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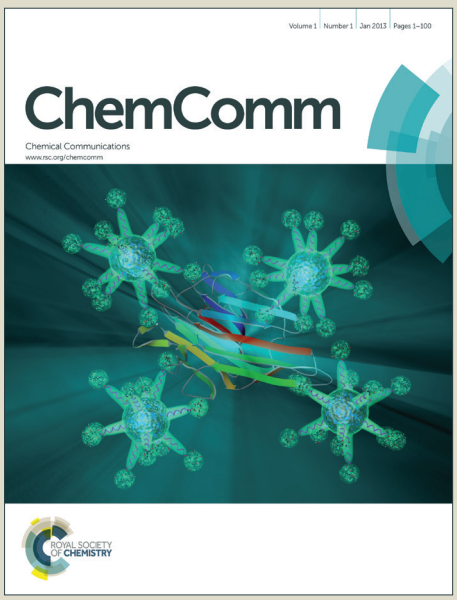

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ARTICLE TYPE

A Cleavable Azide Resin for Direct Click Chemistry Mediated Enrichment of Alkyne-Labeled Proteins

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The direct conjugation of a labeled proteome to a cleavable azide resin utilizing the copper-catalyzed azide alkyne cycloaddition is demonstrated. The procedure omits the classical streptavidin- and biotin-based affinity enrichment step and represents an operationally simpler, cheaper and less contaminated alternative for protein purification.

Activity-based protein profiling (ABPP) is a chemical biological tool that takes advantage of activity-based chemical probes to label, enrich and identify proteins having a specific reactivity of interest. Much work has been dedicated to the optimization of the compounds and systems used for protein profiling.[1] Initially, the chemical probes used for labeling proteins were covalently bound to biotin so that they could be isolated on a streptavidin-linked solid support (beads), exploiting the high affinity between streptavidin and biotin.[2] However, the conjugation to biotin is a significant change in the steric and electronic properties of the probe and could interfere with either the crossing of the cell membrane or the probe's binding to the active site of the target protein.[3]

To overcome these problems a second generation of chemical probes was designed, carrying only a very small and bioorthogonal alkyne or azide tag, making the probes more cell permeable and less likely to interfere with protein binding. Following labeling, the proteins can then be coupled to *e.g.* a biotin-azide linker using the copper-catalyzed azide alkyne cycloaddition (CuAAC). The labeled proteins can then be enriched using streptavidin beads (Fig. 1A).[3]

Usually the biotinylated probe-protein complex is released from the solid support by boiling the resin in sodium dodecyl sulfate (SDS) (Fig. 1A) or by trypsin digestion directly on the resin.[4] However, some proteins contain biotin as a prosthetic group which can lead to false positives in the enriched proteins. Additionally, the harsh conditions used to release the proteins could lead to *e.g.* disruption of subunits. A second generation of cleavable biotin linkers have recently been developed, containing a cleavable moiety such as aryl-azo, disulfide or silyl ethers.[5] This allows for the labeled intact proteins to be cleaved off under mild conditions while the naturally biotinylated proteins remain bound to the resin (Fig. 1B) or alternatively isolation of specific probe-labeled peptides by on-bead trypsin digestion followed by chemical release of the binding site peptides.[6]

The cleavable biotin linkers are still dependent on the strong interaction with streptavidin. Both biotin and the streptavidin-agarose resin are expensive and binding of the biotinylated probe-protein complex to streptavidin resin adds an extra step to the work process. Several research groups have developed resins that

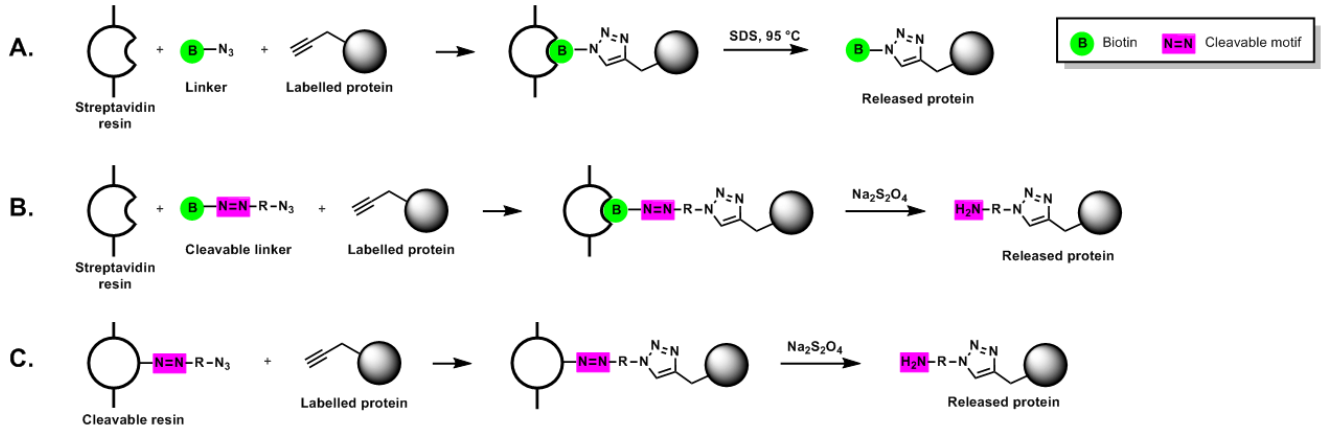

Fig. 1: A – Alkynylated proteins enriched on a streptavidin resin using an azido-biotin linker and released by boiling the beads in a solution of SDS. **B** - Alkynylated proteins enriched on a streptavidin resin using a cleavable azido-biotin linker (illustrated here with the azo-moiety) probe and released by chemical cleavage. **C** - The present novel approach by which alkynylated proteins are enriched by direct reaction with a cleavable azide-bearing resin and released by chemical cleavage.

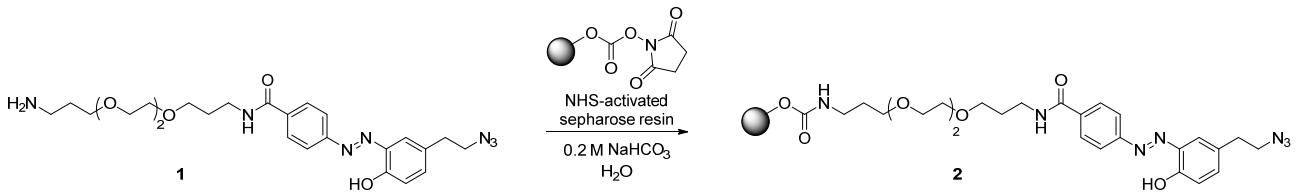

Fig. 2: Synthesis of the cleavable azido-azo beads (**2**) by conjugation of compound **1** to activated sepharose resin

have the probe directly attached to a solid support using a cleavable moiety in the linker or using the alkyne motif to enrich azidonated peptides.[7] While having the probe directly attached to the resin is useful for affinity chromatography, it is not compatible with ABPP applications where it is often desired to work with intact cells or to use several probes in the same experiment. Furthermore, any resin useful for ABPP experiments needs to be able to capture proteins of virtually any size, something that sets high demands for both the resin as well as the conjugation reaction.

We demonstrate here that the well-known CuAAC can be carried out directly between a cleavable solid phase azide and an alkyne-labeled proteome containing a wide range of protein sizes, completely eliminating the need for the classical streptavidin beads and biotin-linkers (conceptually illustrated in Fig. 1C). The cleavable solid phase azide is easily synthesized from inexpensive agarose resin, significantly reducing the cost of protein purification experiments. The ability to carry out the CuAAC directly between the labeled proteome and the resin makes it possible to label proteins *in vivo* before enrichment, thus still permitting experiments with intact cells as well as the use of several different probes in the same experiment.

The aryl-azo motif, which was originally discovered to be useful for ABPP purposes by Bogyo *et al.*, was chosen as the cleavable moiety for our resin because it is stable under a wide range of conditions, easily introduced synthetically and can be cleaved using mild conditions without disrupting protein tertiary structure.[5a,8] We chose sepharose as the solid phase since it can be conveniently activated using *N*,*N*'-disuccinimidyl carbonate and subsequently coupled to amine-containing compounds.[9] A PEG-linker was introduced between the azo/azide motifs and the sepharose resin to improve solubility and accessibility to the functional part of the resin.

The linker-azo-azide compound **1** with a free amine was synthesized in 5 steps from tyramine (12% over all yield, ESI† Fig. S1). Compound **1** was coupled to the sepharose beads under mild conditions to form the azido-azo beads **2** (Fig. 2). After extensive

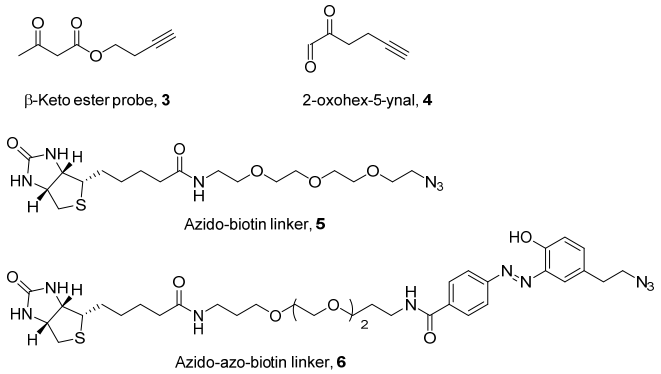

Fig. 3: Structures of probes and linkers

washing, the beads had changed in color from colorless to bright orange, indicating that the aryl-azo compound was now covalently bound to the solid support.

We initially tested the efficiency of the beads **2** by treating HEK293 cell lysates with a β-keto ester probe **3** (Fig. 3) which has been demonstrated to selectively label sulfenic acids.[10] The samples were also treated with 100 μM H_2O_2 to induce oxidation of free cysteins. An aliquot of the treated cell lysates were conjugated by CuAAC to rhodamine-azide,[11] separated using SDS-PAGE and analyzed using in-gel fluorescence to observe labeling. The remaining lysates were conjugated by CuAAC to azido-azo-beads, which were then washed thoroughly before eluting the labeled proteins with $Na_2S_2O_4$ (full procedure given in ESI†). Comparison to rhodamine fluorescence signal and coomassie staining of the full lysates shows a selective enrichment of the labeled proteins with no background labeling in the samples without probe (Fig. 4A). Furthermore, we spiked cell lysates with bovine serum albumin that had been alkynylated with 2-oxohex-5-ynal **4** (Fig. 3) or untreated albumin.[12] Comparing the full cell lysates to the eluted proteins confirms specificity of the binding (Fig. 4B). Please note that the less intense bands in the upper part of the gel are contamination proteins present in the commercially available albumin (ESI,† Fig. S2), which were also alkynylated and thus show up in fluorescence analysis and the enriched sample.

Commercially available streptavidin agarose beads used with biotinylated proteins are able to enrich proteins of practically any size with a cut-off around 10 kDa, according to supplier documentation. To be useful for general proteomics experiments, the azido-azo beads **2** would have to be able to enrich a similar range of protein sizes. To test this, we treated full cell lysates with a high 15 mM amount of 2-oxohex-5-ynal for 1 h. We have demonstrated earlier that this probe labels a wide range of cellular proteins at high levels (>5 mM).[12] For comparison, labeled and unlabeled lysates were reacted with the two known biotin-azide linkers, as well as the azido-azo beads **2** under normal CuAAC conditions.[8] The two biotin-based linkers (Fig. 3) are **5 -** a simple linker that has to be boiled off the streptavidin beads in SDS, and **6 -** a cleavable linker based on the azo-motif similar to the resin reported here. The proteins, now conjugated to these linkers, were enriched on streptavidin-linked agarose beads and eluted using standard procedure.[8] The samples were analyzed using SDS-PAGE (Fig. 4C)

We observe that there is little difference between streptavidin beads with a biotin linker (**5** or **6**) and azido-azo beads **2**, and all three methods are able to enrich and purify proteins ranging in size from less than 15 kDa to over 250 kDa. The same volume of beads was used with each system, indicating a similar capacity for binding alkynylated proteins. These findings indicate that the CuAAC can be carried out directly on the resin with as high

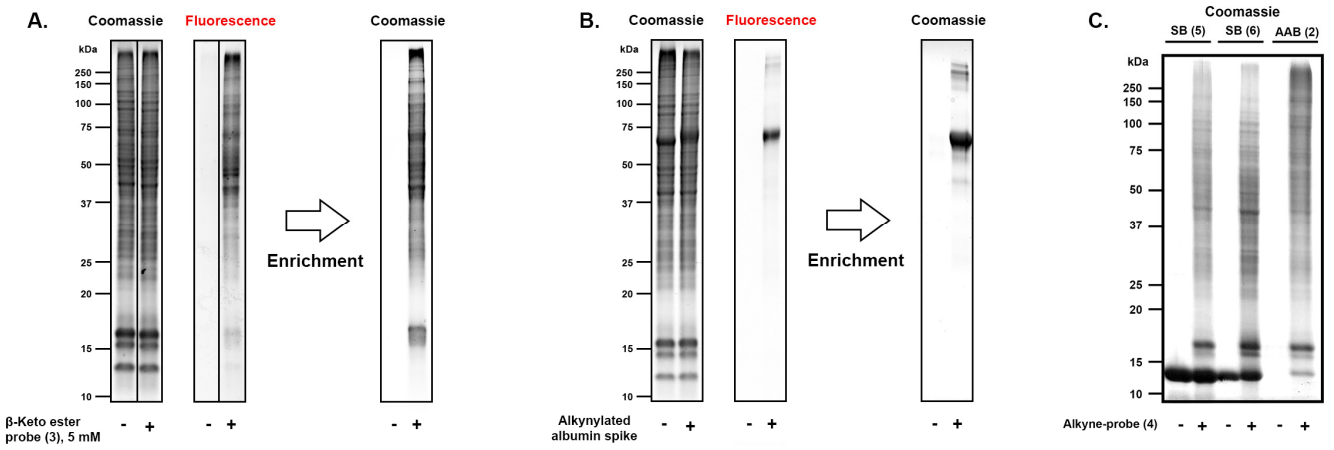

Fig. 4: A – Enrichment of proteins labeled with β-keto ester probe. **B –** Enrichment of lysates spiked with alkynylated albumin. **C –** Comparative enrichment of lysates alkynylated by probe **4** and enriched with linkers **5** and **6**/streptavidin beads (SB) and azido-azo beads **2** (AAB).

efficiency as the classical two step procedure using biotin-azide and streptavidin beads. Denatured streptavidin subunits are furthermore observed as a false positive in both samples using streptavidin (the strong band at ~15 kDa) but not when using azido-azo beads **2,** adding a further advantage of using them.

Since the proteins are bound covalently to the beads, the method is not dependent on the streptavidin-biotin bond and is thus compatible with harsh conditions such as high detergent concentration and chaotropic reagents, as long as these do not interfere with the azo-moiety or other chemical groups in the protein-resin complex (ESI,† Fig. S3). The beads are not compatible with high temperatures, which can lead to gelation of the beads.

In this work we have described the synthesis and biological evaluation of a cleavable azide resin that can be used to isolate alkyne-labeled proteins of virtually any size, making it useful for general proteomics experiments *e.g.* ABPP. The enrichment process is simplified requiring fewer precipitation and incubation steps, and contamination of samples by naturally biotinylated proteins and streptavidin subunits from the resin itself is avoided. Furthermore, the cost is reduced since streptavidin beads are not used. The synthesis requires no more work than that of a standard cleavable biotin-linker and can be carried out on a large scale. For these reasons we believe that azido-azo beads can be highly useful for chemical biological applications and in the future substitute the classical two step approach.

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Notes and references

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