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Published in:
Chem. Commun.

DOI:
10.1039/C7CC07001B

Publication date:
2017

ChemComm

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: J. Lohse, A. Schindl, N. Danda, C. P. Williams, K. Kramer, B. Kuster, M. Witte and G. Médard, *Chem. Commun.*, 2017, DOI: 10.1039/C7CC07001B.

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View Article Online
DOI: 10.1039/C7CC07001B

Journal Name

COMMUNICATION

Target and identify: triazene linker helps identify azidation sites of labelled proteins via click and cleave strategy

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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A method for identifying probe modification of proteins via tandem mass spectrometry was developed. Azide bearing molecules are immobilized on functionalised sepharose beads via copper catalysed Huisgen-type click chemistry and selectively released under acidic conditions by chemical cleavage of the triazene linkage. We applied this method to identify the modification site of targeted-diazotransfer on BirA.

Proteins are modified post-translationally with a large variety of chemical groups. Besides the enzymatic introduction of phosphates, glycans, lipids, or ubiquitin-like proteins, also endogenous small molecule electrophiles modify proteins.[1] Furthermore, inhibitors and probe molecules have been prepared that react covalently with proteins of interest. Studying these protein modifications has been an active yet challenging field of research over the past 20 years[2] that has led to valuable insights into the role of these modifications in many biochemical processes, for instance those underlying pathological states of cells and tissues.[3] Holistic proteomics approaches enable the global study of protein modifications and these techniques provide unparalleled insight into the effect of modifications on the organisation of the cell or entire organisms of unforeseen complexity.[4] At the same time, targeted proteomics approaches have evolved that dissect the function of modifications on individual protein(-groups) in complex mixtures,[5] thereby tendering two valuable systems that complement each other.

Targeted chemical proteomics relies predominantly on *in situ* selective and site-specific modification of proteins with chemical probes combined with modified proteome detection by tandem mass spectrometry. Despite the recent advances in the field it is still challenging to directly identify peptide-probe adducts amongst the background of an entire proteome, especially if these peptides are low abundant, ionize poorly or are too small/large to be reliably detected. To reduce the sample complexity, it has been shown that peptide-probe adducts can be enriched by affinity-purification with biotin-(strept)avidin.[6] Under optimized conditions, this system has recently enabled the detection of 4217 peptides from azidohomoalanine labelled cell digests.[7] However, it has been suggested that the biotin-(strept)avidin system bears several drawbacks (false-positives, timing, cost, (strept)avidin peptide noise in the MS). Some of these can be overcome by incorporating a cleavable linker into a probe that allows selective release of the probe-adducts.[8] Solid supported reagents that can be used to capture-and-release the probe-peptide adducts chemically also provide an alternative to the biotin-(strept)avidin system.[9] An added advantage is that these solid supported reagents may be used in conjunction with reported systems and that they enable introducing new functionalities. Design of such novel reagents therefore is still in demand. Ideally, the solid supported reagents should be straightforward to synthesize, allow efficient enrichment of the modified peptides and introduce a functional group that improves the detection.[10] With this mind, we prepared a novel clickable and cleavable resin based on the acid labile triazene group[11] and employed it to study azidation of proteins by our recently reported targeted diazotransfer probe.[12] Cleavage of the triazene moiety, known as an protecting group for secondary amines,[13] in a complex biological environment is feasible, as was demonstrated by the work of Hejesen *et al*. on DNA-directed chemistry.[14] We therefore synthesized *m*-benzoic acid propynylpiperazine triazene **1**, a minimal molecule containing three functional moieties: a carboxylic acid for immobilization onto a solid matrix, a terminal alkyne for click-capture of azido-biomolecules and, to tether these two handles, a linker containing the triazene group (Fig. 1A). The propynylpiperazine moiety constitutes both the reagent reacting bioorthogonally with the azide minitag and the secondary amine for the triazene protecting group. This design

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Electronic Supplementary Information (ESI) available: experimental details, supporting figures, spectral data. See DOI: 10.1039/x0xx00000x

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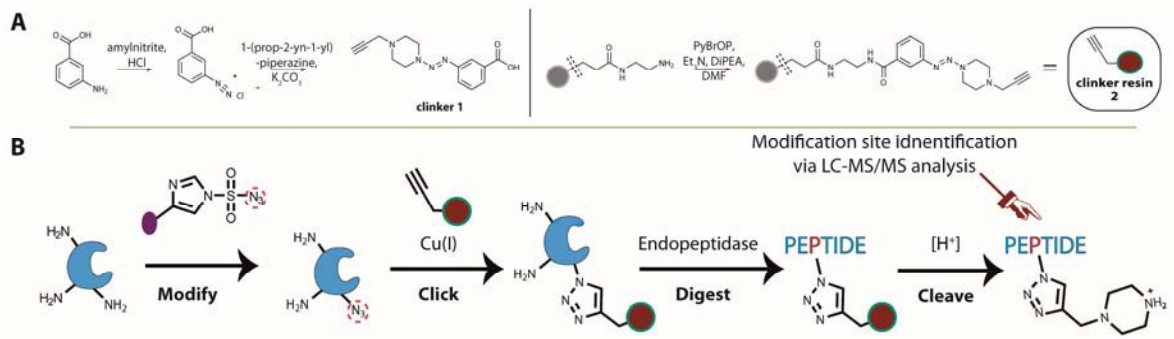

Fig. 1 The clinker resin **2** enables immobilisation and controlled release of azide bearing molecules. (A) Synthesis of the clinker resin **2**. (B) Concept of the target and identify strategy: targeted diazotransfer reagent installs an azide onto a protein of interest. The clinker resin **2** is used to capture the azide-containing proteins. Modified peptides remain covalently attached to the resin after on-bead digestion with an endopeptidase and a washing step. Acidification of the resin to a pH of 1 cleaves the triazene linkage between resin and peptide and releases the peptide-clinker adduct for further analysis with tandem mass spectrometry.

allows for covalent enrichment of the target on protein or peptide level by Huisgen-type click chemistry[15] followed by extensive and possibly denaturing washing steps to remove non-specifically bound species. Upon acidic release of the proteins or peptides from the resin, the target molecule is tagged with a triazolylmethylpiperazine adduct. Through this design, an additional positive charge is introduced in the modified biomolecule under acidic conditions, thereby improving ionisation and helping identification by mass spectrometry. We here demonstrate that molecule **1**, which we coined "clinker", a clickable and cleavable linker, facilitates straightforward identification of ligand-directed labelled sites in proteins: we show that the modified sites of streptavidin and BirA targeted by our diazotransfer probe could be efficiently determined using this strategy.

The synthesis of clinker **1** was carried out in two steps (Fig. 1A) by diazotisation of 3-aminobenzoic acid using the method described by Knoevenagel[16] and subsequently adding the dissolved diazonium salt directly to a basified aqueous solution of 1-(2-propynyl)piperazine. Amine functionalized sepharose beads[17] were then equipped with **1** to yield the clinker resin **2**. Functionalisation of the resulting resin with BODIPY-azide **3** using copper catalysed alkyne-azide cycloaddition (CuAAC)[18] proceeded uneventfully, demonstrating that on-bead CuAAC is indeed feasible (Fig. S1A). The resulting fluorescently labelled resin was used to determine the sensitivity of the triazene linker towards acidic cleavage. The linker was stable up to pH 2, but lowering the pH further led to cleavage of the triazene as could be visualised by the loss of fluorescence of the beads and concomitant appearance of colouration of the supernatant (Fig. S1B). To quantify the efficiency of retrieval and release under the optimal clinker cleavage conditions, we employed the fluorogenic 3-azido-7-hydroxycoumarin **4**.[19] This coumarin derivative has been used extensively in the study of CuAAC reaction parameters:[18a,20] Fluorescently quenched in its unreacted form, the fluorescence of **4** is activated upon cycloaddition with an alkyne. The retrieval and cleavage efficiencies could therefore be determined by measuring the fluorescence intensity of **6** after clicking coumarin **4** onto the resin and subjecting the resin to acid (Fig. 2). Treatment with different acidic solutions confirmed the triazene stability down to a pH of 2. We observed sharp cleavage upon acidification to a pH of 1 with a solution of 0.1 M aqueous hydrochloric acid (Fig. 2), conditions under which the sepharose beads remain stable (further acidification to pH 0 leads to disintegration of the resin material and obliteration of fluorescence altogether). Loading of the resin was concentration dependent and quantification of the fluorescence of the cumulative amount of released material revealed that the yield over two steps is above 90%, based on the trendline (Fig. S2).

Clinker resin **2** was subsequently tested in a protein enrichment experiment (Fig. 3A). Subjecting proteins to the cleavage conditions did not result in degradation (Fig. S3). Therefore, we introduced selectively and site-specifically an azide group on streptavidin employing our recently reported probe DtBio **7** (Fig. 3B)[12] and immobilized it onto the beads via triazole formation. The capture-and-release of the modified

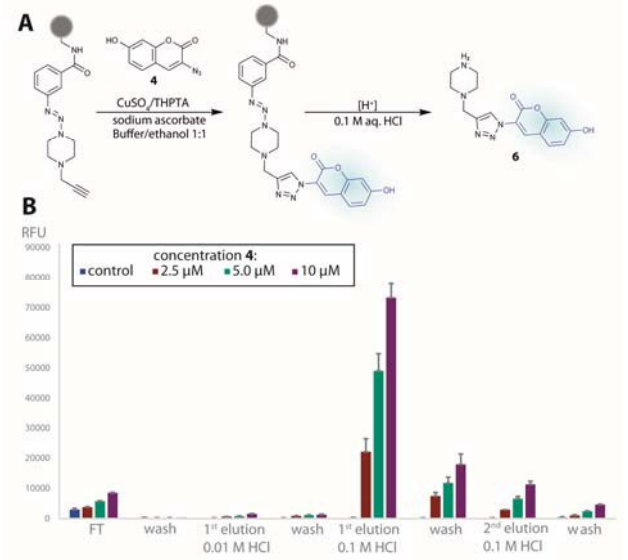

Fig. 2 Analysis of the capture-and-release efficiency with 3-azido-7-hydroxycoumarin **4**. Bar diagram depicts the observed fluorescence intensity under the given condition. The control reaction contains 10 µM **4** and lacks copper, error bars indicate standard deviation, measurements in triplicates.

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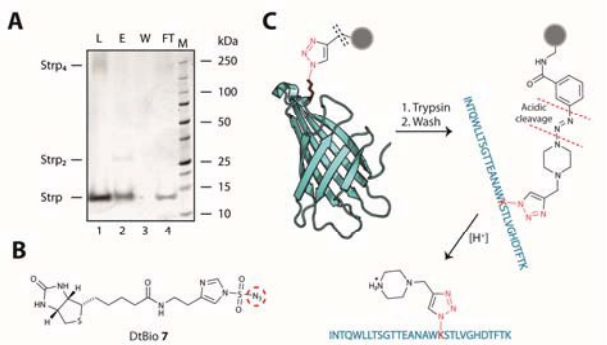

Fig. 3 Capture-and-release of diazotised streptavidin. (A) Coomassie stained SDS-PAGE gel of retrieved diazotised streptavidin: L – protein loading, E – eluate, W – wash, FT – flow-through. (B) Diazotransfer probe DtBio **7**. (C) On-bead digestion of diazotised streptavidin immobilised on clinker resin.

proteins was followed by analysis on a coomassie stained SDS-PAGE gel. Comparing the eluate to the input material indicates that approximately 60% of the streptavidin is recovered over three steps (i) modification via diazotransfer, (ii) immobilisation via clicking and (iii) release via cleavage, when using a protein-to-probe ratio of 1 to 1. The majority of the material remaining in the flowthrough was not modified, as judged from fluorescent labelling with DBCO-TAMRA, indicating that capture-and-release efficiency is considerably higher (Fig. S4). To test the target and identify strategy, we opted for on-bead digestion of immobilised streptavidin with trypsin using standard protocols (Fig. 3C). After post-digestion washing steps with Tris-HCl, 30% acetonitrile in water and water, only those peptides that have been diazotised on protein level are expected to be retained on the resin thanks to the covalent bond established by the clinker. In the next step these peptides were eluted using a solution of 0.1 M aq. HCl and sonication. The released peptides, now bearing the piperazine clinker fragment ($C_7H_{10}N_4$, 150.0906 Da) on the modified lysine, were then analysed with tandem mass spectrometry. Analysis of the raw data using the software MaxQuant[21] identified lysine K121 to be the only lysine bearing a 150.0906 Dalton mass gain as variable modification confirming our previous reported result,[26] yet with a significantly simplified data set (5% of the identified peptide species compared to the full digest) and shortened measuring time (60 min instead of 2 h), conditions with enhanced potential for future applications (Fig. S5, ESI Excel table).

To demonstrate the general applicability of the clinker-aided target and identify approach, we endeavoured to study the labelling of the 33.5 kDa bifunctional ligase/repressor BirA from *E.coli* with DtBio **7**. This enzyme functions as a biotin transferase and as an auto-feedback regulator, binding to the biotin operon in its dimeric state and thereby repressing gene transcription, once it gets saturated with biotinyl-5'-adenylate. The enzyme's affinity for biotin was determined to be 50 nM.[22] This enzyme and derived mutants have proven invaluable in many biotechnological applications, notably for proximity-labelling.[23] We performed a qualitative molecular docking study, which suggests that DtBio **7** can bind to the active site of BirA (Fig. 4A). According to the calculated binding model, two lysine residues in proximity of the sulfonyl azide group can act

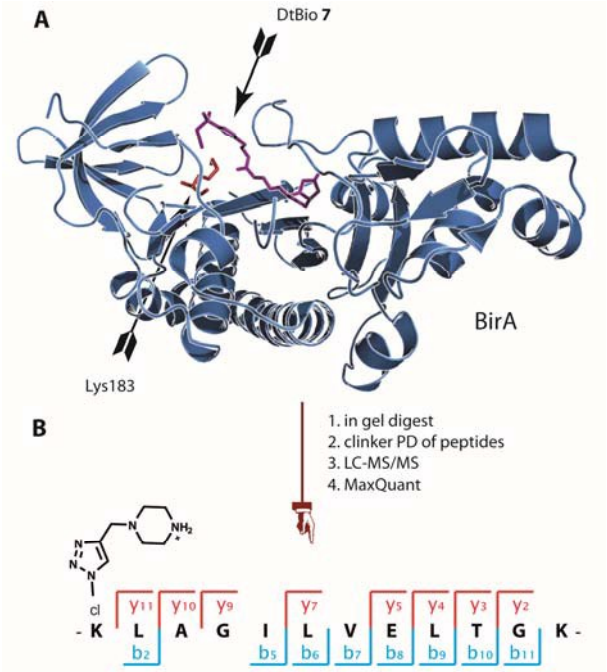

Fig. 4 Clinker pull-down of BirA peptides bearing an azide modification. (A) Model depicting DtBio **7** (deepurple) bound to the active site of BirA (PDB code: 4WF2; skyblue; lysine K183 in firebrick). (B) Workflow for the identification of modified peptides. The sequence of the only detected peptide from BirA that bears the clinker adduct (mass: 1390.8660 Da, mass gain: 150.0906 Da, Andromeda score: 125) is depicted. The modification is located in the b1/y12 position of the sequence and identifies as Lys183.

as potential diazotransfer acceptors. In this pose, the ε-amino group of K183 is in closer proximity than that of K172 (Fig. S6) and we therefore considered K183 to be the most likely site of modification if diazotransfer was to happen. To investigate whether DtBio **7** indeed does label BirA *in vitro*, the protein was expressed in *E.coli* and purified using standard protocols. Incubating BirA with 100 μM of DtBio **7** and 500 μM of copper sulphate for 1 h followed by labelling with BODIPY-alkyne for in gel fluorescent visualisation revealed that diazotransfer had occurred (Fig. S6). Akin to what we observed for streptavidin,[12] BirA labelling is outcompeted by the addition of biotin to the reaction mixture, suggesting active-site affinity-based binding. This result not only encouraged us to use BirA for the target and identify approach but also suggests DtBio **7** or derivatives thereof as potential probes for BirA related applications. The azide modified peptides obtained from labelling purified BirA with DtBio **7** and subsequent in-gel tryptic digestion were clicked onto the clinker resin **2**. Stringent washing of the resin to remove unmodified, non-specifically bound peptides, was followed by elution of the covalently bound peptides with 0.1 M aq. HCl and sonication. Analysis of the eluted peptides by LC-MS/MS and MaxQuant resulted in the identification of lysine K183 as the sole modification site of the labelling pair DtBio-BirA (Fig. 4B). LC-MS/MS analysis of the complete digest of BirA after modification with **7** corroborates the result for

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identification of the modification site from the clinker pull down (ESI Excel table).

In summary, we have demonstrated that the immobilisation of azide bearing species with the newly developed clinker resin is possible for small molecules, peptides and proteins. Furthermore, we have established two new 'target and identify' workflows that allowed the identification of the regioselective diazotransfer modification sites. The modified amino acid of DtBio labelled streptavidin and the novel target BirA could be respectively identified (1) after CuAAC on the protein level and on-bead digestion and (2) after performing the click reaction on the peptide level i.e. the azido-peptide could be enriched from a BirA in-gel digest.

As a simple, yet versatile chemical tool, the clickable and cleavable resin should prove valuable for a myriad of chemical proteomics applications. It leverages the potential of targeted labelling by diazotransfer probes by enabling straightforward identification of the targets and binding sites of ligands. In association with metabolic insertion of e.g. azido-lipids or -amino acids, biosynthetic pathway alterations will be evidenced. Stable isotope-based multiplexing with different flavours of propynylpiperazine can also be conceived to compare e.g. treated vs. non-treated cells or organisms or to perform time-course experiments and we therefore envision an adoption by chemical biologists interested in enriching azido-labelled biomolecules.

The Netherlands Organisation for Scientific Research (NWO-VENI grant number 722.012.003 to M.D.W, NWO-VIDI grant number 723.013.004 to C.P.W.) and a Marie Sklowdoska Curie-CIG (CIG PCIG14-GA-2013-631057 to M.D.W) are acknowledged for financial support. B. Ruprecht is thanked for insightful discussions. There are no conflicts to declare.

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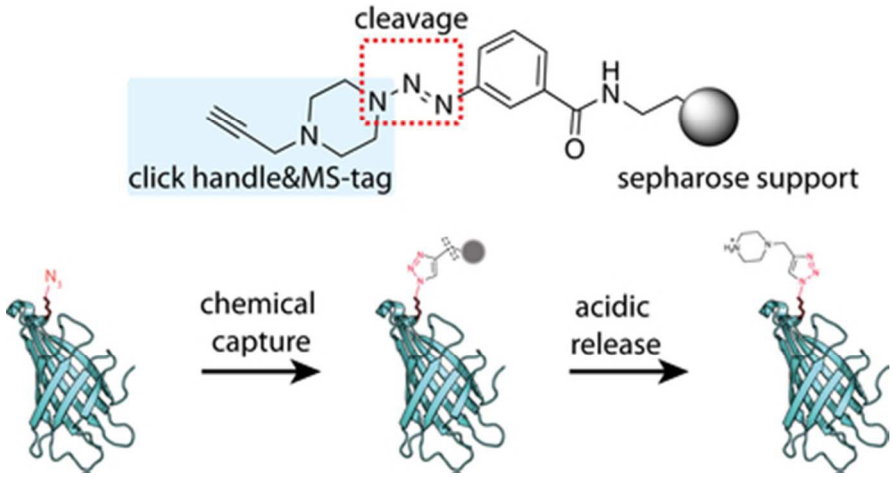

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