Disulfide conjugation chemistry: a mixed blessing for therapeutic drug delivery?

“...we anticipate that conjugate linkers ... will enable site-specific drug release and most certainly will lead to a new generation of well-defined and efficacious nanomedicine.”

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Therapeutics against cancer and viral infections commonly suffer from several drawbacks including nonselective cytotoxicity, high immunogenicity and low circulation half-lives. In order to alleviate these challenges, therapeutics developed in the last decade have embraced conjugation of synthetic polymers and antibodies to remedial and cytotoxic payloads. Among the plethora of these (bio)hybrid therapeutics, attachment of poly(ethylene glycol) (PEG) to biological pharmaceuticals as well as antibody–drug conjugates are prominent examples approved for clinical application [1,2]. Despite providing stealth, prolonging circulation life times and reducing immunogenicity of these therapeutics, clinical limitations of PEG conjugation (PEGylation) include its nondegradability. Additionally, maintaining biological activity after conjugation remains a challenge particularly when conjugating polymers to peptides, proteins with macromolecular substrates or proteins that bind substrates requiring a large proportion of its solvent-exposed surface [3,4]. Similarly, permanent polymer conjugation to small molecule inhibitors often results in reduced potencies [5].

The conjugation chemistries employed to attach polymers can also lead to profound effects on the ultimate activity of the biological therapeutic. Conventional polymer conjugation techniques were typically randomly performed on peptides and proteins. However, this nonspecific (random) conjugation

may lead to detrimental effects on biological activity. Random conjugation chemistries also increase heterogeneity of antibody–drug conjugates. For example, the antibody–drug conjugate, trastuzumab–emtansine (Kadcyla®) is produced via conjugation of maleimide linkers to lysine residues. This leads to a high degree of heterogeneity due to incomplete conjugation of the drugs to maleimide linkers, resulting in average drug to antibody ratio of 3.5 [6]. Alternatively, thiol couplings have allowed for site-specific conjugations and have permitted development of homogeneous antibody–drug conjugates. Brentuximab–vedotin (Adcetris®) is synthesized via the attachment of a cathepsin B-cleavable antimetabolic agent monomethyl auristatin E to a cysteine obtained via partially reducing interchain disulfides on the antibody [7]. The drug conjugation method employed in the development of the brentuximab–vedotin conjugate also resulted in drug-to-antibody ratios variability averaging about eight drug molecules per antibody due to the nonselective nature of the reduction process.

Thiol–thiol couplings, also known as disulfide conjugation, has been a tremendously popular conjugation strategy, in part due to the orthogonality it possesses in lieu of other conjugation chemistries [8]. Not only does the orthogonality of disulfide-based chemistry permit site-specific polymer attachment, it is also amenable to biological

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and nonbiological therapeutics and allows for modulating the strength of the conjugation depending on the sterics induced by groups adjacent to the disulfide. This ultimately allows for tuning the release of the therapeutic under specific reducing environments such as when a therapeutic is taken up by a cell. The ease of conjugation of polymers and antibodies via disulfide bonds have been overshadowed by mixed results obtained from *in vitro* and *in vivo* experiments. This editorial briefly highlights recent failures and successes of disulfide chemistry when applied to therapeutic drug conjugation to polymers and antibodies.

Failures of disulfide conjugation chemistry

Early thiol conjugation methods involved a direct coupling to thiol moieties present on the therapeutic. Antibodies known as *thiomabs* consisting of one or two cysteine residues have been developed to assist in this type of drug conjugation [9,10]. While this is an elegant way to generate conjugates via site-specific conjugation, the process to engineer antibodies to contain extra cysteine residues can be laborious and could cause complications during protein folding. Nevertheless, thiol couplings with other engineered proteins has been explored, for example, site-specific modifying proteins through thiol-exchange of naturally existing disulfide bonds. Successful PEGylation of L-asparaginase through a native disulfide bond while maintaining biological activity was demonstrated, although immunogenicity was retained [11]. Conversely, site-specific PEGylation through a native disulfide bond on IFN- α 2b resulted in a 92% reduction in its antiviral activity and was therefore comparable to the loss in antiviral activity obtained by random PEGylation of lysine residues on IFN- α 2b [12]. One major disadvantage of this type of disulfide conjugation is that it is not directly amenable to most synthetic (small molecule) therapeutics as they do not have thiol moieties that can be modified. Thus in lieu of direct thiol couplings, linkers possessing a free thiol moiety are attached to the abundant amine groups on the protein or targeting antibody to gain access to nonpermanent (reversible) therapeutic attachment. One such method includes the modification of the therapeutic with a linker that exposes a thiol moiety, which could subsequently be attached to a polymer via formation of a disulfide bond.

Another conjugation method is to bind to the disulfide by forming a bridge between the two resultant thiols. For example, dibromomaleimide modified cytotoxic drugs have been linked to disulfide bonds on antibodies, allowing the preservation of stable linkages between the Fab and Fc regions [13]. The dibromomaleimide coupling strategy has also been applied to the conjugation of polymers to proteins. This disulfide

coupling technique, however, results in a chemical tag, irreversibly modifying the antibody or protein, and may induce immunogenicity (e.g., [14]).

The use of self-immolative linkers has permitted temporary drug conjugation to polymers and antibodies. In the case of antibody–drug conjugates, these linkers can be engineered to act as ‘traceless’ bond from which cleavage results in the native antibody and unmodified drug [15]. Self-immolative linkers containing disulfides have hypothesized to specifically permit the release of the drug in its unmodified form through reduction processes that are contained within cells. For example, the tripeptide glutathione is present in high (millimolar) concentrations in cells whereas only submicromolar concentrations are present in the extracellular space [16]. As such, antibody–drug conjugates incorporating disulfide linkers can be targeted to the tissue, ultimately resulting in glutathione-mediated drug release in close proximity to its therapeutic target. Lewis Phillips and co-workers assayed a number of trastuzumab–maytansinoid antibody–drug conjugates using several traceless linkers, which had methyl groups to progressively investigate disulfide linker hindrance on the biological activity of the conjugates [17]. However, the maytansinoid linked by a nonreducible maleimide-thioether linkage showed superior activity compared with trastuzumab–maytansinoid (DM1) conjugates made with disulfide linkers with increasing hindrance induced by methyl groups. The conjugate with the least hindered disulfide linker was shown to be rapidly cleared and thus contradicts the hypothesis that selective intracellular release by disulfide reduction favors the efficacy of the antibody–drug conjugate. Another antibody–maytansinoid conjugate – cantuzumab–mertansine (C424-DM1) – utilized a methyl hindered disulfide linker, demonstrated high activity in xenograft tumor models [18]. Unfortunately, the disulfide linker utilized in the humanized antibody conjugate proved to be relatively unstable *in vivo* [19].

Successful disulfide conjugates

Converse to the superior activity of the permanent trastuzumab–maytansinoid conjugates described as above, Kelly *et al.* found that humanized antibody huB3F6–maytansinoid (DM4) conjugate with a dimethyl hindered disulfide linkage exhibited higher antitumoral activity in xenograft models in mice than a permanently linked antibody–drug counterpart [20]. Studying a comparable DM4 conjugate using a huC242 antibody, the higher activity exhibited by the dimethyl hindered disulfide conjugate was attributed to the generation of an *S*-methyl maytansinoid derivative following lysosome processing. This bystander metabolite was found to be highly toxic to

cells and proved very effective. By contrast, a lysosome-processed lysine maleimido–maytansinoid derivative demonstrated very low cell killing potency [21].

Self-immolative disulfide linkers used in antibody–drug conjugates have also been applied as side chain linkers in polymer–drug conjugates. The disulfide linked antiviral prodrugs have shown high stabilities in fetal bovine serum [22]. As human serum albumin comprises a single active cysteine residue (Cys-34) and constitutes up to 80% of serum thiols, potentially undergoing disulfide exchange prematurely causing antiviral prodrug release before cell uptake. Polymer–drug conjugates with unhindered disulfides linked to antiviral prodrugs showed stability in the presence 0.5 mM human serum albumin at 37°C and in turn suggests that drug release is selective upon cell internalization [23].

Conclusion & future perspective

Both hindered and unhindered disulfide-linked conjugates have shown to generate promising leads as useful selectively cleavable linkers in drug delivery applications. However, most disulfide-based conjugate literature focuses on demonstrating drug efficacy rather than elaborating on stability of the disulfide-based conjugates in serum. Conjugate stability in serum and in the circulation, after all, is of utmost importance particularly for conjugates whose aim is to attain prolonged drug lifetimes (e.g., polymer–drug conjugates) or drug targeting effects (e.g., antibody–drug conjugates). In addition, it is equally important to understand the effects of linker cleavage on toxicity and immunogenicity, especially those in drug conjugates that are released prior to cell internalization. For

example, the generation of free thiols on antibodies as a result of drug release prior to cell uptake could potentially lead to antibody aggregation, a process that has been linked to immunogenicity [24]. Another aspect to take into account is that long-term studies are required to determine whether the conjugates are indeed selectively released upon cell internalization or are cleaved through a slow degradation process. Additionally, several conjugates, particularly comprising minimally hindered disulfide linkers, have shown promising applications in early stages of research but exhibit slow premature drug release when assessed at longer time scales. Nevertheless, disulfide chemistry has demonstrated to be a popular, versatile conjugation technique that can bind a plethora of (pro)drugs to polymers and antibodies resulting in efficacious conjugates, particularly if hindered disulfides are utilized. As such, we anticipate that conjugate linkers – including the linkers beyond the scope of this editorial – will enable site-specific drug release and most certainly will lead to a new generation of well-defined and efficacious nanomedicines.

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