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Review

Current strategies for in vitro protein glycosylation

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Abstract

Natural glycoproteins are mixtures of multiple glycoforms possessing identical peptide backbones, but differing in composition of glycan parts. This microheterogeneity presents a serious challenge in pharmaceutical applications of glycoproteins, where well-characterized and reproducible structural characteristics are required. Due to the growing importance of biopharmaceutical glycoconjugates, a wide variety of practical approaches to the preparation of glycosylated proteins and oligopeptides have been developed in recent years, with emphasis on strategies for selective glycosylation. This review presents a concise summary of concepts and synthetic approaches currently employed in glycoprotein chemistry.

Keywords: Glycoproteins; Glycopeptides; Chemoenzymatic synthesis; Selective modification; Biopharmaceuticals

Contents

1. Importance of selective protein glycosylation

The majority of protein pharmaceuticals being developed for human therapy are glycoproteins, with oligosaccharides covalently attached to the polypeptide backbone [1]. Oligosaccharides can influence the physicochemical and biological properties of proteins to which they are attached by affecting their folding, modifying intrinsic activity and modulating their interactions with other biomolecules [2]. Glycosylation often results in significant improvement in therapeutic and physicochemical properties of protein pharmaceuticals [1,3]:

1. Better binding with biological targets due to uptake mediated by carbohydrate-specific receptors.
2. Improved immunogenic and allergenic properties due to masking of existing antigenic sites on the peptide backbone.
3. Better pharmacokinetic profile due to the improved persistence of proteins in circulation.

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4. Higher stability due to the prevention of non-specific aggregation, resistance to protease attack, and improved conformational stability.
5. Improved solubility in aqueous solutions and biological fluids.

One of the most significant problems associated with pharmaceutical applications of glycoproteins is their natural microheterogeneity [4,5]. Unlike the biosynthesis of proteins and nucleic acids, the biosynthesis of glycans (oligosaccharide parts of glycoproteins) is not template-driven and there is no mechanism for proof-reading and correcting differently glycosylated biomolecules. The structure of glycans is influenced by the competition of glycosylating enzymes for the same substrate, substrate specificity of these enzymes and substrate availability. As a result, glycoproteins are produced in a number of forms (glycoforms) that possess the same peptide backbone, but differ in both the nature and site of glycosylation.

The heterogeneity of therapeutic glycoproteins, including those produced using the recombinant DNA technology, presents a serious regulatory challenge for pharmaceutical industry [1]. While most recombinant proteins intended for human therapy are currently accepted with some degree of glycosylation heterogeneity, it is very important to define what critical recombinant protein heterogeneity is important to the efficacy of a product, and to design an appropriate analytical test to ensure that the heterogeneity is produced consistently between lots [3]. Meeting these requirements may present a formidable task because glycoproteins cannot be adequately defined by composition analysis alone and there is, as yet, no simple and rapid means to conduct a complete structural analysis of a complex glycoprotein. Moreover, sometimes the exact mechanism of action of a product is not well-established, and slight alterations in glycosylation patterns, which may escape detection, can cause significant variations in therapeutic profile. Therefore, there is an urgent need for sources of homogeneous glycoconjugates [4].

The quickly growing interest to protein glycosylation and expanding research effort in this area has generated over the past decade a vast number of research reports addressing a wide variety of different synthetic strategies and technologies related to the production of glycoproteins and glycopeptides. The goal of this short review is to provide a concise roadmap to important concepts, underlying principles and synthetic approaches currently employed in glycoprotein chemistry, using selected experimental results to illustrate key points. Numerous additional examples of specific applications can be found in several excellent detailed reviews referenced in relevant sections.

2. Protein glycosylation in vivo

2.1. *N*-Glycosylation

N-Glycosylation is the most abundant type of glycan attachment to proteins in nature [6]. The reaction is highly specific and occurs only at Asn embedded in the consensus sequence Asn–Xxx–Ser/Thr, where Xxx is any amino acid except Pro [7]. The glycosyl donor for this reaction is a dolichyl pyrophosphoryl oligosaccharide, which contains the ubiquitous pentasaccharide core $Man_3GlcNAc_2$ found in all N-linked glycoproteins (Fig. 1). As a result of the reaction, catalyzed by enzyme oligosaccharyltransferase (OST), the glycan is transferred to the Asn moiety forming a GlcNAc–(β-N)Asn bond. This initiating event is followed by the "trimming" process, in which various glycosidases hydrolytically remove several monosaccharide units from the attached glycan to expose the pentasaccharide core. The resulting glycoprotein then undergoes further processing by a variety

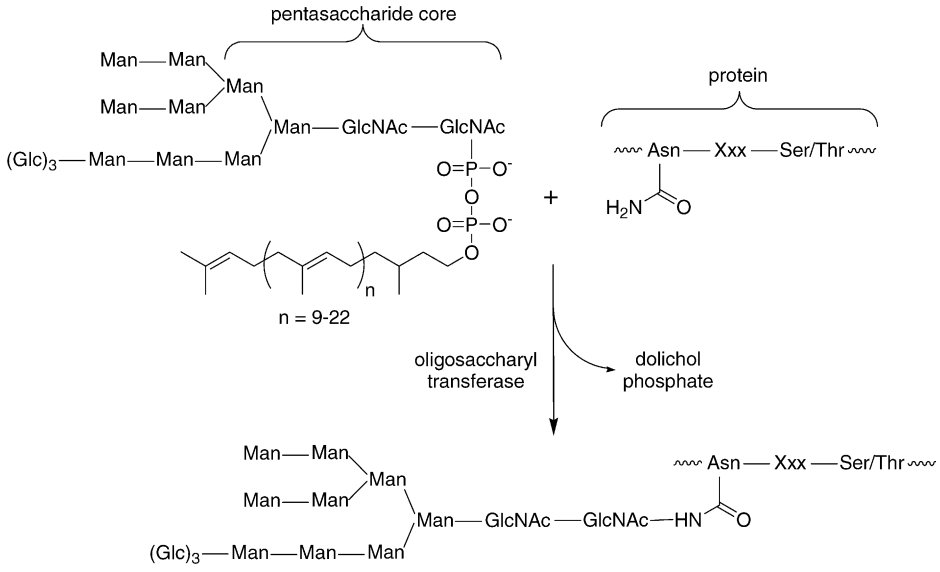

Fig. 1. *N*-Glycosylation of proteins catalyzed by oligosaccharyltransferase.

of glycosyltransferases that add various sugars to the pentasaccharide to produce a wide diversity of oligosaccharide structures [8,9]. The whole process occurs co-translationally, i.e., during protein synthesis but before the final protein molecule is formed and adopts its final conformation.

Oligosaccharyltransferase is a highly intricate membrane-bound multienzyme complex consisting of at least nine subunits [10]. OST concentration in tissues is very low, and it is difficult to isolate [11]. Since the enzyme is designed to work co-translationally with a nascent polypeptide chain, attempts to use it for glycosylation of whole, fully folded proteins have been unsuccessful. It was shown that in vitro glycosylation using OST requires unfolding of the polypeptide chain or even disruption of the secondary structure [12–14]. In combination with the virtual unavailability of the enzyme and its sophisticated glycosyl donor, this makes OST of little practical value for in vitro glycoprotein synthesis.

2.2. *O*-Glycosylation

O-Glycosylation of proteins is generally a simpler process than *N*-glycosylation, since a complex lipid-linked oligosaccharide precursor for transfer to protein is not required. The initiating event in *O*-glycosylation is the transfer of *N*-acetylgalactosamine (GalNAc) from UDP–GalNAc to Ser or Thr in the polypeptide chain. In contrast to *N*-glycosylation, a consensus sequence of amino acids in the polypeptide chain has not been found for GalNAc addition, although some predictive algorithms do exist [15]. The reaction is catalyzed by a polypeptide *N*-acetylgalactosaminyl transferase and occurs post-translationally [9–11,15]. Once the first saccharide is attached to the protein, subsequent monosaccharides can be added at the C-3 and/or C-6 hydroxyl groups of GalNAc by a family of different glycosyltransferases, thus building the glycan structure and resulting in a wide variety of glycoprotein structures. In general, these enzymes are different from those involved in the *N*-glycosylation pathway [11].

Polypeptide *N*-acetylgalactosaminyltransferase, the enzyme that catalyzes the addition of the initiating monosaccharide to the protein in the process of *O*-glycosylation, has been isolated from different sources and well-studied. It occurs in a variety of isozyme forms, many of which have been cloned and expressed in mammalian cells, appears to have quite high tolerance to variations in amino acid sequences flanking Ser or Thr in the polypeptide chain, and shows fairly good stability in purified form [10,15,16]. The enzyme is difficult to isolate and it is not commercially available. The use of polypeptide *N*-acetylgalactosaminyltransferase for practical in vitro synthesis of glycoproteins has not been reported.

3. Synthetic strategies for protein glycosylation in vitro

3.1. Convergent and sequential strategies

The formation of a native-like glycosidic bond between polypeptide and glycan parts is very difficult to achieve using conventional synthetic methods because they require strictly anhydrous conditions incompatible with most proteins and unfeasibly complex series of protection/deprotection steps of both carbohydrate and polypeptide side chains [9]. Furthermore, glycosylated amino acid residues are unstable under acidic and basic conditions required for protection and deprotection operations [4]. Because the glycosidic linkage between oligosaccharide and protein is very difficult to make, most glycoprotein analogs prepared in vitro bear unnatural linkages between glycan and polypeptide parts. These glycoconjugates are termed neoglycoproteins [17].

In general, synthetic strategies for preparation of neoglycoproteins fall into two major categories, convergent and sequential. In the convergent strategy, the glycan moiety of the future glycoprotein is either assembled separately using chemical and/or enzymatic methods, or obtained from natural sources, and then attached to the polypeptide as a single block. In the sequential strategy, a single mono- or disaccharide unit is first introduced into the polypeptide structure, and then, the oligosaccharide structure is built upon this initiating sugar using a sequential series of glycosyltransferases capable of selectively attaching various sugars to the growing glycan structure. Both convergent and sequential strategies have been used to produce neoglycoproteins with non- or site-selective attachment of glycans to the polypeptide chain.

3.2. Non-selective protein glycosylation

The most straightforward approach to the synthesis of neoglycoproteins involves direct chemical conjugation of glycans with polypeptides using reactive amino acid side chain already present in the protein. This relatively simple strategy has been used to prepare the vast majority of neoglycoproteins reported in the literature [4,9,18]. For example, neoglycoconjugates of bovine serum albumin were produced using high-temperature Amadori reaction with dextran [19], or by reacting the protein with natural mannan activated by periodate oxidation to create aldehyde groups [20]. A variety of commercially available linkers can also be used for conjugating albumin with simple saccharides [21]. Similarly, ribonuclease A was conjugated with a naturally derived mannopentaose via reductive amination of the oligosaccharide followed by coupling using an azide heterbifunctional reagent [22], and neoglycoconjugates of tetanus toxoid were prepared by chemical coupling of the protein with oligosaccharides obtained by selective depolymerization of bacterial polysaccharides [23]. Similar convergent strategy can be applied to prepare neoglycoproteins using fairly complex synthetic glycans prepared chemically [24–26], enzymatically [27–29], or chemoenzymatically [30]. Additional non-sugar linkers can be introduced on the surface of protein molecules to facilitate the reaction with glycans [29].

In an alternative approach, the protein molecule is first chemically modified by covalently attaching mono- or disaccharides to create ligation sites for subsequent elaboration of the oligosaccharide chain using specialized enzymes. A

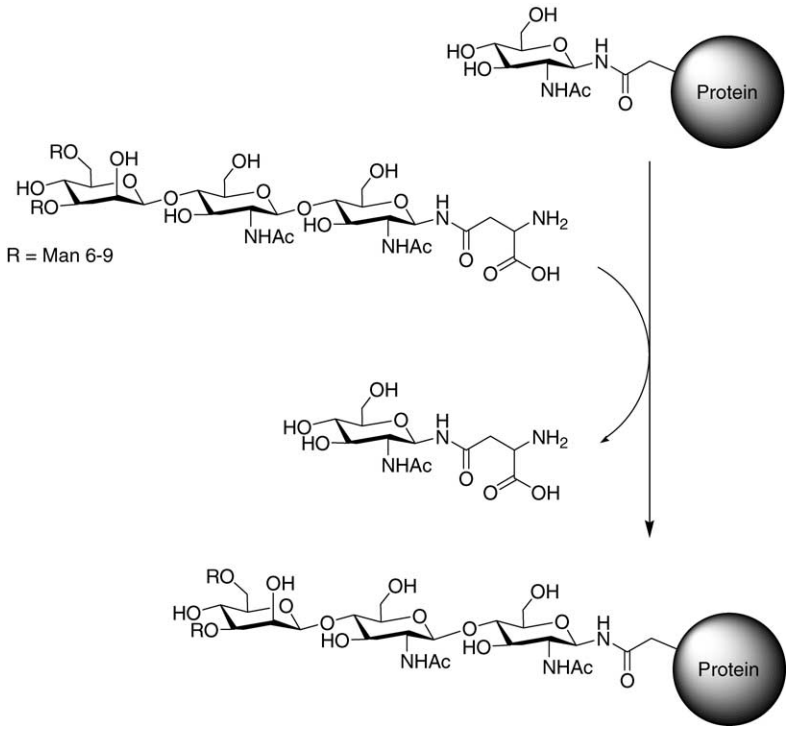

Fig. 2. Typical transglycosylation reaction catalyzed by endo-glycosidase.

very wide variety of synthetic methods for introducing different initiating sugars in oligo- and polypeptide chain have been developed over the past three decades, mostly targeting Lys amino groups [4,8,9,18]. Once the sugar ligation site has been introduced, the glycan structure can be further built using either the convergent or sequential synthetic strategy.

In the convergent strategy, endo-glycosidases have been successfully applied for attaching oligosaccharides to oligo- and polypeptides via a transglycosylation reaction (Fig. 2) [31]. The most widely used enzymes from this group include so-called Endo-A, Endo-M and Endo-F, which are obtained from microbial sources and have different specificities [32–34]. These endo-glycosidases strictly require an Asn-linked glycan donor for transglycosylation and have been employed in the construction of complex glycopeptides and glycoproteins of defined structure in a single transformation, albeit in a relatively low yield [5,33,35,36]. The convergent strategy employing Endo-M was successfully applied to prepare glycosylated derivatives of calcitonin [36] and fragments of nicotinic acetylcholine receptor [37].

In the sequential synthetic strategy, the elongation of the glycan structure from the initiating sugar is achieved by sequentially applying a range of different glycosyltransferases, each possessing exquisite linkage and substrate specificity. Most of these enzymes belong to the class of so-called Leloir transferases, which are responsible for the synthesis of most cell-surface glycoforms in mammalian systems [7]. These enzymes transfer a given carbohydrate from the corresponding sugar nucleotide donor substrate to a specific hydroxyl group of the acceptor sugar (Fig. 3). Due to the unique selectivity of glycosyltransferases, it is possible to design and execute the synthesis of fairly complex glycans composed of various monosaccharide building blocks linked together in a predetermined manner [38,39]. Hundreds of different glycosyltransferases are known, and a number of them are available commercially, including several galactosyltransferases, sialyltransferases, fucosyltranferases, and mannosyltransferase. Application of these enzymes for synthesis of glycoconjugates is well-documented in several extensive recent reviews [5,40].

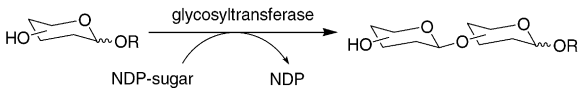

Fig. 3. General scheme of the reaction catalyzed by glycosyltransferases of the Leloir pathway. NDP, nucleotide diphosphate.

The utility of other glycosylating enzymes, not belonging to the class of Leloir glycosyltransferases, has also been demonstrated for the preparation of neoglycoproteins using the sequential strategy. For example, levansucrase was successfully used to elaborate the glycan part by sequentially transferring multiple fructose units onto sucrose covalently attached to Lys amino groups of chymotrypsin, lipase, and lysozyme [39,41].

The vast majority of synthetic strategies for the preparation of glycoproteins described above rely on the formation of covalent linkages with lysine amino groups on the protein. Lysine is one of the most abundant amino acids occurring in proteins, and most of these polar residues are located on the

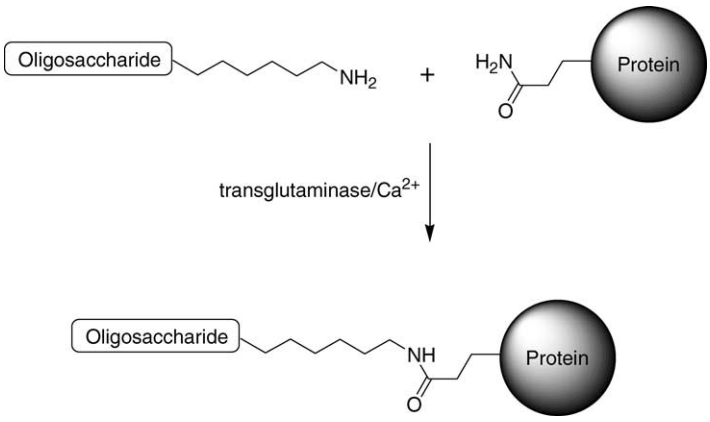

Fig. 4. Conjugation of amino saccharides with glutamine residues in proteins by transglutaminase catalysis.

protein surface [42]. For this reason, any chemical modification targeting lysine amino groups in proteins almost invariably gives a broad distribution of products, differing in the degree of modification of the amino groups. Synthesis of neoglycoproteins is not an exception from this general rule. Attempts to attach glycans to polypeptides via lysine amino groups have resulted in heterogeneous mixtures of various glycoforms, even at low concentrations of reagents used for protein modification [19,20,28,30,39,41]. The heterogeneity of the product mixture can be significantly reduced by using a very high excess of the modifying reagent, resulting in complete modification of all available amino groups [22]. However, in this case, the problem of site-selective glycan attachment remains unsolved.

A slightly better degree of selectivity can be achieved in neoglycoproteins produced by transglutaminase-catalyzed conjugation of aminoderivatives of saccharides with glutamine residues on the protein (Fig. 4). This approach utilizes the fairly relaxed specificity of transglutaminase towards alkylamine donor and strict requirement for glutamine as acceptor. The enzyme has a strong preference for straight-chain aliphatic amines of six carbons, and has been successfully used for conjugation of 6-aminohexyl derivatives of oligo- and monosaccharides to casein and vegetable proteins [43,44], as well as synthesis of trypsin–cyclodextrin conjugates [45]. While transglutaminase offers a certain degree of selectivity, reflecting different accessibility of multiple glutamine residues on the protein surface, the resulting glycoprotein still shows appreciable distribution in regards to the number of attached glycan residues.

In rare cases, selective protein modification and formation of a homogeneous glycoconjugate can be achieved if the protein or oligopeptide happens to have only a single reactive amino acid residue targeted by the modifying agent. For example, this approach was successfully applied for the conjugation of thiol-reactive maleimidosugars with bovine serum albumin [46] and 36-mer HIV-1 peptide T20 [47], both of which have only one reduced cysteine in the molecule. Similarly, the cysteine in bovine serum albumin was chemically conjugated with thioaldoses, resulting in a single glycan attached to the protein with disulfide bond [48]. In a recently reported Glyco-SeS strategy, homogeneous glycoconjugation between cysteine-containing proteins and 1-thiosaccahrides was achieved using (phenylselenenyl)sulfide intermediates, prepared either from the SH-protein or 1-thiosaccharide [49]. This fast, high-yielding reaction was successfully used for conjugation of mono- and oligosaccharides to proteins bearing one or two reduced cysteine residues. In general, however, strategies relying on a single attachment point on the protein molecule can hardly be considered a general method of preparation of homogeneous glycoconjugates because proteins typically bear multiple reactive groups on their surface.

3.3. Selective protein glycosylation

In order to solve the problem of the site-specific preparation of homogeneous neoglycoproteins and glycopeptides, a number of sophisticated strategies have been developed over the past decade. The principal focus of these strategies is to introduce a well-defined specific ligation site in the primary sequence of an oligo- or polypeptide, which can be subsequently used for highly selective attachment of a glycan moiety. Once such a ligation site has been created, the glycan part can be built off that site using one of the sequential or convergent synthetic strategies described in the preceding section.

Perhaps the most straightforward approach to create a well-defined glycan ligation site utilizes oligosaccharide attachment points already present in natural glycoproteins. According to this approach, the native glycoprotein is treated with a specific endo-glycosidase, which can selectively hydrolyze the link between the first sugar adjacent to the protein and the rest of the glycan [35,38]. In this way, heterogeneous glycans typically present in the native glycoprotein [4,5] are removed, leaving a homogeneous protein bearing single monosaccharides as “tags” to the well-defined sites of subsequent glycosylation. A new, homogeneous glycan of desired structure and composition can be then built on this site using either the sequential [38] or convergent [35] enzymatic

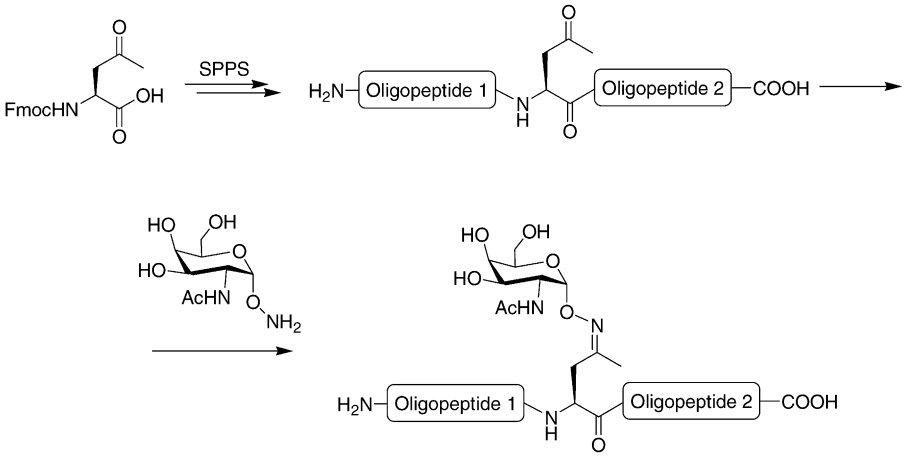

Fig. 5. The principle of chemoselective ligation.

synthetic strategies. In a variation of this strategy, instead of complete removal of glycan, it can be partially trimmed using glycosidases and a modified oligosaccharide chain can be built off the remaining portion using glycosyltransferases [50].

One of the most widely used approaches to introduce a well-defined glycan ligation site in the oligopeptide sequence is based on the solid phase peptide synthesis (SPPS). Typically, a singly glycosylated peptide is made by SPPS, the sugar is selectively deprotected, and the oligosaccharide is built up in a stepwise fashion using the sequential method [18,51,52]. Attempting to use an amino acid with a large pendant oligosaccharide in a SPPS scheme results in a rapid drop-off of coupling yields as the oligosaccharide increases in size and branching [53]. The SPPS approach requires preparation of individual glycosyl amino acids to be used as building blocks in SPPS and involves multiple protection/deprotection steps, although modern FMOC-based peptide synthesis methods are sufficiently mild that the sugar moiety remains intact throughout the synthesis [17,52].

An alternative technique for the site-specific attachment of carbohydrates to peptides is based on the concept of chemoselective ligation [9,54]. In this method, an unnatural amino acid with a unique reactive side chain is introduced into the oligopeptide sequence using SPPS, thus creating a ligation site for a monosaccharide bearing a functional group that can react only with the unnatural amino acid. Chemoselective ligation reactions are highly selective, and therefore, no protecting groups are needed. An example of the reaction that has proven useful for chemoselective ligation is the coupling of the aminooxy group on a monosaccharide with the ketogroup of aminolevulinic acid (Fig. 5) [55].

The SPPS-based methods are typically applied for the preparation of small or moderately sized glycopeptides. The extension of these methods to larger oligopeptides or full-length glycoproteins has proved more troublesome, largely due to limitations inherent to linear, step-wise SPPS. Large polypeptides are difficult to obtain using this technique due to poor yields and accumulating byproducts [17]. Other limitations of SPPS methods include high costs and limited scale-up opportunities.

In order to circumvent the limitations of SPPS on the size of glycopeptides, several semi-synthetic strategies have been developed recently, which rely upon the ligation of polypeptide and glycopeptide fragments to produce relatively large homogeneous neoglycopolypeptides. The ligation can be achieved by enzymatic condensation of two oligopeptide fragments, at least one of which is a glycopeptide synthesized using SPPS [37,56]. The condensation is catalyzed by proteases under conditions favoring peptide bond formation instead of hydrolysis, for example, in the presence of high concentration of organic cosolvents such as glycerol or *N*,*N*-dimethylformamide.

An alternative approach, so-called native chemical ligation (NCL), involves a chemoselective reaction between two unprotected peptide segments, one bearing a C-terminal thioester and the other an N-terminal cysteine residue [57]. The reaction takes place in aqueous solution at neutral pH

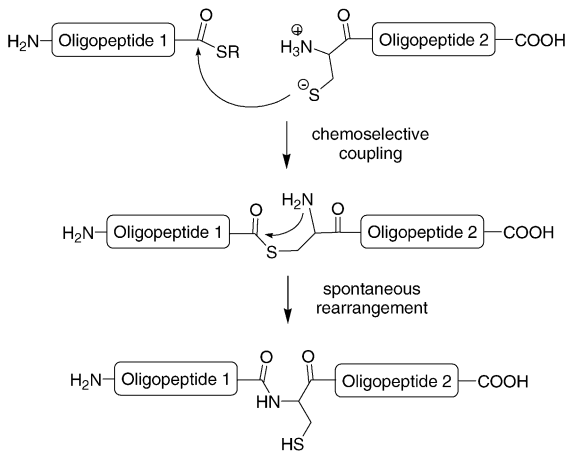

Fig. 6. The principle of native chemical ligation.

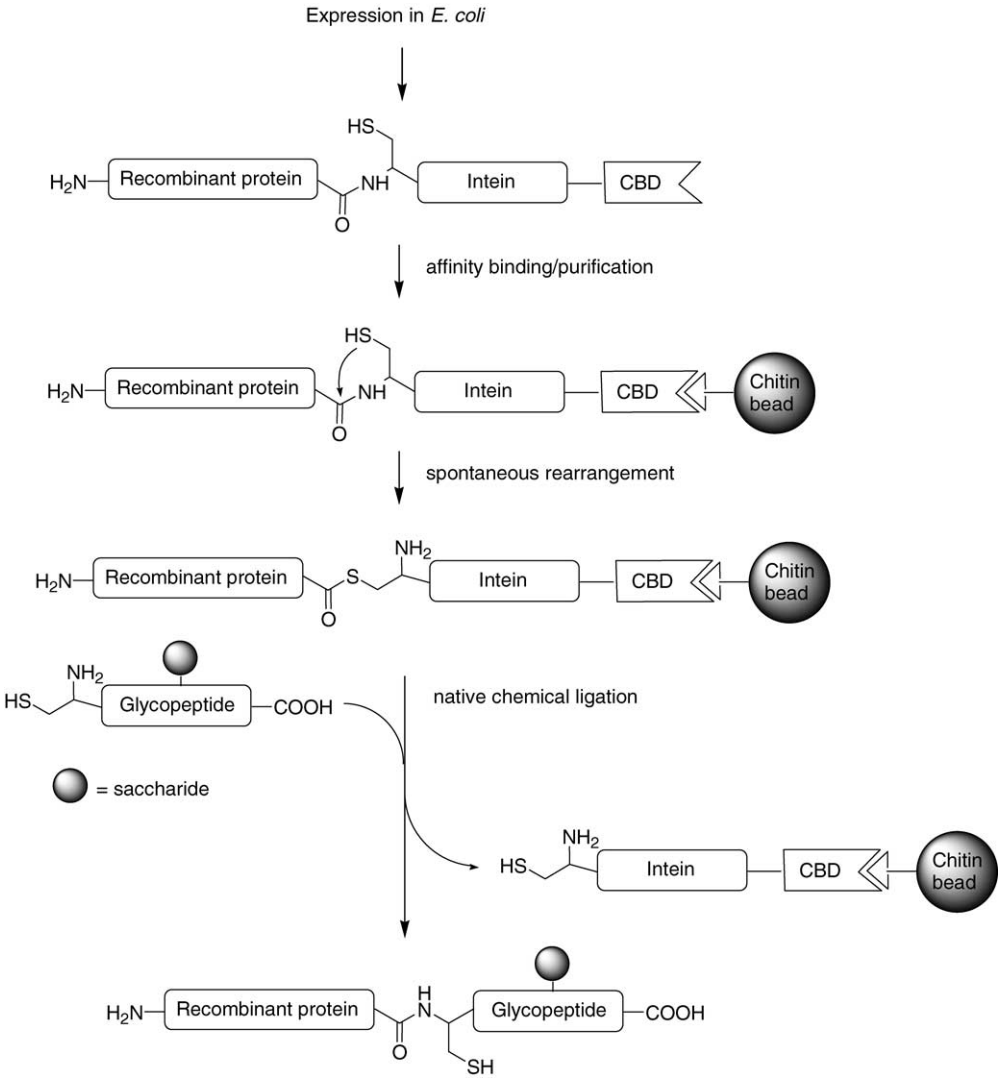

Fig. 7. Expressed protein ligation. The target recombinant protein is expressed in *E. coli* as a fusion construct with an intein and chitin binding domain (CBD). The fusion protein is purified by affinity binding to chitin beads, followed by conjugation with oligopeptide via native chemical ligation.

under conditions compatible with carbohydrates and native proteins, and results in the product oligopeptide possessing a native peptide bond at the ligation site (Fig. 6). Native chemical ligation was successfully applied for the total synthesis of diptericin, an antimicrobial O-linked glycoprotein containing 82 amino acids [58], and lymphotactin, a 93-residue chemokine [59]. In both cases, the synthesis was performed from two separate oligoglycopeptides synthesized by SPPS followed by the native chemical ligation procedure.

While NCL provides access to synthetic glycoproteins of substantial size, inherent limitations of SPPS typically used to prepare oligopeptide building blocks still make it difficult to synthesize proteins with molecular weights larger than 20 kDa (approximately 120 amino acids) [60,61]. The limitation in size can be circumvented by using a closely related strategy, termed expressed protein ligation (EPL) [62]. In this extension of the NCL methodology, the C-terminal thioester required for chemoselective ligation of peptide fragments is formed from a specially designed recombinant fusion protein containing intein, a self-splicing polypeptide that is able to excise itself from proteins posttranslationally. In the presence of added oligoglycopeptide bearing N-terminal cysteine, the thioester group linking intein and recombinant protein undergoes thiolysis analogous to the NCL process, resulting in the release of intein and conjugation of the cysteine-bearing oligopeptide to the C-terminus of the recombinant protein (Fig. 7). This elegant technology provides access to selectively glycosylated proteins of virtually any size, limited only by the ability of the expression system to produce the desired recombinant polypeptide segments. The cysteine-bearing oligopeptide can be either synthesized chemically (e.g., by SPPS) or produced using the recombinant expression [61]. In addition to glycoprotein synthesis [60,63], EPL has been successfully used for other selective modifications of proteins, such as phosphorylation, introduction of fluorescent probes and isotope labels, and other applications [61,64].

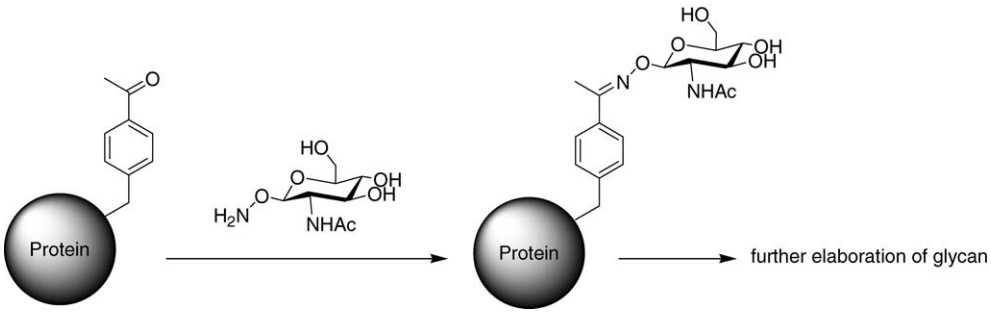

Fig. 8. Selective glycosylation of recombinant proteins bearing an unnatural keto-containing amino acid.

The major component of the EPL technology—the fusion protein expression system—is available commercially [65].

Application of the recombinant DNA technology offers other ways to introduce unique ligation sites for selective glycosylation directly into the primary structure of target proteins. In a recent example, consensus amino acid sequences recognized by oligosaccharyltransferase during *N*-glycosylation in vivo were inserted into various positions in the recombinant human erythropoietin and the corresponding proteins were expressed in mammalian cells. The presence of inserted sequences triggered *N*-glycosylation during expression, resulting in recombinant proteins bearing glycans at predetermined sites of the polypeptide chain [66]. In a related strategy, an unnatural keto-containing amino acid, *p*-acetyl-L-phenylalanine, was genetically encoded at a specific site in the polypeptide sequence of staphylococcal protein A. Since the keto group is absent from the side chains of natural amino acids, it creates a unique ligation site, which can be selectively targeted by saccharides derivatized with an aminooxy group, following either sequential or convergent strategies (Fig. 8) [67]. In a similar approach, cysteine residues were introduced at preselected positions in subtilisin [68–70] and immunoglobulin G [71] using site-directed mutagenesis and then conjugated through their thiol groups with thiol-selective reagents, resulting in selective modification of the protein with sugars attached via disulfide bonds. In a recent significant extension of this strategy, GlcNAc-modified serine was genetically encoded in *E. coli* to produce mutant recombinant myoglobin in which the monosaccharide-bearing amino acid was cotranslationally incorporated into the protein with excellent selectivity and good yield. The incorporated GlcNAc-modified serine could serve as a primary glycosylation site for sequential addition of further saccharides to the neoglycoprotein using a galactosyltransferase [72].

In conclusion, the problem of selective and controlled glycosylation of proteins in vitro has attracted significant and growing attention in recent years, and numerous synthetic methodologies have been developed to address this problem. Analysis of current glycosylation strategies reveals that substantial progress remains to be made in order to achieve the goal of creating a robust protein glycosylation technology. One of the main difficulties is attaining the selectivity of glycosylation while keeping the method reasonably simple. Relatively straightforward methods of protein glycosylation almost invariably result in heterogeneous glycoforms, whereas the selectivity can only be achieved at the high price of using sophisticated and/or labor-intensive technologies, such as genetic engineering or multistep solid phase synthesis involving complex protection group strategies. Identifying relatively simple and reliable methods for producing selectively and uniformly glycosylated proteins will remain the focus of future research in this fascinating area.

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