Expert Opinion

Applications of glycosyltransferases in the site-specific conjugation of biomolecules and the development of a targeted drug delivery system and contrast agents for MRI

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Background: The delivery of drugs to the proposed site of action is a challenging task. Tissue and cell-specific guiding molecules are being used to carry a cargo of therapeutic molecules. The cargo molecules need to be conjugated in a site-specific manner to the therapeutic molecules such that the bioefficacy of these molecules is not compromised. *Methods*: Using wild-type and mutant glycosyltransferases, the sugar moiety with a unique chemical handle is incorporated at a specific site in the cargo or therapeutic molecules, making it possible to conjugate these molecules through the chemical handle present on the modified glycan. *Results/conclusions*: The modified glycan residues introduced at specific sites on the cargo molecule make it possible to conjugate fluorophores for ELISA-based assays, radionuclides for imaging and immunotherapy applications, lipids for the assembly of immunoliposomes, cytotoxic drugs, cytokines, or toxins for antibody-based cancer therapy and the development of a targeted drug delivery system.

Keywords: bioconjugation through glycan residues, cytotoxic agents, drug delivery systems, glycotargeting, lipids, MRI agents

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1. Introduction

Targeting biologically active molecules to the site where they need to act has been a challenging task [1]. They need guiding molecules that have to cross the endothelium, which is dependent on several factors including the molecules' molecular weight and size. Small molecules are taken up by a cell, by a process that generally involves a specific transport mechanism or by a passive diffusion process. By contrast, uptake of macromolecules by a cell involves receptor-mediated endocytosis or pinocytososis [2-4]. These processes involve interactions between the ligand and the receptors [5,6]. Monoclonal or single-chain antibodies against ligands or receptors [7-9] and tumor-homing [10] or cell-penetrating peptides [11] are being developed, which act as tissue and cell-specific guiding molecules that have potential to carry biologically active molecules. The guiding molecules need to be conjugated with the biologically active agents in a site-specific manner.

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2. Methods of bioconjugation

Various methods are being used with mixed success in conjugating peptides or antibodies with the cargo molecule [12,13]. Often, the primary amine groups present on the surface of the protein molecule are used for conjugation; however, as every lysine residue present on the protein molecule would be reactive, this method results in undesirable heterogeneous conjugation and even loss of antibody activity. Recently, methods have been introduced for the site-specific coupling of biomolecules that include site-directed introduction and chemoselective ligation of Cys residues to convert non-glycoproteins to glycoproteins [14], and site-directed introduction of azido/alkynyl-tagged Met analogues into proteins [15]. In the former method, either engineered or naturally present Cys residue, which is highly reactive in basic pH, requires special handling of the protein to prevent its free Cys residue from undergoing undesired oxidation. In the latter method, all the Met residues present in the protein are modified. Carrico *et al.* have described a method for introducing genetically encoded aldehydes at either terminal end of proteins, which can then be used for conjugation [16]. The sugar moieties present on the glycoproteins, such as IgG, upon periodate oxidation provide aldehyde groups that have been used for coupling [17]. However, the heterogeneous nature of the glycan moiety on the recombinant glycoprotein causes poor coupling. Various investigators are developing chemoenzymatic methods for the site-specific conjugation of biomolecules [18,19].

2.1 Bioconjugation using mutant and wild-type glycosyltransferases

Oligosaccharides are attached at unique sites in proteins and glycolipids. They bring about specific geometries of interactions between the two glycoconjugates, resulting in the correct and efficient molecular interplay between various molecular structures [20]. Glycosyltransferases assemble the oligosaccharide structures on glycoconjugates. Recently, the structural information of several of these glycosyltransferases has become available [21], making it possible to engineer these enzymes so that they can transfer a non-preferred sugar residue [22] or a sugar residue with a chemically reactive unique functional group [23]. The presence of a modified sugar moiety with a chemical handle on a glycoconjugate makes it possible to link biologically active molecules through a modified glycan chain.

Based on structural information, we have developed the β-1,4-galactosyltransferase mutant enzyme Tyr289Leu-Gal-T1 (Y289L-Gal-T1) that transfers 2-acetonyl-2-deoxygalactose (2-keto-Gal) or 2-*N*-acetyl-azide (GalNAz) from their UDP derivatives to the *N*-acetylglucosamine residue (GlcNAc), which is present at the non-reducing end of the glycans of glycoproteins [24]. After the transfer of the modified sugar residue, the chemical handle is used for selective conjugation with various molecules [23]. Using this method, we have shown that even the *N*-glycan moiety of the IgG molecule can be used as the substrate for the transfer of 2-keto-Gal sugar by the mutant Y289L-Gal-T1 [24]. However, this method requires the presence of a free GlcNAc moiety at the non-reducing end of the glycan chain of the glycoprotein and cannot be used for non-glycoproteins, such as single-chain antibodies or bacterial toxins expressed in *Escherichia coli*.

To conjugate non-glycoproteins through glycan residues, we have taken advantage of the polypeptide α-*N*-acetylgalactosaminyltransferase enzyme (ppGalNAc-T), which transfers *N*-acetylgalactosamine sugar (GalNAc) from UDP-GalNAc to the Thr/Ser residues on an acceptor polypeptide that is at least 11 amino acids long [25]. Using glutathione *S*-transferase (GST) protein as a model system, we have engineered the fusion peptide sequence at its C terminus, with a single Thr residue, which is the acceptor substrate for ppGalNAc-T2. The ppGalNAc-T2 not only transfers GalNAc but also GalNAz or 2-keto-Gal from its UDP derivative to the single Thr residue present in the fusion peptide tag of GST. Furthermore, we have expressed the soluble form of ppGalNAc-T2 in *E. coli* as inclusion bodies and developed an *in vitro* folding method to make large quantities of active ppGalNAc-T2 [25]. Therefore, using ppGalNAc-T it is possible to glycosylate non-glycoproteins, such as single-chain antibodies (scFv), bacterial toxins, with a modified sugar carrying a unique chemical handle that can be used for conjugation.

3. General utility of the technique of linking through glycan residue

The structural information available for glycosyltransferases has made it possible to design novel glycosyltransferases with broader and requisite donor specificities. Several mutant glycosyltransferases have been generated that can transfer a sugar residue with a chemically reactive functional group (e.g., 2-keto-Gal or GalNAz) from their UDP derivatives to the *N*-acetylglucosamine residue of glycoconjugates, such as to the oligosaccharide chain of IgG [24]. Based on this technique, it was proposed that two glycoproteins with modified sugars having unique chemical handles may be conjugated with crosslinkers with orthogonal chemical reactive groups, thus enabling the design of novel immunotoxins and MRI contrast agents (**Figure 1A**) [26]. Non-glycoproteins can be glycosylated by engineering a C-terminal peptide tag that can be glycosylated with a modified sugar and coupled to a biomolecule that carries an orthogonal reactive group, making the method very useful in many nanobiological applications [25]. Single-chain antibodies, instead of their full-length IgG counterparts, are increasingly used for immunotherapy [27], and they are easily expressed in large amounts in *E. coli* as soluble proteins.

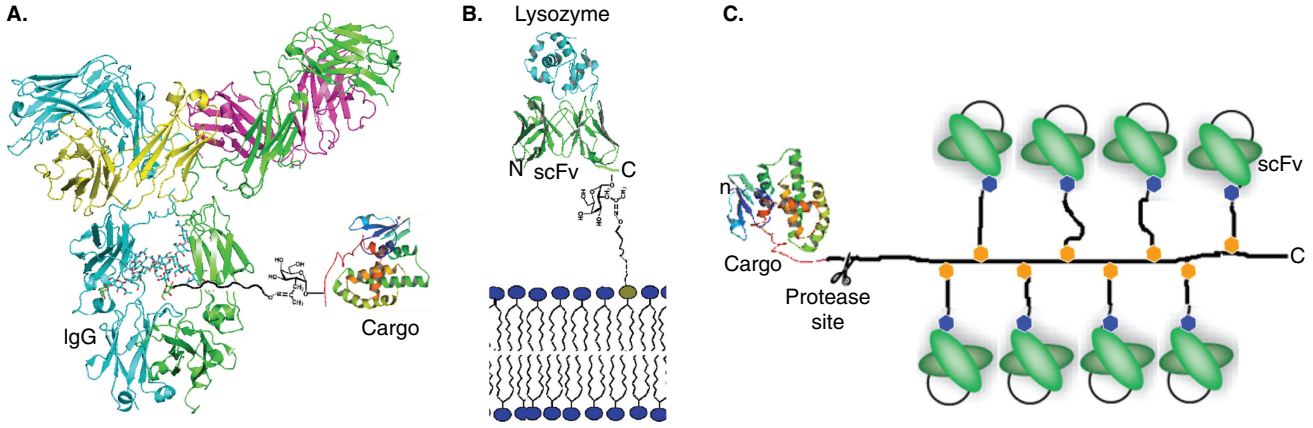

Figure 1. Schematic diagram showing immunoconjugate molecules conjugated through modified glycans. A. Using the Y289L-Gal-T1 mutant, the modified sugar 2-keto-Gal can be transferred to the free GlcNAc residue present at the non-reducing end of the *N*-glycan naturally present on the Fc domain of IgG. The modified Gal residue can be conjugated to the therapeutic or toxin molecule having a modified sugar present at its C-terminal end through its glycan moieties. **B.** Using ppGalNAc-T, the scFv molecule with a fusion peptide at its C-terminal end can be glycosylated with a modified sugar that can be conjugated with a lipid molecule with the corresponding reactive group. Thus, these scFv conjugates can be used for the formulation of the immunoliposomes for the delivery of therapeutic molecules. **C.** Cargo molecules, such as the toxins, with repeats of the fusion peptide sequence at their C-terminal ends, have multiple glycosylation sites and can be conjugated with many scFv molecules, thus enabling a multivalent recognition of the target site.

A fusion peptide attached at the C-terminal end of the scFv molecule can be glycosylated with a modified sugar at a unique site in the fusion peptide with ppGalNAc-T2 [25], and then conjugated with a biomolecule having an orthogonal reactive group, such as aminooxy or alkynes. This method of linking is made more specific by simply glycosylating a fusion peptide attached at the C-terminal end of scFv, or homing peptides or cell-penetrating peptides, similar to the GST-tag protein. The glycan moiety with the modified sugar may then be conjugated with a lipid molecule having a corresponding reactive group, such as aminooxy or alkynes. Thus, the cargo molecules with lipid attached at their C-terminal end through their modified sugar moiety mimic the glycosylphosphatidylinositol (GPI) anchor of proteins, and they can be used for the formulation of the immunoliposomes [28]. Such C-terminal modification in most scFv proteins seems to be feasible since their C-terminal end is away from their antigen-binding site (Fab) (**Figure 1B**). Furthermore, repeats of fusion peptide sequence can be engineered at the C-terminal end of the cargo molecule, generating multiple glycosylation sites for conjugation (**Figure 1C**). The linking techniques described [24,25] demonstrate that the sugar moieties with chemical handles incorporated at specific sites into mAb or scFv molecules by chemoenzymatic methods using glycosyltransferases can be used for linking various biomolecules at specific sites.

4. Expert opinion

Delivering drugs or contrast agents to a specific target site for medical imaging is highly desirable for the diagnosis and treatment of many diseases including cancer. For this purpose, target-specific monoclonal antibodies, single-chain antibodies, affibodies, and tumor-homing or cell-penetrating peptides have emerged as important guiding molecules that can carry a cargo of therapeutic molecules to the desired site. Deploying these carrier molecules for the site-specific delivery of therapeutics molecules requires site-specific conjugation of these molecules with the cargo molecules. Using wild-type or mutant glycosyltransferases to transfer a modified sugar residue with a chemical handle to a specific sugar moiety on a glycoconjugate or to a specific amino acid residue on a polypeptide chain, respectively, allows one to exploit these chemical handles for the bioconjugation of biomolecules. The monoclonal antibodies whose *N*-linked glycan moieties have been modified with the mutant β-1,4-galactosyltransferase to have 2-azido- or 2-keto-Gal can be conjugated with a biomolecule having a corresponding orthogonal reactive group, such as alkynes or aminooxy. Similarly, a fusion peptide attached at the C-terminal end of a single-chain antibody, affibody, or a tumor-homing or cell-penetrating peptide can be glycosylated at a unique site in the fusion peptide with a modified sugar; the glycosylated fusion peptide can then be conjugated with a biomolecule carrying an orthogonal reactive group. In addition, a cargo molecule having multiple conjugation sites at its C-terminal end can be used to conjugate many scFv or affibodies, introducing multimeric antibody interactions at the target site. In the currently prevailing methods, generally a bifunctional crosslinker is used to cross-link two proteins at random sites to a protein residue distributed at several places on

the protein surface. This method of crosslinking often blocks the functional sites on the protein and, thus, reduces the bioefficacy of the protein. The method of linking through glycan residues introduced at a specific site in the guiding molecules, such as mAb, scFv molecules, homing peptides or cell-penetrating peptides, makes it possible to conjugate cytokines, cytotoxic drugs, toxins for antibody-based cancer therapy [28], lipids for the assembly of immunoliposomes for developing a targeted drug delivery system [29], fluorophores for ELISA-based assays, and radionuclides for imaging and immunotherapy applications.

Declaration of interest

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