CHEMISTRY
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Accepted Article

Title: Site-selective labeling of native proteins by a multicomponent approach

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To be cited as: *Chem. Eur. J.* 10.1002/chem.201605938

Link to VoR: http://dx.doi.org/10.1002/chem.201605938

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Site-selective labeling of native proteins by a multicomponent approach

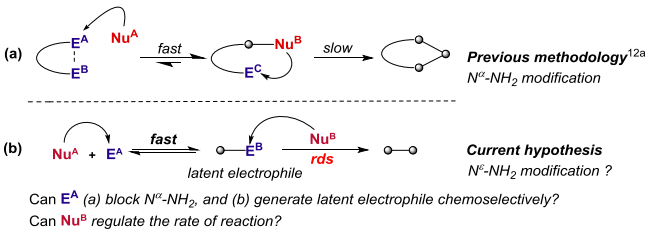
Maheshwerreddy Chilamari[a], Landa Purushottam[a] and Vishal Rai*[a]

Abstract: Chemical functionalization of proteins is an indispensable tool. Yet, selective labeling of native proteins has been an arduous task. The limited success of chemical methods allows N-terminus protein labeling, but the examples with side-chain residues are rare. Here, we surpass this challenge through a multicomponent transformation that operates under physiological conditions in the presence of protein, aldehyde, acetylene, and Cu-ligand complex. The methodology results in labeling of a single Lysine residue in nine distinct proteins.

Attachment of tags to the proteins draws wide attention as they provide utility platforms for biologics, biomaterials, and biophysics.[1] Technology that can deliver single-site labeling of endogenous proteins would be ideal to meet these requirements. However, in the absence of enabling methods, the engineered biosynthetic pathways have led the way. The gateway to such protein conjugates is through nonsense suppression technique,[2] chemoenzymatic transformations,[3] and ligations.[4] In addition to unnatural amino acids, engineering Cys at the desired position of protein or antibody sequence has led to their chemoselective labeling.[5,6] These methods have evolved with time, but they do not operate with native proteins and are limited to systems regulated by exogenous gene expression.[2] On the other hand, native protein modification has been largely dependent on chemoselective transformations.[7] The low-frequency residues such as Cys, Tyr and Trp are utilized to limit the number of labeled sites.[7,8] The naturally abundant proteinogenic residues with high average relative accessibility such as Lys are off-target as they lead to a heterogeneous mixture.[9] For instance, five Lys residues of bovine serum albumin undergo acetylation to result in a mixture of proteins within 3% conversion.[10] The task of site-selective labeling of native proteins has proved daunting, yet its immense importance has encouraged efforts in this direction.[11] In this perspective, N-terminus α-amine (N^{α}-NH_2) has been a confidence instilling target due to its high reactivity.[12] However, it also obviates the additional challenge associated with the modification of a Lys residue (N^{ε}-NH_2) in presence of free N^{α}-NH_2.

Protein is a multifunctional organic molecule that offers an array of nucleophilic residues on its surface. The pre-requisite of the labeling method involve the development of a chemical transformation that would be effective in near neutral aqueous buffer at ambient temperature. Besides, Lys residue (N^{ε}-NH_2) has to compete with the other nucleophilic functionalities (chemoselectivity) and all N^{ε}-NH_2 residues (site-selectivity). The focal challenge revolves around the tenet that difference in nucleophilicity of multiple copies of an amino acid residue is significantly low. In addition, the pKa of Lys residues can vary considerably owing to its microenvironment.[13] Recently, we reported labeling of N^{α}-NH_2 with high degrees of chemo- and site-selectivity (Scheme 1a).[12a] In this case, the site-selectivity is compromised upon elevating the reaction time or stoichiometry of electrophile in an attempt to increase the conversions. Limitation of our previous method and other related protocols[12c-d] was primarily due to the absence of a regulatory tool that can allow control over reactivity without compromising the selectivity.

We argued that an apt electrophile (E^A) can react in a fast reversible step chemoselectively and render latent electrophiles (Scheme 1b). Subsequently, the stoichiometry of nucleophile (Nu^B) in a rate determining step can serve as a desired regulatory tool. It is an imperative pre-requisite to block the N^{α}-NH_2 group reversibly. This will create an opportunity to investigate whether one N^{ε}-NH_2 can be labeled selectively amongst multiple Lys residues. Besides, if the electrophile (E^A) itself can decimate the nucleophilicity of N^{α}-NH_2, additional site-selective protection, and bio-orthogonal deprotection steps can be avoided. In this perspective, here we report a chemo- and site-selective coupling where a primary amine of protein (Nu^A), formaldehyde (E^A) and phenylacetylene (Nu^B) assemble to result in single-site labeling of the protein. The formaldehyde blocks the N^{α}-NH_2 in the form of imidazolidinone through neighboring group participation of backbone amide. Additionally, it creates the latent electrophile with Lys side-chain residues in a reversible reaction. The phenylacetylene serves as the nucleophile after activation by the copper catalyst. The methodology offers excellent selectivity at low micromolar concentrations in physiological conditions. A single N^{ε}-NH_2 residue is labeled selectively in the presence of all the nucleophilic proteinogenic amino acids, N^{α}-NH_2, and multiple copies of N^{ε}-NH_2. The methodology operates efficiently with a structurally diverse set of nine proteins. The mild operational conditions allow enzymes to retain their activity post-modification



Scheme 1. Native protein modification through latent electrophile

The coupling reaction involving amines, aldehyde, and alkyne (A^3 coupling) result in propargyl amines that serve as synthetic intermediate and natural product fragment.[14] Metal catalysis has often led to the success of this three-component reaction.[15] However, there are multiple challenges for its translation into a protein compatible transformation. (a) The success of A^3 coupling is dependent on the use of secondary amine as one of

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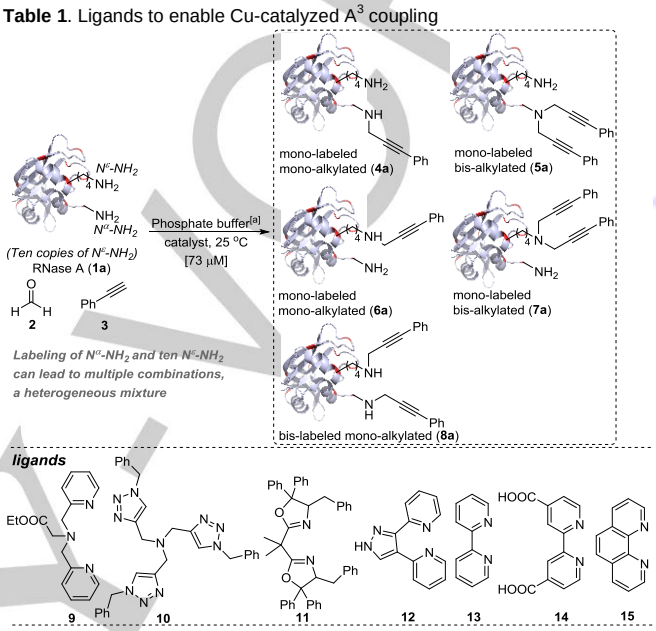
the components. Unfortunately, the primary amines proffered by proteins constitute the class of highly challenging substrates. (b) This multi-component process requires high reaction concentration (0.3 M to solvent free)[16] and organic solvents. Both the parameters are detrimental for experiments with protein as it results in their aggregation and denaturation. (c) The aerobic aqueous conditions required by proteins poison several catalytic systems applied in these reactions.[17] (d) The presence of numerous binding sites in the protein can lead to substantial metal-protein binding.[18]

The re-discovery of A^3 coupling was imperative for its application to proteins in physiological conditions. Initially, we employed a broad range of catalysts[15] [$AuCl_3$, $AuCl(PPh_3)$, $RuCl_3$, $InCl_3$, $NiCl_2$, $Ni(COD)_2$, and $CdCl_2$] for examining their efficiency with RNase A (73 μM), formaldehyde and phenylacetylene in aqueous conditions (Table S1, Figure S1 in Supporting information). Unfortunately, these catalysts were found to be unsuccessful in resulting transformation to the desired product. Next, we generated Cu(I) in-situ with $CuSO_4$ and sodium ascorbate for regulated oxidation of Cu(I) to Cu(II) (entry 1, Table 1). Here, RNase A reacted within 2 h but with poor selectivity and resulted in mono- and bis-A^3 coupling products along with several unidentifiable compounds. Whereas CuI led to no conversion (entry 2), CuCl as a catalyst resulted in <5% conversion over a period of 2 d (entry 3). All the initial attempts of catalytic transformations with proteins were either marred with the lack of reactivity or selectivity. The experiments with a model substrate, benzylamine (Scheme S1 in Supporting Information), routed for the possibility of over-alkylation of amine residues (**5a**/**7a**). Besides, formaldehyde can lead to the formation of reactive intermediates primed for multiple reaction pathways (Figure S2 in Supporting information).[19] Such intermediates are also known to react with R, Y, H, W and Q residues to result in protein-protein conjugates.[20] We observed multiple unidentified adducts upon incubating protein with 50-100 equivalents of formaldehyde. However, all of them were found to be reversible during dialysis and do not inhibit or alter the course of the reaction. On the other hand, adducts formed with 200 equivalents HCHO require additional assistance of hydroxylamine or acidic conditions (pH 4) for reversibility. The interplay of HCHO concentration and reaction time allows a window to operate without the interference from protein-protein conjugates (Figure S3 in Supporting Information). Besides, the alkyne is inert for proteinogenic nucleophiles in presence or absence of a catalyst (Table S2 in Supporting Information). Next, we argued that the poor reactivity might be culminating from the binding of Cu with protein. The identification of protein-copper complex (MALDI-ToF MS, Figure S1 in Supporting Information) in reaction mixture supports this attribute. We hypothesized that a suitable ligand might allow us to fine-tune the reactivity of catalyst,[21] reduce its binding to the protein and favorably alter the course of disproportionation. We identified CuCl and CuI for the next stage screening. $CuSO_4$/sodium ascorbate is excluded owing to the potential alternative pathways[22] in aerobic conditions and capabilities of ascorbate to alter the pKa of N^{ε}-NH_2 unpredictably.[23]

From a pool of ligands **9**-**15** (Table 1), 1, 10-phenanthroline (**15**) resulted in excellent conversion with RNase A (**1a**) in the presence of CuCl (>99% conversion, entry 5, Table 1) and CuI (90% conversion, entry 12). It was exciting to note the formation of mono-labeled mono-alkylated product in both the cases. The treatment of product (**4a** or **6a**, entry 5, Table 1) with hydroxylamine and EDTA removes formaldehyde and Cu from their adducts by competitive substitution. The structure of the labeled RNase A is unperturbed after the transformation (CD, Figure S4 in Supporting Information). Next, the product (**4a** or **6a**) is vortexed with proteolytic enzymes. It was surprising to note the severely diminished activity of trypsin and α-chymotrypsin. The control experiments (Figure S5 in Supporting Information) confirmed that presence of CuCl compromises the proteolytic activity of enzymes and hinders RNase A (**1a**) digestion.

Table 1. Ligands to enable Cu-catalyzed A^3 coupling



Entry[b]	Catalyst	Time	%Conversion (**4a** or **6a**)[c]
1	$CuSO_4$/Na ascorbate	2 h	Complex mixture
2	CuI	2 d	0
3	CuCl	2 d	<5
4	CuCl, **9** or **10** or **11** or **12** or **13** or **14**	2 d	<5
5	CuCl, **15**	14 h	>99
6	CuI, **9**	3 d	-[d]
7	CuI, **10**	3 d	<5
8	CuI, **11**	3 d	7
9	CuI, **12**	3 d	30
10	CuI, **13**	3 d	38
11	CuI, **14**	3 d	60
12	CuI, **15**	3 d	90

[a] Phosphate buffer (pH = 7.8, 0.1 M):DMSO = 9:1. [b] RNase A (**1a**, 73 μM), HCHO (**2**, 7.3 mM), phenylacetylene (**3**, 7.3 mM), CuI (7.3 mM), CuCl (7.3 mM) and ligand (29.2 mM). [c] Product is **4a** or **6a**. Reactions were monitored by using MALDI-ToF MS. [d] Unidentified products.

These results shifted our focus to CuI catalyzed transformation. The digestion of labeled protein (**4a** or **6a**) with α-chymotrypsin went smoothly in this case. The ε-amine of K31 residue (K31-N^{ε}-NHR, **6a**) was identified by peptide mapping and MS-MS (Figure 1b) to be the exclusive site of modification. The site of the modification remains unaltered in the presence of different ligands (entries 9-12). It is exciting to note that the N^{α}-NH_2 did not participate in any irreversible process. We can attribute this feature to the reversible formation of imidazolidinone that can be traced by NMR or MALDI-ToF MS of a dipeptide Ala-Ala (Figure S6 in Supporting information) and RNase A respectively (Figure

1a).[12b] Phosphate buffer is the choice of solvent, and its concentration (0.1 M) was selected to prevent the formation of Cu-phosphate complexes. The co-solvent (DMSO) is required to attain a homogenous solution. However, it is restricted to 10% in the buffer so as to avoid protein denaturation and curb competitive binding to Cu^{I}. The optimized ligand-Cu ratio of 4:1 resulted in an efficient transformation (Table S3 in Supporting Information). The stoichiometry of formaldehyde, alkyne and copper iodide (100 equivalents each) were optimized to achieve high reactivity (Table S4 in Supporting Information). As hypothesized, the stoichiometry of Cu-alkyne complex was critical for enhancing the conversions without compromising the selectivity (Table S4 in Supporting Information). Under the optimized conditions, we do not observe the formaldehyde-mediated labeling of Tyr.[24] Even though the reaction conditions are aerobic, the desired multicomponent pathway outcompetes Cu-catalyzed oxidation, oxygenation, and Glaser-Hay coupling.[25] The operational simplicity of the optimized process is noticeable.

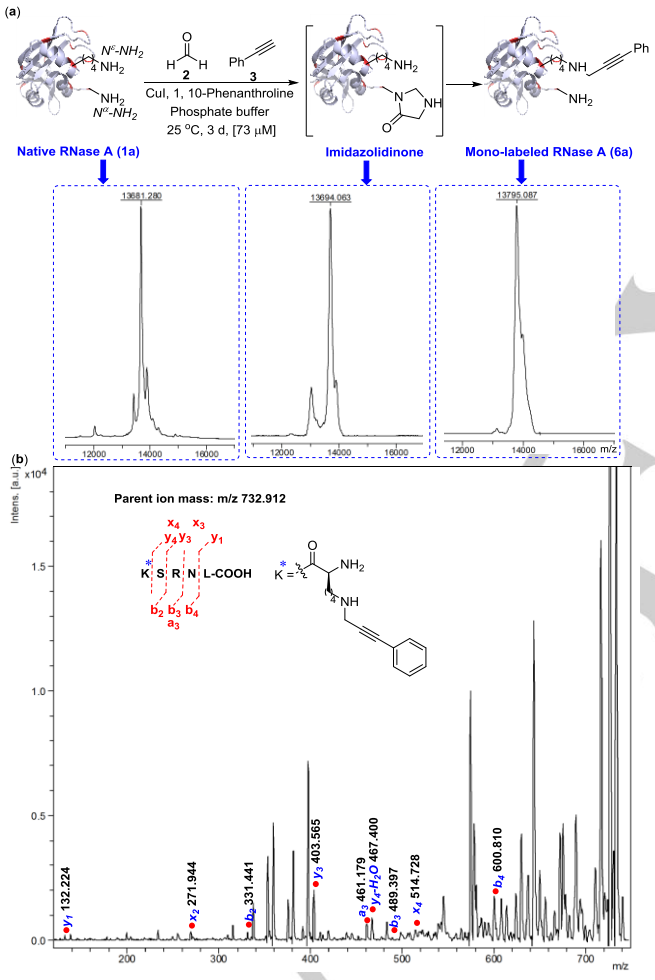
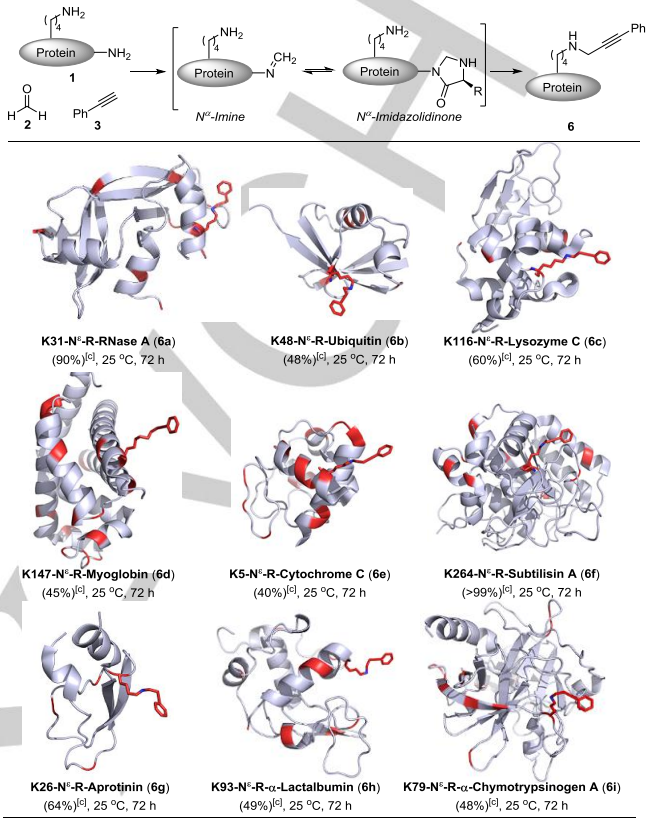


Figure 1. [a] Representative scheme for RNase A modification with MALDI-ToF MS of RNase A (**1a**), imidazolidinone of RNase A, and mono-labeled RNase A (**6a**). [b] MS-MS spectra of the modified peptide fragment (K*SRNL) of RNase A after digestion with α-Chymotrypsin.

RNase A served as a model protein as it offered a highly reactive N^{α}-NH_2 and ten competing N^{ε}-NH_2 groups. The secondary structure of protein remains unaffected (CD experiment, Figure S4 in Supporting Information) in the process of site-selective labeling under the optimized conditions. It is potentially due to the unperturbed charge distribution on the protein surface throughout the process (Schiff base intermediate and secondary amine product).

Table 2. Expanding the concept of site-selective native protein modification[a,b]



R = -$CH_2C{\equiv}CPh$, [a] Phosphate buffer (pH = 7.8, 0.1 M):DMSO = 9:1. [b] Protein (**1**, 73 μM), HCHO (**2**, 7.3 mM), phenylacetylene (**3**, 7.3 mM), CuI (7.3 mM) and 1, 10-phenanthroline (**15**, 29.2 mM). [c] Conversions based on MALDI-ToF MS analysis. Site of modification is identified by protein sequencing using MALDI-ToF MS. (*refer Section 10 in SI*).

In the pursuit to understand the generality of this methodology, it was imperative to investigate proteins that can offer nucleophilic residues with the diverse microenvironment. We selected Ubiquitin (76 residues, N^{α}-NH_2, seven N^{ε}-NH_2) that provides a pair of highly reactive and competing N^{ε}-NH_2 residues (K48 and K63). It was exciting to note that the reaction resulted in K48-N^{ε}-NHR (**6b**) with excellent chemo- and site-selectivity. Next, we selected Lysozyme C (129 residues, N^{α}-NH_2, six N^{ε}-NH_2) that bears N^{α}-NH_2 with reduced reactivity likely due to its coordination with aptly positioned E7. A single site modification resulted in K116-N^{ε}-NHR (**6c**) without interference from competing pathways within the reaction time. In particular, no Trp-formaldehyde adduct is observed even in the presence of six Trp residues. Subsequently, we selected two proteins with very high Lys frequency. Myoglobin (153 residues, N^{α}-NH_2, nineteen N^{ε}-NH_2) results in site-selective modification of K147-N^{ε}-NHR (**6d**), whereas K5-N^{ε}-NHR (**6e**) is the single site of modification in Cytochrome C (N^{α}-$NHCOCH_3$, nineteen N^{ε}-NH_2). The efficiency of transient N^{α}-NH_2 protection in Myoglobin is similar to the protected N-terminus in Cytochrome C. To investigate the competitive formation of Tyr-formaldehyde adducts, Subtilisin A (286 residues, N^{α}-NH_2, nine N^{ε}-NH_2) with thirteen Tyr residues was considered adequate. It was pleasing to note the single-site labeling of K264-N^{ε}-NHR (**6f**) with the quantitative conversion.

The MS analysis did not trace any Tyr-formaldehyde adduct. Subsequently, we investigated three proteins of varying sizes, Aprotinin (58 residues, N^{α}-NH_2, four N^{ε}-NH_2), α-Lactalbumin (123 residues, N^{α}-NH_2, twelve N^{ε}-NH_2), and α-Chymotrypsinogen A (245 residues, N^{α}-NH_2, fourteen N^{ε}-NH_2). The single-site labeling results in K26-N^{ε}-NHR (**6g**), K93-N^{ε}-NHR (**6h**), and K79-N^{ε}-NHR (**6i**) respectively. It is noteworthy that the site of labeling in all the cases (**6a**-**6i**) are solvent accessible. At the end, the labeled RNase A was examined for its capability to degrade Ribonucleic acid (RNA, **16**). In parallel, lysis of Micrococcus lysodeikticus cells (**17**) served as a benchmark for the activity of labeled Lysozyme C. The enzymatic activity of both the examples remains largely unperturbed after the chemical modification (Figure 2).

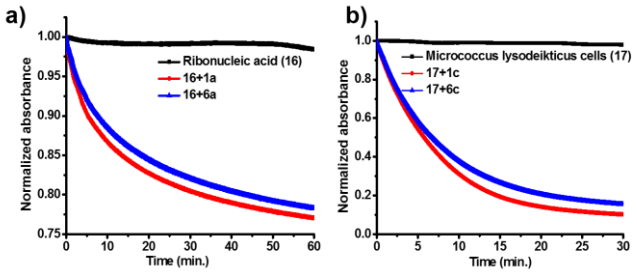


Figure 2. Normalized UV-Vis spectra of enzymatic assay. [a] Comparison of enzymatic activity between native RNase A (**1a**) and labeled RNase A (**6a**) at 300 nm. [b] Comparison of enzymatic activity between native Lysozyme C (**1c**) and labeled Lysozyme C (**6c**) at 450 nm. (*refer Section 11 in SI*).

In summary, we have developed a chemical methodology that enables chemoselective and site-selective modification of Lys residue in a native protein. The protocol addresses two key selectivity challenges related to the native protein bioconjugation, viz. N^{α}-NH_2 versus N^{ε}-NH_2 and N^{ε}-NH_2 versus N^{ε}-NH_2. In a multicomponent approach, we construct a C-C and C-N bond appending propargylamine motif into the protein. The success of this approach resides in finding a suitable ligand that can inhibit the binding of Cu catalyst to the protein while modulating the reactivity favorably. This feature allows the transformation to operate at low micromolar concentrations. The role of aldehyde as a transient N-terminus protecting group provides the platform for probing the selectivity of Lys side-chain residues. The mild nature of the reaction condition is apparent through examples of enzymes that retain their activity post-modification.

Keywords: Multicomponent reaction • Protein modification • Chemoselectivity • Site-selectivity • A3 coupling reaction

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10.1002/chem.201605938

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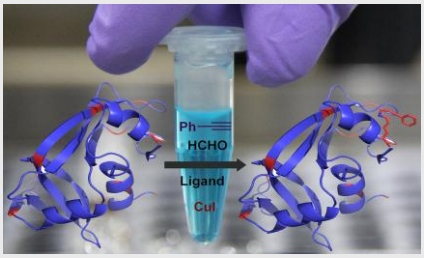
Chemoselective and site-selective coupling of Lysine residue, aldehyde, and alkyne enable single-site native protein labeling. The mild reaction conditions do not perturb the structure or activity of the protein.



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Site-selective labeling of native proteins by a multicomponent approach