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Supramolecular strategies for protein immobilization and modification

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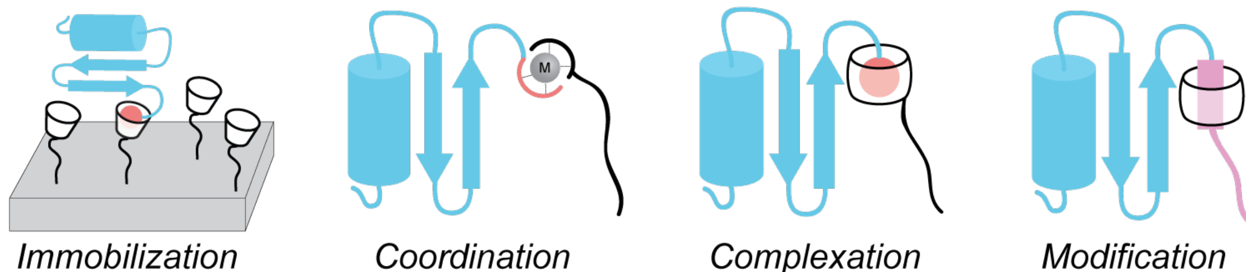
Abstract

Protein immobilization and modification are widely used techniques in the fields of chemical biology and biomaterials science. While covalent strategies based on small molecules are traditionally used, supramolecular chemistry offers numerous useful opportunities for guiding the modification locations on complex protein landscapes and introducing different degrees of reversibility into the products. In this opinion, we highlight recent advances in using supramolecular interactions, particularly host-guest chemistry, for controlling protein modification and immobilization. We discuss supramolecular strategies for protein-conjugate purification and capture, as well as for protein modification via host-guest interactions and metal coordination. Lastly, we address recent advances in utilizing supramolecular interactions to direct covalent protein modification. These examples of supramolecular chemical biology present opportunities to advance a wide range of applications, including proteomics and drug delivery.

Highlights

- Supramolecular strategies can offer advantages over traditional covalent approaches
- Metal coordination and host-guest chemistry provide bio-orthogonal couplings
- Host-guest complexation can be used for protein capture and subsequent release
- Supramolecular chemistry can direct covalent modification to a single protein site



Introduction

Protein modification and immobilization are important techniques that are used in a variety of fields, including drug delivery, environmental science, and proteomics.[1–4] For these applications, protein modification reactions should offer chemical orthogonality to other functional groups that are present in complex biological mixtures or on protein surfaces. It is also frequently required that proteins only be modified at a single site of interest, resulting in a homogeneous product instead of a heterogeneous mixture that can display nonuniform properties.[1] While covalent strategies have been the standard approach for protein modification and immobilization, new advances in supramolecular chemistry could afford alternative and complementary approaches to achieving controllable bioconjugation. Supramolecular chemistry, and particularly host-guest chemistry, allow for highly controllable interactions with tailorable binding affinities.[5–8] Additionally, many supramolecular interactions with synthetic macrocyclic hosts are highly specific in nature, and therefore mitigate side reactions that interfere with downstream applications. By taking advantage of these properties, researchers have expanded the scope of bioconjugation reactions, and have developed protein conjugates with properties that would be difficult to replicate using traditional covalent approaches. Supramolecular bioconjugation also offers the potential for new developments in protein-based materials, as these modification strategies can afford unique constructs that combine the function and precision of proteins with the responsive behavior of supramolecular assemblies.

In this opinion, we highlight recent advances in using supramolecular interactions for protein immobilization and modification. While host-guest chemistry has dominated the field of supramolecular protein modification and immobilization, other supramolecular interactions such as metal coordination, π-π stacking, and hydrogen bonding have seen recent use in these applications. Additionally, we highlight emerging advances in using supramolecular interactions to direct the covalent modification of proteins, affording controllable site-selective reactivity.

Protein purification through host-guest interactions

The use of biotin-avidin interactions could be considered the first supramolecular strategy widely used in protein chemistry.[9,10] However, the exceptionally high binding affinity of biotin for avidin, estimated at 10^{15} M^{-1},[11] poses challenges in separating heterogeneous mixtures of biotinylated proteins.[10] As alternatives, several examples of more tunable purification strategies have been reported, which rely on weaker host-guest interactions between synthetic molecules conjugated to proteins and resins modified with macrocyclic hosts.

In 2006, Francis and coworkers reported a strategy for the purification of fluorescently-labeled biomolecules using cyclodextrin host-guest chemistry.[12] Proteins were modified with fluorescent dyes and purified with cyclodextrin-modified beads, which selectively bound to the protein conjugates. Since that initial report, other examples of cyclodextrin-mediated protein purification have been reported,[13] including a more general approach reported by Francis and coworkers in 2015.[14] Proteins were modified heterogeneously with an azobenzene derivative that has a high binding affinity for β-cyclodextrin. Chromatographic purification of the proteins with a cyclodextrin column achieved the separation of unmodified, singly, and doubly modified

proteins. After purification, the azobenzene was cleaved with sodium dithionite, exposing an aniline on the surface that could undergo an orthogonal reaction for site-selective modification. While certain bioconjugation reactions can afford homogenous protein conjugates via site-specific covalent modification, oftentimes these reactions require specific amino acid residues that might not be available on the protein surface, or require reagents that can be difficult to synthesize. In those cases, supramolecular protein conjugate purification offers a strategy to extract homogenous protein conjugates from heterogeneous mixtures.

Supramolecular protein immobilization and detection

The interactions between synthetic hosts and native protein residues have been used for protein recognition and analysis. The binding interaction ($K_a = 1.5x10^6\ M^{-1}$) between the N-terminal phenylalanine of insulin and cucurbit[7]uril (CB7) macrocycles was first reported by Urbach and coworkers in 2011.[15] Since that report, other synthetic hosts have been used as receptors for protein binding and recognition, with potential applications in proteomic analysis. In particular, Urbach, Kim, and coworkers demonstrated this possibility by using N-terminal Phe-CB7 interactions as an MS/MS enhancement technique for the detection of proteins and protein digests.[16] Other synthetic receptors have also been developed for selective binding to post-translational modifications, including methylated lysine and arginine residues.[17,18] These synthetic receptors are advantageous over covalent tags, as they do not alter the protein or peptide structure, and therefore allow for noncovalent capture and subsequent competition-mediated release of native protein structures for further study.

In 2011, Kim and coworkers reported a robust strategy for the enrichment and analysis of synthetically modified proteins in cell culture using ferrocene-CB7 interactions (Figure 1).[19**] Membrane-bound proteins were modified with a ferrocene NHS-ester derivative with a CB7 binding affinity of $\sim10^{12}\ M^{-1}$. The modified proteins were then enriched with CB7-functionalized beads, and were released for analysis by competition against a small molecule ferrocene derivative. This technique overcame several challenges associated with biotin-mediated enrichment, including cross-contamination from naturally biotinylated proteins and avidin contamination during the enrichment process. In a follow-up study, the same research group used a bifunctional probe for the proteomic analysis of intracellular proteins. In this work, one segment of the reagent labeled the proteins of interest, while the other side contained a ferrocene moiety for CB7-binding.[20] This strategy can be applied to a variety of applications, with a ferrocene tag substituted for the traditional biotin handle.

In addition to protein capture for proteomics, protein immobilization on surfaces has been used in applications ranging from regenerative medicine to the study of signaling pathways. While nonspecific adsorption of proteins on surfaces and covalent modification strategies are traditionally used, supramolecular approaches to protein immobilization offer opportunities for modulated and reversible binding.[21–24] In one example, Francis and coworkers used host-guest interactions to enable enzyme recycling.[25**] Sortase A was modified site-specifically at the N terminus with a lithocholic acid (LA) derivative. LA has a reported binding affinity of $10^6\ M^{-1}$ for β-cyclodextrin (βCD). This mid-range affinity allowed for binding of sortase A to βCD-modified resin, followed by competition-mediated release. The enzyme retained its activity after LA

modification and was easily removed from solution by bead capture for repeated use. In another example reported by Francis and coworkers, DNA hybridization was used as a supramolecular means of protein immobilization.[26] DNA strands were templated onto a surface through photopatterning, followed by hybridization to a complementary DNA strand covalently modified to an enzyme of interest. This supramolecular protein templating allows for facile protein recycling through DNA strand melting, as well as photolithographic means of producing complex patterns for protein arrays.

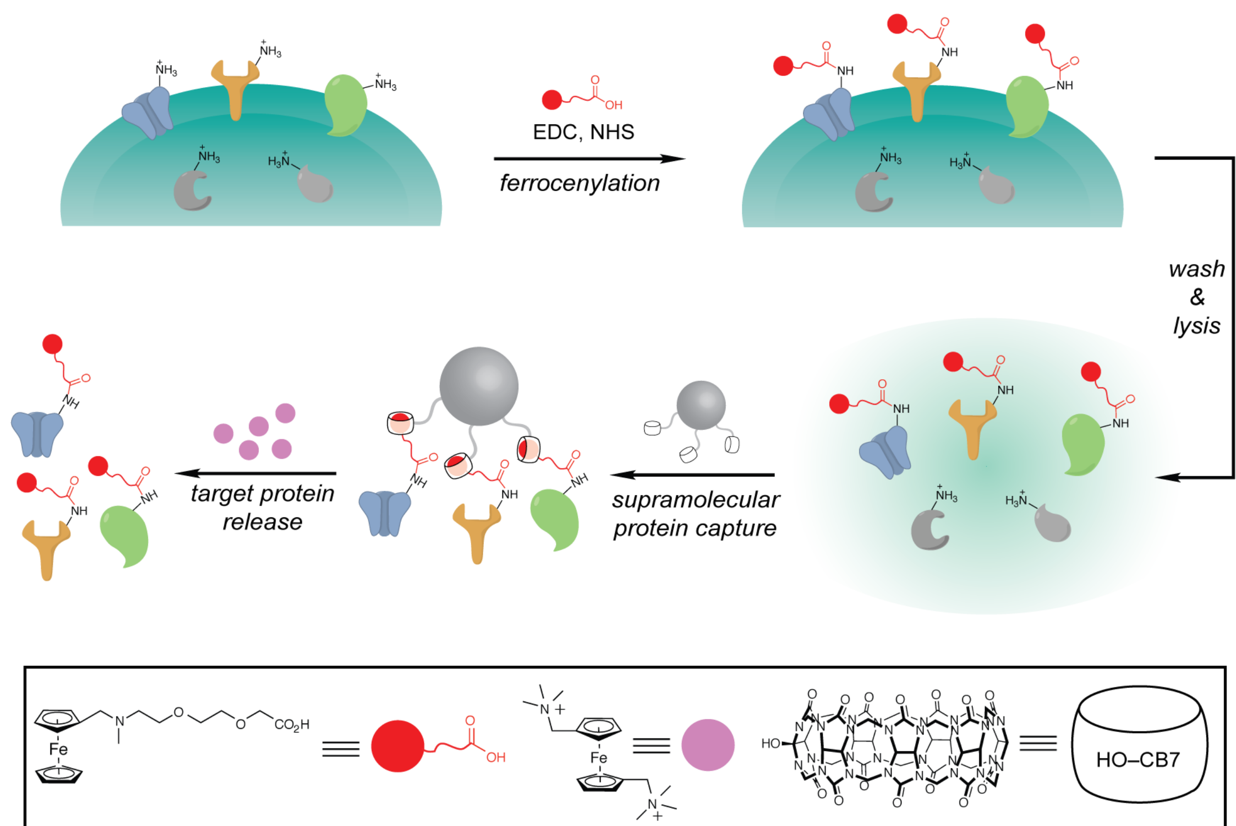


Figure 1. Supramolecular protein capture in complex cellular environments mediated by ferrocene-CB7 host-guest chemistry. Figure adapted from [19**].

Supramolecular protein modification

Coordination chemistry offers a powerful approach to modify proteins with synthetic substrates. The most well-known example of synthetic coordination in the realm of protein chemistry is the affinity of hexahistidine peptide sequences for nickel.[27] This affinity has been used extensively in biochemistry for the purification of proteins containing a 6xHis tag at either the N or C terminus via Ni-NTA column chromatography. Recent work has taken advantage of this interaction for applications in protein modification.[28,29] In one example, Lee and coworkers expressed hexahistidine-tagged proteins and modified them site-specifically with Ni-NTA functionalized polyethylene glycol (PEG) polymers.[30] These PEGylated proteins displayed improved half-lives and *in vivo* efficacies over unmodified proteins. This approach is particularly promising, as many

proteins tolerate 6xHis tag incorporation. Additionally, this strategy adds functionality to a protein tag that is oftentimes already required for purification.

In another example of coordination-driven protein modification, Gothelf and coworkers used the 6xHis to Ni-NTA interaction to direct covalent modification of proteins with DNA-NHS esters.[31**] Typically, NHS ester modification results in a heterogeneous mixture of protein conjugates, with reactions occurring with the N terminus and any surface-exposed lysine residues. The researchers expressed GFP with a 6xHis tag at the N terminus in order to bind a Ni-NTA-modified strand of DNA. The Ni-NTA-DNA was then hybridized to its complementary DNA strand, which contained an NHS ester moiety. Because of the hybridization, the NHS ester only reacted with the lysine residue in closest proximity to the 6xHis tag. Thus, a combination of supramolecular interactions, namely metal coordination and Watson-Crick-Franklin base pairing, led to site-specific covalent lysine modification.

Synthetic host-guest pairs are oftentimes completely distinct from other functionalities that are found on protein surfaces, providing highly specific binding events, and therefore opportunities for site-specific protein functionalization through non-covalent interactions. Two basic approaches have been explored in this regard: (1) the protein (modified or native) forms a binary complex with a respective partner bound to a synthetic substrate,[32–36] or (2) the protein and substrate have each been modified with synthetic guests that are brought together in a ternary complex with an additional macrocyclic host.[37–40] These strategies have been used to modify proteins with both synthetic cargo and biomolecules.

As an example of the first approach, in 2016, Langer, Anderson, and coworkers reported the supramolecular PEGylation of biopharmaceuticals. In this work, the researchers associated PEG polymers with protein N-termini using cucurbit[7]uril host-guest chemistry (Figure 2a).[32**] CB7-functionalized PEG derivatives were synthesized and used to target insulin as a particularly interesting target. PEGylation of insulin with CB7-PEG at the N-terminal phenylalanine led to a marked enhancement in stability and therapeutic properties. Unlike covalent attachment strategies, this method did not alter the native structure of insulin, demonstrating a potential advantage with supramolecular PEGylation.

In 2011, Scherman and coworkers reported a ternary approach to protein PEGylation, whereby a ternary complex was formed within a cucurbit[8]uril (CB8) cavity between methylviologen (MV)-modified proteins and naphthalene-modified PEG (Figure 2b).[38**] MV and CB8 form a 1:1 binding complex exclusively, but upon addition of an electron-rich substrate, such as a naphthalene derivative, a 1:1:1 complex comprising MV, the naphthalene moiety, and the CB8 host is formed. Interestingly, electron-rich substrates such as naphthalene and anthracene do not bind to CB8 alone, and only bind in the presence of the MV guest.[7] Thus, this strategy allows for a well-controlled complexation between two distinct substrates without competition from other binding pathways. Ternary complexation with CB8 has been used to induce protein-protein dimerization and higher order assemblies,[39–43] and this topic has been highlighted in recent reviews.[44**,45] While host-guest chemistry offers unique advantages for protein modification, all host-guest interactions used thus far have displayed binding constants on the order of 10^3-10^6 M^{-1}. It is therefore important for follow-up studies to determine the stability of these conjugates in biologically-relevant conditions, where protein-conjugates are likely to be present in the

nanomolar to low micromolar concentration range. While all of the protein-conjugates developed through host-guest means have displayed improved activity, the half-lives of these conjugates in biological conditions remain unreported. Additionally, it is unclear if other biomolecules such as sugars, peptides, and small molecules will compete with the host-guest protein-conjugate pair and promote decomplexation. In the coming years of this new area of research, it will be imperative to address these challenges if host-guest protein-conjugates are to be used in pharmaceutical applications.

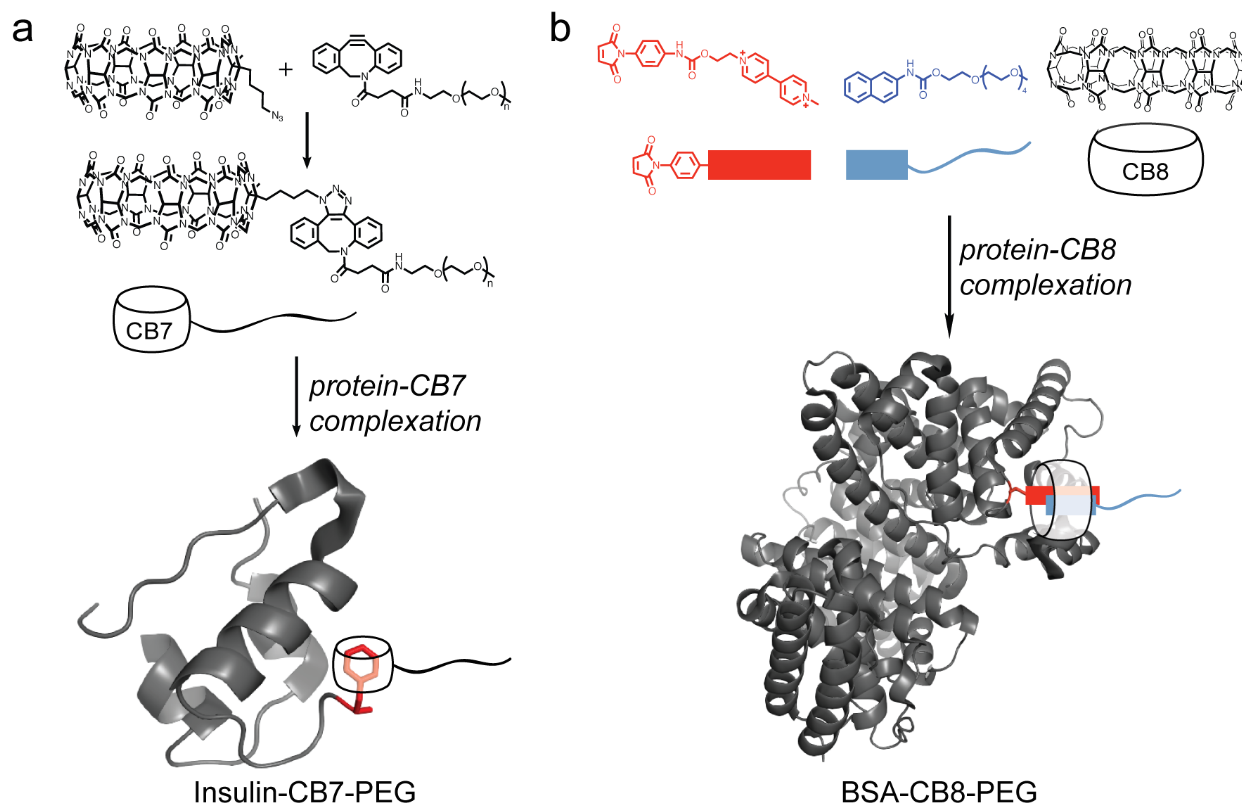


Figure 2. PEGylation of proteins using cucurbituril host-guest interactions through (a) binary Phe-CB7 or (b) ternary MV-Napthalene-CB8 complexation. Figure adapted from [32**, 38**].

Controlling covalent protein modification through supramolecular chemistry

Supramolecular chemistry offers a unique toolkit to control chemical reactions in biological environments. Several examples of host-guest controlled proteins have been reported,[46,47] as well as the first report of a protein-rotaxane conjugate,[48] which may facilitate future stimulus-responsive control over enzymatic activity. In the realm of protein modification, supramolecular interactions have seen recent use as a means of controlling chemical reactivity.[31] In 2016, Pentelute and coworkers reported a supramolecular means of controlling cysteine arylation.[49**] When cysteine is located within an FCPF peptide sequence, a π-clamp is formed with a perfluoroaromatic compound. This places the small molecule reactive handle in close proximity to the cysteine thiol in the sequence, promoting the reaction at that specific site (Figure 3). The

researchers used this technique to modify several proteins site-selectively, including a reduced antibody that contained over 20 cysteines on its surface. In a subsequent report, the researchers determined how salt effects can be used to tune the reactivity of the π-clamp cysteines.[50]

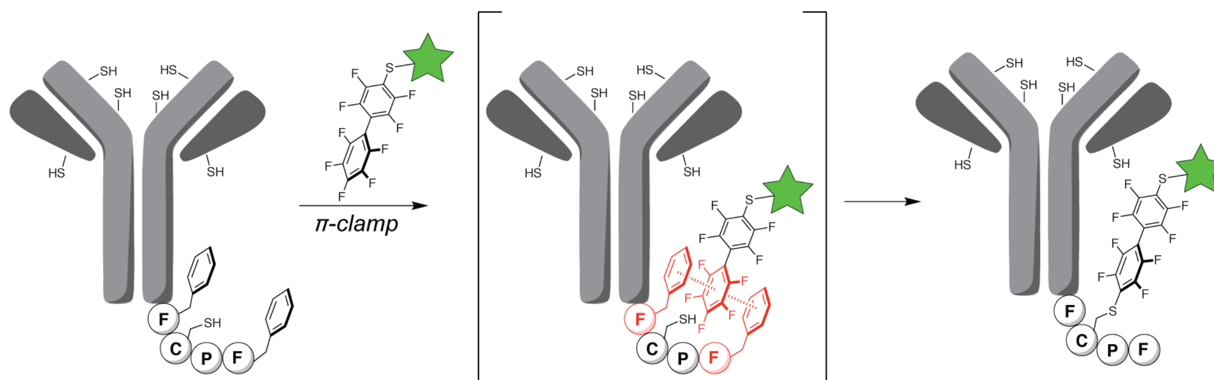


Figure 3. Supramolecular π-clamp directed cysteine arylation led to the selective modification of antibodies with biotin and drug cargos. Figure adapted from [49**]

In 2017, Francis and coworkers reported a supramolecular-controlled azide-alkyne cycloaddition for protein modification.[51**] Cucurbit[6]uril (CB6) promotes the cycloaddition between propargylamine and azidoethylamine derivatives, leading to the 1,4-substituted triazole product selectively (Figure 4). While CB6-click chemistry had been used for rotaxane synthesis, it had not been used for protein modification, presumably due to substrate and reaction condition limitations.[52] The researchers developed new substrates for the CB6 click that do not contain cross-reactive functional groups, and optimized reaction conditions for bioconjugation. CB6-click chemistry led to near-quantitative conversion of azide-modified proteins with propargylamine coupling partners without the use of copper catalysts or cyclooctynes. Francis and coworkers tested this reaction on a panel of substrates, including small molecules, synthetic polymers, DNA oligomers, and peptides. Additionally, because CB6 only binds to azidoethylamine and propargylamine derivatives, it was possible to modify azidoethylamine-functionalized proteins via CB6 click chemistry with high selectivity in the presence of other alkylazide-modified proteins (Figure 4b). Thus, the supramolecular specificity of CB6-promoted click chemistry offers complementarity to copper-catalyzed and strain-promoted azide-alkyne cycloadditions.

While the field of supramolecular-promoted protein modification is relatively new, these examples showcase the strengths of supramolecular chemistry in controlling the site and reactivity of canonical amino acids, as well as functional group handles that are installed synthetically. New types of supramolecular interactions could be developed for enhanced control over covalent modification, such as increased hydrogen-bonding between a specific protein site and a synthetic reactive handle. Additionally, supramolecular interactions such as those presented here are particularly sensitive to pH, salt, and buffer concentration conditions. While this may limit certain avenues of research, this increased sensitivity could offer a unique toolkit to fine-tune reaction conditions to achieve specific degrees of modification.

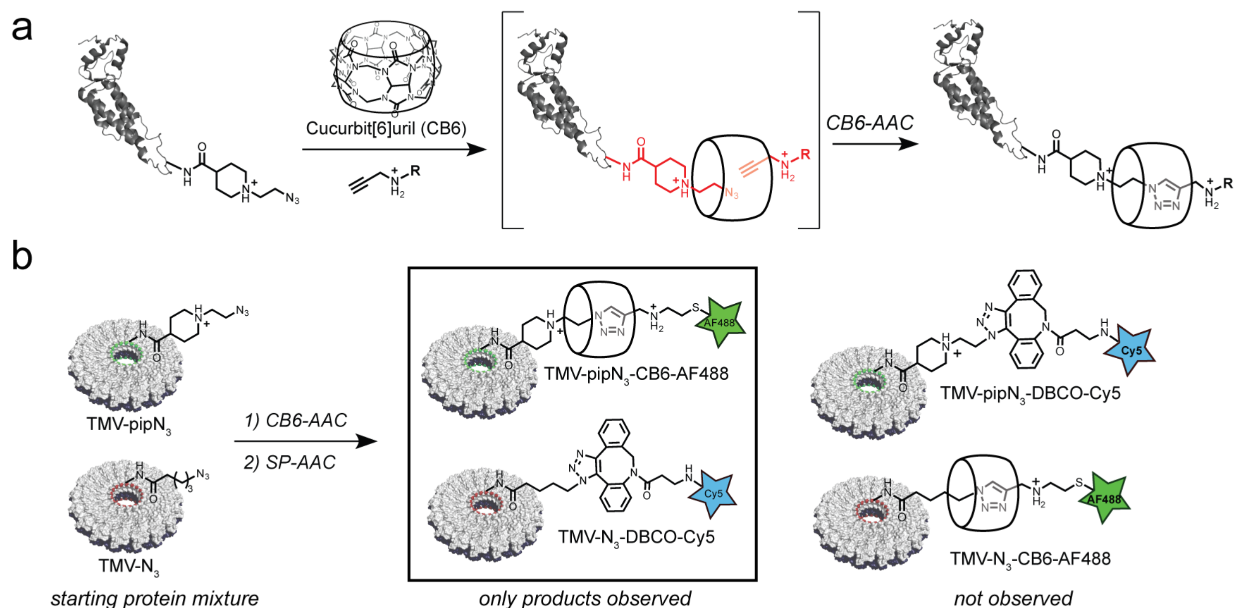


Figure 4. (a) Cucurbit[6]uril-promoted azide-alkyne cycloaddition (CB6-AAC) as a copper-free click bioconjugation technique. (b) Specific CB6 binding interactions led to complementarity with strain-promoted click. Figure adapted from [51**].

Conclusions and future outlook

Noncovalent interactions such as biotin-avidin have been used for decades in protein research, but only recently have synthetic supramolecular strategies received attention in chemical biology. Supramolecular interactions display the bio-orthogonality that is necessary for protein modification in complex mixtures, and can undergo complexation at the low concentrations, physiological pH values, and moderate temperatures that are necessary for biological applications. Complexes with mid-range binding affinities can be tailored for protein immobilization, purification, and recycling, while tight binding pairs are used for protein modification. Additionally, researchers have shown that supramolecular interactions can be used to direct covalent modification strategies, enabling the site-specific modification of natural amino acids, or promoting reactions with non-native functionalities. While metal coordination and host-guest chemistries are predominantly reported for protein modification, other supramolecular interactions such as hydrogen bonding, DNA hybridization, and π-π stacking have been used successfully. These alternative interactions demonstrate that other types of supramolecular chemistries could be used to generate protein conjugates with unique properties.

It should be noted that the current strategies for protein modification rely on a small subset of the rich and rapidly growing number of supramolecular interactions that are known. So long as the components are water soluble, in principle virtually any of these designs could be used. It is also possible that multiple supramolecular interactions could be used in concert to afford highly complex materials while retaining homogeneity.

A particularly interesting and underexplored aspect of supramolecular protein modification is stimuli-responsive protein capture and release. Host-guest chemistry offers opportunities for responsive behaviors, such as those used in supramolecular materials and molecular machines.[5,6] Stimuli such as light, pH, and redox conditions can be used to dissociate and re-associate host-guest pairs for drug delivery and diagnostics.[8] Additionally, the incorporation of mechanical bonds into proteins could lead to new biomaterials. Responsive rotaxane linkages within a protein network could create protein-based artificial muscles,[53] and the installation of catenanes into protein backbones could lead to enhanced stability.[54] Lastly, host-guest chemistry could allow for greater control over protein function. By installing molecular machines near enzyme active sites, stimuli-responsive shuttling could be used to activate or deactivate the protein. This process could similarly be used to direct binding events of proteins to their receptors for use in diagnostics. While supramolecular interactions have been used in protein modification and immobilization, these strategies should also be directed to solve unique challenges that are unmet by covalent means. By combining developments in responsive supramolecular materials with protein-based assemblies, new advances in chemical biology can be realized.

Acknowledgments

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