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Review

Site-Specific Bioconjugation Approaches for Enhanced Delivery of Protein Therapeutics and Protein Drug Carriers

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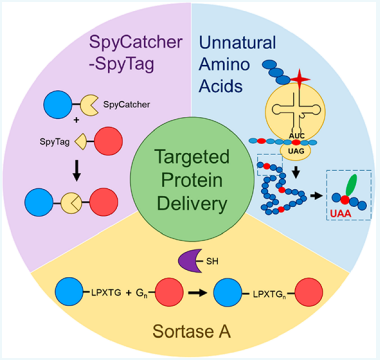
ABSTRACT: Proteins have the capacity to treat a multitude of diseases both as therapeutics and as drug carriers due to their complex functional properties, specificity toward binding partners, biocompatibility, and programmability. Despite this, native proteins often require assistance to target diseased tissue due to poor pharmacokinetic properties and membrane impermeability. Functionalizing therapeutic proteins and drug carriers through direct conjugation of delivery moieties can enhance delivery capabilities. Traditionally, this has been accomplished through bioconjugation methods that have little control over the location or orientation of the modification, leading to highly heterogeneous products with varying activity. A multitude of promising site-specific protein conjugation methods have been developed to allow more tailorable display of delivery moieties and thereby enhance protein activity, circulation properties, and targeting specificity. Here, we focus on three particularly promising site-specific bioconjugation techniques for protein delivery: unnatural amino acid incorporation, Sortase-mediated ligation, and SpyCatcher/SpyTag chemistry. In this review, we highlight the promise of site-specific bioconjugation for targeted drug delivery by summarizing impactful examples in literature, considering important design principles when constructing bioconjugates, and discussing our perspectives on future directions.



INTRODUCTION

Proteins have unprecedented potential to improve human health both as therapeutics and as drug carriers. As therapeutics, the diversity and biospecificity in protein functions impart the ability to treat multiple diseases including cancers,[1−5] autoimmune diseases,[6−8] and metabolic disorders.[9−11] Because of this, the global protein market has grown rapidly in the past decade and includes a variety of new and forthcoming products such as antibody−drug conjugates, nanobodies, enzymes, cytokines, hormones, and inhibitors. Additionally, proteins are ideal building blocks for the fabrication of nanoparticles and other drug carriers because of their intrinsic programmability, biodegradability, and biocompatibility. Protein nanoparticles have been assembled using both naturally derived strategies (e.g., protein cages[12] and virus-like particles (VLPs)[13]) as well as synthetic routes (e.g., amphiphilic nanoparticles[14] and desolvation-driven nanoparticles[10]), and these protein nanoparticles have been extensively explored for the delivery of therapeutic proteins, small molecule drugs,[15] RNA,[16,17] and DNA.[18]

Despite the potential of proteins as therapeutics and drug carriers, their efficacy is limited by poor stability, rapid clearance, and membrane impermeability. To enhance delivery, moieties such as hydrophilic polymers, cell penetrating peptides, and targeting molecules have been conjugated to proteins to enhance circulation properties, internalization, and specificity. Traditionally, reactive side groups on naturally occurring amino acids, e.g., lysine and cysteine, have been used to conjugate delivery moieties to proteins.[19,20] This approach has demonstrated improved delivery compared to proteins in their native form; however, non-site-specific protein conjugation leads to heterogeneities in the number and location of modifications, which can affect the physiochemical properties of the protein and result in undesirable outcomes such as compromised bioactivity for protein therapeutics or particle disassembly for drug carriers.[21]

Site-specific protein bioconjugation approaches allow researchers to build highly tunable protein constructs capable of improving the specificity and efficacy of targeted drug delivery by stimulating cellular internalization and prolonging bioavailability while minimizing negative effects on therapeutic activity. In this review, we highlight recent efforts to improve drug delivery in protein carriers and therapeutics through site-specific modification approaches, placing special emphasis on some of the most promising and versatile techniques: unnatural amino acid incorporation, Sortase-mediated ligation, and SpyCatcher/SpyTag bioconjugation.

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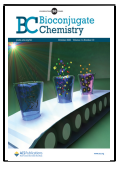

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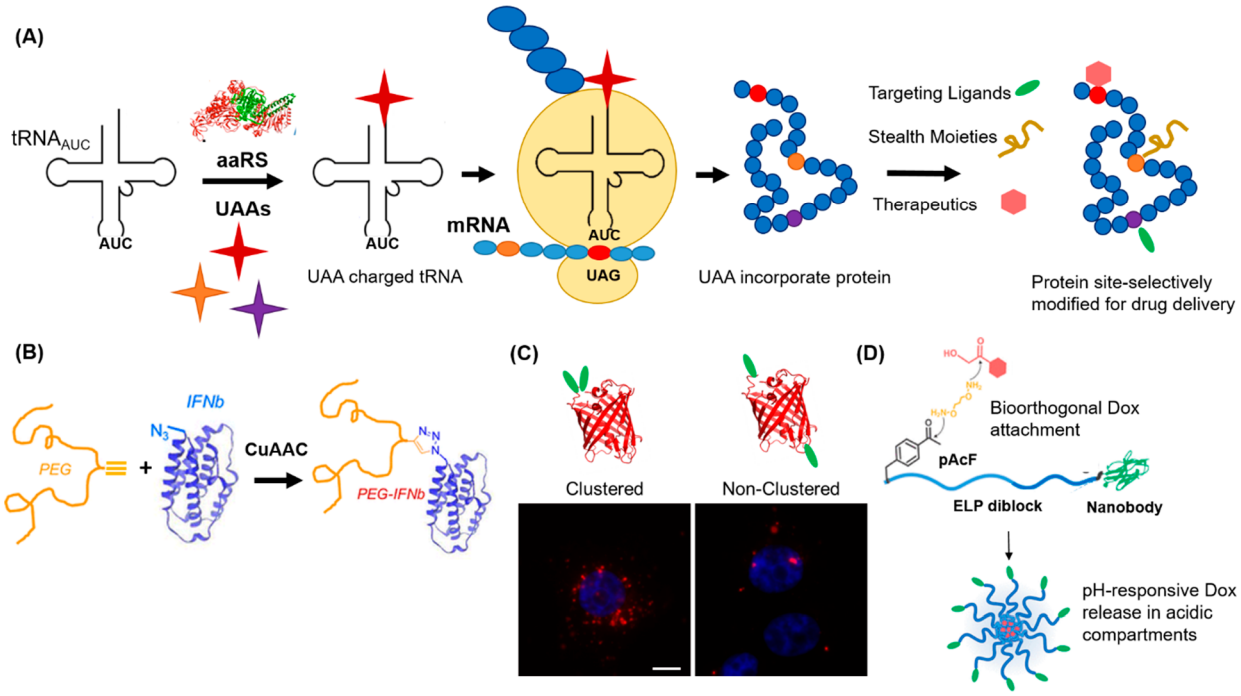

Figure 1. UAA incorporation for targeted drug delivery. (A) UAA incorporation can be used to insert multiple bioorthogonal groups site-specifically into proteins for controlled conjugation of drug delivery moieties. Adapted from from Figure 1 of "Unnatural Amino Acids into Recombinant Proteins in Living Cells" (https://www.labome.com/method/Incorporating-Unnatural-Amino-Acids-into-Recombinant-Proteins-in-Living-Cells.html) with the permission of its copyright owner Labome from ref 27. (B) Azido containing UAA incorporated into IFNb for site-specific conjugation of alkyne-containing PEG through copper-catalyzed alkyne azide cycloaddition (CuAAC). Adapted with permission from ref 47. Copyright 2012, American Chemical Society. (C) Clustering of two EGFR targeting ligands on mCherry through UAA incorporation showed improved internalization in SUM149 cells compared to two ligands attached heterogeneously through ester-amine chemistry on lysine residues. Scale bar represents 15 μm. The nucleus is stained blue and mCherry internalization is represented in red. Adapted with permission from ref 60. Copyright 2019, American Chemical Society. (D) Acetyl containing UAA incorporated into ELP micelles for pH-responsive drug release through oxime hydrolysis. Adapted with permission from ref 70. Copyright 2019, American Chemical Society.

UNNATURAL AMINO ACID INCORPORATION

Unnatural amino acid (UAA) incorporation is a particularly exciting and well-studied bioconjugation method that allows site-specific insertion of unnatural amino acids containing bioorthogonal functional groups. UAAs are most commonly incorporated into the genetic code of cells by using transfer RNA (tRNA) that recognizes the amber stop codon,[22] ochre stop codon,[23,24] or other nonsense codons,[25,26] as well as an aminoacyl-tRNA synthetase (aaRS) that catalyzes the acylation of the tRNA to the UAA. The charged tRNA then inserts the UAA into the growing polypeptide during protein translation (Figure 1A).[27] While UAA incorporation has previously suffered from low incorporation efficiency and diminished expression yields that have limited its use,[28] recent efforts to address these limitations, through cell strain[29−31] and tRNA/aaRS[23,32,33] engineering, have greatly increased the number and types of UAAs that can be incorporated while maintaining high yields. One report demonstrated the insertion of 22 UAAs into a protein sequence while maintaining roughly 35% of the wild-type yield.[34] It is also important to consider design parameters for effective UAA installation, since incorporation efficiency and expression yields can vary greatly based on the site of modification and the type of UAA. A review of the literature demonstrates a series of general guidelines: (1) to prevent protein misfolding and activity loss, folded and active regions within a protein should be avoided as sites of modification, unless altering protein activity is itself the desired outcome;[35] (2) to minimize heterogeneity due to leaky expression, UAAs should not be incorporated immediately following the start codon;[36] and (3) to ensure reactability, UAAs should be incorporated in surface-exposed regions of the protein.[37] By employing these design rules along with advanced cell strains and UAA effectors, researchers have successfully incorporated over 70 different types of UAAs, with unique bioorthogonal chemical handles, into the genetic code of multiple organisms, including yeast, mammalian, and bacterial cell lines.[38] These UAA-modified proteins have been evaluated in applications ranging from protein labeling to biosensing[39] and to vaccine development.[40] Here, we highlight recent applications of UAA incorporation pertaining to targeted drug delivery.

The ability of UAA incorporation to improve bioactivity has been well documented through site-specific bioconjugation of stealth molecules. These stealth molecules function by forming a hydrophilic "shell" around the therapeutic or nanocarrier, enabling it to avoid clearance and extending circulation time.[41] One such stealth moiety, polyethylene glycol (PEG), has been used extensively in the literature to enhance serum half-life, increase protein stability, and mitigate immunogenicity of a multitude of drugs and drug carriers.[42] PEG conjugation (PEGylation) to native amino acids results in heterogeneous products that require complicated purification steps, and unintended modification of certain critical residues can significantly reduce bioactivity. For example, PEGylation at lysine residues of numerous therapeutics, e.g., human growth hormone (hGH),[43] arginine deaminase,[44] and tumor necrosis

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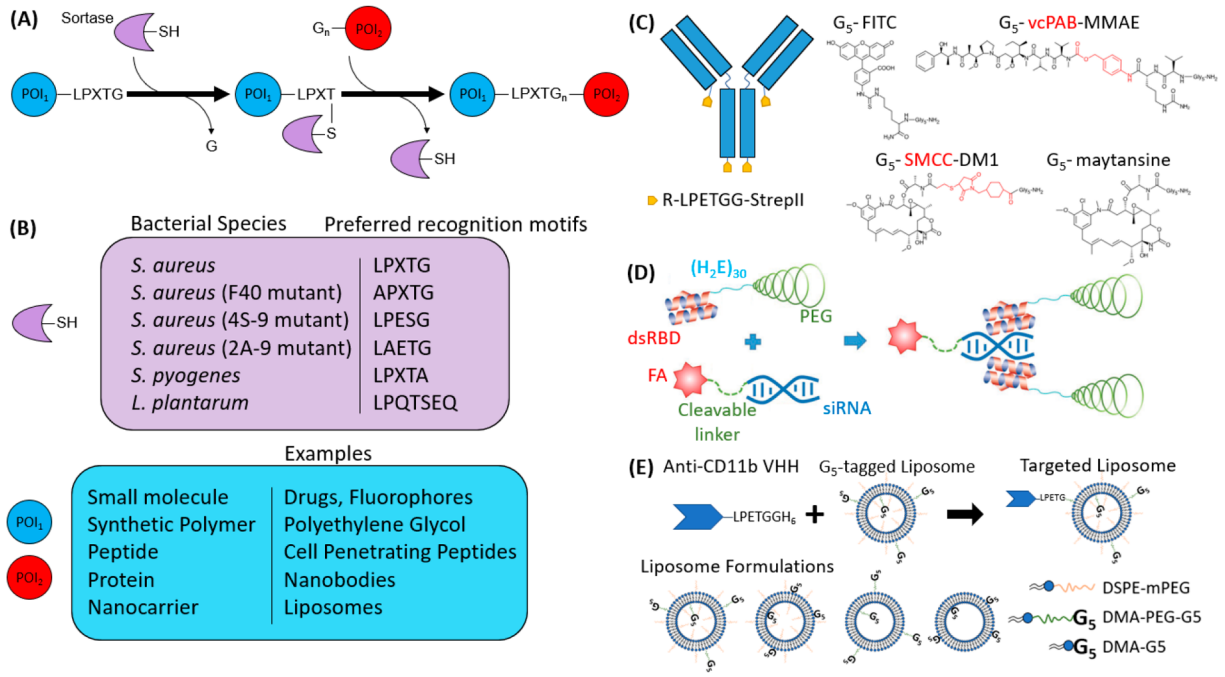

Figure 2. Uses of Sortase-mediated ligation for drug delivery applications. (A) Sortase ligation is a reaction sequence that begins with a nucleophilic attack from the cysteine in the Sortase active site on the T-G peptide bond in the LPXTG recognition motif. The result of this nucleophilic reaction is the formation of an acyl-enzyme intermediate that subsequently reacts further via a second nucleophilic attack from the G_n containing partner to yield the final, ligated product. (B) SML is a molecular toolbox containing many orthogonal sortase enzymes and compatible substrates for versatile and simultaneous modification of protein therapeutics. (C) Sortagging antibody fragments and small molecule drugs can easily produce a library of different antibody−drug conjugates for targeted delivery. MMAE and DM1 have a protease-sensitive dipeptide valine-citrulline-containing linker (vcPAB) and a noncleavable succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate linker (SMCC), respectively (shown in red). Adapted from ref 78 under Creative Commons Attribution license. (D) Conjugating a ribonucleoprotein with PEG via SML can enhance the *in vivo* circulation time and the effectiveness of RNA interference in xenograft tumors in mice. Adapted with permission from ref 86. Copyright 2019, John Wiley and Sons. (E) Liposomes that have G_5 conjugated lipids (DSPE − 1,2-distearoyal-*sn*-glycero-3-phosphoethanolamine, DMA − dimyristyl-amino-propanediol) can be ligated with VHH targeting domains for cell-specific delivery of encapsulated small molecule drugs. Adapted from ref 102. Copyright 2018, with permission from Elsevier.

factor (TNF-α),[45] improved circulation properties but reduced the therapeutic activity by over 50% compared to the unconjugated protein. To site-specifically conjugate PEG molecules onto proteins, Cho et al. used UAA incorporation to insert bioorthogonal acetyl groups into distinct locations on hGH for coupling with oxime-functionalized PEG.[35] A single PEG molecule conjugated to six unique locations on the hGH demonstrated a 3-fold difference in activity in rodents, depending on the location of PEG. Wu and co-workers expanded this approach by using UAA incorporation to compare the efficacy of mono-PEGylated and di-PEGylated hGH.[46] The location of the PEG molecules significantly impacted the pharmacokinetics and activity of hGH, particularly when multiple PEG molecules were attached, with the best performing analogue, a di-PEGylated hGH, enhancing circulation time and stability while maintaining activity similar to the levels of mono-PEGylated hGH. Furthermore, the versatility of UAA incorporation has been demonstrated with site-specific PEGylation to interferon β-1b (IFNb) (Figure 1B)[47] and an anticancer fusion toxin,[48] with both studies showing significant enhancements in pharmacokinetic properties of the PEGylated therapeutic without a significant reduction in bioactivity.

Human serum albumin (HSA) has also shown the propensity to prolong serum half-life,[49,50] and recent work has utilized UAA incorporation to site-specifically conjugate HSA to therapeutic proteins.[51−53] Bak and co-workers used UAA incorporation to conjugate HSA to three specific sites on glucagon-like peptide 1 (GLP-1), a therapeutic peptide used to treat type 2 diabetes.[53] All three HSA conjugated GLP-1 variants exhibited an increase in circulation half-life of approximately 160-fold as compared to wild-type GLP-1. Despite this, only two analogues exhibited improved glucose tolerance in mice compared to wild-type GLP-1, with the best variant demonstrating over a 6-fold increase in activity compared to a C-terminal fusion of HSA to GLP-1. These results further emphasize the importance of site-controlled protein modification.

An additional design variable to consider when building protein therapeutics and carriers is the display of active targeting moieties capable of binding to cell surface receptors to promote cellular internalization. Control of targeting ligand orientation, valency, and spacing can improve targeted internalization by encouraging multivalent ligand−receptor interactions through ligand clustering[54−56] and synergistic receptor binding through dual ligand functionalization.[57,58] In our own work, we used UAA incorporation to cluster varying numbers of the epidermal growth factor receptor (EGFR) targeting peptide, GE11,[59] onto azido-modified mCherry. Four GE11 peptides clustered onto the N-terminus of mCherry resulted in an 18-fold enhancement in uptake in EGFR-overexpressing cancer cells, SUM149, as compared to mCherry

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with a single GE11 peptide and a 4-fold enhancement as compared with uptake in healthy breast epithelial cells with basal EGFR expression levels.[60] Additionally, clustering two GE11 peptides to mCherry with UAA incorporation resulted in 5-fold higher uptake in cancer cells as compared to mCherry constructs with two unclustered GE11 peptides conjugated via ester-amine chemistry (Figure 1C). This example highlights the importance of controlling ligand density and clustering for targeted internalization, which can only be accomplished through site-specific bioconjugation. Additionally, UAA incorporation has been used to generate bispecific antibodies to cross-link HER2+ cancer cells and CD3+ cytotoxic T lymphocytes cells to promote T-cell-mediated cancer cell death.[61] UAA incorporation has also been used extensively for the synthesis of site-specific antibody−drug conjugates to improve product homogeneity[62−66] and for functionalizing the surface of protein nanoparticles to control the orientation and density of targeting moieties.[67−69]

The chemistries available through UAA incorporation can also be used to enable stimuli-responsive, targeted drug release. The UAA *p*-acetylphenylalanine (pAcF) was incorporated into an EGFR targeted elastin-like protein micelle and used to attach Doxorubicin (Dox) through oxime bond formation (Figure 1D).[70] Under acidic conditions, e.g., in endolysosomal compartments, the oxime bond underwent acid-catalyzed oxime hydrolysis triggering Dox release, ensuring the drug was released inside cells rather than in the extracellular environment where it could affect healthy tissues. Another example by Guo and co-workers used UAA incorporation to functionalize proteins containing a thrombin cleavage site with an azido group for immobilization onto a PEG hydrogel network through strain-promoted azide−alkyne cycloaddition chemistry.[71] Incubation with the proteolytic trigger, thrombin, resulted in spatially controlled, stimuli-responsive protein release.

Collectively, these examples demonstrate the versatility of UAA incorporation for the bioconjugation of moieties for targeted protein delivery. An advantage of UAA incorporation is the site-specific modification of individual residues within a protein sequence. Additionally, UAA incorporation is a minimally disruptive way to add one or multiple reactive sites to a protein since each reactive site is housed within a single amino acid. This approach also allows for incorporation of diverse bioorthogonal chemistries with a wide array of chemical properties. For these reasons, UAA incorporation has demonstrated particular utility for applications requiring modifications within a protein sequence, multiple modifications in close proximity, modification of delicate proteins, and/or incorporation of unique chemistries. When these design requirements are not necessary, other modification approaches, such as Sortase-mediated ligation and SpyCatcher/SpyTag chemistry, may be more appropriate, since UAA incorporation often comes with the caveat of reduced expression.

SORTASE-MEDIATED LIGATION

Sortase-mediated ligation (SML) is a powerful biomolecular tool for the post-translational modification of a target protein. Sortase A is a transpeptidase that is natively found in Gram-positive bacteria, where it is responsible for anchoring proteins onto the bacterial membrane with small peptide tags.[72−76] The most commonly used sortase enzyme found in *Staphylococcus aureus* catalyzes a peptide bond between an LPXTG motif (where X is any amino acid), commonly placed near the C-terminus of a protein substrate, and an N-terminal G_n motif-containing partner (Figure 2A).[75,77,78] Because sortase processing requires only small peptide tags, SML in proteins is useful for incorporating a broad range of moieties ranging from synthetic small molecules[79−85] to biopolymers[86−89] to conventional peptides[74,90,91] and proteins[92,93] (Figure 2B). Furthermore, adding separate recognition motifs at the N- and C-terminus of the target protein can facilitate multiple modifications[94,95] by using orthogonal sortases, such as those found in *Streptococcus pyogenes*,[75,86,94,96] *Lactobacillus plantarum*,[97] or derivative mutants from *S. aureus*.[75,88,91,98] Using sortase to ligate two sortase-tagged (sortagged) moieties occurs on the order of hours, with minimal scarring (e.g., insertion of amino acids necessary for SML chemistry), and sortase offers reaction conversions of up to 90%.[76,83,96,99] However, transpeptidation reactions are reversible and reaction conversion strongly depends upon the solvent accessibility of the sortags on each sortagged partner, which necessitates reaction optimization and post-reaction purification of unreacted species and enzyme.[78,100,101] Furthermore, sortags must be inserted at the terminus of polypeptides, thereby limiting the quantity and location of potential ligation sites. To address the challenges in reaction reversibility and efficiency, better-performing *S. aureus* sortase A mutants have been developed by truncating the N-terminus[102] and mutating the active site,[103] which offers an improvement of 2 orders of magnitude in k_{cat}/K_M, potentially due to increased steric accessibility of the enzyme active site and affinity of the tags to the enzyme. Aside from engineering a more potent enzyme, design and reaction considerations can also be adjusted to enhance the solvent exposure and structural flexibility of both substrates, and thereby the conversion of the reaction. The C-terminal LPXTG is commonly fused after short, flexible glycine-serine rich linkers with at least one additional residue after the final glycine, and the N-terminal oligoglycine substrate partner typically contains multiple glycines (three to five), with the optimal length of both the glycine-serine rich linker and the oligoglycine substrate requiring empirical determination.[100] Improvements in reaction conversion have also been observed by using the ligation partner in excess to drive the equilibrium reaction toward the products and to reduce the competing acyl enzyme intermediate hydrolysis reaction, as these alternative processes cause the formation of undesired side products and unreactive target proteins.

SML provides an especially ideal technique for conjugating proteins to synthetic molecules, such as small molecule drugs, that cannot be incorporated into proteins via genetic fusion. Small molecule drugs diversify the functionality of a protein, and accordingly expand its range of therapeutic applications; however, these drugs often cause unfavorable side effects due to nonspecific cellular uptake.[104,105] For enhancing the targeted delivery of small molecules to lymphoma or breast cancer cells, Beerli and co-workers synthesized various antibody−drug conjugates (ADC) via SML of an antibody targeting either CD30 (cAc10) or HER2 (Trastuzumab and cFRP5) to a small molecule drug or fluorophore, such as monomethyl auristatin E (MMAE), mertansine (DM1), maytansine, or fluorescein isothiocyanate (FITC) (Figure 2C).[79] SML conjugation provided a more stable ADC linkage as compared to the FDA-approved analogues Adcetris and Kadcyla, which are conjugated via maleimide-based linkers and can be reversed during serum circulation. This reversal is hypothesized to occur when the maleimide-conjugated drug

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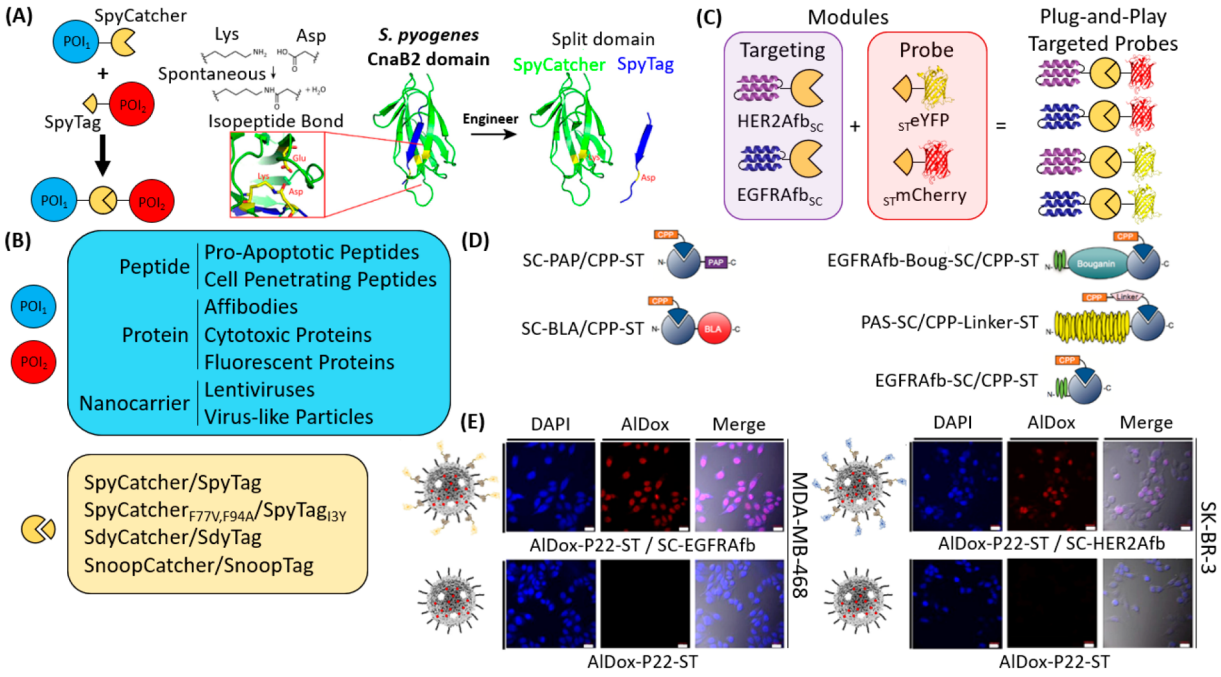

Figure 3. Using SpyCatcher-SpyTag ligation for developing modular protein and peptide drug delivery components. (A) SpyCatcher and SpyTag can be used to ligate two polypeptides via the formation of a spontaneous isopeptide bond between the two moieties. Adapted with permission from ref 111 by the National Academy of Sciences, U.S.A. (B) The use of orthogonal Catcher/Tag moieties derived from similar CnaB2 adhesin-like domains from different bacteria can be used for single or multiple ligations involving various types of protein cargoes and carriers to form more sophisticated therapeutics. (C) Modular fusion protein drugs were developed by adding a SpyTagged affibody targeting module to a SpyCatcher-fused probe for cell-specific uptake[130] (SC − SpyCatcher, ST − SpyTag). (D) SpyCatcher-SpyTag ligation is utilized for cell penetrating peptides incorporation for cytosolic delivery of active biologics. [SC − SpyCatcher protein (blue, three-quarter circle), ST − SpyTag peptide (dark blue, quarter-circle), CPP − cell penetrating peptide (orange), PAP − Pro-apoptotic peptide (purple), BLA − β-lactamase protein (red), EGFRAfb − EGFR Affibody (green), Boug − Bouganin (teal)] Adapted from ref 119 under Creative Commons Attribution license. (E) SpyCatcher-fused affibodies were ligated with SpyTagged P22 VLPs encapsulating AlDox for targeted delivery into MDA-MB-468 and SK-BR-3 cells. Adapted with permission from ref 126. Copyright 2019, John Wiley and Sons.

undergoes a substitution reaction from an antibody cysteine residue to the reactive cysteine-34 in HSA via a retro-Michael reaction mechanism.[106] Beerli and co-workers demonstrated that SML of various small molecule drugs/fluorophores to immunoglobulin heavy and light chains fused with a C-terminal LPETG recognition motif yielded about 80% ligation efficiency for each ADC combination. The combination of ligated drug-containing ADCs developed via SML, cAC10-MMAE, cFRP5-DM1, cFRP-maytansine, Trastuzumab-DM1, and Trastuzumab-maytansine, offered comparable reductions in cell viability of CD30+ and HER2+ cancer cells, in a dose- and cell-specific manner, as their commercial counterparts.

SML has also been utilized to ligate synthetic polymers, such as the stealth agent PEG, to enhance *in vivo* pharmacokinetics and stability. Tai et al. synthesized a PEGylated ribonucleoprotein (RNP) by SML of a double-stranded RNA binding domain (dsRBD)-endosomal destabilization peptide $(H_2E)_{30}$ fusion with a C-terminal LPETGG and a GG-PEG molecule (Figure 2D).[87] The PEGylated dsRBD fusion protein bound a folic acid conjugated anti-polo-like kinase 1 (PLK1) siRNA molecule (siRNA-FA) to prolong circulation half-time and targeted delivery to folate receptor (FR)-overexpression KB cells. Delivered *in vitro*, the RNP exhibited cell specific uptake that was orders of magnitude higher in KB cells as compared to FR-negative DU-145 cells, resulting in an up to 70% reduction of PLK1 mRNA and protein levels in KB cells. Furthermore, intravenous administration of these RNPs in KB tumor-bearing mice resulted in an 18-fold enhancement in circulation half-life as compared to free siRNA-FA, and these RNPs also reduced the tumor mass by more than 50% compared to the approximately 15% reduction from siRNA-FA.

In addition to small molecules and biomacromolecules, SML has also been used to modify larger nanocarriers for targeted cell uptake. Wöll et al. demonstrated SML to enhance delivery of liposomes (Figure 2E).[107] In their work, PEGylated and pentaglycine modified liposomes were ligated to an anti-CD11b single domain antibody fragment (VHH) with a C-terminal LPETG recognition motif, which offered up to 80% conversion and yielded a density of 110−430 VHHs per liposome. The VHH-ligated liposomes demonstrated selective uptake in CD11b receptor expressing CD14+ monocytes and CD15+ granulocytes, with an order of magnitude enhancement in cell internalization as compared to control cells.

Overall, SML provides a versatile post-translational modification technique to couple therapeutic proteins to various types of substrates.[108] It offers rapid ligation kinetics, minimal scarring, and only requires the incorporation of small peptide tags, which can easily be added via genetic fusion or chemical coupling reactions, making it attractive especially for linkage of synthetic molecules to proteins. SML has also been explored extensively for conjugating therapeutic proteins to targeting proteins[92,93,109,110] and cell penetrating peptides[90,111] that facilitate improved intracellular delivery. However, when exclusively modifying proteins or peptides for delivery applications, the limited reaction efficiency and inability to modify proteins at nonterminal sites often reduces the

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applicability of SML compared to other techniques. The SpyCatcher/SpyTag ligation method offers fast reaction and near-completion conversion of reactive "Spy" partners, as well as the capacity for nonterminal protein modification, and thus presents a compelling alternative.

SPYCATCHER/SPYTAG CONJUGATION

SpyCatcher and SpyTag comprise a versatile protein modification tool that is a biologically derived analogue of synthetic click chemistries.[112−114] They are derived by splitting the adhesin domain of the fibronectin binding protein FbaB in *S. pyogenes*, where SpyCatcher is a 116-amino-acid protein and SpyTag is a 13-amino-acid peptide. Mixing the two moieties facilitates a spontaneous isopeptide bond formation reaction between SpyCatcher Lys31 and SpyTag Asp117, catalyzed by the nearby Glu77 residue (Figure 3A).[113] This bioclick reaction for proteins[115−120] and peptides[121,122] offers irreversible, covalent fusion that goes to near-completion with rapid kinetics (ca. minutes) and requires minimal purification post-reaction. Furthermore, this technology offers wide applicability due to its ease of incorporation at both terminal and internal polypeptide sites and the access to an array of orthogonal Catcher/Tag parts, derived from similar adhesin domains of other bacteria (e.g., *Streptococcus pneumoniae*,[115] *Streptococcus dysgalactiae*,[123] and derivative mutants from *S. pyogenes*[124]) (Figure 3B). To incorporate SpyCatcher and SpyTag as fusion partners (e.g., at terminal sites), they should be fused at the N- or C-terminus of proteins with flexible glycine-serine-rich linkers to reduce potential folding problems and avoid undesirable protein−protein interactions.[112] Meanwhile, to incorporate either Spy moiety at a nonterminal site within a protein, the insertion should be placed within an exposed flexible loop of the acceptor protein and flanked by flexible linkers to reduce potential effects on protein folding. Despite its wide applicability and simplicity, the scope of this technology is generally limited to post-translational protein and peptide ligation due to necessary genetic fusion of SpyCatcher and SpyTag, and a large 129-amino-acid "scar" from the reaction process. Efforts have been made to address these challenges with the development of smaller and faster reacting SpyCatcher/SpyTag pairs, SpyCatcher002/SpyTag002[125] and SpyCatcher003/SpyTag003,[126] as well as a ternary SpyLigase/SpyTag/KTag system, where the 11 kDa SpyLigase locks the 13-amino-acid SpyTag and the 10-amino-acid KTag via an isopeptide bond.[127] Other ternary systems have also been developed,[128,129] such as the orthogonal SnoopLigase/SnoopTagJr/DogTag system; however, despite their more minimalist nature, these systems are still predominantly used in proteins and peptides.

The versatility and simplicity of this SpyCatcher/SpyTag family as a tool for post-translational fusion of different proteins has led to its frequent use to develop plug-and-play delivery modules for targeted protein delivery. Moon et al. demonstrated this concept by developing N-terminal Spy-tagged anti-HER2 (HER2Afb) and anti-EGFR (EGFRAfb) affibody targeting modules and C-terminal SpyCatcher-fused mCherry or yellow fluorescent protein (YFP) cargo protein modules (Figure 3C).[130] Reacting the targeting and cargo protein modules resulted in near-complete ligation within 5 min. Subsequent delivery of HER2Afb and EGFRAfb fluorescent protein conjugates demonstrated enhanced uptake to HER2 overexpressing SK-BR-3 cells and EGFR over-expressing MDA-MB-468 cells compared to control cell lines with basal expression of each receptor. Further modifications of the anti-HER2-YFP affibody fluorescent protein conjugate (AFPC) were made to genetically introduce a glutathione transferase (GST) between the SpyTag and the affibody for AlDox (a prodrug of doxorubicin) conjugation and delivery. Ligation of SpyTag-GST-HER2Afb to YFP-SpyCatcher demonstrated enhanced AlDox delivery and cytotoxicity to HER2+ SK-BR-3 cells compared to HER2− MCF10A cells and unconjugated protein AFPC vehicle. In a similar manner, Hoffmann et al. broadened this simple assembly scheme to develop a strategy to screen for their best-performing cell penetrating peptide (CPP) from a library of peptide candidates.[121] Upon doing so, they demonstrated its potency by delivering various biologics (Figure 3D) and demonstrating the bioactivity of the conjugates in a range of cell types. Additionally, this strategy has also been used in our aforementioned, GE11-conjugated mCherry protein for ligation of the prodrug converting enzyme, yeast cytosine deaminase (yCD), for targeted delivery into breast cancer cells.[60]

Aside from therapeutic proteins, SpyCatcher/SpyTag technology has also been utilized in larger nanocarriers, such as VLPs[131,132] and lentiviruses,[133] for site- and orientation-specific conjugation of targeting domains. Kim et al. developed a modular nanocarrier from *Salmonella typhimurium* P22 VLP bacteriophage for targeted delivery by genetically fusing a C-terminal SpyTag to the P22 coat protein and an N-terminal SpyCatcher to an anti-HER2 (SC-HER2Afb) or anti-EGFR affibody (SC-EGFRAfb) (Figure 3E).[131] Up to 40% of the P22 coat proteins were modified with a SpyCatcher-affibody (SC-Afb) fusion with near-complete consumption of the limiting SC-Afb reagent. AlDox was conjugated to the VLP via an acid-cleavable maleimidocaproyl hydrazine linker, facilitating release of the drug in the acidic, late-endosomal compartment. The SC-EGFRAfb and SC-HER2Afb ligated VLPs, loaded with AlDox, demonstrated enhanced uptake in EGFR overexpressing MDA-MB-468 cells and HER2 overexpressing SK-BR-3 cells, respectively. Furthermore, dose-dependent cytotoxicity comparable to the free doxorubicin control was demonstrated when delivering the AlDox-loaded targeted VLPs to cells overexpressing the cognate receptor. Near-basal nanocarrier cytotoxicity levels were observed when AlDox-loaded targeted VLPs were delivered to control cell lines. These findings demonstrate how targeted delivery yielded an important reduction of off-targeted effects.

SpyCatcher/SpyTag is a facile protein ligation technique that can be used to construct sophisticated, multifunctional proteins and nanocarriers for effective drug delivery. Incorporating the pair of mutually reactive moieties can develop different delivery modules that are easily "clicked" together based on the desired functions of the resultant protein. As a ligation strategy, it is most suitable for covalent binding proteins and peptides, which can more easily tolerate SpyCatcher or SpyTag fusion and provides a versatile plug-and-play technology for constructing new "smart" protein therapeutics.

CONCLUSION

Site-specific protein bioconjugation is a notable tool for controlled functionalization of protein therapeutics and drug carriers. Precise control over protein modification allows for highly tailorable display of delivery moieties, which can lead to superior specificity and therapeutic efficacy and will likely be a

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hallmark of the next generation of targeted protein therapies. UAA incorporation provides an avenue to introduce unique functionalities anywhere within the polypeptide backbone. SML allows for conjugation of a broad range of delivery moieties to proteins with minimal scarring, while SpyCatcher/SpyTag offers fast and effective covalent binding for plug-and-play protein and peptide coupling. The examples presented here demonstrate the versatility and utility of UAA incorporation, SML, and SpyCatcher/SpyTag bioconjugation, but are not an exhaustive list, and as the toolbox is enriched with improved efficiency of current techniques and the discovery of new site-specific modification methods, the potential for highly controlled protein drug products is immense. Already, several therapies utilizing these techniques are undergoing preclinical and clinical development,[35,112,134,135] but further work regarding scale up and cost minimization is necessary to realize the full potential of site-specific protein modification in the clinic. The precise control provided by these bioconjugation approaches can be used to identify criteria for selecting modification sites on proteins as well as determining generalizable design principles to maximize protein activity and stability, circulation properties, and targeted cellular uptake. Research applying site-specific bioconjugation has only just begun, and prospective discoveries will likely shape the future of protein drug delivery systems and beyond.

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Notes

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ABBREVIATIONS

aaRS, aminoacyl-tRNA synthetase; ADC, antibody−drug conjugate; Afb, affibody; AFPC, affibody fluorescent protein conjugate; BLA, β-lactamase; Boug, bouganin; CPP, cell penetrating peptides; CuAAC, copper-catalyzed alkyne−azide cycloaddition; DM1, mertansine; DMA, dimyristyl-amino-propanediol; Dox, doxorubicin; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; dsRBD, double-stranded RNA-binding domain; EGFR, epidermal growth factor receptor; FITC, fluorescein isothiocyanate; FR, folate receptor; GLP-1, glucagon-like peptide 1; GST, glutathione transferase; hGH, human growth hormone; HSA, human serum albumin; INFb, interferon β-1b; MMAE, monomethyl auristatin E; pAcF, *p*-acetylphenylalanine; PAP, pro-apoptotic; PEG, polyethylene glycol; PLK1, polo-like kinase 1; POI, protein of interest; RNP, ribonucleoprotein; SC, SpyCatcher; SMCC, succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate; SML, sortase-mediated ligation; SRT, sortase; ST, SpyTag; TNF-α, tumor necrosis factor alpha; tRNA, transfer RNA; UAA, unnatural amino acid; vcPAB, protease-sensitive dipeptide valine-citrulline-containing linker; VHH, single domain antibody fragment; VLP, virus-like particles; yCD, yeast cytosine deaminase; YFP, yellow fluorescent protein

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