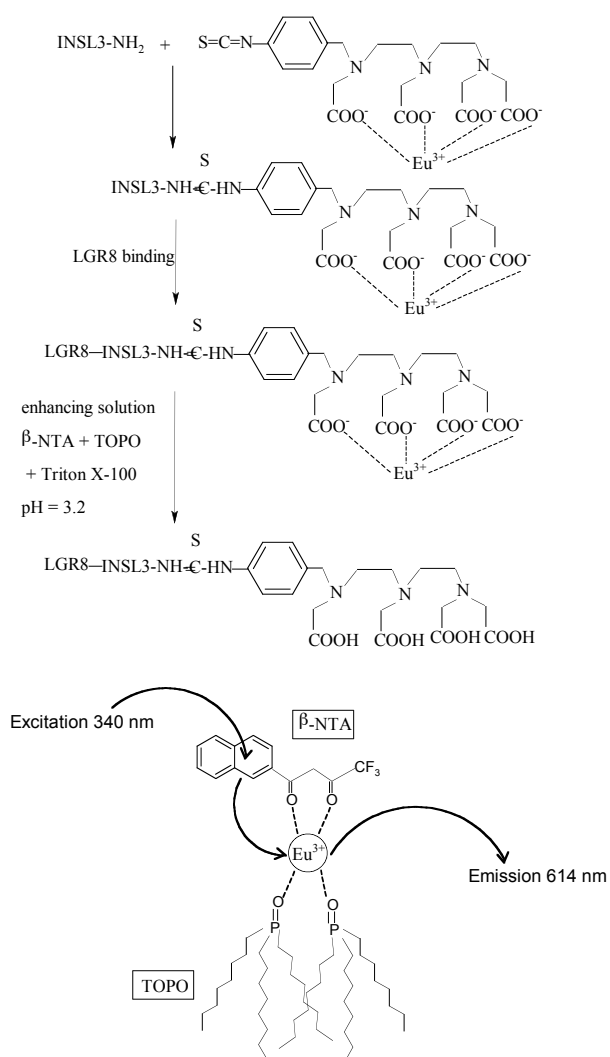


Fig. 3. Reaction of lanthanide-bound chelate with amino group on INSL3 and liberation of Europium from the chelation after binding of INSL3 to its receptor LGR8. Liberated europium binds to beta-NTA and co-ligand TOPO which is then excited at 340 nM. Intense fluorescent light is emitted at 640 nM.

### Acknowledgments

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