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Review article

Bioorthogonal strategies for site-directed decoration of biomaterials with therapeutic proteins

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ABSTRACT

Emerging strategies targeting site-specific protein modifications allow for unprecedented selectivity, fast kinetics and mild reaction conditions with high yield. These advances open exciting novel possibilities for the effective bioorthogonal decoration of biomaterials with therapeutic proteins. Site-specificity is particularly important to the therapeutics' end and translated by targeting specific functional groups or introducing new functional groups into the therapeutic at predefined positions. Biomimetic strategies are designed for modification of therapeutics emulating enzymatic strategies found in Nature. These strategies are suitable for a diverse range of applications – not only for protein-polymer conjugation, particle decoration and surface immobilization, but also for the decoration of complex biomaterials and the synthesis of bioresponsive drug delivery systems. This article reviews latest chemical and enzymatic strategies for the biorthogonal decoration of biomaterials with therapeutic proteins and inter-positioned linker structures. Finally, the numerous reports at the interface of biomaterials, linkers, and therapeutic protein decoration are integrated into practical advice for design considerations intended to support the selection of productive ligation strategies.

1. Introduction

Covalent conjugation of therapeutic peptides or proteins to biomaterials or polymers opens promising new interfaces for the design of functionalized implants with improved pharmaceutical properties [1]. Among these, attachment of targeting moieties to biologics as done with antibody-drug conjugates (ADCs) [2,3], polymer conjugation (e.g. PEG conjugates) [4,5], immobilization on biomaterials [6–8] and preparation of controlled release delivery systems [9] are preeminent examples improving efficacy, pharmacokinetics (PK), distribution and safety of therapeutics. Despite this great potential, current approaches have limitations, particularly resulting from the heterogeneity of formed conjugation products as of unspecific modification. It is this heterogeneity, giving rise to analytical as well as safety and efficacy concerns. For example, some ADCs with heterogeneously attached cancer drugs had reduced clinical efficacy compared to the free drugs which was at least in part linked to unspecific coupling chemistries [3]. Similarly, the development of Mylotarg® (Gemtuzumab ozogamicin) – the first ADC approved by the FDA in 2000 and unspecifically carrying the cancer drug at the antibody's amine groups – was withdrawn from the market in 2010 following a post marketing clinical trial for insufficient clinical benefit and toxic side effects associated with its heterogeneity [10]. It was further detailed that the drug-antibody ratio (DAR) strongly impacted Mylotarg's therapeutic efficacy with the large portion of the unlabeled antibody (of ~50%) serving as a competitive inhibitor to the ADC for cellular uptake which is a prerequisite for its activity [11]. Conversely, a high DAR commonly increases toxicity and hydrophobicity of the ADC, thereby inducing precipitation, reducing stability and accelerating plasma clearance [12,13]. Especially in case of ADCs with cytotoxic payload, statistical conjugation of the highly potent drug affects the therapeutic window of the ADC jeopardizing safe and efficient therapy [3]. Moreover, multiple drug-loaded forms of ADCs (usually with DARs from 0 to 8 [14]) have distinct PK *in vivo* and differ in clinical performance, immunogenicity, clearance and stability [15,16]. Despite narrowing the DAR by purification, the multitude of different conjugation sites – e.g. in case of Kadcyla® (Trastuzumab emtansine) 70 of the 88 lysines were shown to be conjugated – generates a mixture of ADC species with substantial differences in clinical performance and PK properties [17]. Consequently, 'second generation' ADCs were developed with the ultimate goal to reduce product heterogeneity providing a therapeutic tool with predictable properties and batch-to-batch consistency [2,18–21]. Recent effort was placed on (i) identification of regions that are well suited for site-specific drug attachment [22,23], (ii) development of versatile chemical linker strategies to modulate drug release [24] and (iii) pharmacokinetic/pharmacodynamic modeling [25].

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In analogy to this example, these considerations are also promoting the development of conjugates for other therapeutic proteins such as enzymes, growth factors or cytokines for which new synthetic approaches are sought leading to clearly defined physicochemical, biological and pharmaceutical properties. Batch-to-batch quality differences of such heterogeneous products causes concerns as illustrated by the recall of five batches of PEG-asparaginase between 2000 and 2003 due to deviations in activity associated with unspecific coupling [26]. The polydispersity of various species of multi-PEGylated variants and positional isomers involves difficult separation and mitigates biological activity for a significant fraction of the product species. For example, PEGylated Interferon α-2a retains only a fraction of its activity (7%) in comparison to the original protein [27], as it is PEGylated at 9 different lysine residues resulting in 9 positional isomers exhibiting significantly distinct specific bioactivities [28]. Essentially the same was observed for unspecifically surface-conjugated enzymes, such as glycosyltransferase, for which a loss of bioactivity by ¼ was reported whereas site-directed approaches had a loss of $^{1}/_{10}$ only [29]. Due to the convincing body of data demonstrating the advantages of site-directed coupling strategies, a change of direction becomes apparent with the first site-specifically conjugated ADCs entering clinical trials [30,31]. Furthermore, among the recently FDA-approved PEGylated proteins and those in phase I-III clinical trials a growing number is produced by bioorthogonal methods (e.g. Cimzia®, a PEG-anti-TNFα antibody fragment or Neulasta®, PEGfilgrastim). However, site-directed conjugation chemistries are yet to find broad entry into bioengineering of functionalized biomaterials [6,32–39].

In this review, we summarize novel, bioorthogonal chemical and enzymatic coupling strategies and discuss them with a focus on the site-directed decoration of biologics and the functionalization of biomaterials. Pros and cons of each strategy are critically discussed. We are addressing novel approaches integrating the complexity of site-directed coupling of (i) therapeutic proteins, (ii) linkers, and (iii) biomaterials. Ultimately, we try emulating these various strategies into one blueprint/flow chart in an effort to facilitate future selection processes for those who aim for the site-directed conjugation of therapeutics to a biomaterial or polymer with or without inter-positioned linkers.

2. General considerations for protein based bioconjugates

Covalent conjugation of biologics to biomaterials allows for implant materials with lasting bioactivity [40]. The challenges for biologic-decorated biomaterial manufacture are manifold, more importantly the preservation of the therapeutics' stability during the coupling reaction and during storage [41] and – with arguably increasing importance - site-directed decoration for the aforementioned advantages [42]. This is why the focus is on mild reaction conditions to preserve the integrity and biological function of the biologics to the maximal possible extent [43]. 'Biologics' in our context are a diverse set of molecules (e.g. peptides, polypeptide hormones, large proteins, high molecular weight antibodies, but also nucleic acids, blood products and vaccines) and it is this diversity requiring a suite of chemical/enzymatic strategies for synthesis and a guided rationale for selecting the most promising one for conjugate development.

The consequences of unspecific versus specific labeling are illustrated for insulin-like growth factor I (IGF-1) (Fig. 1). IGF-1 has 4 amino groups, three from its 3 lysine residues and one from the N-terminal amino group [45]. Therefore, conventional amine-targeting strategies - including *N*-hydroxysuccinimide (NHS)-chemistry – end up in unspecific conjugation to one, two, three, or all of the 4 different amines. Assuming identical probability of labeling among all 4 amines (which is a theoretical consideration for illustration purposes), the relatively small growth factor IGF-I with 7.4 kDa already allows for $[4^{(n+1)} - 1]$ or 15 species following conjugation through its amines (Fig. 1A). Challenges associated with this random protein orientation include partial or full blockade of a protein's active site and an associated loss of bioactivity [46,47] along with potential unfolding increasing the immunological risk. In case of IGF-I, conjugation through the surface-exposed K27 (Variant #1) and partially also through K65, K68 or the N-terminus (Variants #2–#4), respectively, masks important binding sites to receptors and binding proteins (IGFBPs), thereby impacting efficacy of immobilized growth factor [48,49]. These concerns can be met by introducing non-natural amino acids such as azidohomoalanine at an ideal position into the therapeutics' primary structure resulting in optimum protein conformation for ligand interaction [50]. This chemo- and region-selective ligation method (Fig. 1B) is only one yet quite promising approach selected from the choices outlined in the next section.

3. Chemical conjugation strategies for site-specific protein immobilization

Site-specific covalent coupling of a protein to a carrier requires a distinguishing functional group within the protein. This distinguishing functional group can be a unique proteinogenic amino acid (e.g. a free cysteine) or an introduced unnatural amino acid [50]. The introduction of novel functional groups (e.g. a thiol group) - referred to as site-directed mutagenesis - offers an elegant method for site-specific conjugation, but is restricted to proteins with no [51] naturally occurring free cysteine [52,53]. Cysteine's nucleophilicity at pH 7 features rapid reaction with chemical functionalities such as α-haloacetyl- and maleimide-modified surfaces leading to covalent thioether bonds. Studies describe fusion protein synthesis of therapeutic peptides with serum albumin using this method, as albumin possesses exactly one free thiol group [54]. One common challenge of this approach, however, is the formation of intermolecular disulfide isomers jeopardizing the yield and pharmaceutical properties of the product. Additionally, free cysteines are frequently required within enzymes' active sites. Furthermore, introduced cysteine residues can cause changes in the disulfide bond pattern thereby jeopardizing proper protein folding [55]. Lack of selectivity of cysteine-involving syntheses was also reported. Many of the electrophiles traditionally used with thiols including iodoacetamides, maleimides or vinyl sulfones, are subject to competing reactions with other nucleophilic amino acids aside of cysteines, typically lysine and histidine [53]. In any case, the formed maleimide bonds suffer from instability due to hydrolysis or retro-addition reactions with free thiols after administration of the therapeutic, e.g. to serum albumin [53] causing some concerns for *in vivo* application of these conjugates [43].

The challenge of disulfide isomers and unspecific reaction with lysines/histidines and instability of the maleimide bonds is met by introducing novel functionalities into the protein sequence by 'genetic code expansion' (Fig. 2) [56,57]. Thereby, artificial or unnatural amino acids (uAA) are incorporated into proteins and it is these uAA that are site-specifically conjugated for homogenous product outcome (e.g. as outlined for IGF-I in Fig. 1B) [58,59]. Alternatively, several uAA can be introduced using 'post-translational mutagenesis' into a suitable cysteine mutant of recombinant target proteins [60]. The biorthogonal chemistries available for l modification are now discussed.

In general, chemical conjugation strategies can be divided into three main categories: (i) metal-catalyzed bioorthogonal reactions, (ii) photocatalytic reactions and (iii) bioorthogonal reactions proceeding without a need for catalysts.

The most relevant metal-catalyzed bioorthogonal reaction is the Huisgen 1,3-dipolar cycloaddition linking an azide with an alkyne to form a 1,2,3-triazole, typically catalyzed by copper(I) [33,34] (Fig. 2A). This copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) proceeds pH-independently at considerably rapid rates [61,62]. As azide and alkyne groups are essentially absent from biological systems, they are truly orthogonal in their reactivity. A number of Cu(I) sources are available but the catalyst is preferentially prepared *in situ* by reducing Cu(II) salts, such as $CuSO_4$, with TCEP or sodium L-ascorbate while THPTA or TBTA are added as Cu(I)-stabilizing agent [63]. The presence