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N-Sulfanylethylanilide-based traceable linker for enrichment and selective labelling of target proteins

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An *N*-sulfanylethylanilide-based traceable linker, developed to facilitate identification of target proteins of bioactive compounds, was introduced into an alkynylated target protein. Subsequent adsorption onto streptavidin beads allowed it to be treated with a cysteine-fluorophore conjugate in the presence of phosphate. This induced the *N*-*S* acyl transfer reaction of the *N*-sulfanylethylanilide unit. The subsequent native chemical ligation of the fluorophore resulted in cleavage of the linker for target elution and fluorescent labelling of the target, allowing it to be distinguished from non-target proteins.

Many bioactive ligands such as natural products and synthetic small molecules exhibit their biological activity via the formation of specific binding interactions with their target proteins such as enzymes, receptors and ion channels. The unambiguous identification of the target proteins involved in these ligand-mediated biological processes is therefore essential for targeted drug development and chemical biology research to clarify the biological signalling pathways affected by the binding of such ligands. Target proteins are generally identified as follows: (1) the attachment of a tag moiety to the target protein using a suitably functionalized ligand; (2) tag-based enrichment of the target; and (3) sequence analysis of the target protein using proteomics, Edman degradation and/or mass spectrometry (MS).[1] The first steps generally involves the covalent attachment of a tag moiety (e.g., an alkyne), which is usually achieved by photo-affinity labelling using a photo-irradiation[1a,b,2] or activity-based probe approach involving a chemical reaction.[1c-e] The tagged target protein can then be connected to a biotinylated linker molecule for purification over streptavidin beads based on the strong biotin-streptavidin interaction.[1,3] The subsequent release of the target protein from the streptavidin beads is then required for sequence analysis. However, this process can be hampered by the high strength of the biotin-streptavidin interaction (K_d = 10^{-15} M),[4] and several cleavable linker molecules have therefore been developed to overcome this issue. The use of a cleavable linker for the efficient elution of a target protein from streptavidin beads has been successfully applied.[5] However, this use can sometimes result in contamination of the target protein with non-target materials derived from non-specific adsorption processes.[6] Contamination of the target in this way can hamper its subsequent identification and several research groups, including our own, have recently reported the development of traceable linkers as advanced cleavable systems for the selective labelling of target proteins. In this way, it is possible to discriminate the target protein from non-target materials.[7] Cleavage of the traceable linker leads to the formation of an orthogonal functional group that is not seen in the non-target proteins. This orthogonal functional group can then be selectively labelled with a reporter unit such as an isotopically-enriched probe or fluorescent dye that can contribute to its identification by MS or sodium dodecyl sulfate (SDS)-PAGE analysis, respectively. Previously reported traceable linkers are based on reversible reactions, including (i) hydrazone/hydrazone[7e] and hydrazone/oxime[7d] exchange reactions for linker cleavage and labelling; and (ii) oxime formation between an aldehyde-based reporter and an aminoxy group-containing cleavage product or vice versa.[7a-c] The oxyme and hydrazone linkage is stable under carefully optimized conditions.[7d] But more robust linkage that can be applicable to various working conditions without optimization is preferred to remove the potential risk of the linker being accidentally cleaved and/or the reporter being removed. The cleavage of the linker or the removal of the reporter would lead to a decreased detection limit of the analytical method. It was therefore envisioned that the development of a traceable linker capable of the cleavage and introduction of a suitable reporter via an irreversible reaction would represent an effective analytical tool. Herein, we report the development of an *N*-sulfanylethylanilide (SEAlide)-based traceable linker that

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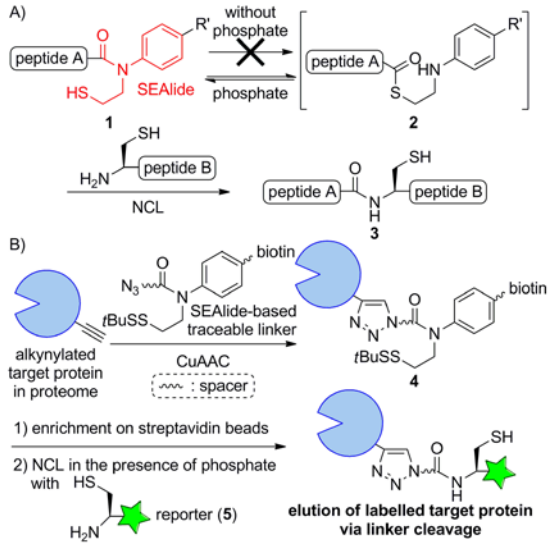
can be used to connect a target protein to a reporter unit via the formation of a stable amide bond.



Scheme 1 Design of the SEAlide-based traceable linker. A) Phosphate catalysed activation of the SEAlide unit via *N*–*S* acyl transfer followed by NCL with a cysteinyl peptide; B) Enrichment and selective labelling of a target protein using the SEAlide-based traceable linker.

We previously reported the SEAlide unit as a precursor for C-terminal peptide thioester to be used in chemical protein synthesis (Scheme 1A).[8] Peptide **1** bearing an amide-type SEAlide unit remained stable in the absence of phosphate, whereas the addition of phosphate led to its activation to give thioester **2**.[9] In situ generated **2** was subsequently reacted with a peptide bearing an N-terminal cysteine residue to give the amide-linked peptide **3** via native chemical ligation (NCL).[10] We envisaged that the cleavage of the SEAlide unit from peptide **1** followed by an amide-bond forming reaction with an N-cysteinyl peptide could be used to generate an irreversible traceable linker.

With this in mind, we designed a SEAlide-based traceable linker consisting of a SEAlide unit attached to an azide group and biotin (Scheme 1B). In this study, the thiol moiety on the SEAlide unit was protected as S*t*Bu disulfide, which could be removed under the NCL conditions to avoid any complications resulting from disulfide formation or the accidental cleavage of the linker. The subsequent copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC)[11] reaction of the linker with an alkynylated target protein in the proteome would give conjugate **4**, which could be enriched over streptavidin beads. The NCL of the molecules of **4** immobilized on the beads with the cysteine-derived reporter unit **5** in the presence of phosphate would allow for the elution of the amide-connected target protein-reporter conjugate via the removal of the S*t*Bu group and the subsequent cleavage of the linker. SEAlide-based traceable linker **6** shown in Fig. 1 was prepared using Fmoc-based solid phase peptide synthesis (Fmoc SPPS) (Scheme S1). Prior to using this linker with a protein, we investigated its CuAAC reaction with the alkynylated small molecule **S5**,[12] followed by the cleavage of the linker with cysteine to give the desired products, as shown in Fig. S1. We then proceeded to investigate the reaction of traceable linker **6** with an alkynylated bovine serum albumin (BSA), which was used as a model alkynylated target protein.[7a] The CuAAC of traceable linker **6** and the alkynylated BSA afforded the corresponding biotinylated BSA, as shown in Fig. S2. The product was subsequently adsorbed on streptavidin beads and its elution was evaluated. In the presence of 1 M phosphate and 100 μM cysteine-fluorescein conjugate **8**,[13] successful elution of the fluorescently labelled BSA was observed as shown in Figure S3. Effect of concentration of phosphate on the elution efficiency was next examined, and efficient elution was observed when 400 mM phosphate was employed. When the resin obtained after the linker cleavage was boiled in the sample buffer, we detected a weak (400 or 100 mM phosphate) or moderate (1000 mM phosphate) fluorescence signal. This signal might be attributed to the labelled BSA that was adsorbed on the streptavidin beads through non-specific interactions because high concentration of phosphate can induce salting out of proteins. Next, the traceable linkers **S8** and **S9**, their linker length between the SEAlide and the azide is different to that of **6**, were also examined but their elution efficiency was similar to that of original **6** (Fig. S4). Similar elution conditions were also applied to the product resulting from the CuAAC reaction of the model alkynylated BSA with the *S*-alkylated negative control **7**, which could not be cleaved via an *N*-to-*S* acyl transfer reaction (Fig. 1 and S5). In this case, small amount of the protein was eluted from the beads, presumably because of the SDS-mediated denaturation of streptavidin. However, elution efficiency of the negative control was lower than that of the corresponding traceable linker **6**. These results therefore demonstrate that linker **6** may be used to achieve an efficient elution profile by the cleavage of the linker. Furthermore, the eluted protein was detected by fluorescent imaging when **6** but not **7** was used as the linker.[14]

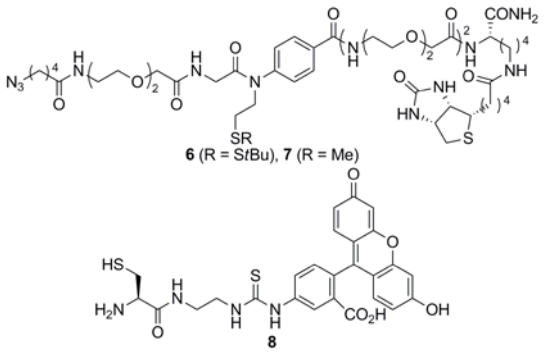


Fig. 1 Structures of the SEAlide-based traceable linker **6**, negative control **7**, which could not be cleaved via thioester formation, and cysteine-fluoresceine conjugate **8**.

Finally, we examined the enrichment and selective visualization of the alkynylated protein using a mixture of the alkynylated BSA and red blood cell lysate (Fig. 2). This mixture was treated with traceable linker **6**, allowing for the CuAAC reaction. The resulting mixture was then treated with streptavidin beads, and the adsorbed proteins were eluted. The eluted mixture was analysed by SDS-PAGE and the

proteins were visualized by silver staining or fluorescent imaging. When the elution was performed by the denaturation of streptavidin in a similar manner to that used with conventional linkers, the enrichment of the BSA was observed (Fig. 2, silver staining, lane 4). The elution of the proteins by the cleavage of the linker in the presence of cysteine-fluorescein conjugate **8** gave slightly better enrichment results compared with those obtained by the denaturation of streptavidin (Fig. 2, silver staining, lane 5). The traceable linker allowed for the visualization of the target, which could be used to identify specific targets (Fig. 2, fluorescence imaging, lane 5). When traceable linker **8** was applied to enrichment and labelling of the alkynylated BSA in lysate of HCT116 cells, contamination of the target with non-target proteins was observed but the target selective labelling enabled discrimination between the target and non-target materials (Fig. S7).

In conclusion, we have developed a SEAlide-based traceable linker as an advanced cleavable linker for the identification of specific protein targets. This new system not only allowed for the cleavage of the linker and the enrichment of the target in a similar manner to conventional cleavable linkers, but also allowed for the selective labelling of the target. In this way, it was possible to distinguish the desired target from any undesired non-target proteins. The results obtained in this study therefore demonstrate that this newly developed SEAlide-based traceable linker could be used for the facile identification of the target proteins of bioactive compounds, including drug candidates. Work toward the application of this traceable linker to identify the targets of target-unknown bioactive compounds is currently in progress.

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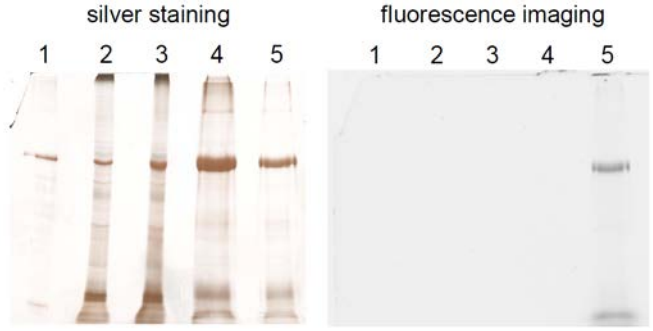

Fig. 2 Enrichment and selective labelling of the alkynylated BSA in red blood cell lysate using traceable linker **6**. Details of the conditions are shown in the supporting information. Lane 1: alkynylated BSA; Lane 2: red blood cell lysate; Lane 3: a mixture of the alkynylated BSA and red blood cell lysate; Lane 4: eluted proteins after the CuAAC reaction of the mixture with **6**, adsorption of the biotinylated material onto streptavidin beads and the treatment of the beads with sample buffer at 100 °C for 5 min (elution by denaturation of streptavidin); Lane 5: eluted proteins after the CuAAC reaction of the mixture with **6**, adsorption of the biotinylated material onto streptavidin beads and the treatment of the beads with cysteine-fluorescein conjugate **8** in the presence of phosphate (elution conditions: 100 μM **8**, 50 mM MPAA, 40 mM TCEP, 0.1% SDS, 400 mM Na phosphate buffer, pH 7.4, 37 °C, 24 h); Fluorescence imaging, λ_{ex} = 460 nm, λ_{em} > 515 nm.

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result demonstrates that efficient labelling of the target was achieved.