



Universiteit
Leiden
The Netherlands

Bioorthogonal peptide enrichment from complex samples using a rink-amide-based catch-and-release strategy

Leeuwen, T. van; Doelman, W.; Kieboom, R.W.R. van den; Florea, B.I.; Kasteren, S.I. van

Citation

Leeuwen, T. van, Doelman, W., Kieboom, R. W. R. van den, Florea, B. I., & Kasteren, S. I. van. (2023). Bioorthogonal peptide enrichment from complex samples using a rink-amide-based catch-and-release strategy. *Chembiochem*, 24(8). doi:10.1002/cbic.202300082

Version: Publisher's Version

License: [Creative Commons CC BY-NC-ND 4.0 license](https://creativecommons.org/licenses/by-nc-nd/4.0/)

Downloaded from: <https://hdl.handle.net/1887/3656815>

Note: To cite this publication please use the final published version (if applicable).

Bioorthogonal Peptide Enrichment from Complex Samples Using a Rink-Amide-Based Catch-and-Release Strategy

Tyrza van Leeuwen^{+, [a]} Ward Doelman^{+, [a]} Robin W. R. van den Kieboom,^[a] Bogdan I. Florea,^{*, [a]} and Sander I. van Kasteren^{*, [a]}

Uptake and processing of antigens by antigen presenting cells (APCs) is a key step in the initiation of the adaptive immune response. Studying these processes is complex as the identification of low abundant exogenous antigens from complex cell extracts is difficult. Mass-spectrometry based proteomics – the ideal analysis tool in this case – requires methods to retrieve such molecules with high efficiency and low background. Here, we present a method for the selective and sensitive enrichment of antigenic peptides from APCs using click-antigens; antigenic proteins expressed with azidohomoalanine (Aha) in place of methionine residues. We here describe the capture of such

antigens using a new covalent method namely, alkyne functionalized PEG-based Rink amide resin, that enables capture of click-antigens via copper-catalyzed azide-alkyne [2 + 3] cycloaddition (CuAAC). The covalent nature of the thus formed linkage allows stringent washing to remove a-specific background material, prior to retrieval peptides by acid-mediated release. We successfully identified peptides from a tryptic digest of the full APC proteome containing femtomole amounts of Aha-labelled antigen, making this a promising approach for clean and selective enrichment of rare bioorthogonally modified peptides from complex mixtures.

Introduction

Mass spectrometry-based chemical proteomics is a technique to characterize the function, modifications and interactions of proteins in their native environment.^[1] One of the challenges in this field is the enrichment of very low abundant proteins of interest from complex mixtures as this requires very high efficiency of the retrieval reaction, and an elution protocol that is essentially background-free.^[2] This becomes particularly poignant in the field of antigen processing and presentation, as here large antigen presenting cells (APC) take up exogenous antigens, that are degraded to peptides that are loaded on APC major histocompatibility complexes (MHCs) for presentation to T cells.^[3] Antigen presentation to T cells is exquisitely sensitive, with as few as 1–2 copies of a peptide loaded on MHC molecules being enough to activate a T cell.^[4] The precise rate and nature of proteolysis during the antigen processing process can profoundly affect the efficiency of antigen presentation and T cell activation.^[5] Certain proteolytic events can, for example, result in the liberation of a particular peptide for MHC-

loading,^[6–7] whereas in other instances, a specific proteolytic event can lead to the destruction of a particular T cell epitope, leading to low T cell activation.^[8–9] Post-translational modification of antigen, for example through citrullination, can shift this balance leading to altered processing. In case of self-antigen this can lead to the presentation of neo-epitopes, which can lead to auto-immune disorders.^[10–11]

The precise degradation mechanisms of antigen processing can be studied *in vitro*,^[12] although this does not recapitulate the topological complexity of the processes that occur within the antigen presenting cell. Antigens are routed in a concerted fashion through various compartments that contain different protease activities which process the antigen to peptides that are subsequently loaded on either MHC-I or MHC-II type complexes.^[13] Studying this process inside APCs would therefore be of prime importance to the field. We have recently reported the application of “click antigens” as tool to visualize antigen routing and processing in the context of living APCs.^[14] These are recombinant antigen proteins containing bioorthogonal handles that are specifically reactive only in so-called ‘click’ reactions.^[15] Click reactions are highly efficient bioorthogonal reactions, that enable selective conjugation of (bio)molecules labelled with a certain reactive group, without influencing the non-labelled molecules present in the cell or lysate. The eponymous click reaction is the copper catalyzed azide-alkyne cycloaddition (CuAAC);^[16] this reaction involves the formation of a triazole ring by cycloaddition between an azide function and a (terminal) alkyne, mediated by copper(I) catalysis. Both of these functional groups are uncommon in nature but readily introduced into biomolecules via synthetic means, and are typically referred to as ‘click handles’.

In the case of protein antigens, azide labelled variants are produced recombinantly by the replacement of methionine residues by azidohomoalanine (Aha) during the bacterial

[a] T. van Leeuwen,⁺ Dr. W. Doelman,⁺ R. W. R. van den Kieboom, Dr. B. I. Florea, Prof. S. I. van Kasteren
 Division of Bio-organic Synthesis
 Leiden Institute of Chemistry, Leiden University
 Einsteinweg 55, 2333 CC, Leiden (The Netherlands)
 E-mail: b.florea@chem.leidenuniv.nl
 s.i.van.kasteren@chem.leidenuniv.nl

[†] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cbic.202300082>

© 2023 The Authors. ChemBioChem published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution Non-Commercial NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

expression of the protein,^[17–19] using a technique called bio-orthogonal non-canonical amino acid tagging (BONCAT).^[17,20–21] The introduction of this azide-bearing amino acid as a click handle will allow for selective targeting and conjugation of the exogenous antigen inside the antigen presenting cell. We previously demonstrated that visualization of internalized click antigens could be achieved by CuAAC mediated fluorophore conjugation.^[14] Incorporation of bioorthogonal amino acids introduces only minor changes in the protein structure,^[14,22] while azide remains stable inside the lysosome for up to 24 hours, allowing for the imaging of antigen processing even when the antigen is degraded.^[23]

We hypothesized that the click antigen approach might also find application in a chemical proteomics platform. The click-reactive groups could then be used for retrieval, rather than visualization, providing information on the intactness of an antigen during routing and proteolytic processing inside the APC. The archetypal approach would entail the CuAAC-mediated conjugation of the tagged antigens to biotin, followed by enrichment with avidin-agarose. The biotin-avidin interaction is specific, strong and can only be broken by heating above 70 °C.^[24] A downside of this method is the unwanted capturing of endogenously biotinylated proteins causing identification of many false positives. The method also suffers from non-specific bound protein background that is irreproducible and difficult to remove by detergents or high ionic strength washing steps. These drawbacks are of particular concern considering the very low amounts of antigen that may be present in antigen presenting cells during the processing events and, when studying the processing of self-antigens (which can underpin auto-immune disease) any background resulting from endogenous antigens present in the antigen presenting cell is a complicating factor.

One way to overcome these issues is to remove the biotin-avidin step by covalently binding the clickable antigens directly to a functionalized solid support, preferentially one that can be cleaved again in order to release the enriched peptides. The solid support resin beads have a high chemical stability that permits extremely stringent washing in order to reduce the background signal of non-specifically bound peptides. Previous work in this area involved the development of agarose,^[25–26] Sepharose^[27] and polyacrylamide based supports.^[28] Inorganic supports, in the form of silica,^[29] and cobalt^[30]-based functionalized particles have also been used as solid supports for the selective enrichment of bioorthogonally tagged peptides and proteins. Examples of cleavable linkers are levulinoyl esters,^[31] disulfides,^[32–33] diazobenzenes,^[34] acid- or photo-cleavable linkers.^[35–36] However, in practice, the use of these approaches is hindered by the fact that the sensitivity of such approaches is often low due to poor chemical release of the linker.

Here, we present an alternative approach using the acid-cleavable linkers commonly used in solid phase peptide synthesis. By employing commercially available poly(ethylene glycol)-based resins modified with alkynes linked via an acid-labile Rink-amide linker as the basis of an azide-selective capture medium. The method presented here is highly efficient for retrieval of nascent Aha-labelled peptides, showing greater

efficiency of capture compared to established methods. The methodology is fully bioorthogonal, is compatible with high salt buffers, denaturing conditions, and whole cell lysate to recover peptides present in low nanomolar concentrations. We further show that after internalization of azido-tagged antigen by dendritic cells, our enrichment method yields retrieval of antigen peptides with essentially no background, proving the suitability of the method to study antigen processing on peptide level.

Results and Discussion

Synthesis and evaluation of alkyne functionalized resins

Our aim was to produce an inexpensive alkyne-modified solid support for the selective enrichment of azide-modified peptides. We set our sights on the resin beads commonly used in solid support peptide synthesis (SSPS). While the original polymer supports introduced by Merrifield^[37] consisted of hydrophobic polystyrene material, modern developments have produced more water compatible supports, typically consisting of polyethyleneglycol (PEG) chains grafted onto a polystyrene core.^[38] More recently solid supports consisting of only PEG have been reported and commercialized.^[39] The water-compatibility, chemical stability and ready availability of these latter supports, made them excellent candidates for an acid-cleavable retrieval resin.

To facilitate release of the captured peptides from the resin, we again looked at the chemistries used commonly in the field of peptide synthesis. Liberation of synthesized peptide from the support is typically achieved by acidolysis of an acid-labile linker function. Several different linkers have been developed, with the most popular linkers requiring high concentrations (50–95%) of trifluoroacetic acid (TFA) in aprotic solvent to mediate cleavage. TFA itself is an excellent solvent for unprotected peptides, and TFA salts are not expected to hinder MS analysis, two desirable properties for our goals. The two main TFA-labile linker functionalities used in peptide synthesis are the Rink-amide linker,^[40] and the para-hydroxybenzyl (PHB)-based linkers.^[41] Between these, the Rink-amide had our preference, as cleavage of this moiety leads to the formation of an uncharged carboxamide, that is favored for positive mode electrospray ionization during mass spectrometry analysis over the carboxylic acid formed after acid-mediated cleavage of a PHB-based linker.

As a proof of concept, we selected two different commercially available, aqueous solvent compatible, solid supports, Tentagel S and NovaPEG, both pre-functionalized with the Rink-amide linker. These two supports represent the two most popular hydrophilic SPPS resins, with Tentagel S being of the PEG-grafted polystyrene variety and NovaPEG consisting of pure PEG-based material. We derivatized these into CuAAC ready supports 1–4 (Figure 1A) by functionalization with two different alkynes: 5-aminohexynoic acid and a triethyleneglycol (TEG) containing alkyne. Loading, as specified by the manufacturer, was similar for both resins. We rationalized that the

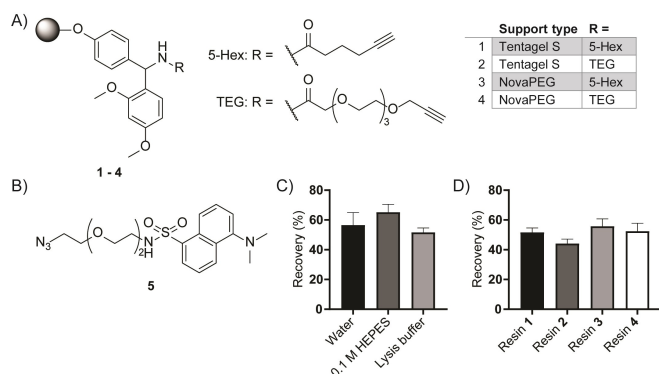


Figure 1. Overview of the different alkyne modified resins produced and their evaluation. Structures of alkyne modified solid supports 1–4 (A). Structure of azide-functionalized dansyl 5 for recovery efficiency determination (B). Evaluation of buffer compatibility using a CuAAC reaction between alkyne-resin 1 and azide 5 (C). Recovery (%) was determined from peak integration of LC-UV using unmodified 5 as an internal standard. Comparison of the efficiency of the recovery of azide 5 using alkyne-resins 1–4 from lysis buffer (D). The recovery (%) was determined as in C. Experiments are the average of $n=3$ and displayed as mean \pm SD.

lipophilic character of the Rink-amide linker could hinder the aqueous click reaction, that might be partially alleviated using the more water-soluble TEG alkyne. We first evaluated the effectiveness of resin 1 in the retrieval of fluorescent azide 5 from aqueous buffer (Figure 1C). After Cu(I)-mediated click, followed by washing and release of the clicked molecule with TFA, around 50% recovery was achieved (as determined by LC-UV analysis) when the reaction was carried out in water, with a similar result when adding small or large amounts of salts to the reaction solvent, in the form of 100 mM HEPES buffer (pH 7.2) or a high salt lysis buffer (8 M urea, 1 M NaCl, 200 mM TRIS pH 8.0, 4% (w/v) CHAPS detergent). Encouraged by these results, we evaluated supports 1–4 in the same manner (Figure 1D). We found that the four different supports performed equally well in this assay. However, the samples obtained using resins modified with the TEG-based alkyne (2,4) showed additional signals in the mass analysis and were therefore not considered applicable for proteomic experiments (Figure S1). We selected the NovaPEG-based support 3 to continue the experiments.

Next, we studied the recovery of azide-modified peptides. The introduction of an azide functionality was accomplished using the unnatural amino acid azidohomoalanine (Aha). For these experiments, we synthesized three peptides (6–8, Figure 2A), the first two were derived from the myelin auto-antigen myelin oligodendrocyte glycoprotein (MOG),^[42,43] and the third derived from vinculin,^[44] an auto-antigen implicated in rheumatoid arthritis. We modified these peptides with the fluorescent dansyl-group to aid detection of the peptides by LC-UV. As our end-goal was to recover azide-modified peptides from complex cell lysates, we first evaluated the ability of resin 3 to recover these peptides from lysis buffer. We dissolved the different peptides into the previously described lysis buffer (final peptide concentration 10 μ M) and added these solutions to resin 3. We performed the Cu(I)-mediated click reaction for two hours,

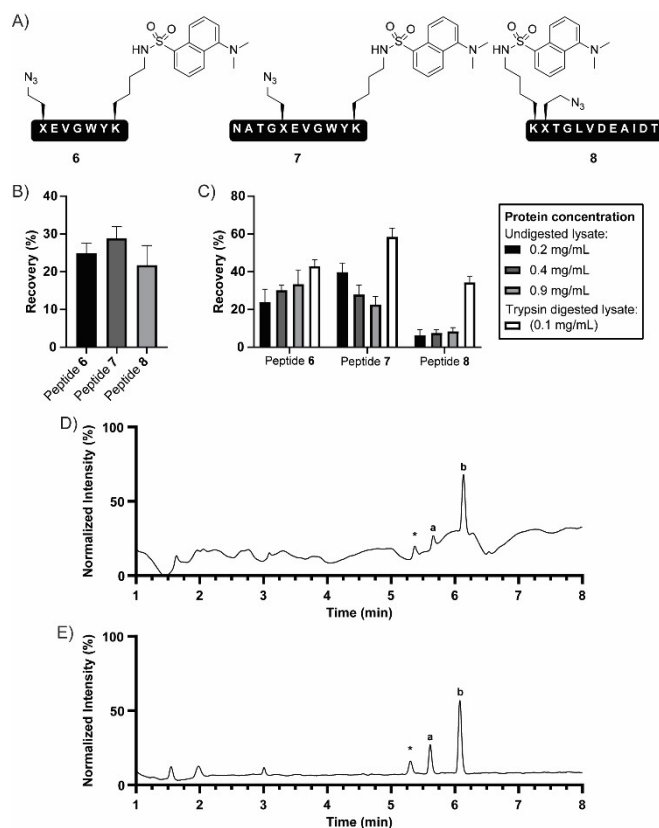


Figure 2. Recovery of dansyl-modified Aha-containing peptides from buffer and cell lysates using resin 3. Schematic structure of Aha-containing and dansyl modified peptides 6–8 (A). Recovery of peptides 6–8 using alkyne resin 3 (B). Recovery was determined by the area the peak corresponding to the detected peptide on LC-UV, compared to internal standard. Recovery of peptides 6–8 from various concentrations of BMDC lysates containing whole proteins, as well as a tryptic digest (C). The specified concentrations refer to the concentration of irrelevant non-labelled lysate proteins. Comparison of partial LC-UV traces of recovered peptide after lysate click and TFA release when using either MilliQ (D) or *N,N*-dimethylformamide (E) to wash the support after CuAAC reaction. ^arecovered peptide ^binternal standard ^{*}resin specific impurity.

followed by washing with MilliQ. The captured peptides were then released by treatment with TFA and the recovered peptides were analyzed by LC-UV to determine the recovery efficiency. In Figure 2B, the % recovery for these three peptides is shown. While the total recoveries here are lower than those found for fluorophore 5 (Figure 1C), the fact that peptide sequence seems to have little effect on the recovery shows that the method is unbiased for peptide identity. Further optimization of the recovery was attempted, but no major improvement was seen (Figure S2). We proceeded to testing recovery from cell-lysate where peptide was spiked into the solution (Figure 2C). The effect of this complex mixture of biomolecules on the observed recoveries varied greatly between the peptides. Gratifyingly, when instead of intact proteins the lysate was first digested by overnight incubation with trypsin, the % recovery increased for each peptide (Figure 2C).

We also investigated non-specific interactions between the resin and the proteins in the mixture (Figure 2D–E). Figure 2D

shows a partial LC-UV trace when, between click reaction and TFA release, the support was washed only with MilliQ. Here, the noisy character of the baseline indicates the presence of large amounts of unidentified background molecules. By simply washing the support three times with *N,N*-dimethylformamide (DMF), the interference of the non-specific background was greatly reduced (Figure 2E).

The application of resin 3 to retrieve low abundant Aha-peptides from complex mixtures

Having established the suitability of resin 3 for the pulldown of simple peptides, we next tested the retrieval of BONCAT-labelled *E. coli* proteome in a direct comparison with a commercially available alkyne-agarose containing the hydrazine-labile linker 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde-Alk-agarose). *E. coli* B834(DE3) cells were transformed with a plasmid containing the native auto-antigen vinculin₄₃₅₋₇₄₁ (Vin) under control of the isopropyl β -D-thiogalactopyranoside (IPTG)-inducible T7 promoter.^[45] The cells were grown on methionine depleted medium in the presence of Aha to incorporate azides at all methionine sites in newly synthesized proteins. During this phase, no IPTG was added to prevent overexpression of Vin, but also no additional glucose was added to the cells so that minor amounts of leaky expression of Vin could occur,^[46,47] thereby being minorly abundant in *E. coli* cells. The cells were then lysed and incorporation of Aha was verified by reacting the lysate via CuAAC with AF647-alkyne and subsequently resolving the labelled proteins by SDS-PAGE (Figure S3). Varying amounts of Aha-labelled *E. coli* lysate were then mixed with lysate of unlabeled bone marrow-derived dendritic cells (BMDCs) to provide a source of irrelevant background proteins. The mixtures were digested with trypsin, followed by retrieval of Aha-containing peptides using resin 3 or Dde-Alk-agarose. The amounts of resin were adjusted so that equal amounts of available alkyne handles (0.2 μ mol) were present in each experiment. Retrieved peptides were measured by MS-MS and identified by PEAKS software (FDR < 1%). Retrieval with resin 3 yielded 17.5 times more Aha-containing peptides compared to Dde-alkyne-agarose (Figure 3), indicating the efficiency of bioorthogonal peptide retrieval with resin 3. In total, circa 1300 unique Aha-containing *E. coli* peptides were retrieved from 100 μ g Aha-*E. coli* lysate using resin 3. Dde-Alk-agarose by comparison yielded only circa 70 peptides. Aha-containing peptides emerging from *E. coli* proteins, as well as the low abundant Aha-Vin (Figure 3B, Tables S1–2), could be detected from 1 μ g *E. coli* lysate spiked into 99 μ g of BMDC lysate, thereby also showing the high efficiency on retrieval of very low abundant proteins from complex mixtures. The method also proved very selective with only minor amounts of unmodified peptides (1.6% of total) identified (Figure S4).

To investigate whether our method could also be used to isolate and identify small amounts of labelled antigen from complex lysate, we diluted different amounts of the Aha-labelled auto-antigen vinculin (Aha-Vin) in 100 μ g BMDC lysate (Figure 4, Table S3). In theory, five Aha-containing Vin peptides

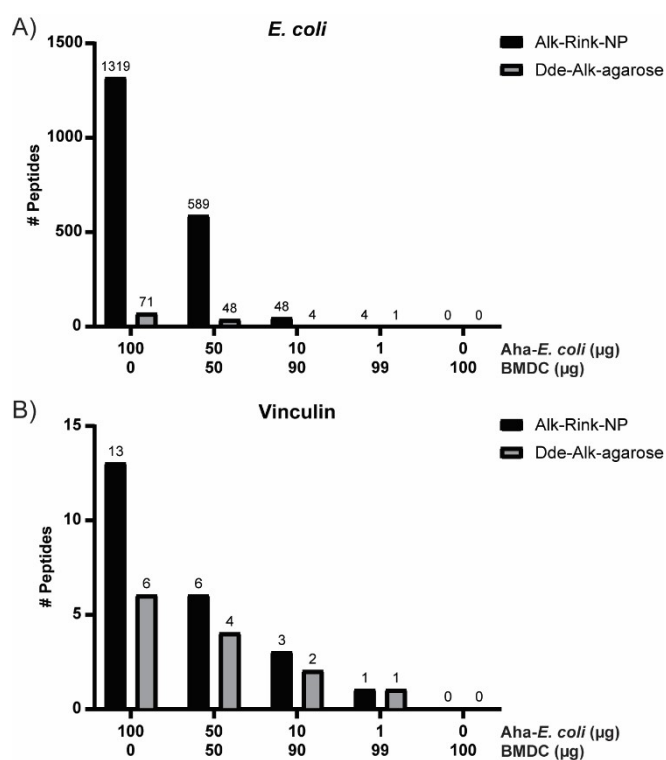


Figure 3. Amount of unique Aha-labelled peptides identified after enrichment by resin 3 or Dde-Alkyne-agarose from complex mixtures of BMDC and Aha-labelled *E. coli* lysate. The amount of confident (FDR < 1%) nascent Aha-labelled peptides emerging from *E. coli* proteins (A) or vinculin expressed by *E. coli* cells (B) enriched on either resin 3 (black bars) or Dde-Alk-agarose (grey bars).

are formed after tryptic digest, which can potentially be enriched from the mixture (Figure 4A). The detection limit of Aha-containing Vin peptides proved to be 1 ng (28 pmol) Aha-Vin in 100 μ g lysate, where still one tryptic Aha-Vin peptide was identified after enrichment using resin 3 (Figure 4B). Peptide #1 (HMLGEISALTSK) could not be retrieved in any of the experiments. This is likely due to often missed cleavages of trypsin after acidic residues such as aspartic acid.^[48] Full coverage of four tryptic Aha-peptides could be retrieved by spiking at least 0.1 μ g Aha-Vin in lysate (2.8 nmol). Stringent washing of the resin with DMF was proven to be very efficient as no murine or unmodified-Vin peptides were identified from any of the samples.

Finally, we investigated if our enrichment method could be applied to study cellular processes as antigen processing by retrieval of auto-antigens from APCs. BMDCs were pulsed with Aha-Vin (1.35 μ M) for two hours, then washed and lysed, which was followed by full proteome digest with trypsin and enrichment using resin 3 (Figure 4C, Table S4). Two out of four tryptic Aha-Vin peptides were retrieved without any background of endogenous proteins, proving the suitability to retrieve rare antigen from cells. In the future, our resin can be applied to study sub-cellular processes such as antigen processing of low abundant Aha-labelled antigen on peptide level.

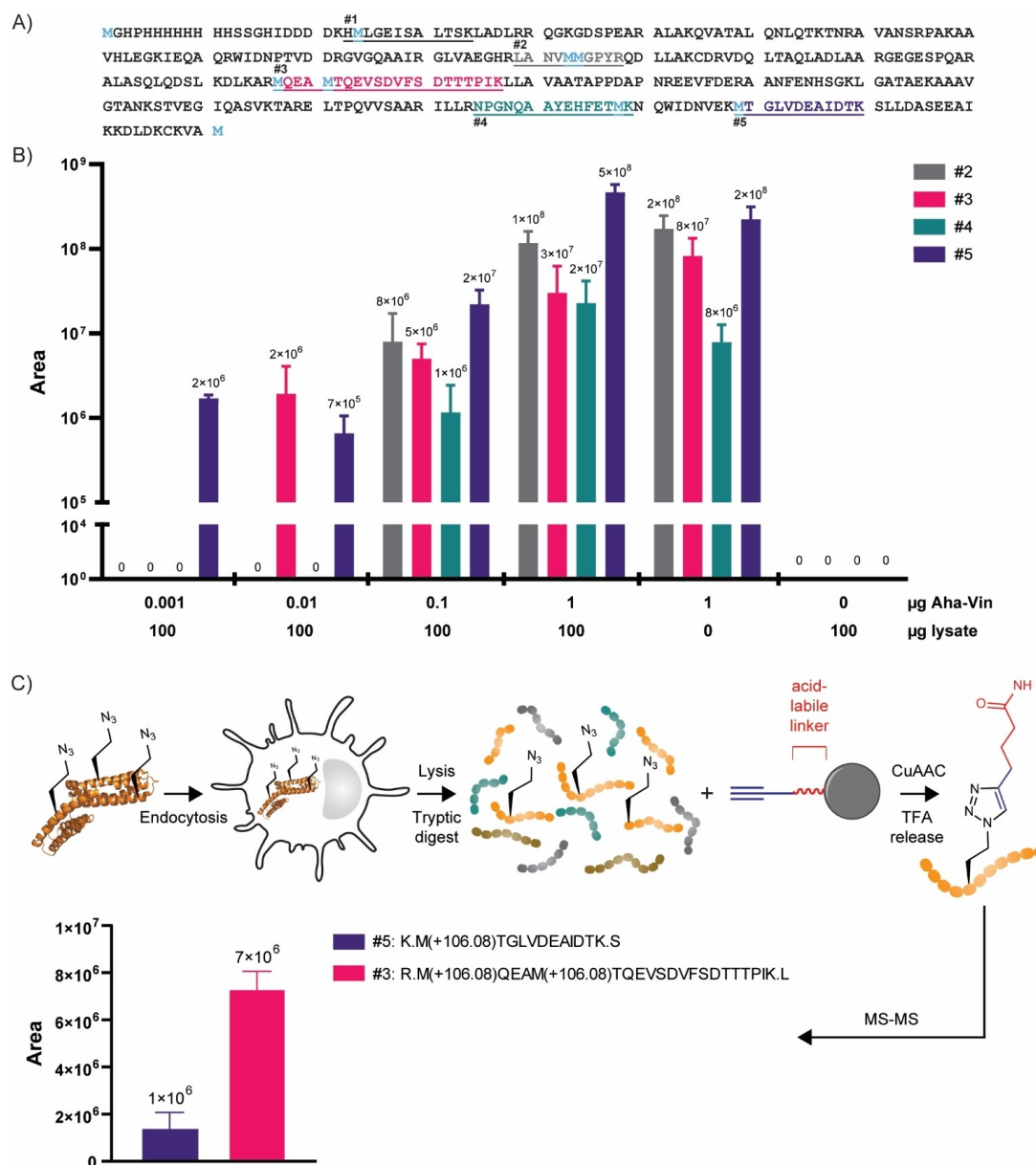


Figure 4. Retrieval of Aha-containing Vin peptides from lysate or live BMDCs ($n = 3$). Amino acid sequence of vinculin showing five theoretical tryptic peptides (A, #1–#5). Retrieval of Aha-containing Vin peptides after spiking different concentrations of Aha-Vin in BMDC lysate (B) or after pulsing live BMDCs with Aha-Vin for two hours (C). The peak area (y-axis) of the parent ion of the detected peptide was used as a measure of peptide abundance. Experiments are the average of $n = 3$ and displayed as mean \pm SD.

Conclusion

In this paper we describe the development of a cost-effective and highly efficient copper-click enrichment method for retrieval of low abundant azide-containing peptides from complex mixtures. We show that by modifying a commercially available poly-(ethylene glycol)-based resin, commonly used for the solid support synthesis of peptides, with an alkyne functionality, a reagent capable of retrieval of low abundant azide-containing peptides could be produced. The high chemical stability of the polymer allowed for stringent washing with DMF, which greatly reduced background signal of non-specific

ally bound molecules. Furthermore, the acid-mediated cleavage reaction is highly compatible with the purifications steps required in proteomics workflows. One potential downside of the acid-mediated release is that some acid-labile post-translational modifications (PTMs), like tyrosine sulfation or histidine phosphorylation, could be unintentionally destroyed during the acidic treatment, making this approach unusable in studies focused on detecting these PTMs. Fortunately, many PTMs relevant to autoimmune disorders, like citrullination^[49] and/or O-glycosylation,^[50] are known to be stable to these acidic release conditions. Regardless, in the future we aim to develop

additional methods for the enrichment of Aha-modified peptides with a broader PTM compatibility.

We show that the method works well on entire labelled bacterial proteomes, even in the presence of large quantities of unlabeled irrelevant peptides. Additionally, the method allowed for retrieval of protein antigen fragments from dendritic cell lysate, showing that this procedure is usable for the study of antigen processing in a cellular context. The solid supports and other reagents used in this study are widely available and commonly applied in many chemical biology laboratories, making this method easily accessible compared to alternatives. Furthermore, no complex chemical synthesis is required for the production of the modified resin itself, unlike many other examples of selective peptide capturing reagents. In conclusion, we have developed an inexpensive solid support based on widely commercially available materials for the selective enrichment of Aha-containing antigenic peptides.

Experimental Section

Chemicals: Chemical reagents for buffer preparation and chemical synthesis were purchased from Acros (Belgium), Chem-Lab (Belgium), Honeywell Riedel-de Haën (Germany), Merck (The Netherlands), Novabiochem (The Netherlands), Sigma Aldrich (The Netherlands), Sigma Life Sciences (The Netherlands) or Sphaero Hispanagar (Spain) and used without further purification unless stated otherwise. Fluorophores (Alexa Fluor 488 Azide, Alexa Fluor 488 Alkyne, Alexa Fluor 647 Azide and Alexa Fluor 647 Alkyne), were purchased from Thermo Fisher Scientific.

Bone Marrow Dendritic Cell Differentiation: Bone marrow derived dendritic cells (BMDCs) were isolated and cultured as previously described.^[14] On day 2, 5 ml fresh medium was added and on day 4 loosely adherent cells were split in fresh medium (10×10^6 cells/15 cm dish) and adherent cells were discarded. On day 8, differentiated BMDCs were used for experiments after expression of CD11c, MHC II, CD86 and CD115 were analyzed by flow cytometry.

Azidohomoalanine labelling of *E. coli* B834(DE3): A single colony of *E. coli* B834(DE3) transformed with pET3d-Vin₄₃₅₋₇₄₁ was grown overnight in 10 mL LB augmented with 1% w/v glucose and Ampicillin (50 µg/mL). The next morning, the culture was diluted 1:50 with fresh LB media augmented with 1% w/v glucose and Ampicillin (50 µg/mL) and cells were grown to an OD₆₀₀ of 0.3–0.5. The resulting culture was then centrifuged (2000 rcf, 10 min), washed and resuspended in SelenoMetTM media (Molecular Dimensions, USA) without additional methionine. The culture was incubated at 37 °C for 30 min, after which L-Azidohomoalanine (Aha, 4 mM final concentration) was added. After 1 h, the culture was centrifuged, washed with PBS and pellets were stored in –80 °C until further use.

Synthesis of alkynyl functionalized NovaPEG-based Rink-amide resin: Functionalization of NovaPEG resin was carried out using typical Fmoc–SPPS methodology. Briefly, 100 mg of NovaPEG Rink amide resin (loading 0.22 mmol/g, Novabiochem) was swelled in 2 mL of DMF for 15 minutes before the Fmoc-group was removed by treatment of the resin with a 20% (v/v) solution of piperidine in DMF (2 × 5 min), followed by thorough washing of the solid support (5 × 2 mL). 5-hexynoic acid (5 equiv., 0.1 mmol, 11 µL) and HCTU (5 equiv., 0.1 mmol, 41 mg) were dissolved together in DMF (200 µL), followed by addition of DiPEA (10 equiv., 0.2 mmol, 35 µL). This solution was added to the resin and incubated for 90 minutes. Completion of the reaction was verified by negative Kaiser test. The

resin was drained and washed with DMF (3 × 2 mL), DCM (3 × 2 mL) and methanol (3 × 2 mL) and stored at –20 °C until further use.

Synthesis of (S)-4-azido-2-aminobutanoic acid (Azidohomoalanine, H–Aha–OH): Azidohomoalanine was produced in-house as previously described.^[14]

Synthesis of Fmoc–Aha–OH: H–Aha–OH (0.72 g, 5 mmol, 1.0 equiv.) was dissolved in 1:1 H₂O:dioxane (50 mL). Sodium carbonate (0.53 g, 5 mmol, 1.00 equiv.) and Fmoc–OSu (1.69 g, 5 mmol, 1.00 equiv.) were added and the solution was stirred at room temperature (RT) for 4 h, after which TLC (1:9 EtOAc:DCM) showed complete consumption of the starting compound. The dioxane was removed under reduced pressure and the remaining aqueous solution acidified with 1 M HCl to pH 2–3. This aqueous solution was then extracted with ethyl acetate and the organic layer was collected, dried over MgSO₄ and concentrated. The residue was purified using silica gel column chromatography (10% EtOAc in DCM). Fmoc–Aha–OH was obtained as a pale-blue solid (1.12 g, 3.07 mmol, 61%) ¹H NMR (300 MHz, CDCl₃) δ 7.73 (dd, J = 7.4, 1.1 Hz, 2H, Fmoc–Ar), 7.59–7.52 (m, 2H, Fmoc–Ar), 7.41–7.33 (m, 2H, Fmoc–Ar), 7.28 (tt, J = 7.5, 1.0 Hz, 2H, Fmoc–Ar), 4.47 (d, J = 6.8 Hz, 3H, α-CH, Fmoc–CH₂), 4.20 (t, J = 6.5 Hz, 1H, Fmoc–CH), 3.35 (s, 2H, γ-CH₂), 2.25–1.85 (m, 2H, β-CH₂). ¹³C NMR (75 MHz, CDCl₃) δ 175.5, 156.4 (C=O), 143.9, 143.8 (Fmoc–Cq), 141.6, 128.0, 127.3, 125.1, 120.2, 120.2 (Fmoc–Ar), 67.6 (Fmoc–CH₂), 52.0 (α-CH), 47.9 (γ-CH₂), 47.5 (Fmoc–CH), 31.6 (β-CH₂).

Synthesis of Dansyl-PEG₂-azide (5): Azido-PEG₂-amine (27 mg, 0.16 mmol, 2.0 equiv.) was dissolved in 0.4 mL dry DCM. In a separate flask, Dansyl chloride (22 mg, 0.081 mmol, 1.0 equiv.) was dissolved in 0.8 mL dry DCM and this solution was added dropwise to the dissolved amine. The bright orange solution was stirred overnight at RT. The reaction mixture was diluted with 50 mL DCM and washed with 50 mL sat. aq. NaHCO₃ and 50 mL brine. The organic layer was dried over MgSO₄, filtered and concentrated. The residue was purified via column chromatography (1:1 DCM/EtOAc) producing a green, fluorescent oil (27 mg, 0.067 mmol, 85%). ¹H NMR (400 MHz, CDCl₃) δ 8.56 (dt, J = 8.5, 1.1 Hz, 1H), 8.33 (dt, J = 8.7, 0.9 Hz, 1H), 8.27 (dd, J = 7.3, 1.3 Hz, 1H), 7.57 (ddd, J = 19.5, 8.6, 7.4 Hz, 2H), 7.21 (dd, J = 7.7, 0.9 Hz, 1H), 5.36 (t, J = 6.0 Hz, 1H), 3.65–3.60 (m, 2H), 3.53–3.48 (m, 2H), 3.44–3.36 (m, 6H), 3.13 (td, J = 5.7, 4.6 Hz, 2H), 2.91 (s, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 152.08, 135.10, 130.51, 130.01, 129.76, 129.52, 128.43, 123.31, 118.97, 115.29, 70.45, 70.37, 70.15, 69.37, 50.69, 45.53, 43.18, 29.81. HRMS calcd for C₁₈H₂₅N₅O₄S [M + H]⁺: 408.1700, found 408.1699

Fmoc–SPPS synthesis of peptides: SPPS of peptides was carried out using manual synthesis on a 50 µmol on Tentagel S RAM resin (Rapp Polymere GmbH, Germany) when a C-terminal carboxamide was desired, or on chloro-(2'-chloro)-trityl (CTC) polystyrene resin when a C-terminal carboxylic acid was intended. Fmoc protected amino acids were purchased from either Novabiochem or Sigma-Aldrich. All standard sidechain protection groups were applied, with the exception of Lys(Mmt) used for on-resin fluorophore introduction. Fmoc deprotection was accomplished by repeated treatment with 20% piperidine in DMF (3 + 7 min). To extend the growing peptide chain, 5 equiv. of Fmoc-amino acid was mixed together with an equimolar quantity of 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU) in DMF at a concentration of 0.5 M, together with 10 equiv. of diisopropylethylamine (DiPEA). Coupling reactions were carried out for 30–45 minutes. Fmoc–Lys(Mmt)–OH was coupled using 2 equiv. of amino acid mixed together with an equimolar quantity of 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU) in DMF at a concentration of 0.2 M, together with 4 equiv. of diisopropylethylamine (DiPEA). These reactions were left to couple for 90 minutes. Global deprotection

and resin cleavage of peptides was accomplished using a 95:2.5:2.5 mixture of TFA/TIS/H₂O for 3 h, followed by precipitation from cold diethyl ether (1:9 ratio TFA to ether) and recovery of the precipitate by centrifugation. Crude, tryptophan containing peptides were dissolved in MilliQ water and lyophilized overnight in order to remove the residual carboxylate. Preparative reverse phase HPLC on a Waters AutoPurification system (eluent A: H₂O + 0.2% TFA; eluent B: ACN) with a preparative Gemini C18 column (5 μ m, 150 \times 21.2 mm) yielded the final products. Peptides were characterized using electrospray ionization mass spectrometry (ESI-MS) on a Thermo Finnigan LCQ Advantage Max LC-MS instrument with a Surveyor PDA plus UV detector on an analytical C18 column (Phenomenex, 3 μ m, 110 \AA , 50 mm \times 4.6 mm) in combination with buffers A (H₂O), B (MeCN), and C (1% aq TFA). Quality of crude and purified peptides was evaluated with a linear gradient of 10–90% B with a constant 10% C over 10 minutes. Preparative reverse phase HPLC on a Waters AutoPurification system (eluent A: H₂O + 0.2% TFA; eluent B: ACN) with a preparative Gemini C18 column (5 μ m, 150 \times 21.2 mm) yielded the final products. All peptides were purified to at least 95% purity as determined by HPLC-UV analysis.

Chemoselective deprotection of Lys(Mmt) and introduction of the Dansyl fluorophore: After the synthesis of the peptide sequence was completed, the resin was washed 3 times with DCM. Chemoselective deprotection of the Mmt group was achieved by addition of a mixture of acetic acid and trifluoroethanol (TFE) in DCM (AcOH/TFE/DCM 1:2:7) to the resin, which was incubated under gentle agitation for 2 h. After draining the resin and thorough washing with DCM, the resin was treated twice with 10% Et₃N in DCM (v:v) for 10 min. Residual Et₃N was removed by washing 5 times with DCM. Dansyl chloride (2 equiv.) and DIPEA (4 equiv.) were dissolved in DCM (1 mL / 50 μ mol resin loading) and added to the resin. The suspension was shaken overnight at RT. The excess solution was drained and the resin was washed 5 times with DCM and 3 times with DMF. Removal of the final Fmoc group and release of the peptide was carried out according to the general SPPS procedure.

Recombinant expression and purification of Aha-*vinculin*: Aha-*vinculin*⁴³⁵⁻⁷⁴¹ (Aha-Vin) was expressed and purified as previously described.^[14]

Preparation of Aha-labelled *E. coli*- and BMDC lysate: Aha-labelled *E. coli* lysate and BMDC lysate were prepared by incubation of cell pellets in lysis buffer (50 mM HEPES pH 7.2, 150 mM NaCl, 8 M UREA, 0.1% SDS, 1% IGEPAL, 1x EDTA-free protease inhibitor, 10 U benzonase) for 1 h on ice and subsequent probe sonication (4 \times 10 sec, 30% amplitude). Lysates were centrifuged (5 min at 10,000 rcf) to remove cell debris and protein concentration was measured by Bradford assay. The concentration of lysates was adjusted to 1 mg/mL and stored in -80°C .

Capture-and-release of fluorophore 5 or peptides 6–8 with alkyne modified solid supports: 1 mg of alkyne-modified resin was transferred to an Eppendorf vial. A 20 μ M solution of fluorophore 5 or peptide was prepared in MilliQ or buffer, which was first degassed for 15 minutes by bubbling N₂ through the solution. 100 μ L of the probe/peptide solution was added to the resin and the vial was shaken for 1 h at RT. To a separate vial CuAAC click mix was added in order: 2 μ L 0.1 M CuSO₄ solution, 2 μ L 1.0 M sodium ascorbate solution, 10 μ L 0.1 M THPTA solution and 2 μ L 1.0 M aminoguanidine solution. The solution was thoroughly mixed and diluted to 100 μ L with MilliQ or buffer. The click mix was added to the resin suspension and was shaken for 2 h at 25 $^{\circ}\text{C}$. The suspension was centrifuged and the supernatant was removed and discarded. The resin was subsequently washed three times by addition of MilliQ, followed by recovery of the resin by centrifugation and careful aspiration of the supernatant. Release of the retained molecules was initiated by

the addition of 50 μ L of release cocktail (TFA/TIPS/MilliQ 95:2.5:2.5) to the dry resin. The release reaction incubated for 2 h at RT, followed by quenching by the addition of 150 μ L MilliQ, followed by the addition of 2 μ L 1 mM probe/peptide solution as an internal standard. The suspension was centrifuged and the supernatant was collected and analyzed by LC-MS/UV-VIS ($\lambda = 280$ nm, 10 \rightarrow 50% B, mass range: 400–2000). Peptide recovery was quantified by the ratio of the area under the UV peak @ 280 nm of the released molecule (identified by MS) and the standard.

For experiments carried out in cell lysate, the labelled peptide was spiked into the lysate as 2 μ L of a 10 mM solution. The resin was first incubated with this solution for 1 h, followed by initiation of the click reaction as described above.

Enrichment of nascent Aha-labelled *E. coli* peptides using resin 3: Samples were prepared by diluting varying amounts of Aha-labelled *E. coli* lysate in BMDC lysate. Mixtures were reduced with DTT (5 mM final) for 15 minutes at 65 $^{\circ}\text{C}$ and free thiols were alkylated with iodoacetamide (12 mM final) for 30 min at RT in the dark. Mixtures were diluted 10 \times in 100 mM NH₄HCO₃, 1 mM CaCl₂, pH 8.0 and proteins were digested by 3 μ g trypsin (Sequencing grade, Promega) overnight by shaking at 950 rpm, 37 $^{\circ}\text{C}$. Trypsin was inactivated by incubation for 5 min at 95 $^{\circ}\text{C}$. 1 mg alkyne-modified resin resin was added to the samples and incubated for 1 h. Then, CuAAC click mix (1 mM CuSO₄, 10 mM sodium ascorbate, 1 mM THPTA ligand, 10 mM aminoguanidine, 100 mM HEPES pH 8.0) was added and samples were incubated for 2 h, RT at 1000 rcf. The resin was first washed 3x with MilliQ, then 3x with DMF and 3x with MilliQ by centrifugation at 4000 rpm for 3 min. Samples were incubated with 50 μ L TFA for 1 h to release peptides and dried by N₂ for 2 min. Eluted peptides were dissolved in 0.5% (v/v) formic acid solution in MilliQ and desalted over StageTips,^[51] then dried by SpeedVac (2 h at 45 $^{\circ}\text{C}$) and reconstituted in 30 μ L 97:3:0.1 solution (H₂O, ACN, CHOOH) for MS measurements.

Dde-alkyne-agarose enrichment of nascent Aha-labelled *E. coli* peptides: Mixtures were prepared as described above. 100 μ L of Dde-alkyne-agarose (50% slurry, Click Chemistry Tools) was washed with MilliQ (2 min, 1000 rcf) and added to the digested lysate mixtures. Then, CuAAC click mix was added and samples were incubated for 2 h, RT at 1000 rcf. Samples were transferred to micro Bio-SpinTM columns (Bio-Rad) and the resin was washed 5x with SDS wash (100 mM Tris, 1% SDS, 250 mM NaCl, 5 mM EDTA, pH 8.0), 5x with 8 M urea/100 mM Tris pH 8.0 and 5x with 20% ACN. Peptides were released by incubation with 200 μ L 2% (v/v) hydrazine for 1 h at RT with agitation. Eluted peptide mixtures were acidified by addition of 5 μ L formic acid and desalted over StageTips,^[51] then dried by SpeedVac (2 h at 45 $^{\circ}\text{C}$) and reconstituted in 30 μ L 97:3:0.1 solution (H₂O, ACN, CHOOH) for MS measurements.

Enrichment of Aha-containing Vin peptides from BMDC lysate: Varying amounts of Aha-Vin were spiked in 100 μ g BMDC lysate, reduced with DTT (5 mM final) for 15 minutes at 65 $^{\circ}\text{C}$ and free thiols were alkylated with iodoacetamide (12 mM final) for 30 min at RT in the dark. Samples were diluted 10 \times in 100 mM NH₄HCO₃, 1 mM CaCl₂, pH 8.0 and proteins were digested by 3 μ g trypsin (Sequencing grade, Promega) overnight by shaking at 950 rpm, 37 $^{\circ}\text{C}$. Aha-containing peptides were enriched by CuAAC reaction with 1 mg resin 3 for 2 h. The resin was first washed 3x with MilliQ, then 3 \times with DMF and 3x with MilliQ by centrifugation at 4000 rpm for 3 min. Samples were incubated with 50 μ L TFA for 1 h to release peptides and dried by N₂ for 2 min. Eluted peptides were dissolved in 0.5% (v/v) formic acid solution in MilliQ and desalted over StageTips,^[51] then dried by SpeedVac (2 h at 45 $^{\circ}\text{C}$) and reconstituted in 30 μ L 97:3:0.1 solution (H₂O, ACN, CHOOH) for MS measurements.

Enrichment of Aha-Vin peptides from live BMDCs: 5×10^6 BMDCs were seeded onto 10 cm dishes (non-tissue treated) in 10 mL IMDM supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma, ref# F0804, lot# 015 M3344), 2 mM Glutamax™ (GIBCO, ref# 35050-038), 20 μ M β -Mercaptoethanol (Gibco, ref# 31350010), 50 IU/mL penicillin and 50 μ g/mL streptomycin, and recombinant GM-CSF (20 ng/mL, Peprotech, ref# 315-03). After resting for 2 h, cells were pulsed with 1.35 μ M Aha-Vin for 2 h. Cells were washed with PBS (2x) and harvested by scraping. Cells pellets were collected in Eppendorf tubes and stored in -20°C until further use.

The cell pellets were lysed in lysis buffer (50 mM HEPES pH 7.2, 150 mM NaCl, 8 M UREA, 0.1% SDS, 0.1% IGEPAL, 1x EDTA-free protease inhibitor, 10 U benzonase) for 30 minutes at RT and subsequent probe sonication (4×10 sec, 30% amplitude). Lysates were centrifuged (5 min at 10,000 rcf) to remove cell debris. Denatured proteins were reduced with DTT (5 mM final) for 15 minutes at 65°C and free thiols were alkylated with iodoacetamide (12 mM final) for 30 min at RT in the dark. Samples were diluted 10x in 100 mM NH_4HCO_3 , 1 mM CaCl_2 , pH 8.0 and proteins were digested by 3 μ g trypsin (Sequencing grade, Promega) overnight by shaking at 950 rpm, 37°C . Aha-containing peptides were enriched using resin 3 as described above.

LC/MS/MS measurement and data analysis: The desalted peptide solution was separated on an UltiMate 3000 RSLCnano system set in a trap-elute configuration with a nanoEase M/Z Symmetry C18 100 \AA , 5 μ m, 180 μ m \times 20 mm (Waters) trap column for peptide loading/retention and nanoEase M/Z HSS C18 T3 100 \AA , 1.8 μ m, 75 μ m \times 250 mm (Waters) analytical column for peptide separation. The column was kept at 40°C in a column oven. Flow gradient used for analysis was a steep (45 min) gradient of mobile phase A (0.1% formic acid (FA) in ULC-MS grade water (Biosolve)) and mobile phase B (0.1% FA in ULC-MS grade acetonitrile (ACN, Biosolve)) controlled by a flow sensor at 0.3 μ L/min with average pressure of 400–500 bar (5500–7000 psi). Samples were injected (1 μ L) on the trap column at a flow rate of 15 μ L/min for 9 min with 99%A, 1%B eluent. The gradient was programmed with linear increment to 1% B from t_0 to t_2 min, 10%B to t_5 min, 30%B at t_{25} , 90%B at t_{26} to t_{33} and 1%B at t_{34} to t_{45} min. The eluent was introduced by electro-spray ionization (ESI) via the nanoESI source (Thermo) using stainless steel Nano-bore emitters (40 mm, OD 1/32", ES542, Thermo Scientific). The QExactive HF was operated in positive mode with data dependent acquisition without the use of lock mass, default charge of 2+ and external calibration with LTQ Velos ESI positive ion calibration solution (88323, Pierce, Thermo) every 3–5 days to less than 2 ppm. The tune file for the survey scan was set to scan range of 350–1400 m/z, 60,000 resolution, 1 microscan, automatic gain control (AGC) of $1e6$, max injection time of 50 ms, no sheath, aux or sweep gas, spray voltage ranging from 1.7 to 3.0 kV, capillary temp of 250°C and a S-lens value of 80. The sensitive MS method settings were: the survey scan was taken at 120,000 resolution, AGC target of $3e6$, maximum IT time of 100 ms, and scan range of 350 to 1400 m/z. For the 10 data dependent MS/MS events the loop count was set to 10 and the general settings were resolution to 15,000, AGC target $1e5$, max IT time 50 ms, isolation window of 1.6 m/z, fixed first mass of 120 m/z and normalized collision energy (NCE) of 28 eV. For individual peaks the data dependent settings were $1.00e3$ for the minimum AGC target yielding an intensity threshold of $2.0e4$ that needs to be reached prior of triggering an MS/MS event. No apex trigger was used, unassigned, +1 and charges $> +8$ were excluded with peptide match mode preferred, isotope exclusion on and dynamic exclusion of 10 sec. In between experiment samples routine wash and control runs were done by injecting 5 μ L 97.3:0.1 solution, 5 μ L of 10 fmol/ μ L BSA or enolase digest and 1 μ L of 10 fmol/ μ L angiotensin III (Fluka, Thermo)/oxytocin (Merck) to check the performance of the platform

on each component (nano-LC, the mass spectrometer (mass calibration/quality of ion selection and fragmentation) and the search engine).

Resulting MS RAW. files were analyzed by using PEAKS software 10.0 (Bioinformatic Solutions Inc.) and PEAKSDB search with a focused UniProt identifier database consisting of human vinculin₄₃₅₋₇₄₂, his-tag vinculin₄₃₅₋₇₄₂, BSA, yeast enolase, trypsin, avidin, and streptavidin was performed. Error tolerance was set to 10 ppm for the parent mass and 0.2 Da for fragmented ions. Enzyme specificity was set as 'Trypsin, semi-specific' and 30 PTMs per peptide were allowed. Carbamidomethylation (+57.02) was set as a fixed modification and Aha replacement by Met (−4.99), Met oxidation (+15.99), as variable modifications. The following variable modifications were added per enrichment method: modification of Met to Aha+5Hex (+106.08 Da) for enrichment using resin 3, Met to Aha-Dde (+50.06) for Dde-alkyne-agarose. FDR was set to 1% and only PTMs with ion intensity $> 5\%$ were considered confident. Protein coverage images were obtained by the software and peptide sequences were combined when found in at least 2/3 replicates.

SDS-PAGE analysis:^[52] For SDS-PAGE analysis all samples were heated for 5 minutes at 95°C (exception: samples containing click cocktail). 20 μ L of each sample was loaded onto a 15% SDS-PAGE gel (0.75 or 1.5 mm) and run for ~ 70 min at constant 170 V. Subsequently in-gel fluorescence was measured at indicated wavelength filters for Alexa 488, Alexa 647 or Cy5. For imaging the gels, Biorad Chemidoc Imager and ImageLab 5.2 software (Biorad) was used.

Acknowledgements Conflict of Interest

This work was funded by a Consolidator Grant (Grant number 865175-KineTic) from the European Research Council.

Acknowledgements Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: antigen processing · bioorthogonal chemistry · chemical proteomics · click-antigens · mass spectrometry

- [1] B. F. Cravatt, G. M. Simon, J. R. Yates Iii, *Nature* **2007**, *450*, 991–1000.
- [2] T. C. Walther, M. Mann, *J. Cell Biol.* **2010**, *190*, 491–500.
- [3] B. Ludewig, T. Junt, H. Hengartner, R. M. Zinkernagel, *Curr. Opin. Immunol.* **2001**, *13*, 657–662.
- [4] M. A. Purbhoo, D. J. Irvine, J. B. Huppa, M. M. Davis, *Nat. Immunol.* **2004**, *5*, 524–530.
- [5] S. I. van Kasteren, H. S. Overkleeft, *Curr. Opin. Chem. Biol.* **2014**, *23*, 8–15.
- [6] L. Delamarre, M. Pack, H. Chang, I. Mellman, E. S. Trombetta, *Science* **2005**, *307*, 1630–1634.
- [7] L. Delamarre, R. Couture, I. Mellman, E. S. Trombetta, *J. Exp. Med.* **2006**, *203*, 2049–2055.

- [8] B. Manoury, D. Mazzeo, L. Fugger, N. Viner, M. Ponsford, H. Streeter, G. Mazza, D. C. Wraith, C. Watts, *Nat. Immunol.* **2002**, *3*, 169–174.
- [9] C. X. Moss, J. A. Villadangos, C. Watts, *Eur. J. Immunol.* **2005**, *35*, 3442–3451.
- [10] S. M. Anderton, *Curr. Opin. Immunol.* **2004**, *16*, 753–758.
- [11] H. A. Doyle, M. J. Mamula, *Curr. Opin. Immunol.* **2012**, *24*, 112–118.
- [12] I. Z. Hartman, A. Kim, R. J. Cotter, K. Walter, S. K. Dalai, T. Boronina, W. Griffith, D. E. Lanar, R. Schwenk, U. Krzych, R. N. Cole, S. Sadegh-Nasseri, *Nat. Med.* **2010**, *16*, 1333–1340.
- [13] S. van Kasteren, H. Overkleeft, H. Ovaas, N. Jacques, *Curr. Opin. Immunol.* **2014**, *26*, 21–31.
- [14] T. van Leeuwen, C. Araman, L. Pieper Pournara, A. S. B. Kampstra, T. Bakkum, M. H. S. Marqvorsen, C. R. Nascimento, G. J. M. Groenewold, W. van der Wulp, M. G. M. Camps, G. M. C. Janssen, P. A. van Veelen, G. J. P. van Westen, A. P. A. Janssen, B. I. Florea, H. S. Overkleeft, F. A. Ossendorp, R. E. M. Toes, S. I. van Kasteren, *RSC Chem. Biol.* **2021**, *2*, 855–862.
- [15] C. R. Bertozzi, *Acco. Chem. Res.* **2011**, *44*, 651–653.
- [16] Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless, M. G. Finn, *J. Am. Chem. Soc.* **2003**, *125*, 3192–3193.
- [17] K. L. Kiick, D. A. Tirrell, *Tetrahedron* **2000**, *56*, 9487–9493.
- [18] J. C. M. van Hest, K. L. Kiick, D. A. Tirrell, *J. Am. Chem. Soc.* **2000**, *122*, 1282–1288.
- [19] K. L. Kiick, E. Saxon, D. A. Tirrell, C. R. Bertozzi, *Proc. Nat. Acad. Sci.* **2002**, *99*, 19–24.
- [20] K. L. Kiick, R. Weberskirch, D. A. Tirrell, *FEBS Lett.* **2001**, *502*, 25–30.
- [21] J. C. van Hest, K. L. Kiick, D. A. Tirrell, *J. Am. Chem. Soc.* **2000**, *122*, 1282–1288.
- [22] F. Lehner, D. Kudlinzki, C. Richter, H. M. Müller-Werkmeister, K. B. Eberl, J. Bredenbeck, H. Schwalbe, R. Silvers, *ChemBioChem* **2017**, *18*, 2340–2350.
- [23] T. Bakkum, T. van Leeuwen, A. J. C. Sarris, D. M. van Elsland, D. Poulcharidis, H. S. Overkleeft, S. I. van Kasteren, *ACS Chem. Biol.* **2018**, *13*, acschembio.8b00355–acschembio.00358b00355.
- [24] A. Holmberg, A. Blomstergren, O. Nord, M. Lukacs, J. Lundeberg, M. Uhlén, *Electrophoresis* **2005**, *26*, 501–510.
- [25] F. Landi, C. M. Johansson, D. J. Campopiano, A. N. Hulme, *Org. Biomol. Chem.* **2010**, *8*, 56–59.
- [26] H. Hahne, N. Sobotzki, T. Nyberg, D. Helm, V. S. Borodkin, D. M. F. van Aalten, B. Agnew, B. Kuster, *J. Proteome Res.* **2013**, *12*, 927–936.
- [27] C. Sibbersen, L. Lykke, N. Gregersen, K. A. Jørgensen, M. Johannsen, *Chem. Commun.* **2014**, *50*, 12098–12100.
- [28] M. A. Nessen, G. Kramer, J. Back, J. M. Baskin, L. E. J. Smeenk, L. J. de Koning, J. H. van Maarseveen, L. de Jong, C. R. Bertozzi, H. Hiemstra, C. G. de Koster, *J. Proteome Res.* **2009**, *8*, 3702–3711.
- [29] S. Wang, W. Xie, X. Zhang, X. Zou, Y. Zhang, *Chem. Commun.* **2012**, *48*, 5907–5909.
- [30] E. M. Schneider, M. Zeltner, V. Zlateski, R. N. Grass, W. J. Stark, *Chem. Commun.* **2016**, *52*, 938–941.
- [31] P. P. Geurink, B. I. Florea, N. Li, M. D. Witte, J. Verasdonck, C.-L. Kuo, G. A. Van der Marel, H. S. Overkleeft, *Angew. Chem. Int. Ed.* **2010**, *49*, 6802–6805; *Angew. Chem.* **2010**, *122*, 6954–6957.
- [32] C. A. Gartner, J. E. Elias, C. E. Bakalarski, S. P. Gygi, *J. Proteome Res.* **2007**, *6*, 1482–1491.
- [33] Z. Hou, X. Han, Z. Wang, S. Ghazanfar, J. Yang, H. Liu, *J. Chromatogr. B* **2021**, *1165*, 122527.
- [34] S. H. L. Verhelst, M. Fonović, M. Bogoy, *Angew. Chem. Int. Ed.* **2007**, *46*, 1284–1286; *Angew. Chem.* **2007**, *119*, 1306–1308.
- [35] H. Y. Kim, K. A. Tallman, D. C. Liebler, N. A. Porter, *Mol. Cell. Proteomics* **2009**, *8*, 2080–2089.
- [36] J. Szychowski, A. Mahdavi, J. J. L. Hodas, J. D. Bagert, J. T. Ngo, P. Landgraf, D. C. Dieterich, E. M. Schuman, D. A. Tirrell, *J. Am. Chem. Soc.* **2010**, *132*, 18351–18360.
- [37] R. B. Merrifield, *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154.
- [38] E. Bayer, B. Hemmasi, K. Albert, W. Rapp, M. Dengler, in: *Peptides: Structure and Function*, Proc. 8th Amer. Pept. Symp. (V. Hruby and D. Rich, Hrsg.), Ann Arbor Science Publ., **1983**, 87–90.
- [39] F. García-Martín, M. Quintanar-Audelo, Y. García-Ramos, L. J. Cruz, C. Gravel, R. Furic, S. Côté, J. Tulla-Puche, F. Albericio, *J. Comb. Chem.* **2006**, *8*, 213–220.
- [40] M. S. Bernatowicz, S. B. Daniels, H. Köster, *Tetrahedron Lett.* **1989**, *30*, 4645–4648.
- [41] S.-S. Wang, *J. Am. Chem. Soc.* **1973**, *95*, 1328–1333.
- [42] M. Reindl, C. Linington, U. Brehm, R. Egg, E. Dilitz, F. Deisenhammer, W. Poewe, T. Berger, *Brain* **1999**, *122*, 2047–2056.
- [43] C. Araman, M. E. van Gent, N. J. Meeuwenoord, N. Heijmans, M. H. S. Marqvorsen, W. Doelman, B. W. Faber, B. A. t Hart, S. I. Van Kasteren, *Biochemistry* **2019**, *58*, 763–775.
- [44] J. van Heemst, D. T. Jansen, S. Polydorides, A. K. Moustakas, M. Bax, A. L. Feitsma, D. G. Bontrop-Elferink, M. Baars, D. van der Woude, G. J. Wolbink, T. Rispens, F. Koning, R. R. de Vries, G. K. Papadopoulos, G. Archontis, T. W. Huizinga, R. E. Toes, *Nat. Commun.* **2015**, *6*, 6681.
- [45] J. W. Dubendorf, F. W. Studier, *J. Mol. Biol.* **1991**, *219*, 45–59.
- [46] T. H. Grossman, E. S. Kawasaki, S. R. Punreddy, M. S. Osburne, *Gene* **1998**, *209*, 95–103.
- [47] S. K. Lee, J. D. Keasling, *Protein Expression Purif.* **2008**, *61*, 197–203.
- [48] J. A. Siepen, E.-J. Keevil, D. Knight, S. J. Hubbard, *J. Proteome Res.* **2007**, *6*, 399–408.
- [49] Z. Baka, B. György, P. Géher, E. I. Buzás, A. Falus, G. Nagy, *Joint Bone Spine* **2012**, *79*, 431–436.
- [50] C. Reilly, T. J. Stewart, M. B. Renfrow, J. Novak, *Nat. Rev. Nephrol.* **2019**, *15*, 346–366.
- [51] J. Rappsilber, M. Mann, Y. Ishihama, *Nat. Protoc.* **2007**, *2*, 1896–1906.
- [52] U. K. Laemmli, *Nature* **1970**, *227*, 680–685.

Manuscript received: February 1, 2023

Revised manuscript received: February 22, 2023

Accepted manuscript online: March 7, 2023

Version of record online: March 28, 2023