Native Chemical Ligation

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Traceless Purification and Desulfurization of Tau Protein Ligation Products**

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Abstract: *We present a novel strategy for the traceless purification and synthetic modification of peptides and proteins obtained by native chemical ligation. The strategy involves immobilization of a photocleavable semisynthetic biotin–protein conjugate on streptavidin-coated agarose beads, which eliminates the need for tedious rebuffering steps and allows the rapid removal of excess peptides and additives. On-bead desulfurization is followed by delivery of the final tag-free protein product. The strategy is demonstrated in the isolation of a tag-free Alzheimer's disease related human tau protein from a complex EPL mixture as well as a triphosphorylated peptide derived from the C-terminus of tau.*

The development of native chemical ligation (NCL) and expressed protein ligation (EPL) revolutionized the accessibility of proteins bearing challenging posttranslational modifications.[1] Both chemoselective ligations proceed via a capture step between an N-terminal cysteine moiety of one peptide and a C-terminal thioester of a second peptide or protein, followed by an intramolecular rearrangement to yield an amide bond linking the two peptides. As one or both of the portions can be constructed synthetically, posttranslational modifications or other functional labels can be introduced. NCL and EPL have paved the way to a deeper understanding of protein structure–function relationships,[2] allowed novel protein design,[3] enabled the determination of complex protein structures by NMR spectroscopy[4] and X-ray crystallography,[5] and shed light on the role of posttranslational modifications in dynamic biological processes.[6] Despite these successes, the processing and purification steps of NCL and EPL reactions can be time-consuming and often low-yielding, especially in cases in which multiple complex postligation steps are required. For example, desulfurization, which is often performed to replace the cysteine that results at the ligation site with an alanine, requires different reaction conditions from those of NCL. Non-native thiol moieties can be removed by either heterogeneous reductive[7] or homogeneous radical desulfurization.[8] In particular, homogeneous radical-initiated desulfurization has become widely popular in protein synthesis due to mild reaction conditions and improvements in recovery of material, which is otherwise often lost by adsorption when transition metal catalysts are used. Nevertheless, desulfurization conditions often require buffer exchange and intermediate HPLC purification. While a one-pot ligation and desulfurization procedure is possible in the presence of excess alkyl thiols like sodium 2-sulfanylethanesulfonate (MESNa) and glutathione, an excess of highly activating aryl thiols like thiophenol and 4-mercaptophenylacetic acid (MPAA) would inhibit the desulfurization reaction, as they act as radical scavangers and thus compete with the desulfurization step.[9] In attempts to overcome these obstacles, several methods to remove aryl thiols from ligation mixtures have been developed to facilitate protein synthesis and allow one-pot ligation/desulfurization. This was done either by liquid/liquid extraction of excess thiophenol from ligation mixtures with diethyl ether or by solid-phase extraction procedures.[10] In this context, Brik et al. used a bifunctional aryl thiol catalyst that can be removed by an aldehyde-bearing solid support.[11] In an alternative approach by Payne et al., trifluoroethanethiol (TFET) was introduced as a novel thiol additive which does not interfere with the desulfurization reaction.[12] Finally, native chemical ligation was shown very recently to be suitable for successive on-resin ligations and desulfurization, allowing the release of a Cys-free ligation product upon treatment with trifluoroacetic acid (TFA).[13]

A challenge for EPL is the small difference in size between the ligated protein product and the expressed protein reactant, which limits the use of conventional chromatographic purification methods. HPLC purification offers highly sensitive separation, but requires organic solvents which are often unfavorable for folded proteins. Affinity tags are frequently used as alternatives;[14] however, these tags are often difficult to introduce synthetically (e.g. the His-tag) or they remain covalently bound to the semisynthetic product, thus potentially influencing the structure or function of the protein. Consequently, researchers have developed cleavable and traceless purification tags, which have aided in the purification of synthetic peptides and expressed proteins by temporary immobilization to a solid support.[15]

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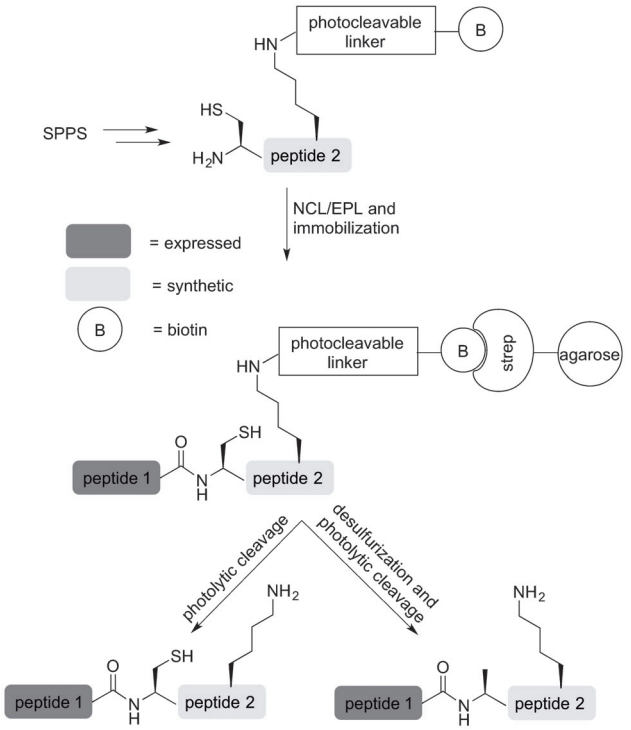

Scheme 1. Ligation, desulfurization, and purification of components involved in NCL and EPL reactions through the use of a photocleavable biotin-linker.

Herein, we introduce a new strategy for the traceless purification of posttranslationally modified peptides and proteins obtained by NCL or EPL, which enables en route a straightforward final desulfurization step, in order to install an alanine residue at the ligation site (Scheme 1). We envisioned that biotin linked by a photocleavable 2-nitrobenzyl group to a Lys side chain of a synthetic peptide would be well-suited for this purification strategy. Despite outstanding efforts in using cleavable linkers in solid-supported methods for iterative protein ligations[13] and the fluorescence labeling of proteins,[15b–d] we did not find examples in the literature where traceless photocleavable biotin (PC-biotin) was used with EPL to produce native full-length proteins or to perform the desired postligation chemistry. Specifically, in the synthesis of the desired peptide conjugate we employed the bifunctional linker **1**, originally introduced by van der Donk et al. for leader peptide cleavage.[16] The synthesis of linker **1** was carried out according to the reported procedure over four steps with an overall yield of 39%. To test whether this linker, when combined with biotin, functions as a good photolabile protecting group, namely showing high affinity to streptavidin, low requirements for light intensity and exposure time, and efficient release of the substrate in native form,[15a] we introduced it to a test peptide, synthesized by Fmoc-based solid phase peptide synthesis (SPPS, Figure 1; see the Supporting Information). The bifunctional linker was coupled to a deprotected Lys side chain through incubation with the peptide and base. Biotin was then added to the resin with hydroxybenzotriazole (HOBt) and *N*,*N*′-diisopropylcarbodiimide (DIC) in *N*-methyl-2-pyrrolidone (NMP) in the presence of tri-*n*-butylphosphine, which initiated the in situ reduction of the azide to an amine. We obtained the model peptide in 7% overall yield and high purity. Finally, we tested the potential for on-bead cleavage through incubation with streptavidin-coated agarose beads. Photolytic cleavage was monitored by fluorescence spectroscopy and revealed an 82% yield of unbiotinylated peptide **2*** in only 5 min (Figure S18 and Table S4). The use of agarose beads was crucial, as photocleaved peptides from azlactone-activated

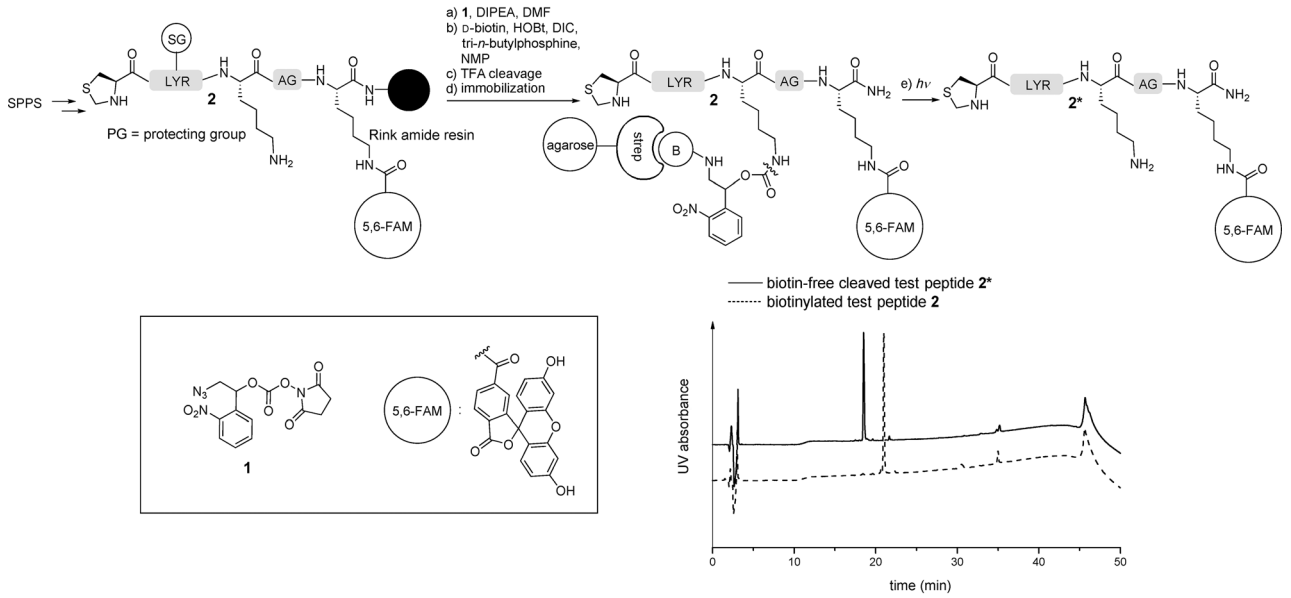

Figure 1. UV cleavage of photocleavable biotin from test peptide **2** (see the Supporting Information for experimental details). a) Coupling of **1**; b) in situ reduction and one-pot biotin coupling; c) TFA cleavage from resin; d) immobilization on streptavidin-coated agarose beads; e) photolytic release by irradiation with UV light ($\lambda = 297$ nm) for 7 min; HPLC-UV traces of test peptide **2** (m/z: 871.5 $[M+2H]^{2+}$, calcd. m/z: 871.4) and cleaved test peptide **2*** (m/z: 654.3 $[M+2H]^{2+}$, calcd. m/z: 654.3).

polyacrylamide resin showed various side products (Figures S27 and S28).

Encouraged by these results, we turned our attention to the applicability of this linker in EPL reactions. We recently reported the first semisynthesis of the longest isoform of human tau (441 amino acids),[17] a key target in Alzheimer's disease related research.[18] In this previous approach, biotin was irreversibly introduced at the synthetic C-terminus of the protein at Lys438 for a final purification step. By applying our traceless purification strategy, we intended to remove covalently bound biotin and facilitate a tag-free semisynthesis of tau through the high-yielding release of the native protein **5** from streptavidin beads under mild conditions in a buffer of choice (Figure 2A). The selected ligation site between Gly389 and Ala390 required a single point mutation of Ala390 to Cys, which has been shown not to alter the ability of tau to initialize tubulin polymerization.[17] The synthetic fragment tau[390-441] (Ala390Cys) (**4**) was obtained by linear Fmoc-based SPPS, in which Lys438 was incorporated with an orthogonal Alloc protecting group for the site-specific introduction of PC-biotin. The peptide was obtained in good purity with a yield of 8.6 mg (3%) from a 0.1 mmol scaled synthesis. The recombinant α-thioester **3** (tau[1-389]SR) was obtained as reported previously in situ by the addition of MESNa to the immobilized intein fusion protein and simultaneous peptide addition to initiate the EPL. After two days the reaction mixture was eluted from the column and analyzed by SDS-PAGE, and a ligation efficiency of approximately 70% was determined (Figure 2B, lane 4). First, the excess peptide (5.8 kDa) was removed by spin filtration (30 kDa cut-off), which allowed a simultaneous buffer exchange for the subsequent immobilization to streptavidin-coated agarose beads. After incubation for 3 h the beads were washed and the ligation product **5** was cleaved off the beads by irradiation with UV light ($\lambda = 297$ nm, 3× 10 min), yielding 0.95 mg of highly pure semisynthetic protein from an expression volume of 0.6 L (see the Supporting Information). To further analyze the product, we confirmed the identity of **5** with an anti-C-terminal tau western blot (Figure 2C) and MALDI-TOF spectrometry (Figure S21). We finally demonstrated the complete cleavage of biotin from the semisynthetic protein by an anti-biotin western blot (Figure 2D, lane 4).

Intrigued by the fact that streptavidin does not contain cysteines, we intended to apply our PC-biotin tool for a convenient, fast, and traceless procedure for NCL and desulfurization with concomitant purification. To test our theory, we synthesized two peptides derived from the C-terminus of tau, namely tau[421-425] (**6**) with C-terminal *N*-acylbenzimidazolinone (Nbz) as the thioester precursor[19] and tau[426-441] (**7**) with Cys instead of native Ala at the N-terminus and equipped with PC-biotin (Figure 3). A successful NCL and subsequent desulfurization between these two peptides would streamline the C-terminal modification of tau by allowing additional ligations at position 426. To test this, we carried out the ligation in a buffer containing 6 M guanidine hydrochloride (Gn·HCl), 200 mM NaH_2PO_4, 200 mM MPAA, and 10 mM TCEP at pH 7.2 with a slight 1.5-fold excess of peptide **6**. The reaction was complete within 90 min, assuring the consumption of the PC-biotin-containing peptide. Dilution of the postligation NCL mixture with water is required to retain the extraordinarily high binding affinity of biotin for streptavidin ($K_d \approx 10^{-14}$ mol L^{-1}), which is lowered at Gn·HCl concentrations of 4 M and higher.[20] Incubating the ligation mixture with an adequate amount of streptavidin-coated agarose beads for 1 h led to complete immobilization of the ligation product. Homogeneous desulfurization was directly carried out on the beads and was complete within 1 h at room temperature. Subsequently, the desulfurization mixture was filtered off, and the beads were washed and then irradiated with UV light ($\lambda = 297$ nm, 3×5 min; see the Supporting Information). We obtained highly pure peptide **8** without a final HPLC purification in an excellent isolated yield of 97% (based on peptide **7**).

We now wanted to extend our NCL disconnection to obtain the synthetically very challenging C-terminal tau peptide [390-441] phosphorylated at serines 396, 400, and 404, positions found to be highly phosphorylated in Alzheimer patients.[21] Previously, we obtained such triple-phosphorylated tau peptides by enzymatic phosphorylation, which, however, yielded heterogeneous mixtures with different phosphorylation patterns.[22] To address this issue we

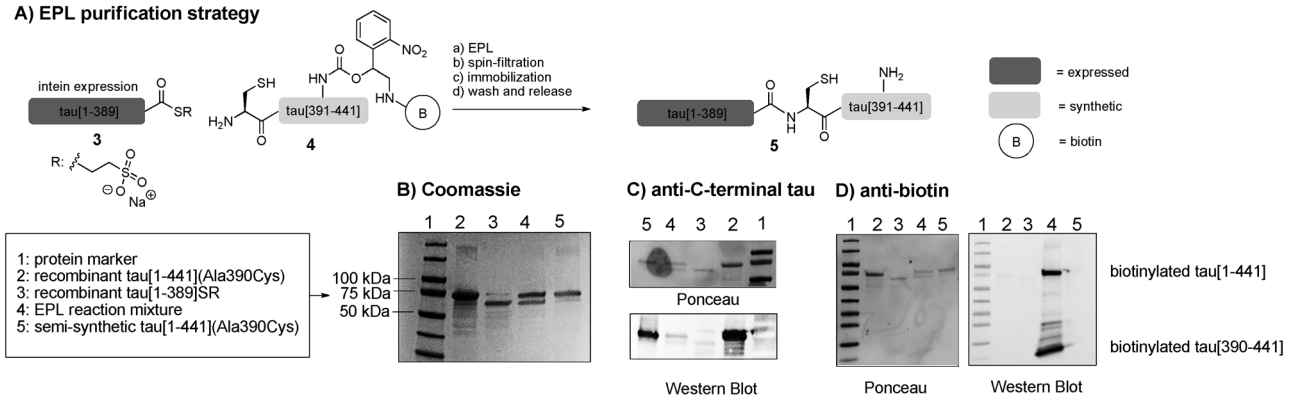

Figure 2. A) Tag-free purification of semisynthetic tau **5** (see the Supporting Information for experimental details): a) EPL; b) spin filtration (30 kDa cutoff); c) capture on streptavidin-coated agarose beads; d) washing and irradiation with UV light ($\lambda = 297$ nm). B) SDS-PAGE gel stained with Coomassie Brilliant Blue. C) anti-C-terminal tau western blot. D) anti-biotin western blot.

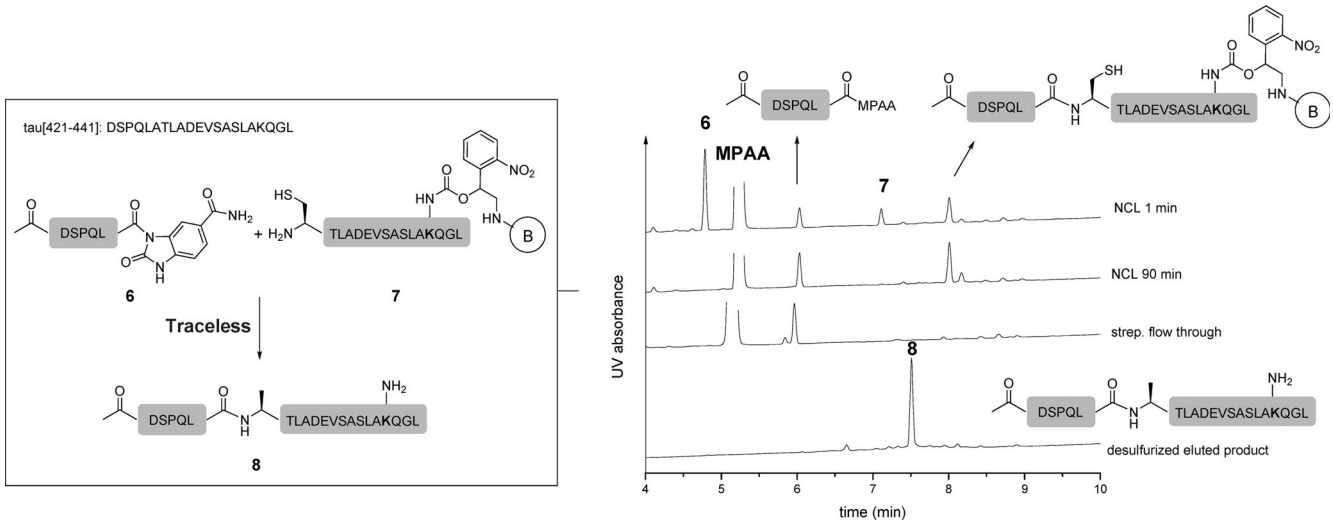

Figure 3. NCL, immobilization, and desulfurization, monitored by UPLC-UV/MS.

utilized NCL at position 426 by reaction of peptide **7** with triple-phopshorylated peptide thioester precursor **9**, which was obtained by standard SPPS (Figure 4). It is important to note that previous attempts to synthesize peptide **10** by a single linear SPPS failed (data not shown). Desulfurization and biotin-based purification yielded peptide **10** in approximately 70% purity according to UV integration. Residual contaminants could be partially traced back to truncated peptides that could not be removed by HPLC after SPPS of the starting materials. After a final HPLC step **10** was isolated in excellent 45% yield and in high purity (>95%).

In summary, we have used a photocleavable biotin-Lys linkage for establishing a convenient protocol to facilitate postligation chemistry and the traceless purification of peptides and proteins. We were able to obtain a tag-free semisynthetic tau protein as well as triphosphorylated 52 amino acid long C-terminal tau peptide. The straightforward synthesis and site-specific implementation into peptides, the favorable adjustment of NCL and desulfurization conditions, and the good cleavage properties make this linker suitable for the described immobilization and traceless workup of NCL reactions. As a result, we believe that this procedure will find broad applicability in the field of peptide and protein synthesis.

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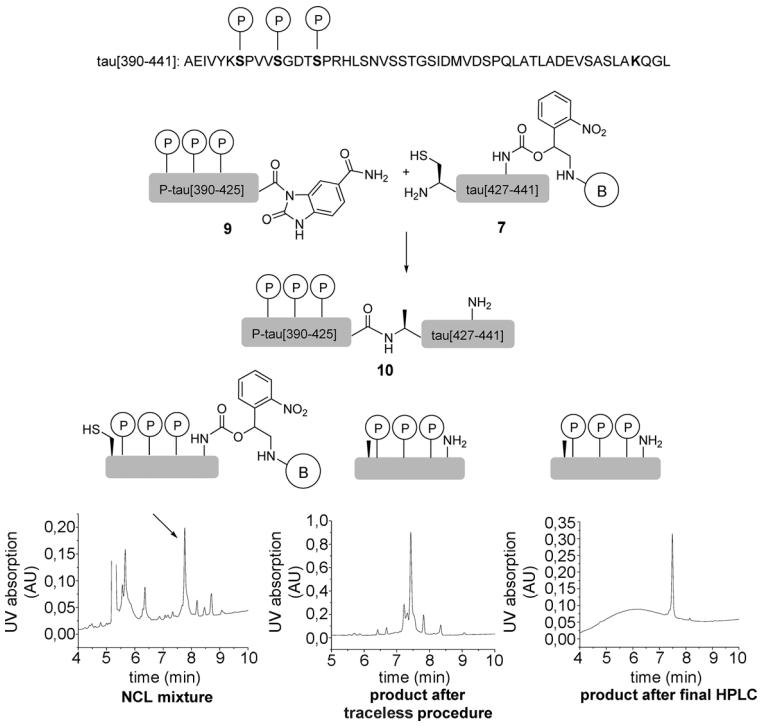

Figure 4. Traceless NCL/desulfurization of peptide tau[390-425]-Nbz (**9**, 1.5 equiv) and tau[426-441] (**7**, 1 equiv) bearing photocleavable biotin. Desulfurized product tau[390-441](pS396/400/404) was purified by C18 HPLC (acetonitrile/water gradient) and isolated in 45% yield.

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