EPSRC Centre for Doctoral Training (CDT) in Molecular Sciences for Medicine (MoSMed)

Delivering Mechanistic Understanding and Novel Approaches to Native Chemical Ligation

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Project overview/context

Native chemical ligation (NCL) is a chemical reaction which is critical in the synthesis of proteins. It has enabled chemists to prepare highly potent peptide-based pharmaceuticals for a range of diseases. Despite its ubiquity, aspects of the reaction's mechanism are still debated, so brute-force reaction screening is commonly employed, which is an expensive and time-consuming process. We will use a quantitative, combined synthetic-kinetic approach to elucidate the mechanism for NCL across a range of amino acids. Using this understanding, we will circumvent current limitations and drawbacks of NCL and deliver a new, more efficient and operationally simple bioconjugation strategy.

Research Project

Background Native chemical ligation (NCL) was a revolutionary development in the synthesis of peptides, which has increased the maximum number of residues in a synthetic sequence from 50 to over 400. NCL allows for the formation of long-sequence peptides through the coupling of smaller fragments. Despite its undoubtable position as a cornerstone of modern synthetic peptide chemistry, mechanistic aspects of NCL are still debated. Owing to the lack of fundamental mechanistic understanding, a brute-force approach is widely used to establish optimal NCL reaction conditions.

The major reaction manifold to achieve NCL is the incorporation of a thiol-containing amino acid at the N-terminus of one fragment and a thioester incorporated into the C-terminus of another. Some mechanistic studies have been carried out on this strategy, but these have not been extensive with many reaction outcomes rationalised in a non-quantitative manner. For example, certain amino acid side chains (e.g., Asp and Glu) have been shown to have a detrimental impact on the reaction rate and yield, which is proposed to be the result of a competing intramolecular cyclisation process, although this has not been verified quantitatively through kinetic analysis.

Another common mechanistic misconception widely assumed in NCL is that a thiol-containing amino acid has a single pK_a value. Our recent published results (*Ref. 1*) dispute the commonly accepted NCL synthetic dogma. Typically, N-terminal cysteine residues are drawn in thiolate form. The preliminary study has revealed that this species only accounts for a maximum of 23% of the total of ionisation states between pH 6 and 8. Owing to this complexity in the nucleophilicity of the N-terminal residue, most NCL strategies have focused on variation at the C-terminus.

[1]O. R. Maguire, J. Zhu, W. D. Brittain, A. S. Hudson, S. L. Cobb and A. C. O'Donoghue "N-terminal speciation for Native Chemical Ligation", *Chem. Commun.* 2020, 56, 6114-6117.

Strategic Vision Probing these mechanistic questions will circumvent the need for brute-force screening, allow for full understanding of the reaction and underpin delivery of a new NCL strategy. By understanding the role of speciation, smarter, more efficient reaction manifolds in which all peptide fragments are added simultaneously can potentially be employed, and their ligation order determined simply by a switch in pH.

Research Plan

Development of Quantitative Mechanistic Understanding: We will quantitatively assess the reaction kinetics for different thiol(ate) species and assess how structural differences between N-terminal

cysteine and thiolated amino acid analogues affect their intrinsic nucleophilicities. Quantitative consideration of both the speciation (*c.f.* Ref. 1) and the kinetics, will enable correlation between abundance of thiolate species and reaction rates. This approach is necessary for a quantitative, predictive understanding of NCL under relevant synthetic conditions and any competing reactions (oxidation, hydrolysis).

Development of a New Ligation Strategy: Utilising the above knowledge, including for non-natural amino acid derivatives, we will evaluate the potential for N-terminus/pH-controlled NCL.

Employment of the New Ligation Strategy: The new pH-controlled bioconjugation strategy will be tested for a variety of targets relevant to the MoSMed CDT *e.g.* the synthesis of peptide and peptoid libraries, and peptide-focused assays.

Training & Skills

Synthesis Training: Dr Brittain will provide training on the synthesis of custom peptide fragments and unnatural amino acids. A range of solid phase and solution phase techniques will be employed to give broad training in synthetic peptide chemistry.

Kinetic Training: Prof. O'Donoghue will deliver training in kinetic method development, reaction monitoring, kinetic analysis and data evaluation. Parallel hands-on training on relevant instrumentation *e.g.* NMR, HPLC, mass spectrometry *etc.* will be provided.

Ligation Strategies: Dr Brittain will provide training in NCL synthetic methods. We will work closely with co-supervisor Prof. Mike Waring and other MoSMed investigators in identifying appropriate targets.

Computational Training: An additional external partner on the project will be Prof. Lynn Kamerlin, University of Uppsala. A visit to Prof. Kamerlin's laboratory through the course of the PhD would be envisaged. This would provide valuable additional, complementary training to the student on high level computational methods.

Further Information

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How to Apply

To apply for this project please visit the Durham University application portal to be found at: https://www.dur.ac.uk/study/pg/apply/

Please select the course code F1A201 for a PhD in Molecular Sciences for Medicine and indicate the reference MoSMed21_11 in the 'Field of Study' section of the application form. Please note that there is no need to submit a Research Proposal with your application however we do require a Covering Letter, CV, an academic transcript, the contact details of two referees and proof of English language proficiency if appropriate.

Should you have any queries regarding the application process at Durham University please contact the Durham MoSMed CDT Manager, Emma Worden at: emma.worden@durham.ac.uk