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Quantitative cross-linking/mass spectrometry using isotope-labelled cross-linkers☆

Lutz Fischer[a,1], *Zhuo Angel Chen*[a,1], *Juri Rappsilber*[a,b,*]

[a]*Wellcome Trust Centre for Cell Biology, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JR, United Kingdom*
[b]*Department of Biotechnology, Technische Universität Berlin, 13353 Berlin, Germany*

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ABSTRACT

Dynamic proteins and multi-protein complexes govern most biological processes. Cross-linking/mass spectrometry (CLMS) is increasingly successful in providing residue-resolution data on static proteinaceous structures. Here we investigate the technical feasibility of recording dynamic processes using isotope-labelling for quantitation. We cross-linked human serum albumin (HSA) with the readily available cross-linker BS3-d0/4 in different heavy/light ratios. We found two limitations. First, isotope labelling reduced the number of identified cross-links. This is in line with similar findings when identifying proteins. Second, standard quantitative proteomics software was not suitable for work with cross-linking. To ameliorate this we wrote a basic open source application, XiQ. Using XiQ we could establish that quantitative CLMS was technically feasible.

Biological significance

Cross-linking/mass spectrometry (CLMS) has become a powerful tool for providing residue-resolution data on static proteinaceous structures. Adding quantitation to CLMS will extend its ability of recording dynamic processes. Here we introduce a cross-linking specific quantitation strategy by using isotope labelled cross-linkers. Using a model system, we demonstrate the principle and feasibility of quantifying cross-linking data and discuss challenges one may encounter while doing so. We then provide a basic open source application, XiQ, to carry out automated quantitation of CLMS data. Our work lays the foundations of studying the molecular details of biological processes at greater ease than this could be done so far.

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1. Introduction

"τά ὄντα ἰέναι τε πάντα καὶ μένειν οὐδέν" — all entities move and nothing remains still [1]. Dynamic aspects of proteins play a pivotal role in many if not all biological processes. Unfortunately, the analysis of protein dynamics remains a technological challenge, as does the analysis of protein structures. Cross-linking/mass spectrometry (CLMS) is finally emerging after many years of method development as a highly successful tool in the structural analysis of proteins and multi-protein complexes [2,3]. Adding quantitative measurements to CLMS would allow expanding this success to the analysis of protein dynamics, such as conformational changes and protein–protein interaction dynamics.

CLMS currently involves chemical or light-induced cross-linking to covalently fix proximities in proteins or multi-protein

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* *Corresponding author at: Wellcome Trust Centre for Cell Biology, Institute of Cell Biology, The University of Edinburgh, Michael Swann Building, Edinburgh EH9 3JR, Scotland, United Kingdom.* Tel.: +44 1316517057; fax: +44 1316505379.
E-mail address: juri.rappsilber@ed.ac.uk (J. Rappsilber).

[1] Authors contributed equally.

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complexes [4–6]. Following proteolytic cleavage of the proteins, the cross-linked peptides are identified by mass spectrometry and database searches. An early analysis of a multi-protein complex, the Ndc80 complex (180 kDa, four sub-units) [7], guided crystallisation trials to success [8]. The approach was benchmarked and found to be highly accurate by investigating the 0.5 MDa RNA polymerase II complex (12 sub-units) and provided reciprocal footprints of the transcription factor TFIIF and Pol II [9]. Integration of multiple data sources including cross-linking has also been used to describe phage packaging motor incorporation [10] and has recently led to a model of the proteasome [11], where classical structure determination tools alone have failed for more than a decade. Some of these studies relied on isotope-labelled cross-linkers to enhance the identification success of cross-linked peptides [3,11] while others utilised the high resolution of modern mass spectrometers [7,9].

Adding quantitation to structural analyses by CLMS is an attractive next step that should benefit from the well-established tools of quantitative proteomics. Proteins and their modifications are quantified using isotope labelling [12–15] or label-free approaches. Signal intensities of peptides as measured by mass spectrometry are proportional to peptide concentration and are used routinely for quantitation. Indeed, CLMS has been used to qualitatively reflect conformational changes of single proteins [2,16] or multiple binding sites [9] through observation of conformation-specific cross-links and also in a label free approach for the quantitation of protein–protein interactions [17]. Isotope labelling for quantitation has been explored for cross-link analysis of conformation changes [18]. However, it has yet to be implemented properly for cross-linked peptides. Software tools will likely be of importance, given the key role MaxQuant software [19] has played in making SILAC a success in quantitative proteomics.

Isotope-labelled cross-linkers were introduced some time ago [20,21], and are used extensively to help identify cross-linked peptides, as any doublet resulting from the simultaneous use of light/heavy labelled cross-linker pair indicates a cross-linker-containing peptide [2,7,22–24]. Identification confidence of cross-linked peptides can be increased e.g.: by pre-filtering MS2 spectra for those precursors that were observed as doublets before the search and thus reducing the noise of database searches [7]; by using the mass shifts observed at MS2 level during the search, when both precursors of a doublet were selected for fragmentation [24]; or by adding confidence after the search by checking if identified cross-links were indeed observed as doublets [2]. Unfortunately, using isotope labelled cross-linkers for identification conflicts with their use for quantitation. Selecting both labelled precursors for fragmentation, a need for reliable identification, becomes less likely as ratios deviate from 1:1, which happens when structural changes are being traced. Here, high-resolution mass measurements of cross-linked peptide masses and their fragments may offer a solution. This label-free approach has proven highly successful in CLMS for the purpose of identifying cross-links thus leaving isotope labels for quantitative purposes [9].

The advantage of quantifying by use of cross-linker over other chemical labels, such as isobaric labels (iTRAQ [25] or TMT [26]), is the added identification confidence in knowing, *a priori*, that one looks at a cross-linker containing species. Furthermore, the additional step of adding e.g. iTRAQ or TMT to introduce the label is avoided. Labelled cross-linkers have the same advantage over SILAC [15] as all chemical labelling schemes of not using labelled proteins. Labelling the proteins for cross-linking during synthesis [27] excludes certain biological materials from analysis, such as human serum. Isotope-labelled cross-linkers might therefore be the most general and practical way of introducing isotopes to cross-linked peptides for quantitation. Obviously, this only holds true as long as a given cross-linker is available in isotope-labelled form.

We set out to test isotope-labelled cross-linkers for quantitation. As a model system, we cross-linked human serum albumin and quantified cross-links at different mixing ratios of heavy and light cross-linker. Quantitation was done either manually or by exploiting MaxQuant. As we observed limitations with either method, we developed an application, XiQ, to prove the technical feasibility of the entire approach by combining the accuracy of manual quantitation with the speed of automated quantitation. This also created a reference data set that may be used to test other established quantitation software. The XiQ application and the mass spectrometric raw data used here are available from http://xiq.rappsilberlab.org.

2. Methods

2.1. *Cross-linking and sample preparation*

Fifteen microgram aliquots of 0.75 M human serum albumin (HSA) (Sigma) in cross-linking buffer (20 mM HEPES-KOH, 20 mM NaCl, 5 mM MgCl2, pH 7.8) were each cross-linked with mixtures of bis[sulfosuccinimidyl] suberate-d0 (BS3-d0) (Thermo Fisher Scientific) and its deuterated form bis [sulfosuccinimidyl] 2,2,7,7-suberate-d4 (BS3-d4) (Thermo Fisher Scientific). For the purpose of quantitation, BS3-d0 and BS3-d4 were mixed with three molar ratios, 1:1, 1:2 and 1:4. The ratio of BS3-d4:HSA was 4:1 (by mass) in all three mixing ratios. Three replicas were prepared for each ratio. The cross-linking reaction was incubated at room temperature (~23 °C) for 1 hour, and quenched by addition of ammonium bicarbonate and incubation for 30 minutes at room temperature. Cross-linked protein samples were isolated on SDS–PAGE gel, and in-gel digested using trypsin following a standard protocol [7]. After digestion, peptide solutions were desalted using self-made C18-StageTips [28], following the published protocol [28] for subsequent mass spectrometric analysis.

2.2. *Mass spectrometry*

We used as analytical column a spray emitter (75-μm inner diameter, 8-μm opening, 250-mm length; New Objectives) that was packed with C18 material (ReproSil-Pur C18-AQ 3 μm; Dr Maisch GmbH, Ammerbuch-Entringen, Germany) by help of an an air pressure pump (Proxeon Biosystems) [29]. Mobile phase A consisted of water and 0.1% formic acid. Mobile phase B consisted of acetonitrile and 0.1% formic acid. Peptides were loaded onto the column with 1% B at 700 nl/min flow rate and eluted at 300 nl/min flow rate with a gradient: 1 minute linear increase from 1% B to 9% B; linear increase to 35% B in 169 minutes; 5 minute increase to 85% B. The eluted peptides

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