Bioconjugation

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Development and Optimization of Glaser–Hay Bioconjugations**

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Abstract: *The prevalence of bioconjugates in the biomedical sciences necessitates the development of novel mechanisms to facilitate their preparation. Towards this end, the translation of the Glaser–Hay coupling to an aqueous environment is examined, and its potential as a bioorthogonal conjugation reaction is demonstrated. This optimized, novel, and aqueous Glaser–Hay reaction is applied towards the development of bioconjugates utilizing protein expressed with an alkynyl unnatural amino acid. Unnatural amino acid technology provides a degree of bioorthognality and specificity not feasible with other methods. Moreover, the scope of the reaction is demonstrated through protein–small molecule couplings, small-molecule–solid-support couplings, and protein–solid-support immobilizations.*

Bioconjugates have found a wide degree of relevance as both diagnostics and therapeutics, along with functional materials.[1,2] Consequently, the development of novel methods for their preparation has far-reaching applications. Various protein bioconjugation reactions are well-known and regularly employed, including reactions involving malamides, isocyanates, NHS esters, and iodoacetamides with nucleophilic amino acid residues.[3] Moreover, novel chemical functionalities have been introduced to further the technology and afford 1,3-dipolar cycloadditions, photo-cross-linking, transition-metal-mediated alkyne couplings, and oxime formations.[4,5] These reactions all must meet several key requirements to be useful in the generation of bioconjugates. These include compatibility with aqueous media, a physiologically stable interaction between the two coupling partners, biologically compatible and mild reaction conditions, and ideally a degree of chemoselectivity.[1,6] Arguably the most widely utilized bioconjugation reaction is the Husigen cyclization reaction involving an alkyne and azide to yield a highly stable triazole linker.[7] This reaction has been employed in the preparation of a wide range of bioconjugates and a range of variant reactions have been developed in the absence of catalysts to increase its biocompatibility.[8] Despite the robust nature of this reaction, further investigations into other bioconjugation strategies are necessary to maximize the utility of bioconjugates.

While bioconjugations dependent on the natural chemical functionality in proteins have found usefulness, they are often associated with a lack of control over both the number of conjugation sites and their location.[9] Thus, the introduction of bioorthogonal handles to proteins proffers an additional degree of control over the reaction. One mechanism that is extremely effective in the introduction of novel functionality is through site-specific incorporation of unnatural amino acids (UAAs). Utilizing an evolved aminoacyl-tRNA synthetase (aaRS)/tRNA cognate pair, a plethora of UAAs have been introduced site-specifically into proteins in response to a mutated TAG stop codon within the mRNA.[10] This method has been employed in numerous applications; however, it is perhaps most useful in the installation of a bioorthogonal handle for bioconjugations.[11] A recent example includes employing a Sonogashira reaction to couple an alkyne UAA within a protein to an aryl halide.[5,12]

Specifically, we became interested in applying the Glaser–Hay coupling of terminal alkynes as a novel biochemical conjugation strategy (Figure 1 A).[13] The Glaser–Hay reaction affords an ideal conjugation strategy as it confers the formation of a highly stable and rigid carbon–carbon bond, can be conducted under mild conditions, does not utilize potentially photosensitive azides, and confers a geometrically well-defined linear conjugate. Moreover, the reagents/catalysts are cost-efficient, numerous alkyne linkers and conjugation partners are commercially available, and the product is a highly oxidized diyne capable of numerous additional reactions.

To assess the feasibility of employing a Glaser–Hay reaction towards bioconjugations, the compatibility of the reaction to aqueous conditions had to be assessed. To our knowledge a Glaser–Hay reaction has not previously been reported in an aqueous solution. Consequently, proof-of-concept couplings were conducted based on previously optimized Glaser–Hay conditions in organic solvents involving the homodimerizaton of either phenylacylene or propargyl alcohol. Gratifyingly, the reaction proceeds to completion in an aqueous solvent after 16 h at room temperature with CuI/tetramethylethylenediamine (TMEDA) in greater than 95% yields.

To utilize this reaction within the context of a protein, an alkyne moiety must be introduced into the protein as a bioconjugation handle. Conveniently an aaRS that recognizes propargyloxyphenylalanine (**1**) has already been evolved, and a convenient synthesis of the alkynyl UAA is known (Figure 1 B).[14] Based on the fluorescent properties of green fluorescent protein (GFP), it is an ideal protein for optimization of the bioconjugation reaction. As such, a TAG codon was introduced at residue 151, which has previously

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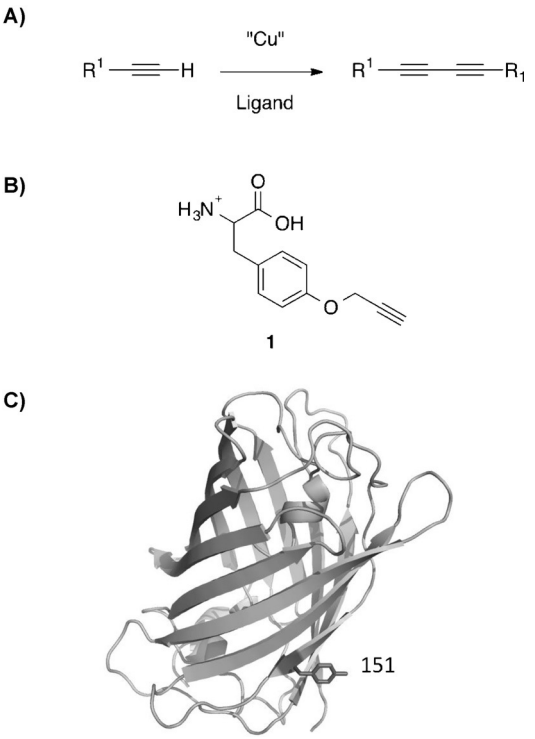

Figure 1. Glaser–Hay reaction and components. A) Standard Glaser–Hay reaction employing catalytic copper to couple a terminal alkyne moiety. B) Propargyloxyphenylalanine (*p*PrF) unnatural amino acid incorporated into proteins for Glaser–Hay bioconjugations. C) Crystal structure of GFP indicating residue 151 targeted for alkyne introduction on the β-barrel, adapted from PDB: 1EMA.[16]

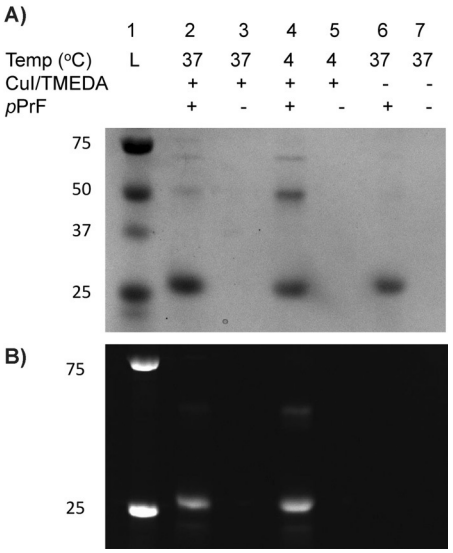

Figure 2. Glaser–Hay bioconjugation at both 37 °C and 4 °C. A) SDS-PAGE analysis after Coomassie staining showing the successful incorporation of *p*PrF into GFP due to presence of a GFP band in lane 2 and lack of GFP in lane 3. B) SDS-PAGE analysis of the identical gel imaged for fluorescence (Ex 280 nm/Em 512) prior to Coomasie staining. The gel indicates the successful Glaser–Hay coupling as fluorescence is present in lanes 2 and 4 indicating the presence of the fluorophore, while the lack of fluorescence in lanes 6 indicate no attached fluorophore in the absence of CuI/TMEDA despite the presence of fluorophore in the reaction.

been demonstrated to be an ideal site for UAA incorporation as it is a surface-exposed position on the rigid β-barrel and does not impact GFP fluorescence (Figure 1 C).[15] BL-21-(DE3) *E. coli* were co-transformed with the pEVOL-*p*PrF and pET-GFP-Y151TAG plasmids, and GFP was expressed in the presence and absence of **1** (Figure 2; Lanes 6 and 7). The lack of full-length protein in the absence of **1** corresponds to the termination of translation at the TAG codon. With a GFP-*p*PrF-151 mutant in hand, Glaser–Hay optimization could proceed.

Initial bioconjugations were attempted between the mutant GFP and an AlexaFluor-488 modified alkyne. The Cu/TMEDA was first prepared by mixing CuI with TMEDA (ca. 50 equiv) in water for 10 min at room temperature. The fluorophore was then added in approximately ten-fold excess followed by the GFP and the reaction was incubated at either 37 °C or 4 °C. While some fluorophore dimerization does occur, this product, the CuI/TMEDA, and excess fluorophore were easily removed by centrifugal concentration accompanied by washings in PBS, and the reactions were analyzed by SDS-PAGE and LC/MS (Figure 2). Gel electrophoresis demonstrated successful Glaser–Hay coupling when comparing the identical gel under fluorescent imaging (Figure 2 B) and Coomassie staining (Figure 2 A). In the presence of the CuI/TMEDA and an alkyne fluorophore, fluorescent labeling of the protein was observed at both temperatures, with 4 °C affording slightly higher fluorescence (Figure 2; Lanes 2 and 4). Control reactions in the absence of CuI and TMEDA demonstrated the presence of protein, but no fluorescent label (Figure 2; Lane 6). Moreover, couplings performed with the wild-type GFP and the fluorophore resulted in no fluorescent signal on a gel. This confirms that the labeling is due to the Glaser–Hay coupling and not non-covalent interactions of the protein with the fluorophore. To validate this comparison, the GFP was also coupled to a SRFluor-680 alkyne under identical conditions. The absorbance at 649 nm that corresponds to the fluorophore ($\varepsilon = 257\,800\ \mathrm{L\,mol^{-1}\,cm^{-1}}$) was then normalized to the GFP absorbance, affording coupling yields of 71 % after 4 h at 4 °C, and 93 % after 6 h at 4 °C (see the Supporting Information). These were roughly in agreement with what was observed from the PAGE results. Moreover, the successful coupling was confirmed by MS analysis (see the Supporting Information).

Extended couplings led to decreased fluorescence and overall lower amounts of GFP upon staining. We hypothesized that this may be a result of Cu^{I} oxidative degradation of the protein over time. To fully optimize the reaction, time courses were performed, sampling the reaction at different time points and comparing the ratios of intact GFP with fluorescence (Figure 3). Based on these results, 6 h at 4 °C appears to be the optimal conditions for the Glaser–Hay bioconjugation. Additionally, reduction of the CuI/TMEDA concentrations, even by a factor of five, resulted in no observable coupling. Attempts to accelerate the reaction by increasing oxygen concentrations, either by bubbling air through the reaction or by leaving the Eppendorf tubes

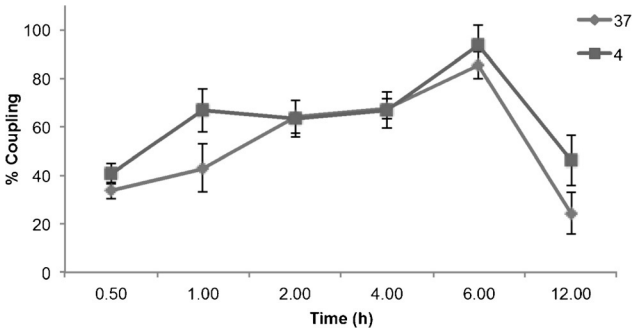

Figure 3. Glaser–Hay time course. Ratio of total protein to fluorescence based on densiometry measurements obtained by SDS-PAGE. Gels were first imaged for fluorescence to ascertain the relative amounts of fluorophore conjugate, then stained to assess the concentration of total GFP protein. Reactions at each time point were conducted in triplicate to generate error bars indicating standard deviation. Results indicate highest coupling and optimum reaction conditions at 4 °C for 6 h. Extended periods of time were found to lead to protein degradation.

open, did not afford the desired effect, as increased protein degradation was observed. This suggests that at these low concentrations of substrate, the CuI/TMEDA is not catalytic, unlike previously observed Glaser–Hay reactions on larger scales.

The Glaser–Hay reaction time is comparable to reported azide–alkyne click couplings, and slightly slower than the previously reported Sonogashira couplings that ranged from 0.5–2 h.[5] However, advantages of the Glaser–Hay approach relative to the Sonogashira is the use of substantially cheaper and more common reagents than palladium catalysts, as well as lower fluorophore equivalents (10 equiv, compared to 50 equiv in the Sonogashira).[12] Interestingly, the Glaser–Hay rate was comparable between the 4 °C conditions and the 37 °C conditions. This may be due to the increased protein degradation observed at the higher temperature, limiting reaction rates via divergent pathways. It appears that as long as the reaction is quenched by the 6 h time point, the oxidative damage is minimal, as confirmed by the mass spectrum of the protein subjected to reaction conditions in the absence of fluorophore remaining identical to protein not subjected to the Glaser–Hay conditions (see the Supporting Information). Oxidative damage was observed by MS after extended reaction times of 10 h. Additionally, based on this result, bioorthogonality was confirmed as no cysteine cross-linking or thiol–yne additions were detected. While some chemoselectivity issues exist, as a consequence of the Glaser–Hay mechanism, these reactions are minimal, or easily addressed by purification. As previously noted, fluorophore dimerization is easily removed by size-exclusion centrifugation, and GFP dimerization occurs in approximately 5–13 % yield as determined by analysis of PAGE protein band intensities (Figure 2 and the Supporting Information). Attempts to enhance and analyze dimerization by reaction of the *p*PrF-GFP mutant under optimized conditions in the absence of the fluorophore failed to produce any increased GFP dimer, and occurred at less than 10 % yield. This is most likely due to the steric bulk of the protein hindering proper orientation and coupling of the two terminal alkyne moieties (see the Supporting Information). Even when protein concentrations were doubled in the reaction, dimerization levels did not increase.

To probe the utility of this novel bioconjugation, we next set out to examine its relevance towards solid-supported reactions. To rapidly assess the method, a propargyl alcohol or a 5-hexyn-1-ol derivitized Sepharose 6B resin was reacted under similar conditions with the AlexaFluor 488 alkyne. Control reactions were performed in the absence of both the fluorophore and the CuI/TMEDA. After several PBS washes, the fluorescence of the resin was assessed, and fluorescence was only detected in the presence of both fluorophore and copper (see the Supporting Information). Based on previous results using UAAs for protein immobilization, attempts were made to translate this reaction to the immobilization of GFP.[15]

A GFP-*p*PrF-151 mutant was expressed and utilized to investigate the ability to conjugate the protein to a solid support via a Glaser–Hay reaction. Using the optimized conditions, the GFP was immobilized on both a propargyl alcohol and 1-hexynol resins with similar effect (Figure 4). Control reactions in the absence of either GFP or CuI/TMEDA yielded little to no detectable fluorescence on the

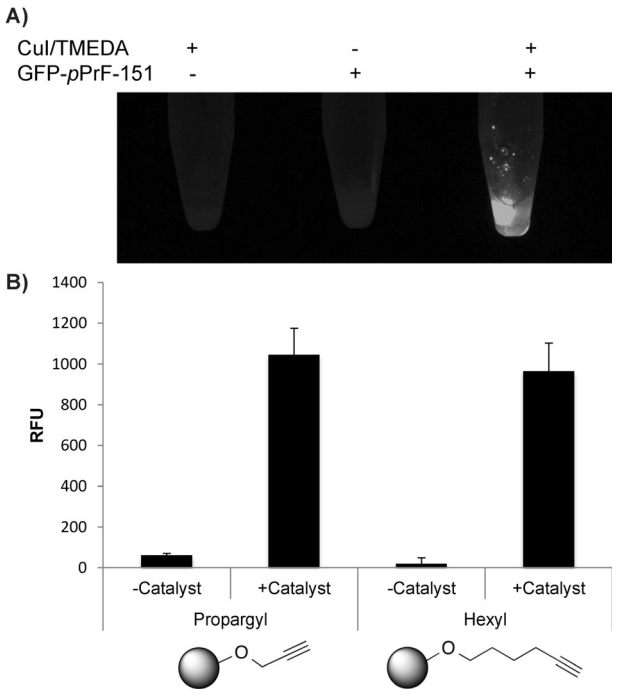

Figure 4. Protein-resin Glaser–Hay immobilization. A) Reaction including GFP-*p*PrF-151 and alkyne derivatized Sepharose 6B resin. Strong fluorescence in the presence of both CuI/TMEDA and GFP-*p*PrF-151 indicate a successful coupling. Lack of fluorescence in control reactions omitting either protein or copper indicate no coupling occurred. B) Fluorescence data of completed reactions with both propargyl alcohol and hexynol loaded Sepharose resins. Controls with no CuI/TMEDA indicate low background fluorescence, while GFP-*p*PrF-151 protein reacted with the CuI/TMEDA and resin displays strong fluorescence. Standard deviations depicted in error bars from triplicate couplings.

resin; however, significant immobilization was detected when the resin was reacted in the presence of both the GFP mutant and the CuI/TMEDA for 6 h at 4 °C. Moreover, controls with wild-type GFP under similar reaction conditions did not result in resin immobilization (see the Supporting Information). Thus, no non-covalent interactions between the protein and resin are occurring and the fluorescence can be attributed to the successful Glaser–Hay bioconjugation.

In conclusion, along with the transfer of the Glaser–Hay reaction to an aqueous environment, a novel bioconjugation has been developed utilizing the reaction and unnatural amino acid technologies. The generation of a new linear carbon–carbon bond affords a highly stable linkage utilizing mild reaction conditions and photochemically inert starting materials. This bioconjugation has been applied to small molecule dimerizations, protein–fluorophore conjugations, fluorophore–resin conjugations, and protein–resin immobilizations, demonstrating the breadth of the reaction substrates and applicability. Future work is underway to expand the scope of the conjugations to biologically relevant applications and therapeutic agents. This technology represents a viable alternative to click conjugations and has the potential to find widespread utility.

Experimental Section

Glaser–Hay aqueous conditions: Aqueous conditions for the Glaser–Hay reaction were optimized by preparing a phenylacetlyene homodimer. TMEDA (10 μL, 0.06 mmol) and CuI (10 mg, 0.05 mmol) were added to a vial containing H_2O (3 mL), forming the copper complex. Phenylacetylene (37 mg, 0.364 mmol) was then added and the reaction was allowed to stir at room temperature for 16 h. The reaction was extracted using EtOAc and H_2O washes (4 × 5 mL), concentrated, and dried in vacuo. The product was obtained as a white solid: 88 mg, 0.349 mmol, 96% yield; ^{1}H NMR (400 MHz; $CDCl_3$): $\delta = 7.38$–7.56 ppm (m, $J = 7.2$ Hz, 10H).

Protein–fluorophore Glaser–Hay bioconjugation: The expressed GFP-*p*PrF-151 was coupled to AlexaFluor 488 alkyne using Glaser–Hay reaction conditions. In an Eppendorf tube, CuI (5 μL, 500 mM) and TMEDA (5 μL, 500 mM) were mixed and equilibrated at 37 °C. After 10 min, AlexaFluor 488 alkyne (10 μL, 1 mM) was added and equilibrated at 37 °C for 10 min. Finally, GFP-pPrF-151 (20 μL, 0.5 mg mL^{-1}) was added. A control reaction was also prepared with the same concentrations of fluorophore and protein, but with the CuI/TMEDA replaced with PBS buffer (10 μL). The reactions were incubated for various times at 37 °C or 4 °C. Reactions were then purified through centrifugal concentration on Spin-X UF colums (Corning), with wash cycles of PBS buffer (5 × 100 μL) until flow-through was free of fluorophore. The protein was then analyzed by SDS-PAGE gel to verify coupling of the fluorophore to the protein. Time-course experiments were analyzed by comparing densitometry of fluorescent bands to their Coomasie-stained bands using a BioRad Molecular Imager Gel Doc XR + system.

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