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Communication

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Site-Specific Protein Immobilization Using Unnatural Amino Acids

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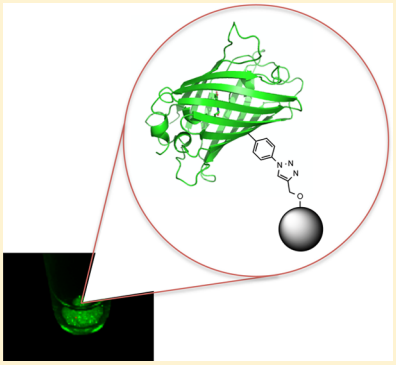
Supporting Information

ABSTRACT: Protein immobilization confers the advantages of biological systems to a more chemical setting and has applications in catalysis, sensors, and materials development. While numerous immobilization techniques exist, it is optimal to develop a well-defined and chemically stable methodology to allow for full protein function. This paper describes the utilization of unnatural amino acid technologies to introduce bioorthogonal handles in a site-specific fashion for protein immobilization. To develop this approach a range of solid-supports, organic linkers, and protein immobilization sites have been investigated using a GFP reporter system. Overall, a sepharose resin derivatized with propargyl alcohol has afforded the highest yields of immobilized protein. Moreover, an unnatural amino acid residue protein context has been demonstrated, signifying a necessity to consider the protein site of immobilization. Finally, a resin-conferred stabilization was demonstrated in several organic solvents.



Proteins are capable of catalyzing a wide range of chemical transformations, and their utility transcends the realm of biology, finding application in organic synthesis, materials chemistry, and alternative energy technologies.[1−3] However, a major limitation to exploiting the utility of proteins in a more chemical setting is their lack of stability and activity in nonaqueous environments.[2,4−10] The immobilization of proteins has proven to be an effective mechanism to expand their utility into nonbiological contexts. Moreover, immobilization confers the ability to recover and reuse relatively expensive enzymatic catalysts, and a better means of reaction control as catalysis can be halted by a simple filtration to remove the immobilized enzyme.[11] Immobilized enzymes have a wide range of applications including organic catalysis, microarrays, imaging probes, and biosensors.[12−17] While many general protocols already exist for protein immobilization (encapsulation, adsorption, and covalent attachment),[18,19] there remains room for significant improvement. For many uses of proteins, covalent immobilization provides a number of advantages including increased thermal stability, better scale up potential, and decreased probability of protein desorption, contaminating the reaction solution. However, many covalent modification approaches rely upon reactive residues (e.g., lysine and cysteine) on protein surfaces for attachment.[20] This can lead to alteration in protein selectivity and activity, and may lead to complete inactivation as the active site may be blocked by the solid support.[19,21] Additionally, there is a general lack of control over how many residues react with the surface and remaining unreacted functionality on the surface, which may impede the catalysis via reaction with substrates and produce undesired heterogeneous mixtures on the solid support.[11]

We set out to address issues associated with nonspecific protein immobilization through the use of unnatural amino acids (UAAs). The site-specific incorporation of UAAs represents a novel mechanism to control protein immobilization via the introduction of a single residue that displays bioorthogonal reactivity. A powerful means of introducing UAAs into proteins involves the exploitation of cellular machinery to genetically encode the UAA.[22,23] This approach is advantageous to other mechanisms to incorporate UAAs, as the technology abrogates issues associated with protein size, scale, and delivery, as well as establishes a permanent, self-sustained system for protein production (not requiring the synthesis of precursors for each protein production).[24−27] The Schultz methodology for the addition of UAAs to the genetic codes of either prokaryotic or eukaryotic organisms involves the engineering of three components to the protein biosynthetic machinery: an orthogonal tRNA-codon pair, an orthogonal aminoacyl-tRNA synthetase (aaRS), and an unnatural amino acid. The aaRS charges a tRNA with the UAA, and is then able to deliver the UAA in response to a codon that does not encode any of the common 20 amino acids (typically TAG, the amber stop codon).[22,23] This approach has afforded the efficient, highly selective in vivo incorporation of a large number of diverse unnatural amino acids.[28,29] Also, in contrast to other auxotrophic methods, this method uniquely specifies additional amino acids at defined sites in the proteome based on the location of the TAG codon.[30] This method has been utilized in the reaction of calmodulin onto carbon nanotubes and nanoparticles via the Staudinger-Bertozzi ligation,[31,32] as well as the immobilization of other proteins to M-20 Dynabeads in a cell-free system and indirectly via streptavidin derivatized gold surfaces with a click reaction.[16,17] Finally, a similar approach was performed using an agarose resin and azido UAA to "catch" proteins for secretome MS analysis; however, in this case the UAA was not site-specifically incorporated into the pool of

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proteins.[33] The combination of these studies demonstrates the utility of site-specific immobilization, and the stabilizing benefits of the solid support; however, a more extensive investigation of immobilization methods and variables has the potential to advance this promising field. Moreover, development of a generalized protocol and better understanding of immobilization conditions is needed to adapt unnatural amino acid technologies to protein immobilizations, and fully exploit this powerful system.

Specifically, the *p*-azidophenylalanine (*p*AzF) UAA was selected due to its ability to participate in bioorthogonal 1,3-dipolar cylcoaddition "click" reactions with an alkyne (Figure 1A). This reaction has found wide use in biological chemistry

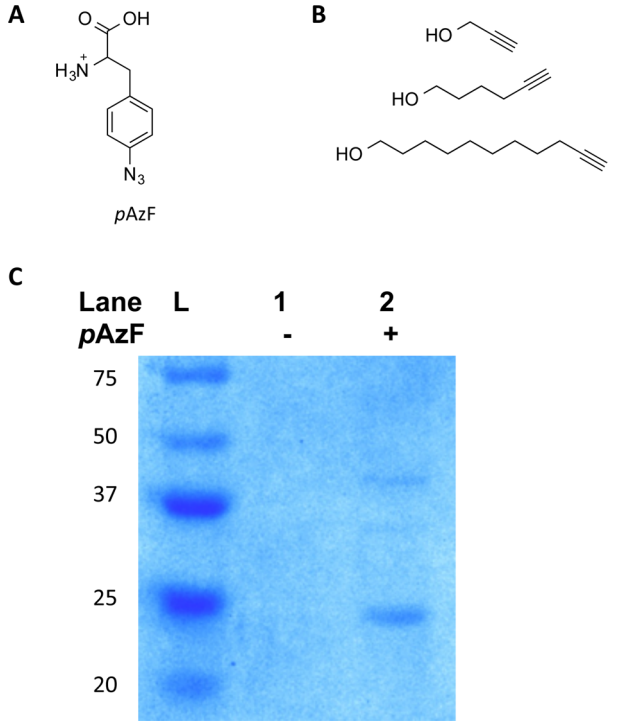

Figure 1. Protein and resin preparation. (A) *p*-Azidophenylalanine (*p*AzF) unnatural amino acid possessing an azide functionality for click conjugations. (B) Series of alkynols used for resin derivitization, varied methylene units confer differential flexibility for protein attachment. (C) SDS-PAGE analysis of *p*AzF-GFP-151 expression. No protein is present in the absence of the UAA (lane 1) suggesting successful incorporation of the UAA (lane 2).

due to its unique reactivity (relative to the natural 20 amino acids), water compatibility, and overall efficiency.[34,35] Fortuitously, an aaRS has already been evolved to facilitate the incorporation of this UAA.[36,37] In order to rapidly and easily assess the protein immobilization, green fluorescence protein (GFP) was selected as a model protein, and expressed with *p*AzF at surface exposed residue 151 using a pET-GFP-Y151TAG plasmid (Figure 1C). The mutated GFP containing the UAA retained its fluorescence relative to wild-type protein, and served as a functional reporter for the immobilization (see Supporting Information Figure S3). Furthermore, as the fluorescence of GFP is not dependent on the orientation of immobilization, GFP was able to serve as a good model protein for the quick assessment of immobilization efficiency when different orientations were considered. It is important to note that this orientational independence does not necessarily translate to enzymes, and additional considerations must be made when adapting the approach.

Initially, attempts were made to ascertain the best material for the solid support. A range of supports was assessed including polystyrene, Tenta-Gel, and Sepharose resins. The solid support was evaluated based on immobilization efficiency, and retention of GFP fluorescence. As a proof-of concept, a representative of each class of support was examined. A trityl-chloride modified polystyrene resin, an epoxide derivatized Sepharose 6B, and a carboxy-modifed Tenta-Gel resin were all derivatized with an alkynyl linker to facilitate click chemistry with the azide on the protein surface (see Supporting Information). Conveniently, each resin is reactive with alcohol functionalities under appropriate conditions, and each was subjected to immobilization with propargyl alcohol, 1-hexynol, or 1-undecynol to vary the length of the carbon tether separating the reactive alkyne from the solid-support (Figure 1B). These linkers were selected due to their low cost and commercial availability. Moreover, the linkers provided a mechanism for the assessment of linker length and immobilization efficiency. Alkyne immobilization and approximate resin loading was determined by cleavage of 15 mg of resin followed by GC/MS and TLC analysis, affording loadings of 0.6−0.9 mmol/g of immobilized alkyne. Resin loadings at these concentrations should ideally be low enough to adequately provide enough spatial protein separation to retain function; however, lower loadings may be necessary if protein function is inhibited. After confirmation of immobilization the different resins were subjected to click conditions: for 16 h at 4 °C. The amount of resin utilized was normalized to the corresponding loading in order to ensure 12 μmol of immobilized alkyne reacted with a normalized 35 μg of GFP protein. In order to prevent aggregation of both the polystyrene and Tenta-Gel resins 10% DMSO was added. To verify that DMSO did not affect protein stability fluorescence spectra were taken in both the presence and absence of 10% DMSO (see Supporting Information S1). Resins were then transferred to spin columns and washed 5 times with PBS buffer. The presence of GFP was assessed both visually on a transilluminator and with a fluorescence plate reader (Ex. 482/Em. 520). While little to no fluorescence was observed on the polystyrene resin, definitive immobilization was noted on both the Tenta-Gel and sepharose resins, with the fluorescence intensity being much greater on the sepharose resin. The decreased immobilization efficiency is most likely due to polystyrene resin aggregation in aqueous solvents. The immobilization of the GFP on the polystyrene and Tenta-Gel resins may be further improved via optimization of organic solvent ratios to accommodate the resin while not denaturing the protein of interest. However, these results do demonstrate that the GFP is capable of being immobilized on a solid support without affecting protein function, as fluorescence is maintained.

While all resins with all three alkynes were analyzed, the resin immobilized with propargyl alcohol reproducibly yielded the highest levels of GFP fluorescence with the sepharose resins (Figure 2). Gratifyingly, resin incubated with wild-type GFP (containing no azide functionality) displayed little to no fluorescence, and conditions involving *p*AzF-GFP-151 in the absence of copper catalyst had similar results. This confirms that the click reaction was the site of immobilization and noncovalent interactions were not responsible for the immobilization event. Also, the absence of fluorescence indicates that the sepharose resin is not prone to noncovalent

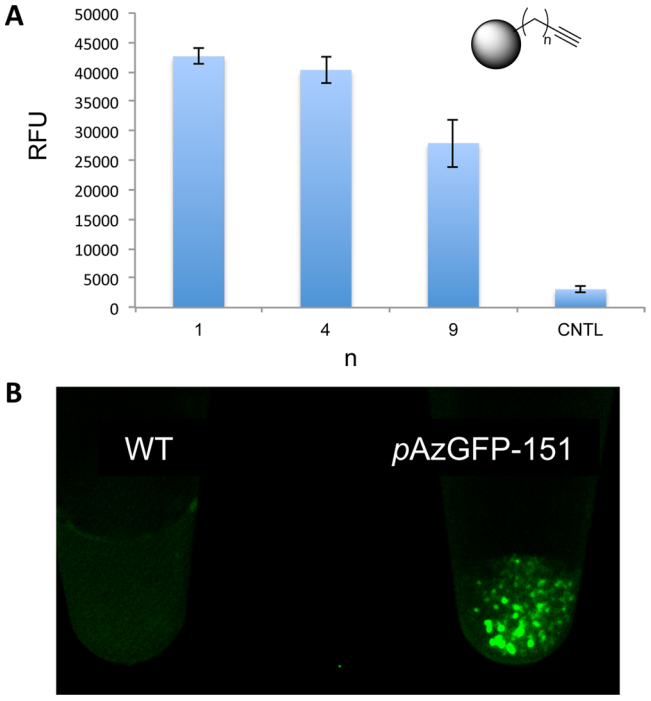

Figure 2. Resin and linker optimization. (A) Fluorescence data from alkyne derivatized Sepharose 6B resins conjugated with the *p*AzF-GFP-151 mutant. Methylene units were varied with the alkyne substrate, and maximal fluorescence was observed with propargyl alcohol. A control resin using WT GFP (no azide functionality) demonstrated very little background fluorescence. All error bars represent standard deviations from three independent experiments. (B) Visualization of resin immobilization, as no fluorescence is observed under identical immobilization conditions using WT-GFP. Resin reacted with the *p*AzF-GFP-151 is notably fluorescent.

"stickiness" and thus only the desired protein can be immobilized. Additionally, the effect of linker length could also be examined in the initial experiments. A trend was observed as decreased immobilization efficiency was correlated to increased methylene units, with undecynol affording the lowest GFP immobilization (Figure 2A). Similar results were observed even with increased immobilization times. It is possible that the increased flexibility of the linker leads to more conformational freedom that inhibits the proper orientation for the conjugation of the polymeric resin with the biopolymer.

With optimized immobilization conditions in hand, the propargyl alcohol derivatized sepharose resin was utilized to ascertain if UAA context is important for protein immobilization. A TAG mutation was introduced into the pET-GFP plasmid at residues 3, 133, and 151, and the corresponding GFP mutants were expressed containing the *p*AzF UAA and diluted to identical concentrations (see Supporting Information Figures S2, S3). These residues were selected due to their differing protein context and variable rotational flexibility, while maintaining surface exposure. To ensure that changes in fluorescence were due to immobilization and not altered spectra, the fluorescence spectra of all mutants were taken (see Supporting Information). The mutants were subjected to click conditions for 16 h at 4 °C as previously described. Visualization of the immobilization indicated that selection of UAA residue is important in the immobilization strategy (see Supporting Information Figure S4). These results were confirmed by analysis of resin fluorescence on a plate reader. Residues 3 and 133 are contained within loop motifs at opposite ends of the β-barrel, while residue 151 is at the terminus of a β-sheet member of the β-barrel (Figure 3A). Due

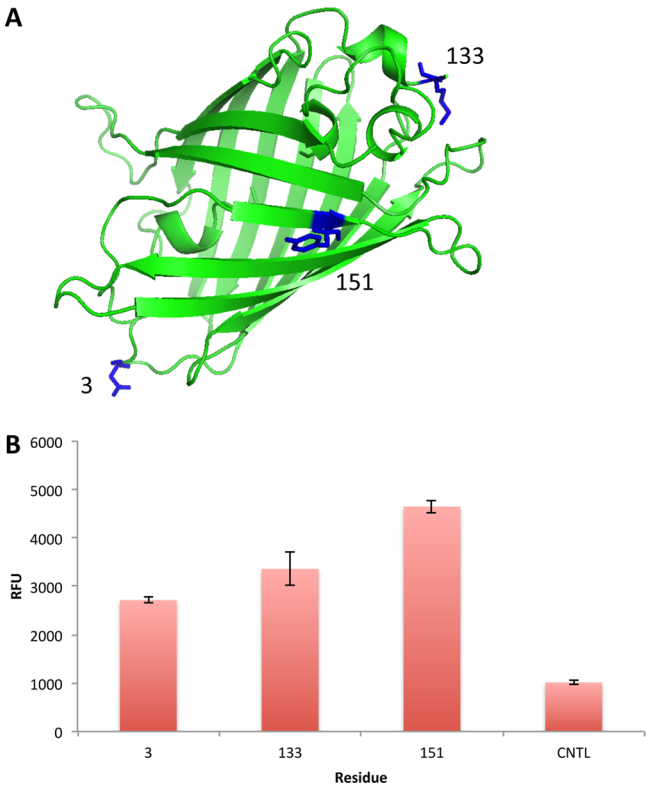

Figure 3. UAA residue context dependence on immobilization. (A) Various GFP *p*AzF mutants were expressed with the UAA at different positions (highlighted in blue), adapted from PDB: 1EMA.[39] (B) Fluorescence data demonstrating maximal immobilization with the *p*AzF-GFP-151 mutant, suggesting some importance of UAA context within the protein for the immobilization. All error bars represent standard deviations from three independent experiments.

to the differential degree of immobilization despite identical conditions and GFP concentrations, there does seem to be a context effect for the placement of the UAA (Figure 3B). The highest degree of immobilization was observed at residue 151, followed by residues 133 and 3, respectively. These trends mimic those observed when selecting optimal linker flexibility with the most rigid residue possessing the highest immobilization efficiency. Residue 3 is at the N-terminus of the protein and in a very unstructured region, possessing a significant degree of conformational freedom. Conversely, residue 151 is extremely structured and very little rotational freedom can be achieved. This rigidity may be useful in facilitating the click reaction to the resin by holding the azide in the proper orientation. Residue 133 has intermediate conformational flexibility, as well as intermediate immobilization yield. Again, little to no fluorescence was observed when the copper catalyst was removed from the reaction as a control. These results indicate that when utilizing UAA bioorthogonal handles for protein immobilization, some consideration must be given to the site of UAA incorporation to maximize immobilization efficiency as well as retain protein function.

In order to demonstrate the utility of resin immobilization, the retention of GFP fluorescence was examined when exposed to different organic solvents. Due to its miscibility, we first

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exposed the resin and free GFP-151 mutant to varied percentages of DMF (25−100%), and incubated the proteins for 2 h, the fluorescence was then measured on a plate reader as an indicator of functional protein. In agreement with literature precedence, GFP fluorescence decreases dramatically in solutions above 50% DMF; however, immobilized GFP fluorescence was retained, even at 100% DMF (Figure 4A).[38]

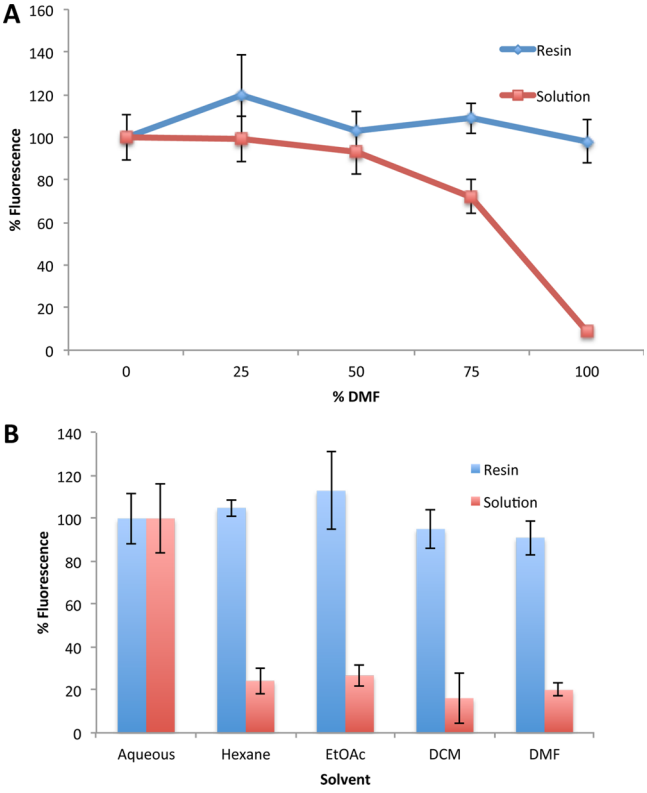

Figure 4. Resin stabilization of GFP in organic solutions. (A) Incubation of both immobilized GFP and GFP in solution with increasing DMF concentration demonstrates a decrease in fluorescence for only the solution phase GFP in increased organic solvent. (B) Incubation of both resin-bound GFP and solution phase GFP with different organic solvents leads to a marked difference in fluorescence values. All error bars represent standard deviations from three independent experiments.

Moreover, when incubated in other pure organic solvents including dichloromethane, ethyl acetate, and hexanes, a significant differential in fluorescence was observed and quantified on a BioRad gel imaging system (Figure 4B, and Supporting Information S5). It should be noted that these observed increases in stability are not limited to the site-specifically immobilized GFP; however, the success of this method does demonstrate its potential usefulness with regard to proteins that are more sensitive to the orientation of immobilization. In addition, while some basic solvation sphere around the protein may exist, the protein remained stable on the resin even when subjected to 48 h incubation times and sonication, while solution based protein readily denatured. In summary, this increased protein stability suggests this methodology can prove useful in the immobilization of a variety of proteins for use in nonaqueous catalysis, and coupled with the specificity of the immobilization technique, could afford heightened enzymatic efficiencies to current technologies.

In conclusion, the utilization of unnatural amino acids has proven to be an effective mechanism for the immobilization of proteins. This has far-reaching implications toward the extension of protein function to nonbiological conditions. Moreover, this methodology is advantageous when compared to existing techniques due to the high level of immobilization control, and its ability to generate covalently linked proteins in a homogeneous fashion. Current studies are underway to immobilize enzymes using this technology in order to expand their catalytic utility in nonaqueous solvents.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental conditions, fluorescence spectra and non-aqueous solvent images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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