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Perspective

Enzymatic Bioconjugation: A Perspective from the Pharmaceutical Industry

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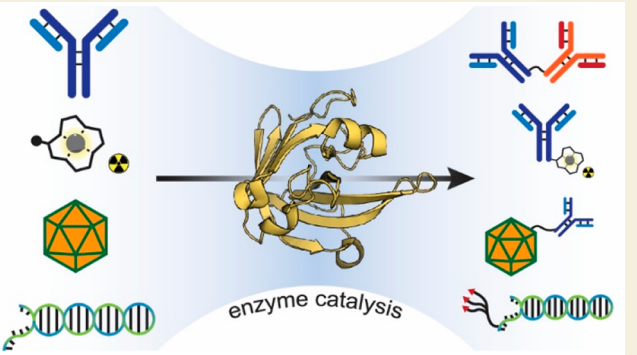
ABSTRACT: Enzymes have firmly established themselves as bespoke catalysts for small molecule transformations in the pharmaceutical industry, from early research and development stages to large-scale production. In principle, their exquisite selectivity and rate acceleration can also be leveraged for modifying macromolecules to form bioconjugates. However, available catalysts face stiff competition from other bioorthogonal chemistries. In this Perspective, we seek to illuminate applications of enzymatic bioconjugation in the face of an expanding palette of new drug modalities. With these applications, we wish to highlight some examples of current successes and pitfalls of using enzymes for bioconjugation along the pipeline and try to illustrate opportunities for further development.



KEYWORDS: *bioconjugation, new modalities, enzymatic synthesis, biocatalysis, site-selective modification*

1. INTRODUCTION

The conjugation of a chemical payload to a biomolecule and the combination of their individual therapeutic effects is a well-established concept that has found a resurgence in the last two decades. For instance, Paul Ehrlich's proposal of a "magic bullet", coupling a highly cytotoxic compound to an antibody for highly selective delivery, has found its way into 12 FDA approved antibody drug conjugates (ADCs) as of 2022.[1,2] At the same time the market value of ADCs is already multiple billion USD and is forecasted to grow within the coming years.[2] Additionally, bioconjugates are not exclusive to antibodies and their derivatives. Attaching payloads and other chemical modifiers to biopolymers is a concept that has been expanded to charging small peptide binders with radioactive payloads,[3] for the formation of multimeric proteins,[4] to decorate RNA molecules with targeting groups,[5] and even to modify the surface of large protein complexes.[6]

Whereas the palette of targets, biopolymers, and payloads has expanded rapidly in the last decades, the bioconjugation chemistry of most clinical candidates has surprisingly remained largely unchanged. Typically, the bio-orthogonal reactivity of maleimides and cysteine, or *N*-hydroxysuccinimide esters and lysine, is being used for coupling reactions.[7] This chemistry is generally robust and leads to high conversion during the process, but it comes with drawbacks. First, proteins of interest need to contain a residue on the surface that can react with the payload. Second, the reactions are often not regiospecific since proteins may contain many nucleophilic residues on their surface. This can lead to a distribution of products with different amounts of payload attached to the target. For ADCs, this is often described as the drug to antibody ratio (DAR). The DAR can be tuned based on the chemical process, but often the need to control the DAR leads to changes in the target protein sequence necessary to introduce reactive residues. Third, lysine is one of the most common surface residues of proteins which can make site-specific coupling and control of the DAR difficult. Cysteine is less abundant compared to lysine, but the thiosuccinimide formed through maleimide couplings is often unstable, potentially leading to premature linker cleavage.[8,9] Fourth, since proteins are usually present in low concentrations and amino acid side chains are often sterically occluded, many equivalents of electrophilic payload are used during the coupling step to control the reaction velocity.[10] Not all target proteins are stable under such harsh conditions. Some of these shortcomings have been addressed with the development of improved small molecule conjugation methods such as next-generation maleimides.[11−13] However, mild and catalytic methods to access chemically defined bioconjugates remain highly desired.

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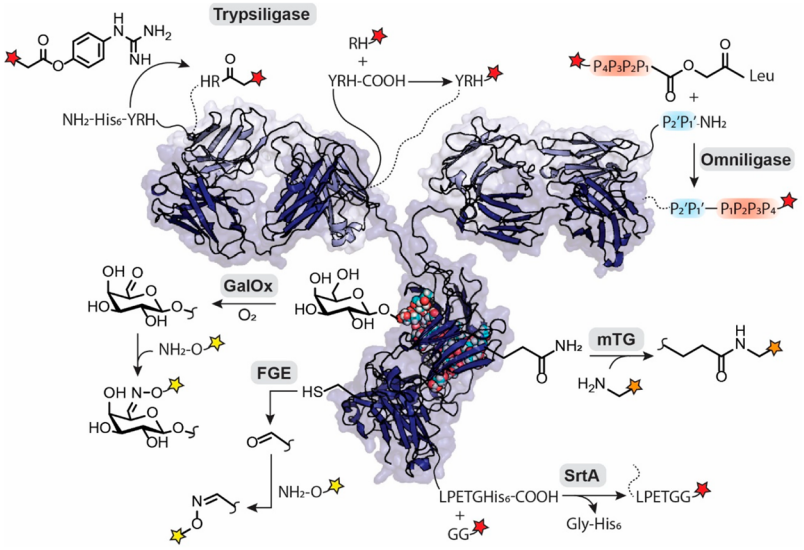

Figure 1. Promising bioconjugation reactions for the creation of ADCs with the payloads color coded: red stars indicate payloads conjugated by peptide bond formation, orange indicates isopeptide bonds, and yellow indicates aldehyde-based conjugation strategies. COOH and NH_2 denote the C- and N-terminus, respectively. Trypsiligase can modify N-termini with a simplified OGp substrate, and C-termini with a RH-dipeptide tagged payload; both cleave a short recognition sequence. Omniligase uses peptides activated with ester leaving groups that can carry a cargo to modify the N-terminus. Microbial transglutaminases (mTG) create an isopeptide bond on a glutamine side chain with an amine functionalized payload. Sortase A (SrtA) performs C-terminal transpeptidation and can cleave affinity tags, e.g., His_6, while coupling Gly_2-labeled payloads. Galactose oxidase (GalOx) and formylglycine generating enzyme (FGE) create aldehydes on the oligosaccharide and cysteine residues, respectively, which can selectively be labeled with a broad range of bioorthogonal chemistries.

Enzyme catalysis can be extremely fast, regio- and stereoselective, and generally works under mild conditions. Unsurprisingly, enzymes have already firmly established themselves as exceptional catalysts in the chemical and pharmaceutical industry.[14−17] In principle, these advantages could also be harnessed for reactions involving macromolecule substrates. An early example from 1979, the production of semisynthetic insulin using carboxypeptidase A and trypsin from porcine, highlights that enzymes can be successfully employed to modify proteins for industrial purposes.[18] Fast forward to 2022, the scientists at Merck reported an engineered penicillin G acylase capable of site-selective deprotection of phenylacetamide derivatives of insulin, demonstrating the advances of biocatalysis in this space.[19] Since the case of semisynthetic insulin, a multitude of enzymes with a broad scope of different bioconjugation mechanisms have been described. The distinct types of bioconjugating enzymes as well as chemical bioconjugation have been extensively reviewed elsewhere.[13,20−23] In this Perspective, we want to focus solely on the types of bioconjugates that have been produced using enzymes with a focus on the macromolecule type. As well as their individual challenges and opportunities.

2. ANTIBODY DRUG CONJUGATES

All ADCs on the market depend, at the time of writing, on chemical coupling of the payload either to lysine or cysteine side chains, apart from Moxetumomab pasudotox, which is a genetic fusion protein.[1] ADCs are the most prominent example of bioconjugates in the clinic and are also the most tested for bioconjugating enzymes. Both site specificity and DAR can have a profound impact on drug efficacy, and thus, enzymes can be an impactful tool.[9,24,25] Protein ligases that belong to the family of serine proteases often serve as platforms for protein chemistry. Most notably, sortase A (SrtA) of *Staphylococcus aureus* performs transacylation by specifically cleaving the sequence, also called sortag, LPXT/G (X being any amino acid and / denotes the site of cleavage) and the acyl intermediate can be attacked by a payload with two N-terminal glycine residues forming a regular peptide linkage (Figure 1).[26] This transformation has proven to be an extremely versatile tool to rapidly functionalize and diversify biomolecules.[27−29] For application to ADCs, this is particularly interesting since C-termini are distant from the antigen binding region; however, genetic modification of the antibody gene is likely needed.[30]

SrtA has since found numerous applications such as labeling and immobilizing antibody single-chain variable fragments (scFvs),[31−33] creating antimicrobial ADCs[34] and monoclonal antibody (mAb) PEGylation,[35] creating nanobody based ADCs and imaging agents,[36,37] and generating artificial bispecific antibodies.[38] Beerli et al. showed that SrtA can be used to generate ADCs of anti-CD30 mAb, analogous to brentuximab vedotin, and several anti-HER2 ADCs.[39] In both cases, the authors inserted recognition sequences at both the C-termini of the heavy and light chain. Addition of a spacer sequence between antibody and tag allowed improvements of transpeptidation. SrtA mediated ligations typically showed high yields of >80%, with DARs of 3−3.5, corresponding to approximately 80% coupling efficiency per site. Additionally, en route to the conjugation, the affinity tag used for purification is removed, which is C-terminal to the leaving group of the sortase tag. The authors managed to show the efficacy of the ADCs with *in vitro* and *in vivo* data. The same authors, and others, showed that this technology can be utilized to introduce a highly toxic payload into full length trastuzumab[40] and proteotoxins to a trastuzumab derived antigen binding fragment.[41]

The application of SrtA for rapid prototyping has been highlighted by the Ploegh group using SrtA to fuse immune

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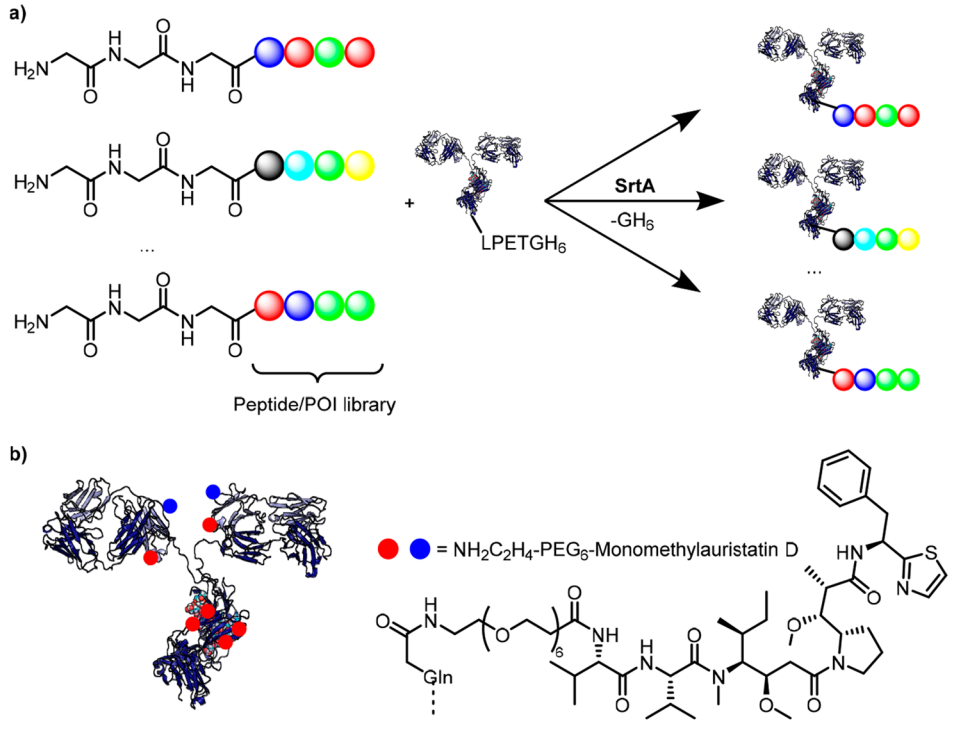

Figure 2. (a) Sortase A enables rapid prototyping of biologics.[42] A single mAb containing the LPETG motif can be functionalized with a whole library of compounds individually. SrtA is acting as an important tool when cloning, expression, and purification of a large number of fusion constructs is impossible. (b) Transglutaminase can attach monomethylauristatin D payloads to Q-tags with high specificity.[74] Strop et al. showed that ADCs with high DARs can be synthesized in a controllable manner, yielding DAR 6 ADCs by targeting Q-tags in the red regions (light chain C-terminus, N297Q, and a native Gln295) and DAR 8 with the addition of Q-tags in green (LLQG inserted at position 135). Numbers are by EU numbering.

stimulatory peptides to an anti-C-type lectin mAb αDEC205 (Figure 2a).[42] By genetically introducing the sortase tag to the C-terminus of the heavy chain the group generated 49 different derivatives of αDEC205. That number of constructs would have been difficult to achieve using genetic engineering only. Importantly, it allows for the introduction of non-natural moieties and removal of the His-tag, analogous to the previous example. This enabled the characterization of the stimulation of dendritic cells *in vitro* and the immune response in a mouse model with a palette of epitopes as well as different linker sequences fused to αDEC205. The same group also combined SrtA with butelase 1, a plant enzyme that works analogously to SrtA that, however, uses a C-terminal N/HV recognition sequence which is transacylated with a N-terminal NH_2-X_1X_2 sequence (X_1 = not proline, X_2 = Leu, Ille, Cys, or Val).[43] By introducing a sortase tag on the light chain and butelase 1 tag on the heavy chain of IgG1 a dual-labeled antibody was produced in a one-pot reaction.

Despite the significant successes of SrtA, it has its limitations. Wild type SrtA is a very slow and highly Ca^{2+} dependent catalyst. Both problems have been tackled with directed evolution, yielding faster and Ca^{2+} independent enzyme variants.[44,45] These improved variants have become the industry standard for SrtA transformations. Additionally, the more recently discovered and functionally similar enzymes from the Asx ligase family butelase 1 and OaAEP1 have significantly higher catalytic proficiencies with minimal engineering necessary.[46,47] A more fundamental limitation of these enzymes is their hydrolytic mechanism, which can suffer from reversibility. Many of the previously mentioned examples circumvent this by using large excess of nucleophile, conditions unsuitable for large scale processes or for high value coupling partners. Another caveat is the production of Asx ligases, since in plants they are expressed in a zymogenic form and often are difficult to express recombinantly. However, this has already been improved in the case of OaAEP1 through expression as ubiquitin fusion proteins as well as structure guided protein engineering to boost the wild type's ligation efficiency.[46,48]

Reaction reversibility can be overcome by substrate and reaction engineering, for example, running ligations in flow conditions,[49] installing activated bonds in the substrate tag,[50,51] self-inactivating leaving groups,[52] using linker regions that form a SrtA unreactive tertiary structure upon ligation,[53] and by adding Ni^{2+} to the reaction to complex the leaving group (requiring a sortase tag containing a His_6 tag).[54] Another common strategy is to engineer serine protease enzymes by replacing the active site serine with a cysteine in combination with a payload that contains an activated depsipeptide or ester bond.[55−57] Cysteine is sufficiently nucleophilic to react with the payload's ester bond and can subsequently perform the transpeptidation, but it renders the enzyme less active for amide bond cleavage, thus reducing the back reaction. Two such engineered enzymes, peptiligase and omniligase-1, have found applications in producing peptidic drugs up to gram-scale (Figure 1).[58,59] Interestingly, these reactions often achieve full conversion in short reaction times (minutes) and the substrates can be used with small excesses (e.g., 1.1 equiv) of depsipeptide. With this methodology being very successful at synthesis of peptides and model substrates,[60,61] similar

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applicability can be expected in future applications with proteins as ligation partner.[62]

Trypsiligase, an engineered trypsin protease, similar to SrtA recognizes a Tyr/Arg-His sequence and transacylate with a Arg-His tagged payload for C-terminal labeling (Figure 1).[63] For N-terminal labeling, payloads activated with a 4-guanidinophenyl ester (OGp), acting as an arginine mimic, can be used which react with N-terminal RH.[64] Importantly, this coupling can also be carried out with *in situ* cleavage of the purification-tag-Y/RH-protein-of-interest (POI) by trypsiligase and subsequent ligation with an OGp-ester. It has since been used to introduce click handles and cytotoxic payloads with a minimal ligation scar into the fragment antigen-binding region of an mAb,[65] as well as the PEGylation of antibodies.[66] The capability of N- and C-terminal tagging makes trypsiligase a very promising tool with possible downsides including: the initial cleavage reaction for N-terminal labeling being time-consuming, the need for a YRH sequence, and the requirement for zinc-(II) making it potentially unsuitable for later described radioligand approaches. However, concomitant cleavage of the purification tag during the ligation by Trypsiligase and the reduced reversibility of the OGp substrates combine the versatility of SrtA with the improved mechanism of peptiligase.

Another well-established enzyme is transglutaminase. It catalyzes the cleavage of ammonium from a glutamate side chain and the concomitant formation an isopeptide bond with a lysine residue's side chain (Figure 1).[67] In particular, microbial transglutaminases (mTGs) are interesting for their ease of production, broad payload substrate scope, and site specificity for the different "glutamine tags", or Q-tags, for instance, LLQG or c-myc-tag,[24,68−70] and in native glutamines.[71−73] Strop et al.[24] produced ADCs based on three mAbs (anti-EGFR, anti-HER2, and anti-M1S1) with mTG giving them tight control over the DAR and used the site specificity to examine how the attachment site influences drug properties. The authors tested 90 sites by genetic introduction of Q-tags in the constant regions of several mAbs. Only 12 positions possessed favorable biophysical characteristics. Conjugation efficiencies also varied depending on the specific mAb used. *In vivo* tests showed that ADCs generated by mTG coupling were similarly effective compared to ADCs prepared with maleimide chemistry. However, pharmacokinetic properties differed significantly. ADCs with payload linked to the C-terminal heavy chain were cleared faster and showed decreased stability compared to conjugates with the payload attached to the light chain.

In an important contribution, the same group improved ADCs with high drug loading (DAR 6 and 8) of monomethylauristatin through site-specific conjugation (Figure 2b).[74] The mAb−drug linkage proved to be more stable than corresponding maleimide-based conjugates with the same DAR, especially at high DARs. Even more importantly, through the ability of introducing the payload at sites that are spatially separated, the group managed to produce a DAR 6 ADC with similar *in vivo* properties to an unconjugated mAb and high efficacy against lowly expressed target compared to similar ADCs prepared with maleimide chemistry. Maleimide-based conjugation led to the payload molecules reacting with cysteines that are located close together. The resulting hydrophobic patches of the payload clustering together is thought to distort the mAb structure and impede its function. Several other examples have shown mTG mediated conjugation to mAbs with diverse payloads such as chelators,[68] biotin,[72] and toxins.[73,75] Conversely to the previous examples, mTG can also tag lysine with a payload containing a glutamine residue.[76] This affords the attachment of two orthogonal payloads to lysine and glutamines with mTG; an application of this will be discussed further in the radioligand therapy section.[77]

Another way of creating intrachain modifications is the use of formylglycine-generating enzyme (FGE, Figure 1).[78,79] It recognizes the CXPXR motif and converts the cysteine residue to formylglycine (fGly). This can be achieved both *in vitro* and by *in vivo* coexpression of FGE. The aldehyde functionality can then be functionalized using aminooxy or hydrazide probes with >90% final conversion. The hydrolytic instability of the respective hydrazone or oxime products, as well as the need for low pH or catalysts for the aldehyde functionalization, have since been circumvented by using the trapped-Knoevenagel reaction,[80,81] Pictet−Spengler bioconjugation,[82] or the hydrazino-*iso*-Pictet−Spengler (HIPS) reaction.[83,84] Additionally, increased copper concentrations boost activity toward less reactive recognition motifs with a proline to alanine mutation (e.g., CTAGR).[85] Genetically introducing two distinct motifs and performing oxidations at different copper concentrations establishes orthogonal control over the sites, which has been used to create dual-labeled antibodies and DARPINs. In an important example, Drake et al. used coexpression of human FGE and genetically engineered trastuzumab, introducing an aldehyde tag either in the light and heavy chain constant regions, and the C-terminus of the heavy chain.[86] Employing HIPS, the group achieved high Cys to fGly conversions between 86% and 98% as well as >90% conjugation efficiency in the heavy chain constructs and 75% for the light chains. Additionally, choosing different conjugation sites tuned the pharmacokinetic parameters while retaining target binding. The C-terminal tag proved to have longer half-life time *in vivo* which ultimately translated to higher efficacy in a mouse xenograft model. An approach to overcome the need for high equivalents of a payload is to first introduce an azide with the HIPS or trapped Knoevenagel reaction and subsequent strain-promoted azide−alkyne cycloaddition (SPAAC) to introduce the payload.[87] Notably, the drug candidate TRPH-222, an anti-CD22-mAb maytansine conjugate drug, which is prepared using the HIPS technology, has recently passed Phase I studies for relapsed and/or refractory B-cell non-Hodgkin's lymphoma.[88]

The use of sortase, FGE, and to a certain extent transglutaminase depend on specific peptide tags of the protein target. This limits the technology for native protein modification. Directed evolution can be used to change recognition sequence acceptance, however, such engineering efforts are often ambitious. For example, sortase has previously been engineered with considerable effort to recognize different sequences.[89,90] For immunoglobulins, glycoengineering can offer a more universal approach to form bioconjugates.[30] Chemical methods of introducing aldehydes in glycans by oxidation of vicinal alcohols exist but long exposure to oxidants may decrease the quality of the final bioconjugate.[91] Oxidases are a good alternative with mild reaction conditions for such reactions which have been applied for bioconjugation (Figure 1).[92] One challenge to overcome is the heterogeneity of glycans that are attached to the antibody.[93] This can be circumvented by developing cell lines with specific glycan structures,[91] or engineering the glycan composition using enzymes *in vitro*.[94,95] The latter approach has been used by

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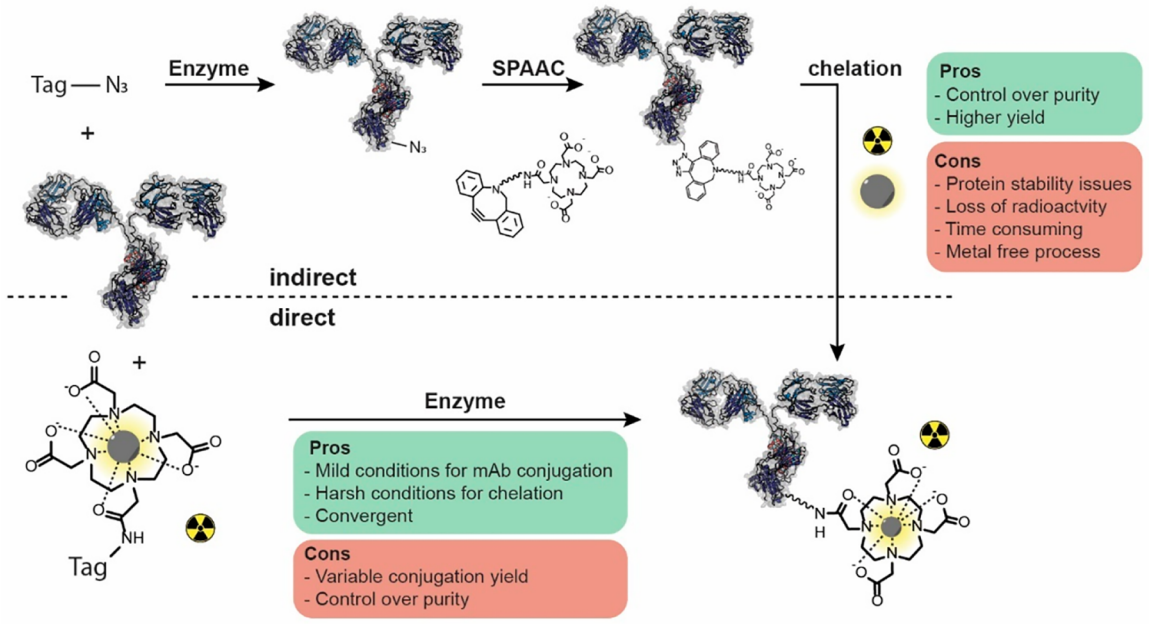

Figure 3. Most strategies in biocatalytic conjugation of RLT payloads to date describe an indirect approach (top), where the protein is first functionalized with the chelator in multiple steps and later charged with the radioactive metal. This process is robust; however, it involves many steps, requires metal-free conditions and long incubation times, and can result in loss of radioactivity. The direct approach, where precharged chelator payload is ligated to the protein of interest, could alleviate most of these issues but requires a very efficient enzymatic step.

Angelastro et al. to introduce galactose at the nonreducing end of the dominant G0F glycoform found on trastuzumab to β-1,4-galactosyltransferase to produce four aldehydes per mAb.[96] A tandem Knoevenagel reaction was used to introduce two azide-containing linkers per aldehyde, resulting in eight potential conjugation sites. The authors showed that this sequence yielded an average of 7.3 azido groups per antibody. Further SPAAC with a dibenzylcyclooctyne-tetramethylrhodamine (DBCO-TAMRA) dye resulted in the main species being DAR 8. Another possibility is to directly introduce azide-labeled glycan mimetics using a glycosyl transferase.[97] The same company developed a hydrophilic linker, which in combination with glycosyl transferases are a promising platform for DAR 4 ADCs with improved stability and therapeutic index.[98] Monoclonal antibodies produced with this technology have also progressed in the clinic to a Phase 1b trial (NCT05389462).

Many additional methods have been explored that we're only going to be able to touch upon briefly. For instance, tyrosinase can form quinones from tyrosine side chains which can undergo strain promoted cycloaddition.[99] Horseradish peroxidase and hydrogen peroxide can be used to modify tyrosine in a single-electron transfer reaction.[100] Both methods proved to be site-selective, without depending on a specific recognition sequence, reacting with solvent accessible residues. Prenyltransferases can transfer non-natural geranyl phosphates to a CaaX motif,[101] with one ADC in clinical trials (NCT03944499).[102] A prenyltransferase substrate, convenient for orthogonal coupling of two payloads, has been reported.[103] Similarly, phosphopantetheinyl transferases (PPTases) can transfer coenzyme-A analogues to an 11 amino acid tag (GDALSWLLRLLN).[104] Subsequent work allowed shortening of the tag sequence and linker length, as well as proving *in vitro* efficacy and showing sufficient serum stability. Potential downsides of prenyltransferases and PPTases are the added synthetic effort for the geranyl phosphate and CoA analogues, respectively.

Mammalian enzymes have been employed for bioconjugation, often using recognition sequences native to the human proteome, potentially less prone to eliciting an immunogenic response. The Bode group, for example, reported a method to form intrachain linkages by repurposing the SUMO conjugating E2 ligase-like enzyme Ubc9.[105] The enzyme was shown to be promiscuous toward linking synthetic LRLRGG peptides simply modified with a C-terminal thioesters to the Lys side chain in a LKSE motif, thus bypassing the need for a cascade reaction with E1 carrier proteins. The system proved to be flexible enough to accept a multitude of POIs with the recognition motif. In one of many proof-of-concept experiments, the group used Ubc9 in tandem with sortase A to label trastuzumab Fab with two different dyes. Purification by size-exclusion chromatography yielded a nearly quantitatively labeled and functional Fab double-conjugate. In another example, the enzyme tubulin tyrosine ligase (TTL) has been shown to be effective in conjugating clickable tyrosine analogues to the C-terminus of VDSVEGEGEEEGEE.[106,107] TTL has proven effective for introducing 4-azide phenylalanine, allowing the subsequent SPAAC with DBCO-conjugated payloads. TTL requires a relatively long tag, but its ATP dependence could be an advantage to push the reaction equilibrium toward the product side.

As seen in these examples, enzymatic bioconjugation can be fast, selective, and high yielding. It has already proven useful for rapid prototyping of ADCs, creating well-defined products to probe the influence of the coupling position and DAR on stability and therapeutic efficacy of ADCs, as well as orthogonal attachment of multiple payloads. With the maturation of these methods, i.e., optimization of payload concentration, addressing reversibility issues, and removing the dependency on long recognition sequences, increasing numbers of ADCs prepared with such coupling methods can

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be expected to enter the clinic. Furthermore, many of the previously mentioned techniques for ADC generation are also applicable to different drug modalities, sometimes with specific caveats. These applications are highlighted in the following sections.

3. RADIOLIGAND THERAPIES

Radioligand therapy (RLT) designates the attachment of a radioactive moiety to a molecule that specifically binds to a target. RLTs can be used to image and effectively treat cancers. Given the transient nature of radionuclides, rapid attachment of the radioactive payload becomes critical. With faster production steps, more active compound is delivered to the biological target after administration. The loading of radionuclides into a chelator can either be done directly, where a chelator is charged with the radionuclide in isolation of the biopolymers and subsequently coupled to the biomolecule afterward, or, coupling is performed first, and the radionuclide then added to the protein-chelator conjugate (Figure 3). Directly coupling a chelator precharged with a radionuclide has the advantage of tolerating more forcing conditions during the chelation step, since the protein target is not present. Moreover, it could reduce the effort in downstream purification. However, reserving the bioconjugation step to the end is often only chosen as a second option if the protein is unstable under radiolabeling conditions.[108] Other challenges for RLT include working with large excesses of radioactive material, which clearly does not scale well and is associated with increasing production volumes. Furthermore, transformations prior to the chelation step must be metal-free to prevent chelation of undesired metals. The process should also be high yielding because labeled and unlabeled drug are often difficult to separate and unlabeled drug will compete for target binding with the active drug, decreasing the therapeutic effect. Here the benefits of biocatalysis could be exploited. Enzymatic couplings can be fast, selective for a specific site in the protein, depending on the enzyme metal-free, with straightforward purification.

Transglutaminase presents an interesting case, since the enzyme can couple relatively simple organic amines. An early example of Jeger et al. showed that it was possible to modify native and engineered glutamines with several payloads, including metal chelators.[68] Building on that work, the same group demonstrated the power of this approach for attaching radioligand payloads to antibodies with high site specificity.[77] By introducing glutamine and lysine residues into proteins, the authors created fully functional modified antibodies, single-chain variable fragments, and antigen-binding fragments (Figure 4). Attachment of *trans*-cyclooctene-tetrazine-PEG_4-NH_3 (TCO-PEG_4-NH_3) using microbial transglutaminase (mTG) from *Streptomyces mobaraensis* to a glutamine residue in a mAb yielded a versatile handle for metal-free conjugation of a metal charged 1,4,7,10-tetrakis(carboxymethyl)-1,4,7,10-tetraazacyclododecane glutaric acid)-anhydride (DOTAGA) metal chelator using the inverse electron-demand Diels–Alder reaction (IEDDA). Additional conjugation of an azide containing peptide to a lysine demonstrated how mTG can orthogonally modify a target with two reactive handles, enabling subsequent one-pot functionalization with two payloads through metal-free click chemistry. Enzyme immobilization eliminated the need for downstream purification. Whereas attachment of a single amine payload to a glutamate usually proceeded with excellent yields, >90% the reaction of

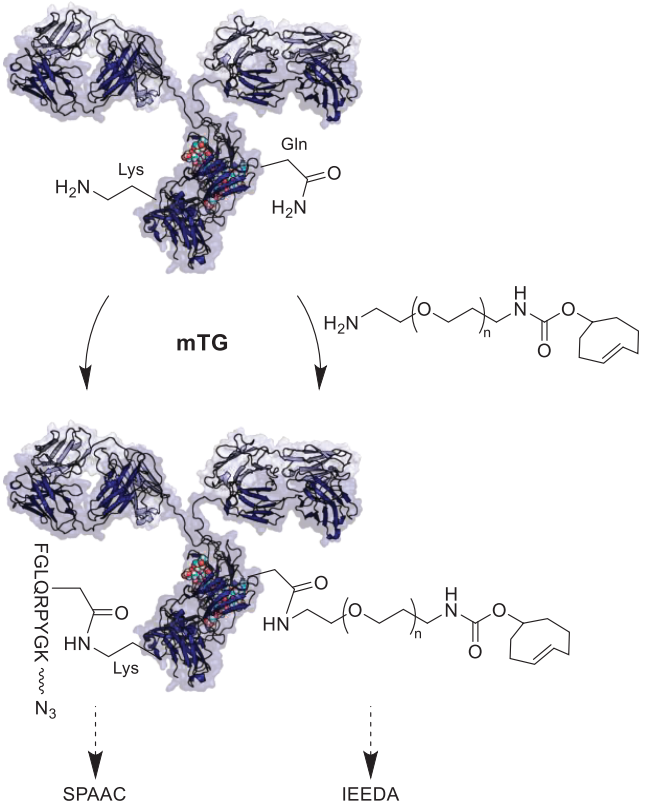

Figure 4. Microbial transglutaminases are capable of orthogonal labeling.[77] Payloads and reactive handles can be attached to glutamine-containing peptides or primary amines. Combining strain-promoted azide alkyne cycloaddition (SPAAC) and inverse electron demand Diels–Alder reactions (IEDDA) provides additional orthogonality for metal-free click chemistry.

lysine with labeled peptides proceeded with significantly lower yields (<70%). The dependence on larger peptide substrates for lysine functionalization and the need for large excesses of substrate are still limitations of mTG which can likely be overcome with directed evolution.

In contrast to transglutaminase, the selectivity of sortase A for a unique C-terminal sequence is in principle an advantage for forming homogeneous bioconjugates. This has for example, been exploited to tag nanobodies with a short peptide GGGYK where a chelator was attached to the lysine's side chain.[109] SrtA is limited by the chemical equilibrium of the reaction, which often necessitates high excesses of payload, sequestering the leaving group for instance by genetic incorporation of a C-terminal His_6 and the addition of Ni-NTA during the reaction or for subsequent purification, and running reactions in flow.[49] This poses a problem for RLT applications since chelators can strip Ni^{2+} from purification columns. Paterson et al. showed that SrtA conjugations can be used to create radiolabeled scFvs in high yields by exchanging the His-tag for a FLAG tag.[110] Additionally, the authors incorporated two tryptophan residues after the sortase tag, which has been shown to slow down the reverse reaction.[111] This enabled yields of up to 90% of a fully functional positron emission tomography tracer after purification with an anti-FLAG column.

A glycan engineering approach was used to make ^{89}Zr-labeled prostate specific membrane antigen-targeting antibodies.[112] First, β-1,4-galactosidase was used to cleave the terminal galactose off the oligosaccharide. Second, uracil diphosphate

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activated N-azidoacetylgalactosamine was coupled to the glycan using galactose transferase. The azide-labeled antibody was reacted with the chelator-cargo in a copper-free SPAAC, and the resulting antibody-chelator conjugate was subsequently radiolabeled with ^{89}Zr. This process turned out to be high yielding and very mild. Nonetheless, the process is time-consuming with multiple 16 h incubation steps, making it potentially impractical for less stable glycoproteins. Additionally, the long incubation times make the process unsuitable for directly coupling a "hot" material to the antibody. All the described processes in this section introduce the radioactive labeling after bioconjugation with a chelator or a click handle. This necessitates metal-free click chemistry and mild chelation conditions in the former case. Immobilized biocatalysts with fast reaction kinetics, such as OaAEP1, are likely a great platform for rapid generation of radioactive therapeutics. It could reduce the need for long reaction sequences under metal-free conditions and extensive downstream purification and give rapid access to radioactive drugs.

4. POLYVALENT CONJUGATES

So far, we focused mostly on the conjugation of small molecules and peptide payloads to proteins. Many of the previously described enzymatic transformations also lend themselves to the creation of bivalent protein conjugates, i.e., the covalent linkage of two proteins. This was recently the topic of an excellent review by the Bernardes group and will only be touched upon here for illustrative purposes for further discussion of conjugates with higher valency.[10] In two publications, sortase A was used to create bispecific binders by linking a mAb with two scFVs.[113,114] Here click handles for SPAAC were introduced in both proteins with SrtA and a C- to C-terminal linkage was created in a subsequent reaction. In another study, a combinatorial library of designed ankyrin repeat proteins (DARPins) was created by preparing a N-terminal glycine residue by TEV protease cleavage and direct transpeptidation with sortagged DARPins creating a C-to-N-terminal linkage.[115] The direct transpeptidation enables very facile generation of libraries without the need for intermediate purification steps. Installing separate click handles adds an additional step to the synthesis, however, it enables using higher substrate loading in the enzymatic step improving overall yield. Therefore, the latter approach is often used for creating conjugates with higher valency, where reaction efficiency is critical.

Polyvalent conjugates, in this context defined as three or more protein-payload attachments, can offer several advantages over their monomeric equivalents. For example, conjugating different binders can enhance the range of targets being neutralized.[116] Receptor engagement with multiple binders can induce receptor clustering and activation of signaling.[117] Additionally, having multiple binders in polyvalent complexes often increases their affinity to the target.[118] As an example, Moliner-Morro et al. used SrtA to introduce DBCO moieties at the C-terminus of a SARS-CoV-2 neutralizing nanobody.[119] The authors created a set of nanobody dimers and tetramers and compared them to the monomer and to nanobodies fused to a IgG heavy chain Fc region, creating a dimer. The dimeric constructs and Fc-fusion were equally potent and, at around 125 pM neutralization IC_{50} in an *in vitro* assay, approximately 150-fold more efficient than the monomeric nanobody. The tetramer showed an additional 10-fold improvement with an IC_{50} of 13 pM. A similar conjugation strategy, albeit with copper catalyzed cycloadditions, has been used to create up to tetravalent conjugates on peptidic dendrimers.[120] The same group has also reported the generation of heptameric proteins attached by click chemistry to an azide-decorated cylcodextrin.[121]

Targeting tyrosine for bioconjugation Minamihata et al. successfully used HRP to cross-link IgG binding peptides.[122] Introducing a GGGGY sequence at the C-terminus allowed the formation of a mixture of dimer and trimer by treatment with HRP and H_2O_2. The group showed that the polyvalent constructs possessed increased affinity for antibodies. However, it proved difficult to control product specificity and the resulting mixture of mono-, di-, and trimer is difficult to separate. In a follow up study, the same group created branched polymers by introducing an additional N-terminal tag.[123] The resulting polymer had a cross-linking degree of 70- to 150-mers.

These examples illustrate current issues with bioconjugation for multivalent complexes. Problems with low yields and specificity are exacerbated when generating polyvalent conjugates which often results in the requirement to perform multiple single conjugations rather than one convergent step. To generate well-defined polyvalent conjugates in a convergent fashion, extremely proficient conjugation reactions are required. Current enzymatic bioconjugation methods do not fulfill these criteria yet. Therefore, if using such processes during discovery phases, the approach might need to be changed when advancing to later development stages, leading to discontinuities in the development process.

5. ADENO-ASSOCIATED VIRUS VECTORS

Cell and gene therapy is a rapidly expanding area with already >20 FDA approved products.[124] Adeno-associated virus vectors (AAVs) are the current delivery method of choice for gene therapies[125] with hundreds of clinical trials ongoing.[126] However, AAVs remain challenging to manufacture and supply, due to their limited shelf life and instability during production and downstream processing.[127] Emerging research indicates that surface modificaton of the AAV capsids can help to (a) stabilize the AAVs and (b) improve their tissue specific target binding. Genetic[128] or chemical[129] modifications of capsids seem inevitable to further improve the therapeutic properties of this vehicle. In addition to peptide insertions, genetic code expansion with unnatural amino acids, serving as a handle for further ligand binding, has been demonstrated.[128] However, introduction of non-natural amino acids to the viral surface remains technically demanding. Ideally, bioconjugation could be performed on native protein structures. Surface-exposed and reactive residues, such as lysines, arginines, and histidines, have also been applied as conjugation sites for nonselective, chemical methods.[129] However, the number of ligands on the capsid surface may be highly relevant for the specific tissue targeting and the PK/PD properties of the virus. Could biocatalysis help to control the density of the ligand coating? How specific could an enzyme be in selecting a ligation (amino acid) site on the capsid surface?

Formylglycine generating enzyme (FGE) was studied for applications in AAV capsid modification.[130] A 13-amino acid concensus sequence LCTPSRAALLTGR was genetically inserted into AAV2 capsid to facilitate an FGE-catalyzed aldehyde formation. The aldehyde functionality then served as a handle for conjugation of fluorescent dyes, gold nanoparticles (Au-NPs), mAbs, and peptides. Using tunnel electron

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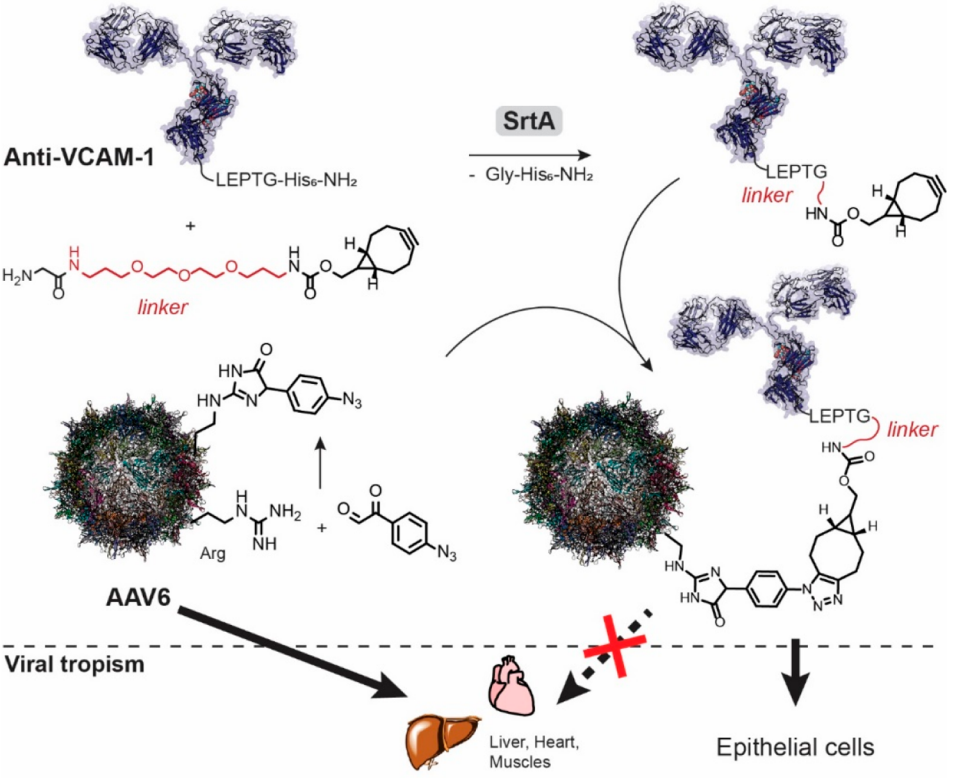

Figure 5. Sortase mediated introduction of a click handle to an antibody anti-VCAM-1 facilitated the synthesis of an antibody-AAV6 conjugate. Whereas AAV6 shows tropism toward liver and heart, conjugation with the para-azido glyoxal removes this tropism. Conjugation with anti-VCAM-1 retargets the virus to infect epithelial cells.

microscopy, the authors showed that on average six to nine Au-NPs were conjugated with a distribution from three to 13. With a single AAV2 containing 60 CXPXR tags, this equates to roughly 12% of all sites being converted to the conjugate. Similar results were obtained for the fluorescent dye conjugation. The near identical conjugation efficiencies of the large nanoparticle and the small dye indicate that steric hindrance of the cargo on the AAV surface is not limiting the conversion, but rather the efficiency of the FGE reaction or the aldehyde conjugation. The authors used this system to couple human leukocyte antigen mAbs to AAV2, which improved transduction by up to 37% in 293T cells. These results are promising for future FGE applications in creating functional virus engineered to target leukocytes. FGE is convenient due to the of *in cellulo* modification of the capsid. Issues with inefficient conjugations leading to wide payload:AAV ratio distributions could be tackled by optimizing the expression of FGE in the host cell.[131]

Similarly, Pearce et al. applied sortase A mediated conjugation of a single-chain antibody (ScFV) targeting vascular cell adhesion molecule (VCAM-1) to the surface of AAV6 (Figure 5).[132] The conjugated antibody facilitated enhanced transduction of AAV6 to endothelial cells. The conjugation strategy included: (a) introduction of a click-handle to AAV6 via an arginine residue in the form of 4-azidophenyl glyoxal, which simultaneously removed AAV6' native tropism, (b) sortase mediated conjugation of a PEG-linked BCN to the anti-VCAM-1 ScFV, and (c) combining the two via a click reaction. For sortase coupling, the anti-VCAM-1 was expressed as an LPETG-His_8 construct. After the sortase reaction, the unreacted anti-VCAM-1, the NH_2-G-His_8 leaving group, and the His-tagged sortase could be removed from the conjugation product via nickel affinity chromatography. This construction elegantly removes prior tropism and introduces selectivity for epithelial cell lines, as demonstrated by *in vitro* experiments. Comparable to the multivalent complexes, this modular approach is more laborious compared to the previous FGE-based method, but it allows for more control over each step.

In another *in cellulo* approach, coexpressed biotin ligase (BirA) from *Escherichia coli* has been used for AAV biotinylation (Figure 6).[133] Additionally, BirA was used to conjugate a ketone analogue of biotin to the surface of genetically modified AAV particles.[134] The attached biotin ketone analogue could serve as a site-specific handle to hydrazide or hydroxylamine probes. Both approaches require

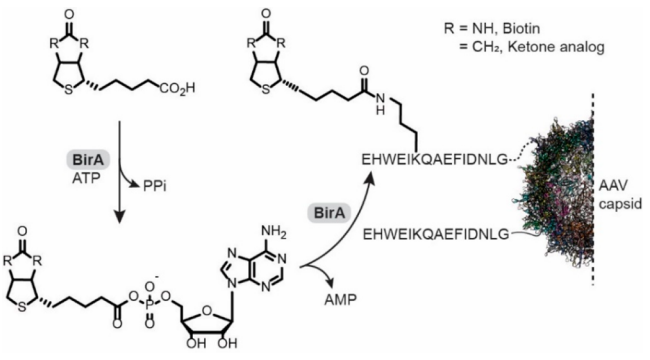

Figure 6. Biotin ligase (BirA) catalyzed biotinylation or biotin analogue ligation to AAV-BAP (biotin acceptor peptide) fusions. The reaction consumes an equimolar amount of ATP.

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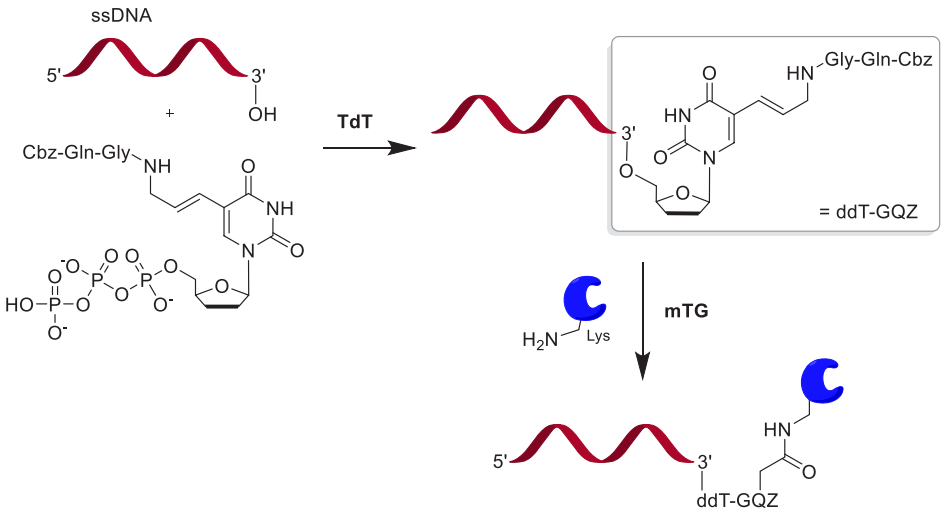

Figure 7. Terminal deoxynucleotidyl transferase mediated incorporation of a Q-tag (Cbz-Gln-Gly) labeled nucleotide dideoxy thymidine analogue (ddT-GQZ) into single stranded DNA oligonucleotide (ssDNA).[153] The lack of a 3′ hydroxy group on ddT-GQZ prevents the polymerization reaction and leads to a selective incorporation of a single tag at the 3′ end of the oligonucleotide. The Q-tag is further used to couple a protein presenting a lysine residue with mTG.

Table 1. Advantages and Limitations of Different Biocatalytic Strategies Which Have Been Used for AAV Bioconjugation

ligating biocatalyst	advantages	limitations
formylglycine generating enzyme	• aldehyde tag enables variety of chemistries	• requires a recognition tag
	• *in cellulo* modification by FGE coexpression	• incomplete conversion results in heterogeneous population of nanoparticles
		• potential side reactions of aldehyde in complex matrices
		• genetic modification necessary
sortase	• sortase has a broad nucleophile scope enabling introduction of variety of conjugate agents (e.g., for labeling and/or tissue targeting)	• requires a recognition tag
	• modular approach	• leaves a scar, e.g., LPET
	• control at each step	• more laborious than labeling during expression
biotin ligase	• biotinylation can be carried out *in cellulo* by BirA coexpression	• requires a recognition tag
	• biotin offers a purification handle	
	• flexibility of conjugate agents	

genetic introduction of the biotin acceptor peptide or also known as Avi tag (GLNDIFEAQKIEWHE) onto the capsid. Five different AAV serotypes were tolerant to the Avi tag insertion and biotinylation and the attached biotin also facilitated viral particle purification via avidin resin. The loading ratio of biotin to capsid was estimated to be 20:1 and no noticeable drop in infectivity was observed after biotinylation. This construct was efficiently transducing cells expressing scavidin, a chimeric biotin-binding membrane protein,[135] whereas unmodified viruses were unable to infect this cell line, even at high virus titers. Key advantages and limitations of biocatalytic strategies for AAV modifications are highlighted in Table 1.

6. OLIGONUCLEOTIDE BASED THERAPIES

Oligonucleotides are a very versatile class of drugs used to modulate gene expression and protein function via multiple modes of action including RNA splicing modulation, gene activation, programmed gene editing, and target degradation.[136] Their potential for treating currently incurable diseases is tremendous, and more than 10 oligonucleotide drugs have already gained FDA approval or are undergoing regulatory approvals.[137,138] Despite their huge promise, tissue targeting represents a major translational challenge. Several strategies have been developed to enhance oligonucleotide drug delivery and have been reviewed elsewhere.[139−141] Previously explored covalent conjugates to oligonucleotides include cholesterol, fatty acids, cell penetrating peptides, aptamers, antibodies, and sugars (e.g., GalNAc). The conjugation of specific molecules to oligonucleotides offers some advantages for targeted delivery over formulations with polymers or other transfecting reagents. Mostly, it provides a defined composition and simplifies analysis and characterization.

Few enzymatic bioconjugations of oligonucleotides have been disclosed. Most of them aim only to generate small quantities for cellular biology studies. For example, to study protein adduct influence on replication, GFP was azide tagged using a farnesyltransferase and subsequently coupled to DNA.[142] Nanobodies have been DBCO-functionalized with SrtA to couple to azide-labeled oligos for super-resolution microscopy.[143] The promiscuous activity of natural and engineered methyl transferases in combination with synthetic *S*-adenosyl methionine analogues to synthesize labeled nucleosides has been studied extensively at the 3′ end.[144] Similar reactivity can be carried out selectively on capped mRNA, directly installing a propargyl group on the first transcribed base.[145] However, the scalability of this chemistry is

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questionable. Often thousandfold excesses are required to drive the reactions toward completion. These limitations can potentially be overcome by clever engineering of cofactor recycling systems, as has already been shown for methylation reactions.[146] The promise of enzyme catalysis has also been recognized for the synthesis of DNA-encoded libraries.[147] For example, Thomas et al. chemically ligated an amine-functionalized DNA duplex to several 2-aminoethyl glycosides.[148] The glycoside was subsequently modified using glycosyl transferases, *trans*-sialidase, and galactose oxidase. Aldehydes resulting from the latter enzymatic reaction were further used for hydrazone ligation and reductive amination to introduce non-natural functional groups. This set of reactions could in the future be leveraged to create DNA-encoded carbohydrate-based ligand libraries with by simple split-and-mix synthesis.

Several approaches using transglutaminase have been tested to link proteins to oligonucleotides. Huggins et al. used transglutaminase to introduce an azide into a mAb and perform SPAAC using a synthetically DBCO-tagged oligonucleotide.[149] A recent applied example of transglutaminase is the conjugation reaction between a Q-tagged antitransferrin camelid antibody and 3-azido-1-propanamine which was subsequently used to link to an oligonucleotide using copper-free click chemistry.[150] The resulting molecule was shown to retain transferrin binding and to be capable of carrying siRNA payloads across the blood-brain barrier *in vivo*. More attempts at direct coupling of proteins and oligos using mTG were made with chemical amination of the 5′ base and subsequent mTG coupling to GFP.[151] Polymerases have been identified for introduction of amide-functionalized bases; however, it is hard to control the site-specificity and stoichiometry of the attachment.[152] The use of template-independent incorporation of Cbz-Gln-Gly-labeled ddUTP by terminal deoxynucleotidyl transferases (TdT) allows for more controlled incorporation of a single tag (Figure 7).[153] Subsequent reaction with mTG and enabled the synthesis of aptamer-protein conjugates.[153,154] Whereas mTG reactions worked well with a relatively low 5-fold excess of POI,[152] the TdT reaction may be limiting when scaling up with 100-fold excesses of nucleoside analogues required. This remains an emerging technology, and TdT efficiency can likely be improved using enzyme engineering.[155] Additionally, TdT only acts on DNA, but alternatives to carry out similar reactions on RNA exist.[156]

It is worth mentioning that enzymes are already used in the synthesis of oligonucleotides, offering an alternative to traditional solid-phase synthesis using phosphoramidite building blocks. As the increased need for synthetic DNA and renewed interest into RNA- and DNA-based therapeutics puts pressure on existing solid-phase synthesis capacities, more sustainable solution-phase approaches are required. These alternatives are represented by RNA/DNA ligases,[157] polymerases capable of accepting non-natural nucleotides,[158] template-independent DNA synthesis dependent on TdT,[159−162] and caged bases that are incorporated by polymerases and random priming.[163] The same applies to synthesis of building blocks of oligonucleotide molecules where enzymatic syntheses of unnatural mono-[164] and cyclic dinucleosides[165] have been developed. As outlined here, enzymatic methods to manufacture therapeutic oligos and their coupling to therapeutic and targeting agents are still in their infancy. With the increased therapeutic application of synthetic oligos, we see a wealth of applications ahead for enzymatic oligo synthesis.

7. CONCLUSION AND OUTLOOK

As highlighted in the previous sections, every therapeutic modality poses its own challenges and opportunities for enzymatic bioconjugation. In parallel to the expanding palette of therapeutic modalities, a multitude of bioconjugation reactions using enzymatic catalysis have been established as important research tools. Sortase A, transglutaminase, and formyl-glycine generating enzyme are the most investigated enzymes and have proven their utility for almost every therapeutic modality.[27,67,166] Importantly, biocatalysts offer unique advantages over chemical conjugation: Sortase A can cleave a purification tag concomitant to the conjugation reaction, transglutaminases are efficient and offer the possibility of dual-labeling, and FGE can be used co-translationally, yielding a protein with an orthogonal reaction handle after expression. More recently discovered enzymes show great improvement in their substrate scope and reaction kinetics.[46,47,56,65,96]

In terms of the individual therapeutic modalities, ADCs are the most explored case for enzymatic bioconjugations. Here, enzymes have proven very useful for rapid prototyping,[42] site-selectivity,[9,24,25] achieving high DARs,[96] creating especially stable linkages,[81,86] and orthogonal control over multiple payload attachments.[43,77] The latter application we expect to be increasingly important with the growing complexity of newer ADC formats, e.g., with multiple payloads.[167] For RLTs, developments in orthogonal and metal-free tagging with transglutaminase are most notable.[77] We expect future applications exploring enzyme immobilization as well as enzymes with faster kinetics to simplify downstream purification and to preserve the final compound's radioactivity. Oligonucleotide conjugates have great potential to improve the activity profile and scope of oligonucleotide-based therapies. For oligonucleotide conjugates, similar strategies as for ADCs and RLTs as well as DNA and RNA modifying enzymes have been explored. Additionally, novel enzymatic oligonucleotide-synthesis methods are expected to play a future role. Applications of enzymatic bioconjugation toward making multivalent conjugates and surface-modified AAVs represent a real challenge. Incomplete transformations often lead to a complex range of products being formed and are difficult to analyze or separate. Additionally, similar conjugation strategies as described in this Perspective may be amenable to other new modalities such as modified peptides, peptide nucleic acids, oligosaccharides, and other chimeric molecules (e.g., proteolysis-targeting chimeras).[168]

As discussed, some challenges still exist: the presented methods are still far from offering a universal and traceless system and may result in a mixture of products. Methods for native protein conjugation are required to avoid introduction of genetically encoded recognition tags, which inadvertently change the protein sequence. Further developments in this direction would decrease molecular cloning efforts and alleviate concerns regarding immunogenicity of the new sequence. Processes with higher efficiencies would be needed to limit incomplete reactions and side-product formation. Until these points are addressed, enzymatic bioconjugation still faces stiff competition from small molecule chemistry. However, in comparison, it is arguably early days for the use of enzymatic bioconjugation in drug development, but enzymes harbor a lot

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of synthetic potential. For example, in synthetic organic chemistry, enzymes have been increasingly applied in the recent decades and nowadays their use is commonplace, from single reactions to entire synthetic cascades.[164,169] We predict a similar trajectory for the use of enzymes in bioconjugation. Additionally, many bioconjugating enzymes have not yet been subjected to and improved by directed evolution or engineering. This will likely enhance the utility of those biocatalysts further and enhance the panel of possible reactions. Illustrative examples are the large array of widely applied SrtA variants,[29] the development of peptiligase,[55] and the improvement of OaAEP1 for bioconjugation.[46]

In some cases, the above-mentioned challenges have already been overcome, as proven by the recent entrance of several biocatalytically produced ADC drug candidates into clinical trials.[7] Patented processes of SrtA conjugations for ADCs report overall yields of up to 76% and reveal the reliance on binding resins to remove the affinity-tagged leaving group and retain unconjugated mAbs.[170,171] Pfizer carried out a Phase 1 study with an antitrophoblast cell-surface antigen-2 ADC produced with mTG.[172] Their patented processes are similar to scientific publications, with >20 equiv of the payload followed by purification with affinity chromatography and hydrophobic interaction chromatography, resulting in conjugation yields of up to 85%.[173] Recently, a glycoengineered ADC has entered clinical trials (NCT05389462). The conjugation process includes partial removal of glycans and addition of an azide-labeled galactose; preparative and analytical scale reactions seem to only differ in the purification methods.[97,174] Processes with >70% overall yield by *in vivo* coexpression of FGE and conjugation using HIPS chemistry have also been patented, and the corresponding products have entered clinical trials.[88,175]

These successes showcase that established biocatalytic processes can already yield sufficient product to support clinical trials. The outcomes of the ongoing trials may inform the development of best practices and protocols for the manufacturing, characterization, purification, and reporting of enzymatic bioconjugations. This progress will be vital as drug modalities increase in complexity and with more widespread application of enzymes. With several new companies founded that either specialize in enzymatic bioconjugation or base their products around a specific biocatalytic platform and more frequent off-the-shelf availability of biocatalysts and corresponding substrates, enzymatic methods are gaining momentum and are easier than ever to apply. Therefore, the broad implementation of enzymatic bioconjugation in research and subsequent development phases remains, in our opinion, merely a matter of time.

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Notes

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