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Phospha-Michael Addition as a New Click Reaction for Protein Functionalization

*Yan-Jiun Lee, Yadagiri Kurra, and Wenshe R. Liu**

Abstract: *A new type of click reaction between alkyl phosphine and acrylamide was developed and applied for site-specific protein labeling in vitro and on live cells. Acrylamide is a small electrophilic olefin that readily undergoes phospha-Michael addition with alkyl phosphine. Our kinetic study indicated a second-order rate constant of 0.07 $M^{-1}s^{-1}$ for the reaction between tris(2-carboxyethyl)phosphine and acrylamide at pH 7.4. To demonstrate its application in protein functionalization, we used a dansyl-phosphine conjugate to successfully label proteins that were site-specifically installed with N^{ϵ}-acryloyl-L-lysine and employed a biotin-phosphine conjugate to selectively probe human proteins that were metabolically labeled with N-acryloyl-galactosamine.*

Many approaches have been developed to modify proteins. A classical method is to target cysteine and lysine residues in proteins albeit with low selectivity and this approach is not applicable for *in vivo* applications.[1-2] The advent of the green fluorescent protein (GFP) technique circumvents this limitation. However, GFP has a large size that potentially perturbs functions of its fused partners.[3] To retain both the small size of residue-based modification and selectivity of the GFP technique, many bioorthogonal labeling approaches have been developed and they exploit orthogonal reactions between chemical group pairs such as azide-alkyne, phosphine-azide, keto-hydrazine/hydroxylamine, nitrilimine-alkene, and tetrazine-alkene in intra- and extracellular milieu.[4-16] For the purposes of developing more robust and rapid bioorthogonal labeling approaches, the alkene functionality has recently drawn a lot of attentions due to its highly inert nature in the biological system and high versatility of its involved chemical transformations.[17] Strained alkenes such as norbornene, trans-cyclooctene and cyclopropene react rapidly with tetrazine or nitrilimine.[18-21] Non-strained olefins also react with tetrazine and nitrilimine readily.[12, 16]

As a highly electron-deficient alkene, acrylamide was originally considered not stable in the biological system due to the existence of high concentrations of cellular glutathione. However, our previous kinetic analysis indicated that it reacts slowly with a free thiol at pH 7.4 with a second-order rate constant as 0.004 $M^{-1}s^{-1}$. An acylamide-containing noncanonical amino acid, N^{ϵ}-acryloyl-lysine (AcrK) was later designed, synthesized, and genetically incorporated into proteins in *E. coli* using an evolved amber suppressing pyrrolysyl-tRNA synthetase-$tRNA^{Pyl}$ pair.[12] A model protein superfolder GFP (sfGFP) with AcrK incorporated at its S2 position (sfGFPS2-AcrK) was synthesized and showed, surprisingly, no Michael addition products at its acrylamide moiety, indicating that the acrylamide functionality is fairly stable in cells. Given its potential reaction with β-mercaptoethanol and dithiothreitol, sfGFPS2-AcrK was later preserved in tris(2-carboxyethyl)phosphine (TCEP), a reductant adapted for various biochemical assays both *in vitro* and on live cells.[22-25] However, in the presence of TCEP, we noticed the deterioration of the acrylamide functionality in sfGFPS2-AcrK. This led to suspecting that TCEP reacts with acrylamide. TCEP is a water-soluble alkyl phosphine that potentially adds to acrylamide via phospha-Michael addition, a well-practiced reaction in organic synthesis.[26] An alky phosphine is also typically used to initiate nucleophilic thiol-ene Michael addition, indicating that its reaction with an electron-deficient alkene is probably more kinetically favored than thiol-ene Michael addition.[27-29] To demonstrate this prospect, we carried out kinetic analysis of the reaction between TCEP and acrylamide. Here, we show TCEP reacts favorably with acrylamide at pH 7.4 and this reaction can be applied for functionalizing proteins both *in vitro* and on living cells.

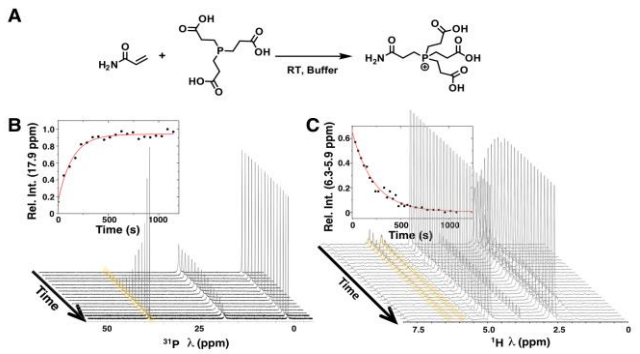

Figure 1. Kinetic analysis of phospha-Michael addition between TCEP and acrylamide characterized by ^{31}P and ^{1}H NMR. (**A**) The reaction scheme. (**B**) Time-course of phospha-Michael addition between TCEP and acrylamide characterized by ^{31}P NMR. The inlet shows the product formation curve. (**C**) The same reaction characterized by ^{1}H NMR at the proton peaks at 5.83-5.74 ppm and 6.35-6.10 ppm. The disappearances of signals were monitored and presented in the inlet.

The reaction between TCEP and acrylamide was investigated in an aqueous buffer at pH 7.4 and 25°C (**Figure 1A**). It was first monitored with ^{31}P NMR. The progression of the reaction was evidenced by the diminishing of the ^{31}P signal of TCEP at 17.6 ppm and the concomitant accumulation of the ^{31}P NMR signal at 37.4 ppm that indicated the formation of a phosphonium product (**Figures 1B**).[30] Data of integrated NMR signal at 37.4 ppm vs. time was fitted well to a one-phase exponential increase equation, resulting a second-order rate constant of 0.060 ± 0.01 $M^{-1}s^{-1}$ (the inset of **Figure 1B**). It is worth mentioning that after an overnight reaction there was no detectable phosphine oxide byproduct observed, suggesting TCEP itself was highly stable in the adopted testing conditions (**Figure S1**). Additionally, a parallel experiment characterized by ^{1}H NMR was performed. The depletion of the sp2 proton signal of acrylamide at 6.35-6.10 ppm and 5.83-5.74 ppm

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were monitored (**Figure 1C**) and the following analysis inferred a second-order rate constant of 0.067 ± 0.002 $M^{-1}s^{-1}$, which agrees well with results from the ^{31}P NMR analysis. Though not as rapid as reactions between a tetrazine and a strained olefin, this phospha-Michael addition reaction is comparable to Staudinger ligation (k = 0.002 $M^{-1}s^{-1}$) and copper-free dibenzocyclooctyne–azide cycloaddition (k = 0.0565 $M^{-1}s^{-1}$), two reactions that have been well endorsed for bioconjugation in live cells.[5, 7, 31-34] In comparison to Michael addition with a thiol nucleophile, acrylamide is more kinetically favored to react with alkyl phosphine, approximately 20 times faster, under same conditions.

After finishing kinetic analysis, we took a step further to examine if the TCEP-acrylamide chemistry can be exploited for protein bioconjugation. The acrylamide moiety has been incorporated into proteins as forms of two noncanonical amino acids, AcrK and *p*-acrylamido-phenylalanine using the amber suppression approach.[12, 35] We took advantage of our existing AcrK-incorporation platform to synthesize sfGFPS2-AcrK. TCEP (2 mM) was then incubated with sfGFPS2-AcrK (50 μM) in a Tris-HCl buffer (pH 6.8 or pH 8.8) at 37 °C for 1 h. 37°C was used to elevate the reaction rate. Following buffer exchange to remove excessive phosphine, the product was subjected to electrospray ionization mass spectroscopy (ESI-MS) analysis. Deconvoluted ESI-MS spectra of modified sfGFPS2-AcrK at both pH 6.8 and pH 8.8 displayed a major peak at 28,013 Da that agreed well with the theoretical molecular weight of the TCEP adduct of sfGFPS2-AcrK (28,014 Da) (**Figure 2**). A majority of sfGFPS2-AcrK (about 80% at pH 6.8 and 90% at pH 8.8) was converted to the addition product. At pH 8.8, more addition product was formed due to less protonation of TCEP (pK_a = 7.6) than at pH 6.8.[25] We previously demonstrated Michael addition between sfGFPS2-AcrK and *β*-mercaptoethanol. *β*-Mercaptoethanol modified sfGFPS2-AcrK very slowly, leading to roughly 50% conversion when 40 mM *β*-mercaptoethanol was used and the reaction was run at 37°C and pH 8.8 with 8 h incubation.[12] Therefore, in comparison to Michael addition between acrylamide and thiol, phospha-Michael addition between acrylamide and alkyl phosphine is more kinetically favored. Given the compatibility of TCEP with proteins, it is well suitable for protein bioconjugation.

To use phospha-Michael addition for biochemical interrogation, we designed and synthesized two functionalized phosphine probes (**Figure 3A**). Briefly, utilizing TCEP as scaffold, TCEP was first methyl-triesterified and followed by treatment of a borane tetrahydrofurane solution to form a phosphine-borane complex in order to reinforce phosphine's ambient stability.

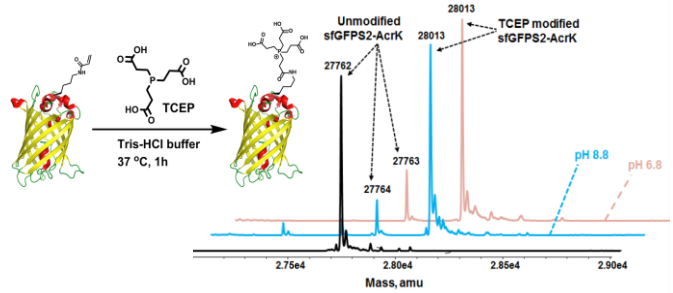

Figure 2. Electrospray ionization mass spectroscopy analysis of sfGFPS2-AcrK modified with TCEP via phospha-Michael addition. Stacked MS spectra of the original and TCEP-treated sfGFPS2-AcrK protein samples at different pHs. Black spectrum is from the original sfGFPS2-AcrK protein without treatment with TCEP (calculated mass: 27,764 Da). Blue and pink spectra are from TCEP-treated sfGFPS2-AcrK samples at pH 8.8 and pH 6.8, respectively, which showed mainly the phosphonium adduct (calculated mass: 28,014 Da).

Stoichiometric hydrolysis of the triester afforded an intermediate **3**. The free acid of **3** was then conjugated with choice of reporters such as fluorophore or biotin using simple amide coupling. Finally, decomplexation of borane by 1,4-diazabicyclo[2.2.2]octane afforded **5a** as a dansyl-phosphine conjugate and **5b** as a biotin-phosphine conjugate. With **5a** in hand, we proceeded to fluorescently label sfGFPS2-AcrK with it. sfGFPS2-AcrK (70 uM) was incubated with **5a** (2 mM) in a Tris-HCl buffer at pH 7.5 and room temperature for 5 h. After fully denatured, the labeled protein was subjected to the SDS-PAGE analysis. In-gel fluorescence detected appreciable signal from sfGFPS2-AcrK treated with **5a**. However, wild-type sfGFP (sfGFP-WT) that was treated similarly with **5a** did not show any positive signal (**Figure 3B**). Interestingly, **5a**-modified sfGFPS2-AcrK migrated slightly farther in the SDS-PAGE gel than sfGFP-WT. This may be attributed to the phosphonium positive charge of **5a**-modified sfGFPS2-AcrK. Similarly, biotin probe **5b** was incubated with sfGFPS2-AcrK and sfGFP-WT. After buffer exchange and SDS-PAGE analysis, the resulting protein samples were transferred onto a nitrocellulose membrane. After sequential treatment with blocking reagents and streptavidin-HRP, the membrane was subjected to chemiluminescent detection. As expected, the sfGFPS2-AcrK showed detectable signal, whereas sfGFP-WT didn't (**Figure 3C**).

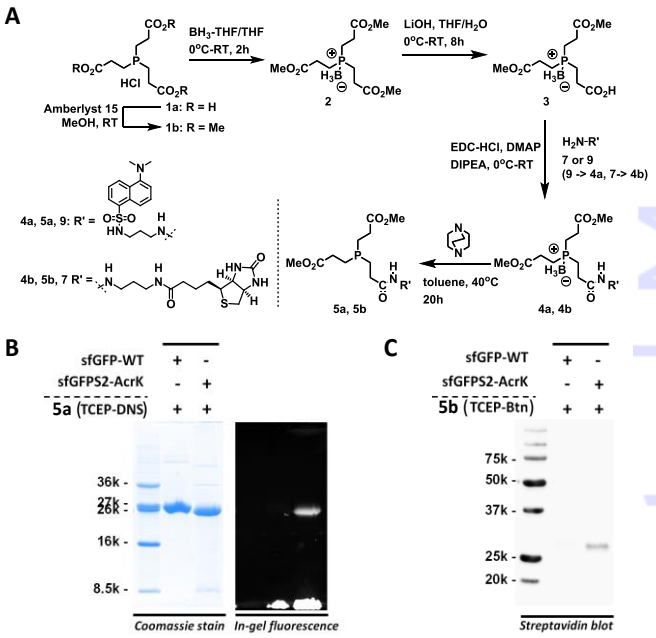

Figure 3. (**A**) The synthetic schemes of phosphine probes **5a** and **5b**. Specific labeling of the sfGFPS2-AcrK protein with (**B**) **5a** or (**C**) **5b**.

With the demonstration of using phospha-Michael addition for protein labeling *in vitro*, we then proceeded to test this reaction to label proteins incorporated with AcrK on live cells. Two previously constructed plasmids pEVOL-pylT-PrKRS and pETDuet-OmpXTAG were used to transform *E. coli* BL21 cells to express an *E. coli* outer membrane protein OmpX with AcrK incorporated at its extracellular domain (OmpXAcrK). Plasmid pEVOL-pylT-PrKRS carried genes for PrKRS and $tRNA^{Pyl}_{CUA}$ and plasmid pETDuet-OmpXTAG contained a gene coding OmpX with an AAAXAA (A denotes alanine and X denotes an amber mutation) insertion between two extracellular residues 53 and 54. PrKRS was selected to acylate $tRNA^{Pyl}_{CUA}$ with AcrK. The expression of the OmpXAcrK protein was performed in LB media supplemented with 1 mM IPTG and 5 mM AcrK (**Figure 4B**).[12] Incubating the lysate of OmpXAcrK-expressing cells with **5a** (3 mM) at room

temperature for 7 h led to a detectable fluorescent band in a SDS-PAGE gel that corresponded well with the Coomassie blue stained OmpXAcrK in the same gel. While many proteins in the lysate were present at higher expression levels, in-gel fluorescence imaging showed that only the expressed OmpXAcrK could be specifically labeled, giving virtually no detectable background. Following the successful cell lysate labeling, experiments were conducted to fluorescently label live cells with **5a** by incubating the PBS-washed OmpXAcrK-expressing *E. coli* with **5a** (4 mM) at room temperature for 1 h. Cells were then washed with an isotonic saline solution and subjected to confocal microscopy imaging. OmpXAcrK-expressing cells showed strong fluorescence (**Figure 4C**). Nonetheless, cells grown in the absent of AcrK were barely fluorescent.

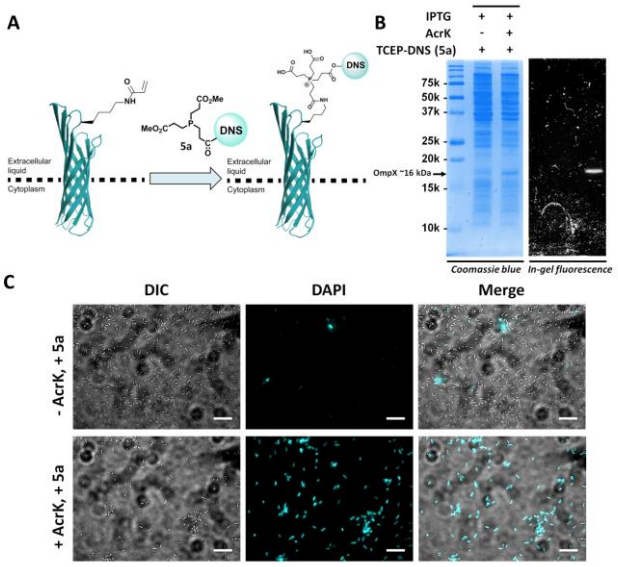

Figure 4. (**A**) The scheme of labeling OmpXAcrK with **5a**. (**B**) Bioorthogonal fluorescence labeling of OmpXAcrK with **5a** in cell lysates. (**C**) Selective labeling of OmpXAcrK-expressing cells with **5a**.

Lastly, to evaluate if alkyl phosphine-acrylamide can serve as a general chemical reporter pair, we chose to demonstrate the metabolic incorporation of a sugar acrylamide to mammalian protein glycans and their labeling with an alkyl phosphine probe. This method involves metabolic labeling of glycans with an acrylamide-functionalized monosaccharide surrogate followed by labeling with a biotin-phosphine probe, allowing the detection of specific glycoconjugates on cells or the selective capture of glycoproteins from cell lysates. It was previously reported that the unnatural monosaccharide *N*-azidoacetylgalactosamine (GalNAz) can be utilized by cells as a substitute for its natural counterpart *N*-acetylgalactosamine (GalNAc).[36-38] Acrylamide structurally highly resembles acetamide. By the same token we speculated the acrylamide version of GalNAc, *N*-acryloylgalactosamine (GalNAcr), will be a close mimic of GalNac and is seamlessly integrated into protein glycans. Therefore, per-*O*-acetylated *N*-acryloylgalactosamine (Ac_4GalNAcr), an acetylated precursor of GalNAcr, was synthesized. HEK293T cells were incubated with Ac_4GalNAcr at a concentration of 50 μM for 36 h or 60 h. The cells were then washed and lysed. The cell lysates were reacted with **5b** (3 mM) for 1 h. Followed by buffer exchange and SDS-PAGE analysis, proteins were transferred onto a nitrocellulose membrane. After sequential treatment with blocking reagents and streptavidin-HRP, the membrane was subjected to chemiluminescent detection. Cells grown in media supplemented with Ac_4GalNAcr displayed stronger signals for a large variety of proteins than control cells that were grown in media without Ac_4GalNAcr supplemented, indicating GalNAcr was globally incorporated into glycoproteins. In comparison to proteins in cells that were incubated with GalNAcr for 30 h, proteins in cell that were incubated with GalNAcr for 60 h displayed reduced labeling signals. This might be due to the gradual scavenging of the acryloyl functionality by thiols inside cells (**Figure 5B**).

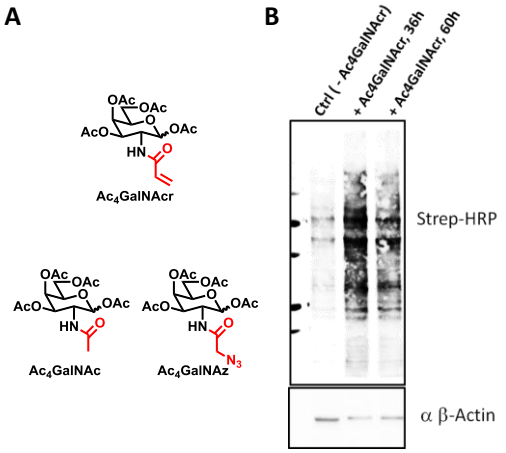

Figure 5. Metabolic incorporation of an acrylamide-conjugated sugar to glycoproteins and their detection with an alkyl phosphine probe. (**A**) The structures of native monosaccharide GalNAc (per-*O*-acetylated form) and non-native monosaccharide surrogates with azide, GalNAz (per-*O*-acetylated) or with acrylamide, GalNAcr (per-*O*-acetylated). (**B**) Detection of the acrylamide-conjugated glycoproteins in HEK293T whole cell lysates by **5b**. The control (Ctrl) represents the whole cell lysate of HEK293T cells grown in media without Ac4GalNAcr provided.

In summary, we discovered that phospha-Michael addition between alkyl phosphine and acrylamide readily undergoes in an aqueous buffer to give a phosphonium product. In comparison to thiol-based Michael addition, phospha-Michael addition between TCEP and acrylamide is much quicker. Using dansyl-phosphine and biotin-phosphine conjugates as probes, we demonstrated that proteins incorporated with an acrylamide moiety could be selectively labeled both *in vitro* and on live cells. We also showed that GalNAcr, an acrylamide-containing mimic of GalNAc could be globally integrated into protein glycans and labeled with a phosphine probe. Therefore, we believe phospha-Michael addition represents a new type of click reaction and it can be readily applied for modifying biomacromolecules for their functional investigations.

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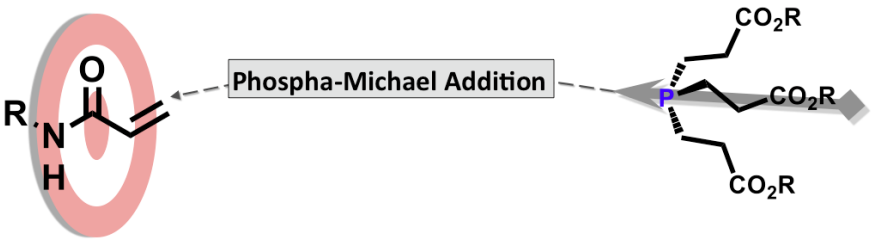
Phospha-Michael Addition as a New Click Reaction for Protein Functionalization



Phospha-Michael addition between alkyl phosphine and acrylamide undergoes favourably in physiological conditions and serves as a new type of click reaction. We demonstrated that this reaction could be applied for cell labeling and glycoprotein profiling.