N-glycosylation, a leading role in viral infection and immunity development

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Abstract

N-linked protein glycosylation is an essential co-and posttranslational protein modification that occurs in all three domains of life; the assembly of N-glycans follows a complex sequence of events spanning the (Endoplasmic Reticulum) ER and the Golgi apparatus. It has a significant impact on both physicochemical properties and biological functions. It plays a significant role in protein folding and quality control, glycoprotein interaction, signal transduction, viral attachment, and immune response to infection. Glycoengineering of protein employed for improving protein properties and plays a vital role in the production of recombinant glycoproteins and struggles to humanize recombinant therapeutic proteins. It considers an alternative platform for biopharmaceuticals production. Many immune proteins and antibodies are glycosylated. Pathogen’s glycoproteins play vital roles during the infection cycle and their expression of specific oligosaccharides via the N-glycosylation pathway to evade detection by the host immune system. This review focuses on the aspects of N-glycosylation processing, glycoengineering approaches, their role in viral attachment, and immune responses to infection.

Keywords Posttranslational modifications · N-glycan · Glycoengineering · Viral infection · Immune response

Introduction

Glycosylation of protein is a ubiquitous and essential form of co-translational and posttranslational modifications involved in numerous biological processes. This is an enzymatic process in which sugar moieties are covalently attached to the side chain of an amino acid residue [1, 2]. Depending on the attachment of sugar moieties, they can be categorized as (1) N-glycosylation (saccharides linked to the amide nitrogen of asparagine (Asn) side chain); (2) O-linked glycosylation (saccharides linked to OH group of serine (Ser) or threonine (Thr), hydroxyproline side chains); (3) Phosphoserine glycan (saccharides linked to the phosphate of a phosphoserine); (4) C-linked glycans (a rare form of glycans added to carbon on tryptophan side chain); (5) Glypiated glycans (addition of a glycosyl-phosphatidylinositol (GPI) anchor that links proteins to lipids through glycan linkages); and (6) Other O-linked glycans found in cytoplasmic/nucleoplasmic proteins, such as O-linked N-acetylglucosamine (O-GlcNAc), O-fucose, and O-mannose [3, 4].

In this review, we focus on N-glycosylation which is predominant among all classes of glycosylation and occurs in all three domains of life. Based on signaling present on protein, the process of N-glycosylation start in the endoplasmic reticulum (ER) and enters to Golgi Apparatus (GA) where specific structural elements such as core fucosylation and branching are introduced [5]. Initially, it was reported that glycosylation is limited to eukaryotes while active processes are also seen in prokaryotes [6]. It was reported that nearly 70% of eukaryotic cells proteins are glycosylated, and 20% of all proteins are lipid-modified [7]. N-glycosylation is associated with protein functioning and its folding [8]. Nowadays RNA also serves as a scaffold for glycosylation.
The non-coding sequence of RNA was seen to be glycosylated and present mostly on the surface of the cell. It is supposed to interact with anti-ds RNA antibodies and receptors belonging to the Siglec family [9].

The addition of sugar moieties by the process of glycosylation is very specific and hence act as a marker on protein surfaces which is required for cell-to-cell recognition, signal transduction [10], and cell-to-extracellular matrix (ECM) interactions, thus controlling their biological activity [11]. The manuscript highlights the importance of glycosylation in immune system regulation and its involvement in viral infection.

**Biosynthesis of N-glycans**

The biosynthesis of N-glycan involves two phases and occurs in the ER and the Golgi [12]. The first phase initiates on the surface of ER or the cytoplasmic site of ER where assembly of the lipid-linked oligosaccharides (LLO) takes place followed by attachment of two N-acetylglucosamine (GlcNAc) and five mannose residues on dolichol phosphate (Dol-P) carrier molecule [13–15]. The assembled oligosaccharides on the Dol-P intermediate are then transferred to the lumen side of ER. In the lumen side of ER, there is the addition of four more mannoses in glycan structure. Glycosyltransferases (GTase) further adds three glucose residues to this growing oligosaccharide chain [16], forming an oligosaccharide precursor, Glc₃Man₉GlcNAc₂ (Supplementary 1) which get transferred to Asn residues in the tripeptide sequences Asn-X-Ser/Thr (where X = Any amino acid excluding proline) of proteins [17] by the catalytic activity of Oligosaccharyltransferase (OST) encompassing complex, a large enzymatic complex on the ER membrane [18]. The first phase of glycosylation ends with removing one or two mannoses residues from Glc₃Man₉GlcNAc₂ ensuing in the existence of Man₈GlcNAc₂ or Man₉GlcNAc₂ structures. As oligosaccharide precursor is bonded to Asn hence, removal of Asn will lead to the termination of the further step of glycosylation. A study on cytokinin IL5 states that loss of Asn196 led to the loss of the function of cytokinin. Such mechanisms are involved in immune regulation and can be a target to prevent the action of any foreign protein.

The second phase of glycosylation begins with the transportation of glycoprotein to the cis-GA where the remaining α-1,2-linked mannoses are removed by α-1,2-mannosidase (Man I), resulting in the formation of glycan Man₉GlcNAc₂ structure. In the GA, N-glycan became more complex in structure due to the addition of a first GlcNAc residue to the Man₉GlcNAc₂ (Fig. 1). The obligatory process is regulated by N-acetylglucosaminyl transferase-I (GnT-I). GA play central role in the processing, trafficking, and check protein folding. The correctly folded protein would be transported to the destiny. The misfolded protein will be sent back to the ER for re-assembly (The mechanism of protein folded would be discussed under the section ‘Role of N-glycosylation’). The process is followed by the role of glycosidasess and glycosyltransferases. Glycosidasess play important roles in the degradation of glycan structures for uptake and metabolism of sugars and are also involved in the formation of intermediates that are used as substrates for glycosyltransferases in the biosynthesis of glycans. Glycosyltransferases catalyze the transfer of sugar moieties and responsible for initiation and elongation of glycan chain. Correctly folded protein is further processed and re-assembled in GA leading to three different classes (High Mannose, Complex, and Hybrid) of glycans sharing a common core structure including the first two N-acetylglucosamine residues and the first three mannose residues. In high-mannose glycans Man residues (5–9 Man residues) only extend the core whereas in complex N-glycan GlcNAc is required. Each of these classes could be elongated with Galβ1-4GlcNAc repeats. In hybrid class of glycan Man extends the Man α1-6 arm of the core and one or two GlcNAc extend the Manα1-3 arm [19]. Further, the glycans moieties helps GA to sort the glycoprotein and its trafficking to the final destination [20]. In GA, glycosylation is affected by the pH, signaling molecules, cellular stress, and integrity of Golgi peripheral membrane proteins [21, 22].

**Glycoengineering approach for improving protein properties**

Glycoengineering is well known from past eras in bacteria, plants, fungi, and mammals. N-Glycan can alter therapeutic glycoproteins’ size, charge, and solubility to prevent rapid clearance from circulation. Additionally, glycoengineering has improved as well as developed new therapeutic modalities by modifying the conformational and folding efficiency [23]. The efficiency to modify the properties of protein is exploited by pharmaceutical, industrial and agricultural industries. Glycoengineering would improve the stability by masking the site for protease activity. Changes in the proteins can be introduced either by using natural or synthetic glycans. Change in the position or pattern of glycans is also preferred to introduce the desirable physical properties in proteins. Knockdown, knock-in, suppression of small molecules and mutation make success to glycoengineering. The introduction of the CRISPER/Cas 9 system has revolutionized the technique and made it cost-effective. Bacteria is a desirable site to conduct glycoengineering as it is a cheaper and fast process for the detection of the success rate of the insert. But to avoid immunological challenges for the proteins to be used in human yeast cells are preferred. Though mammalian-based
cell lines can also be used. All these cell-based glycosylation processes are always at risk for genetic modification inside the cell by natural mechanisms. Further, purification and characterization is another major challenge. As per the stated issues, researchers have introduced organic chemistry-based methods for glycoengineering. It has also been preferred for large scale production. The chemistry-based method is flexible and more precise but needs intensive labor. Later on, intervention of biochemistry for glycoengineering has proved more efficient. The method has extensively used varieties of glycosidase and glycosyltransferases. For example, the treatment of intravenous immunoglobulin (IVIG) with α-2,6- neuraminidase has abolished its anti-inflammatory activity. The mentioned changes have improved its therapeutic efficiency for autoimmune diseases specifically systemic lupus and rheumatoid arthritis. Changes in the position of glycans are also a target to improve the quality of protein. Gidden et al. has successfully improved the ADCC (Antibody Dependent Cell Cytotoxicity) of monoclonal antibody cetuximab by changing the position of glycans present on Fc and Fab region. He implemented the stated method through the combined application of endoglycosidases- Endo-S, Endo-S2, and Endo S3 and 1,6-glucosidase from *Lactobacillus casei* to target N88 and N297 sites of the heavy chain of Fc and Fab domain respectively. The method has resulted in the formation of uniform sialylation of N-glycans at the Fab domain and non-fucosylation at the Fc domain resulting in the improvement of ADCC [24].

Changes in the glycans can also targeted to misdirect the attachment of virus to the host cell. The development of N-acetylmannosamine (ManNAc) analogs like N-butylmannosamine (ManNPr). This hampers the binding of influenza to mammalian cells. Similarly, tributanoylated ManNAc (1,3,4-O-Bu3 ManNAc) acts as an anti-inflammatory [25]. Knock-out and knock-in of xylose and fucose residues from

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**Fig. 1** Processing of *N*-glycan in the ER and Golgi. The arrow line indicates the transition from ER lumen and the Golgi. The biosynthesis *N*-glycan involved different enzymes recognized in plants are depicted by lowercase letters. (a) Oligosaccharyltransferase (OST) containing subunits (b) Glucosidase I and II (c) Mannosidase I (d) *N*-acetylglucosaminyltransferase-I (GnT-I) (e) Mannosidase II (f) Different modification of *N*-glycan structure using enzymes: *N*-acetylglucosaminyltransferase-II (GnT-II), xylosyltransferase, and fucosyltransferase.
plant N-glycan help develop new mammalian glycan structures that improve therapeutic properties [26]. Genetic knock-out of a few glycosylation enzymes like fucosyltransferases and xylosyltransferases has been recognized in Arabidopsis thaliana [27] and the moss Physcomitrella patens via homologous recombination [28]. Homologous recombination with the presence of gene families in plant species is very rare. So another method for gene silencing through RNAi of fucosyltransferase and xylosyltransferases has been developed in several plant species, such as Lemma minor [29], Medicago sativa [30], and Nicotiana benthamiana [31]. The addition of galactose in N-glycan structure with the help of galactosyltransferases forms intermediate galactosylated N-glycan structures in Nicotiana tabacum (Castilho et al. 2010). Absence of xylose and fucose residues in plant N-Glycan structure help in the formation of recombinant glycoproteins [25]. N-glycosylation could modify the solubility of the protein. This property is exploited to increase the half-life of the intravenous drugs. Darbepoetin alfa is the result of addition of two N-linked carbohydrate to erythropoietin which has three fold improved serum half-life [32].

Role of N-glycosylation

Protein folding and quality control

Protein folding is a mechanism of adding functionality to it. The sequence of amino acids determines the nature and function of the protein. Protein is synthesized either on the rough endoplasmic reticulum or in the cytoplasm. In both processes, they need to be folded correctly to enter the functional world. Misfolding of proteins may lead to neurodegenerative disorders such as Alzheimer’s disease (AD) and Parkinson’s disease. Folding is assisted by chaperons and the nature of amino acids present in the protein. The addition of glycans causes steric hindrance, modifies the protein’s folding, and provides a remarkable character to it. The addition of glycans is recruited in ER lumen with the assistance of UDP-Glc-glycoprotein glucosyltransferase (UGGT) [33], calreticulin, calnexin, and Ca$^{2+}$ [34, 35] termed as quality control (QC) system. Calreticulin, calnexin, and Ca$^{2+}$ bring about the establishment of disulfide bonds. However, the completion of the process takes place in the Golgi Complex. UGGT also cross-checks the folding of the protein and exports the misfolded protein in the cytosol for ubiquitination. The addition of glycans prevents it from getting exported, which requires the removal of glucose residues [36]. The presence of a specific sequence of glycans predisposes to be a marker specific for the protein functioning. The ER’s quality control (QC) system is a major monitoring mechanism in the protein maturation process, which confirms the export of properly folded proteins as depicted in Fig. 2 [37].

As discussed above, protein is functional in its tertiary and quaternary structure, which is established by disulfide regulated by calnexin and/or soluble calreticulin [38]. Glycosylation also regulates disulfide bonding in protein. They expose protein to thiol-disulfide oxidoreductase, which assists in the formation of disulfide bonds.

Glycosylation in viral infection

Glycosylation also contributes to viral diseases. Viruses adopt host glycans for their recognition and attachment. After invading the host cell, the virus captures the host cell glycosylation mechanism to replicate and for immune evasion.

The origin of zoonotic viral infection is influenced by three natures of glycosylation: conserved glycosylation, tissue-specific glycosylation, and host specific viral glycosylation. Transmission of viral infection through insect as in the case of Zika and Dengue, the viral protein expresses glycosylation from the invertebrate. After interspecies transmission of these virus to human, there will switching in glycosylation as well. This affects the antigenicity and viral tropism. The conserved nature of glycosylation affects the cross-species antigenicity of the viral infection. Lack of galactose α-1,3-galactose in humans serves the best example for this regard. This is also a reason for delayed-type hypersensitivity to red meat in human and xenograft rejection. The specificity of recognition of α-2,3 and α-2,6-linked sialic acid led to the transmission of influenza A virus hemagglutinin (HA) from avian to human. The virus mostly targets the upper respiratory tract due to tissue specific expression of receptors having α-2,6 sialylated glycan [39]. Hemagglutinin (HA) and neuraminidase (NA) are two major surface glycoproteins are present in the influenza virus. Influenza virus invades host cells specifically through HA and specific to the receptor with terminal N-acetyleneuraminic acid [40]. Glycosylation in HA varies from species to species. It is supposed that the virulence of the influenza virus decreases with an increase in glycosylation [41]. The influenza virus was also proved pandemic in the nineteenth century. In the pandemic year of 1918, 1968, and 1975 the viral strain differs significantly in the glycosylation site in the HA region. HA was having 6, 5, and 9 sites of glycosylation in the respective year of the pandemic [42]. The conserved nature of glycosylation makes some blood groups more prone to certain viral infections. A survey states blood group ‘O’ are more proneness of HIV and Hepatitis B.

Glycosylation of non-structural protein or secretory protein misdirects the humoral immunity by targeting the epitopic site. Lassa virus on invading human cell shed its
glycoprotein subunit-GP-1 which act as an immune decoy for antibody recognition. Like Lassa fever, shedding of gp120 from HIV-I envelope which acts as a receptor for CD4 interrupt T-cell activation. Shedding of glycans, as well as secretion, can mislead humoral immunity. Epstein-Barr virus on infection releases two proteins BARF1, a secretory protein that interrupts innate immunity by targeting macrophages maturation whereas gp42 interfere with the presentation of MHC-II.

In West Nile Virus (WNV) as well glycosylation is involved in the viral assembly along with infection. Removal of glycosylation from the envelope protein (Pre-membrane (prM) and envelope (E)) of WNV decreases the release of viral particles [43]. Coronavirus disease 2019 (COVID-19), a highly infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The genome of SARS-CoV-2 encodes lots of highly glycosylated proteins. Glycosylation in the junction of S1 and S2 subunits of spike protein make human desirable host for COVID-19 [44, 45]. The novel virus shows more than 90% similarity with the SARS-CoV, which erupted in 2002, whereas the spike protein has 75% similarity and has the same binding site to the host cell at ACE receptor (angiotensin-converting enzyme 2). This conservative nature shows that glycans play a central role in the spread of viral infection.


**N-glycans in immunological aspect (vaccine and antigen production)**

Glycosylated proteins play a central role in immune responses both in innate and acquired immunity [46]. Glycans also act as a marker to interact with microbes. ABO blood group typing serves the best example of glycans as immunogenic response markers [47]. Group A and B have α1,3-N-acetylglucosamine (GalNAc) and α1,3-Gal attached to the H antigen’s galactose residue, respectively. The presence of glycans in the blood group not only makes a concern for blood transfusion; but many pathogens also get their vent through these markers. As individuals with blood group O are more prone to *Vibrio cholerae* infection [48].

Glycosylation regulates the maturation, activation, transportation of immune cells to the site of action of immunological response. In the case of innate immunity which has a non-specific response and is considered as first line of defense, glycans are directly associated with the inflammatory response [49], one of the components of innate immunity. Glycans also regulate the migration of neutrophils to the site of infection. During inflammation in the lungs, pathogens’ neutrophils secrete paucimannosidic, an N-glycosylated (mannose<sub>1-3</sub>fucose<sub>0-1</sub>N-acetylglucosamine<sub>2</sub>Asn) proteins from their azurophilic granules into the sputum present in the lungs [50]. This mechanism is involved in pathogen-based inflammation.

Along with innate immunity glycosylation also regulate acquired immunity. Unlike innate immunity, it shows a specific response against the pathogen. Humoral and cell-mediated immunity both serve as a component of acquired immunity. Humoral immunity is considered an antibody-mediated immunity. There are five well-known immunoglobulins or antibodies- Ig M, Ig G, Ig A, Ig E, and Ig D. These are considered as isotypes whose heavy chain (Fc region), as well as antigen-binding site (Fab region), vary concerning glycans and very specific. As in Ig G and Ig A, N-linked glycosylation is at N297 [51] and N394 [52] in heavy chains, respectively. Fc region has a comparatively more fucosylation than Fab [51]. The glycosylation provides specificity in binding the Fc region of Ig to its receptor [52]. Alteration of glycosylation causes autoimmune diseases, inflammatory diseases, and lymphomas [53] (Table 1).

Glycosylation is not only a response to cause diseases rather also helps to fight against them. Mother milk has α-1,2-linked fucosyl moieties that provide an environment

| Disease                          | Alteration in N-glycans                                                                 | References |
|---------------------------------|----------------------------------------------------------------------------------------|------------|
| Inflammatory bowel diseases     | Addition of fucose to core N-linked glycans through α-1,6 linkage                      | [54]       |
| Inflammation of colonic mucosa  | Reduction in α-1,6-mannosylglycoprotein                                                 | [55]       |
| Multiple sclerosis              | Dysregulation of N-glycosylation in Golgi body                                          | [56]       |
| Colorectal tumors               | Increased β-1,6 branching                                                               | [57]       |
|                                | Extension of N-acetyllactosamine                                                       |            |
|                                | Sialylation in N-glycans                                                               |            |
| Malignancy and reduced survival | Increased β-1,6 branching                                                               | [58]       |
| rate of cancer patients         |                                                                                       |            |
| Cirrhosis                       | Hyperfucosylation                                                                      | [59]       |
| HCC (hepatocellular carcinoma)  | Increased α-1,3-fucosylated biantennary glycan                                         | [60]       |
|                                | Decreased α-1,6-fucosylated biantennary                                               |            |
| Systemic lupus erythematosus    | Deficiency of α-mannosidase-II (αM-II)                                                 | [61]       |
| Rheumatoid arthritis and IgA    | Alterations of endogenous IgG glycosylation                                           | [62]       |
| nephropathy                     |                                                                                       |            |
| IgA nephropathy                 | Due to β1,4GalT1 glycosyltransferase deficiency                                        | [63]       |
| Emphysema-like disease          | Deficiency of Fut8 (Fucosyltransferase) led to core 1,6 fucose linkage                 | [64]       |
| Tn syndrome                     | Mutation in COSMC (C1GALT1C1) gene, which functions as β1,3 galactosyltransferase     | [65]       |
|                                | activity in O-glycan biosynthesis                                                     |            |
| PMM2-CDG                        | Mutations in PMM2, which encodes phosphomannomutase which led to a                     | [66]       |
|                                | fault in the conversion of Man-6-phosphate (Man-6-P) to Man-1-P, a precursor for GDP-(Dol-P-Man) synthesis. This is involved in the synthesis of the lipid-bound precursor of N-glycans, Glc₃Man₆GlcNAc₂-P-P-Dol |            |
| MPI-CDG                         | Mutations in MPI, which encodes Man-6-P                                               | [67]       |
|                                | Isozyme causes a defect in the interconversion of Man-6-P and fructose-6-phosphate     |            |
for *Bifidobacteria longum*. This is a beneficiary micro-
biota. Another commensal relation between *Bacteroides spp.* and intestinal cells is a good example which is due to fucosylation.

Researchers have exploited the specific nature of glyco-
sylation in vaccine formulation. Glycogen serves specificity to a pathogen hence, can be conjugated with the vaccine to recruit both T and B cell-based immune responses. Unconju-
gated polysaccharide is not processed by antigen-presenting cells (APC) and since targets only B-cell activation. A sig-
nificant disadvantage of such an immune response is the very short life span of memory cells. However, conjugation of glycans with the protein can hit T cell activation which responds for long-lasting memory and avoid multiple boost-
ers. This concept has proved promising against *N. meningitis* and *S. pneumoniae* conjugated vaccine [68]. Approach tar-
geting glycans have also been taken for the HIV vaccination. The antibody from an HIV patient which targets the envelop trimer cut down the efforts to design a vaccine [69]. Glycan of SARS-CoV-2 is also a target to vaccine and drug design-
ning [70, 71].

Alteration in glycan is another approach to treat diseases. Selectins contribute to cell–cell adhesion. A successful approach has been seen against inflammatory disease and cancer by targeting selectins. Selectin has specific glycans as sialyl-Lewis-X is present in E and P-selectin, whereas 6-sulpho-sLe^\(\alpha^\) is for L selectin. Blocking cell–cell interaction pretends to be the best therapeutic target for inflammatory diseases [72] and prevent tumor development from cancer cells [73].

**Plant as a compartment to produce vaccines**

As discussed in the previous section, *N*-glycosylation has also been a platform to produce subunit vaccines. The plant can also be used as a compartment to produce vaccines [74]. Because of its widespread nature, comparatively less expensive to culture than the animal cells, easy screening methods and ease transfection recombinant technology make the production of antibodies cheaper that is too with mass production. The first attempt to produce antibodies from plants was taken in 1990 and the products are termed as ‘Plantibodies’ [75] and plant *N*-glycan modification by galactose moiety through human β-1,4-galactosyltransferase was first reported in plant *N. tabacum* [76]. The beginning of the twentieth century has brought surprising results of plan-
tibodies. Antibodies produced by plants were homogeneous and performed better in neutralizing antibody (NAb) assays for HIV [77, 78]. But the major challenge to using plantibodies is that being a human protein it may be toxic to other species. Moreover, the glycosylation modification inside the plant may be different from the human glycans sequence which can be allergic by class switching of immunoglobu-
lin to produce Ig E [79]. To overcome the former concern plants like tobacco is preferred which is neither consumed by grazers nor serve as a crop plant and glycoengineering is preferred to solve the latter issue [75]. Beta 1,2-xyllose and alpha 1,3-fucose are the two major core glycans present on the protein produced by plants which act as an immu-
nogen for humans. Removal of these two glycans from the plant protein has produced IgG in the Chinese hamster ovary (CHO) which does have any dissimilarity in enzyme-linked immunoassorbent assay (Table 2(A)). Another way to cheat the plant to prevent the xylene and fucose attachment to gly-
cans is to express an intermediate *N*-glycan which does not serve as a substrate for their attachment. The technique was successfully implemented in *Nicotiana tabacum* by express-
ing mutant galactosyltransferases (Table 2B). Another basic gap between plant and animal glycosylation was filled by the introduction of β-1,4-galactosyltransferase in the plant through recombinant DNA technology. The resultant immu-
noglobulin exhibits partial galactosylated *N*-glycan which is similar to the Ig produced by hybridoma technology in mice (Table 2C). In this galactosylated *N*-glycan Bakker and his colleague have silenced the expression of xylene and fucose using RNAi (Table 2D). Protein sialylation makes another difference between glycosylation in plants and ani-
mals. This is the most challenging task in plants due to the lack of the sialic acid precursor, its transportation, and trans-
ferase enzyme. Though it was successfully done by Castilho et al. by introducing six mammalian proteins in plants that endorse for sialic acid synthesis pathway [80].

Hence, modification of branching *N*-glycan structure in plants seems feasible. However, several times knock-out or knock-in plants *N*-glycan structure by glycoengineering does not show their phenotypic significances. Novel approaches have been carried out in plant species by the glycoengineering process that minimizes undesired phenotypes and helps plants as a host for the development of therapeutic glycopro-
teins [74, 81]. Over the past decades, modification of plant *N*-glycan structure has been a good alternative platform for biopharmaceuticals production and therapeutic properties.
Table 2  Modification of plant N-glycan by glycoengineering to make protein compatible for functioning in humans

| S/N | Amino Acid | Glycomoity | Enzymes involved | Resultants Glycostructure | Application                                                                 | References |
|-----|------------|------------|------------------|---------------------------|-----------------------------------------------------------------------------|------------|
| A   | -ASN-      |            | Xylosyltransferases & fucosyltransferases (Genetic knock-out) |                             | Chinese hamster ovary (CHO)-derived immunoglobulins (IgGs) was produced in plants which do not show any difference in Enzyme-link immunosorbent specificity assay | [82, 83]   |
| B   | -ASN-      |            | Galactosyltransferases |                             | Production of recombinant immunoglobulin which does not serve as a substrate for plant specific xylose and fucose | [74]       |
| C   | -ASN-      |            | β1,4-galactosyltransferases |                             | Production of antibody similar to the one produced by hybridoma technology | [76, 84]   |
| D   | -ASN-      |            | β1,4-galactosyltransferase with RNAi of fucosyl- and xylosyltransferases |                             | Production of human-compatible antibody | [84]       |
Conclusions and future prospects

Research in glycosylation has made huge advances in the last half-century. **N**-glycosylation is a ubiquitous post-translational modification in all three domains of life. It is estimated that nearly 70% of eukaryotic cells protein are glycosylated. Its analysis has provided greater insights into glycans' roles in protein trafficking and folding, viral attachment, and immune responses to infection. The biotechnological potentials of **N**-glycosylation have some technical challenges due to the complex nature of **N**-glycan structures and the difficulties in host-expression systems for glycoprotein production. Glycoengineering could provide abundant future opportunities to improve glycoprotein drugs and produce subunit vaccines by knock-out and knock-in sugar residue in **N**-glycan. **N**-Glycosylation approach helps develop the immunological aspect, e.g., vaccine and antigen production, and can be engineered to produce subunits antigens with high product homogeneity. Moreover, glycosylation of immunogens can play significant roles in innate immune recognition to enhance humoral immunity. The future of research in this area contributes **N**-glycans to reveal paths for new antiviral therapeutics and prophylaxis against a wide range of enveloped viruses containing oligosaccharide-rich membrane glycoproteins that affect the protein function and immunogenicity.

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