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COMMUNICATION

Strategy for catch and release of azide-tagged biomolecules utilizing a photolabile strained alkyne construct†

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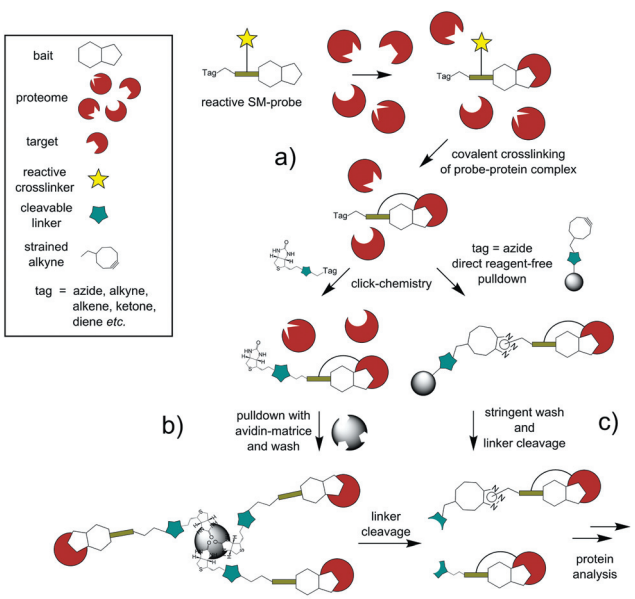
A photolabile *o*-nitrobenzyl linker–cyclooctyne conjugate was prepared, immobilized on poly(methacrylate) beads and utilized as a trap for azide-functionalized compounds. These could be released by 365 nm UV light irradiation in high yield and purity. The "reagent-free" and time economic catch and release protocol was deemed useful for chemical proteomics applications.

In chemical proteomics as well as pharmaceutical drug research, the selective enrichment of a subset of proteins from a complex cellular proteome is an important step towards the elucidation of small molecule (SM)–protein interactions.[1] Among the strategies developed for detection and isolation of target proteins in classical affinity purification[2] or activity-based protein profiling (ABPP; Scheme 1(a) and (b)),[3] methods exploiting the strong specific interaction of SM–biotin conjugates and avidin found the most widespread application.[4] However, precisely because of this strong interaction, biotin, as a small affinity tag, may exhibit significant drawbacks. In fact, harsh elution conditions are sometimes required for protein recovery from avidin matrices, which can lead to loss and damage of the samples or their contamination with avidin. Further, the high background of proteins that bind non-specifically to the affinity matrices hampers proteome analysis or the concrete identification of a SM target. Cleavable linker systems *e.g.* based on disulfide linkage,[5] γ-keto esters,[6] azo-aromatics,[7] acid-labile silanol ethers[8] and photolabile groups[8,9] have been proposed to solve this issue.

With the advent of click-chemistry, the introduction of bioorthogonal functional groups into biomolecules for further conjugation became a versatile strategy for the study of biological systems.[10a] Such tags, like the azide moiety or a terminal alkyne, among others, were grafted onto biomolecules genetically,[10] metabolically,[10,11] chemically or by means of reactive activity- or affinity-based probes (ABPs and A*f*BPs; Scheme 1(a)) in the course of ABPP experiments.[3,12]

The combination of cleavable linker systems and a bioorthogonal ligation reaction facilitates the enrichment of tagged proteins without utilizing biotin–avidin techniques (Scheme 1(b) and (c)). In this regard, reagent-free strain promoted click-chemistry appears to be the method of choice, owing to its excellent reaction dynamics and yields.[11,13]

Indeed, Temming *et al.*[14] and Nessen *et al.*[15] exploited strain promoted click-chemistry in combination with chemically cleavable linkers for the biotin-free catch and release of azide functionalized proteins. In their reports, either a phosphine-cleavable disulfide linker or a hydrazine-cleavable γ-keto ester linker was used for recovery of purified protein samples. However, sterically unhindered disulfide linkers exhibit the tendency of premature cleavage and demand the end-capping of free thioles in order to allow efficient protein recovery. Likewise, treatment of protein samples with hydrazine carries the risk of cleaving target biomolecules. Therefore, photolabile derivatives attracted our



Scheme 1 Enrichment of tagged proteins utilizing click-chemistry and cleavable linkers.

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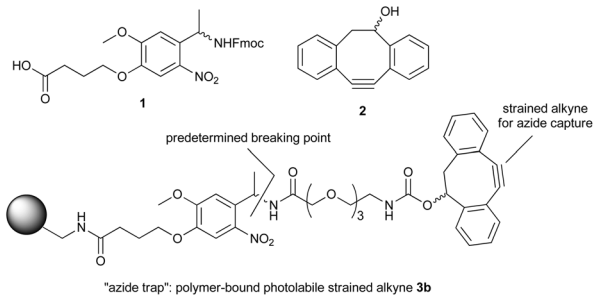
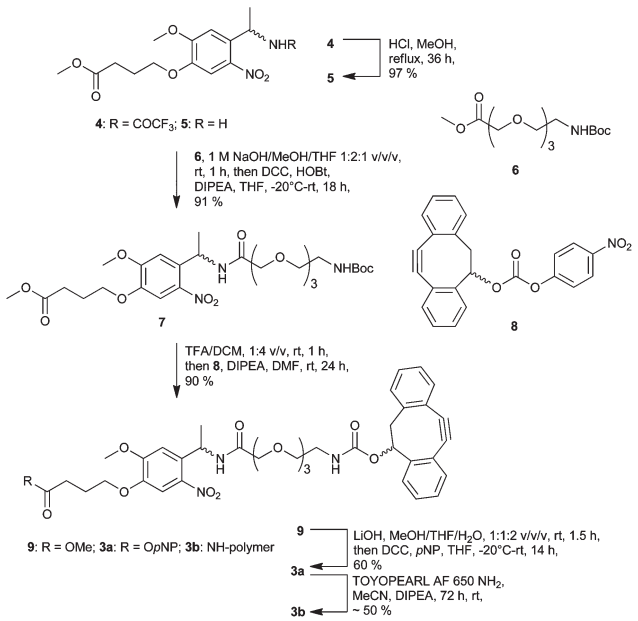


Scheme 2 Building blocks and the photolabile strained alkyne construct.

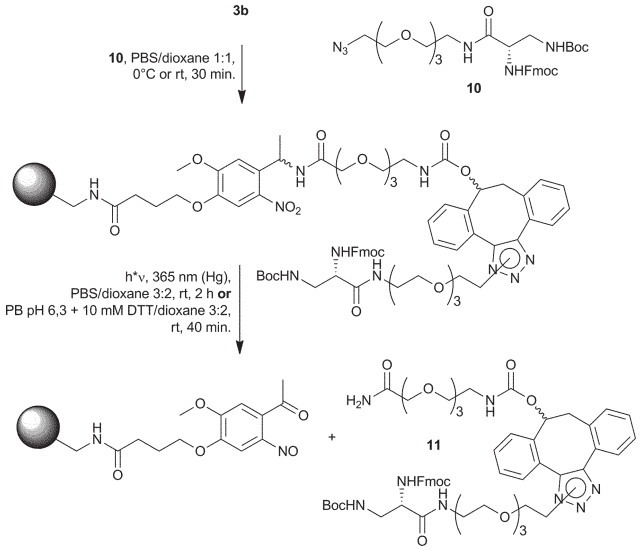


Scheme 3 Synthesis of the photolabile strained alkyne construct.

attention as "reagent-free" cleavable linkers. We hypothesize that combination of a polymer support with a photocleavable linker–cyclooctyne conjugate could yield a useful device for catch and release of azide-tagged biomolecules.

For assembly of the photocleavable cyclooctyne construct **3b**, we utilized *o*-nitrobenzyl-linker **1** (Scheme 2) which was introduced by Holmes and successfully applied in proteomics applications previously.[9,16] Linker **1** exhibits very good cleavage kinetics and absorbs UV light in a range that is relatively uncritical for biological samples (*i.e.*, 340–365 nm). As the appropriate cyclooctyne, the dibenzo derivative **2** (Scheme 2) was considered. Advantageously, alkyne **2** possesses good stability in physiological buffer media and reacts very rapidly with aliphatic azides furnishing the corresponding triazoles in high yields (>90%). Furthermore, **2** can be synthesized rather easily on a large scale starting from dibenzosuberenone in a convenient and high-yielding seven-step sequence.[17]

The synthesis started from nitroaromatic **5** (Scheme 3) obtained in 97% yield from known trifluoroacetamide **4**[9] *via* solvolytic cleavage of the amide moiety with HCl in refluxing methanol. Subsequently, *o*-nitrobenzyl building block **5** was



Scheme 4 Catch and release of model compound **10**.

coupled to the corresponding free acid of ethylene glycol derivative **6** by HBTU-mediated amide formation leading to the *N*-Boc-protected amine **7**.

Removal of the Boc-group and carbamate formation with the activated *p*-nitrophenyl carbonate **8**[11b] yielded 96% of methyl ester **9**. Methyl ester **9** was then saponified with aqueous LiOH in MeOH–THF and re-esterified with *p*-nitrophenol (*p*NP) using DCC-technique to form the activated derivative **3a** in moderate yield (60%).

In order to examine the performance of the proposed catch and release sequence (Scheme 4), active ester **3a** was immobilized on TOYOPEARL® AF 650 amino beads (100 μmol ml^{-1} $-NH_2$) in dry MeCN and DIPEA as the base to yield azide-reactive solid support **3b** (Scheme 3).

The rate of azide capture by strain-promoted click-reaction was evaluated by adding a twofold excess of Fmoc-protected model compound **10** to the beads suspended in a mixture of dioxane–PBS 1 : 1. Depletion of **10** from the supernatant was tracked and quantified by photometric measurement of the Fmoc absorption band at 267.8 nm. Unspecific binding of model compound **10** to the beads was examined by treatment of the alkyne-functionalized beads with excess 2-azidoethanol prior to incubation with **10**. The results show that saturation of the alkyne-functionalized solid support was achieved after 30 min. Non-specific binding of **10** to the beads in the range of 15% was observed (Fig. 1(a)), resulting in a corrected effective triazole loading of ~12.5 μmol per ml of TOYOPEARL-suspension (uncorrected ~14.7 μmol). As indicated by the curves in Fig. 1 (a) azide capture proceeded almost equally fast at rt and 0 °C.

For examination of *o*-nitrobenzyl linker cleavage and liberation of the linker fragment **11** from the solid support, two distinct experiments were conducted. According to the finding that *o*-nitrobenzyl-linker cleavage is greatly accelerated in acidic reducing buffer media,[18] one portion of the beads was suspended in a mixture of dioxane and Na-phosphate pH 6.3 buffer containing 10 mM DTT. Another portion was suspended in dioxane–PBS (see above) and both samples were irradiated with a 150 W medium pressure mercury lamp. We utilized a <350 nm cut-off

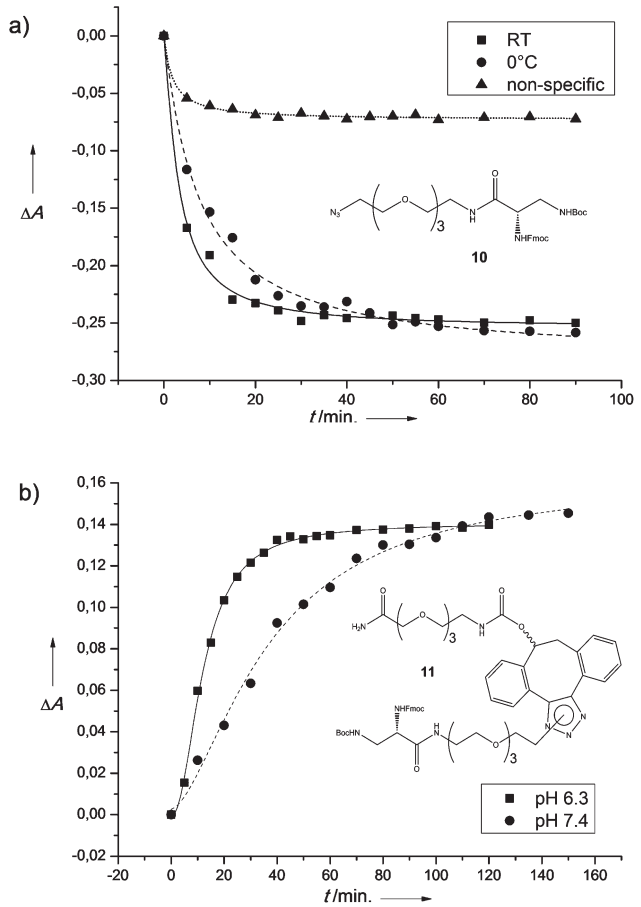


Fig. 1 Photometric monitoring of the catch and release dynamics.

filter solution to shield UV-light <350 nm, which would be detrimental for biomolecules (see ESI†).[9,16] Progression of the cleavage reaction was tracked and quantified by photometric measurement of **11** in the supernatant. As seen from the curve (Fig. 1(b)), liberation of linker fragment **11** was complete after 2 h in dioxane–PBS. In the acidic reducing buffer, cleavage was significantly accelerated (~3-fold) and complete after 40 min. Quantitative analysis indicated 85% recovery of **11** in 98.2% purity (HPLC) based on the total amount of **10** depleted from the supernatant upon binding to the beads. Given that the amount of **10** non-specifically bound to the beads did not contribute to triazole formation, a corrected recovery rate of nearly 100% would be obtained.

The transfer of our catch and release approach to complex protein mixtures (*e.g.*, cell lysates) in regard to enrichment of azide tagged biomolecules is the subject of our current investigations. We expect that the improved time economy and mild reaction conditions of our "reagent-free" approach may offer some advantages over related concepts reported in the literature.[14,15] The recovery yields and cleavage times found in our test system are comparable to the most efficient chemically cleavable linkers, *i.e.* linkers based on disulfides,[5] azo-aromatics[6] or silanol ethers.[7] The stable triazole bond formed in the course of azide-tagged protein immobilization and the chemical robustness of Holmes's linker[9] (amide bond) should allow stringent washing of the beads *e.g.* heating in buffer systems containing SDS and thioles. This should lead to the elimination of all non-covalently bound contaminant proteins and thus to a significant improvement of sample quality. Finally, since no reagent has to be added in the course of the catch and release protocol (UV-light is in fact used), this approach should be suitable for automation.

Whether the reported[13,14] covalent, non-specific addition of proteins by means of residual enzyme activity or protein affinity towards the strained alkyne moiety also occurs in our system was not yet investigated. Further, the reaction dynamics might change when immobilizing an azide tagged macromolecule instead of the small linker fragment **10**. The issue of non-specific addition could be met by using a denaturing capture buffer in order to abrogate residual enzyme activity. Likewise, the use of more hydrophilic or more sterically hindered strained alkynes could suppress covalent protein binding to the catch and release construct.[13] In case reaction dynamics are impaired significantly, change of the solid support to *e.g.* TentaGel® or applying the principal multivalent reactive moieties[19] to improve the yields of strain promoted click-reaction might be beneficial.

The relatively large 450 Da mass tag left on the proteins after photorelease is not expected to interfere with MS/MS sequencing of tryptic peptides obtained from the corresponding biomolecules. Nonetheless, the mass tag might lead to loss of activity and hence hamper functional studies on isolated proteins or their further use in pharmaceutical applications. Whether this holds true for biomolecules isolated by our technique remains to be elucidated.

In summary, we were able to realize a "reagent-free" catch and release process *via* proper assembly of easily accessible photoreactive and strained-alkyne building blocks. The simple protocol utilizes easy-to-handle and inexpensive UV equipment and may represent a versatile tool for enrichment of azide tagged molecules. Time economy and yields can compete with some of the most efficient catch and release protocols presently available. The use of biotin and its disadvantageous implications is circumvented. We therefore believe that our photolabile strained alkyne construct could become a useful tool in chemical proteomics research.

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