British Journal of Applied Science & Technology
18(1): 1-16, 2016; Article no.BJAST.29679
ISSN: 2231-0843, NLM ID: 101664541

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Impact of Glycosylation on Therapeutic Glycoproteins

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MM and MD wrote the first draft of the manuscript. Author AM managed literature searches. Authors MD and MM managed the study searches, arrangement and correction under author AM supervision. All authors were equally contributed in this study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJAST/2016/29679
Editor(s):
(1) Ya-mei Gao, College of life science and Technology, Heilongjiang Bayi Agariculture University, Daqing, Heilongjiang, China.
Reviewers:
(1) C. V. Rao, Herbert Wertheim College of Medicine, Florida International University, Miami, Florida, USA.
(2) Muhammad Ramzan, Institute of Molecular Sciences and Bioinformatics (IMSB), Pakistan.
(3) Ana Carolina Urbaczek, University of São Paulo – USP, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history/16929

Review Article

Received 23rd September 2016
Accepted 9th November 2016
Published 16th November 2016

ABSTRACT

Therapeutic glycoproteins, which are produced in different expression systems are effective as biopharmaceuticals in medical applications and glycan engineering technology can open a new world to facilitate production of these proteins to overcome some pharmacological problems by humanizing the biopharmaceuticals. Also, the recognition of the role of glycosylation upon proteomics inconstancies of therapeutic proteins under manufacturing is critical for safety and non-immunogenic production. In this review, we tried to describe some important therapeutic glycoproteins and focused on some proteomics challenges of these proteins, which are improved by glycosylation.

Keywords: Therapeutic glycoproteins; glycosylation; glycan engineering.

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1. INTRODUCTION

Utilization of proteins as pharmaceutical agents has increasingly developed the field of molecular pharmacology as therapeutically desirable properties, such as higher target specificity and pharmacological potentiality, compared to traditional small molecule drugs [1]. Unluckily, one of the biggest challenges attributed to the pharmaceutical use of them is the structural inconstancy consequences which are generally manifested by this class of protein, as these can negatively affect on their final therapeutic efficiency [1,2]. The macromolecular nature of proteins has led to the structural complexity and diversity which has hindered the expansion of predictive methods and generalized strategies pertaining to their chemical and their physical stabilizations, as opposed to traditional small molecule drugs whose physicochemical properties and structural stabilities are frequently much simpler to foresee and control [3,4]. Although the protein primary structure is prone to the similar chemical instability consequences like traditional small molecule therapeutics (e.g. acid-base and redox chemistry, chemical fragmentation, etc.), owing to the non-covalent nature, the higher levels of protein structure (e.g. secondary, tertiary) often essential for therapeutic efficacy can also bring about additional physical instability consequences (e.g. irreversible conformations changes, local and global unfolding [1,5,6]. Since these proteins are inherently tended to undergo structural alterations in addition to the fact that there is a slight difference in thermodynamic stability between their folded and unfolded states, there is an important obstacle for the long-term stabilization of protein pharmaceuticals. This is resulting from the principle that a thermodynamically stabilized protein could still inactivate kinetically even at the relatively low temperatures used during storage [6,7]. Also, because of their colloidal nature, proteins are may be affected by temperature, pH, and concentration dependent sedimentation, surface sorption, and compression of non-native super molecule [8,9,10,11,12,13]. These inconstancy consequences are further added to that, depending on the physicochemical environment in which the protein is subjected. The diverse levels of protein structure can become disturbed differently [1]. This is particularly relevant in pharmaceutical production environmental settings that may cause destabilization during production, purification, storage, and delivery of proteins. Development of strategies has been emphasized in order to have efficacious long-term stabilization of pharmaceutical proteins owing to these stability problems [8,14,15,16,17]. These consist of external stabilization by affecting the features of the surrounding solvent via the use of stabilizing excipients (e.g. polyols, sugars and amino acids) and inner stabilization by altering the structural properties of the protein using chemical modifications (e.g. mutations, glycosylation, regulation). Despite successful formulation of many protein pharmaceuticals using stabilizing mutations, excipients, and regulation, their use can sometimes be troublesome owing to restrictions. These restrictions can be indicated in brief such as rapidity of distinctive chemical (like aspartate isomerization) and physical inconsistency by excipients (like sucrose, sorbitol or glycerol, sucrose) foretelling the stabilizing nature of amino acid replacements, cross-reactions between excipients and the numerous chemical functionalities of proteins, protein/excipient phase segregation on freezing, the occurrence of protein and excipient dependent non-generalized stabilization effects, and security fears considering the long-term use of regulated proteins in vivo owing to feasible PEG enforced immunogenicity and chronic accumulation toxicity because of its decreased degradation and clearance rates, discovering interferences which are operated by sugar excipients within different protein analysis methods [14,16,18,19,20,21].

These restrictions make it necessary to develop further protein stabilization strategies [1]. Engineering the molecular stability of proteins through manipulation of glycosylation parameters such as the structural composition and the size of glycan, and also glycosylation degree is a part of the promising approaches among the chemical modification methods [16,22]. It has been reported that glycosylation simultaneously stabilizes a diversity of proteins in contradiction of most of the main physicochemical instabilities faced during their pharmaceutical employment. Although there is solid evidence that glycosylation can bring about improved molecular stability and therapeutic effectiveness for protein pharmaceuticals, a comprehensive viewpoint on this subject is still missing owing to the lack of an extensive review of the literature. Therefore it is aimed to further the field of protein glycol engineering by enhancing the overall conception of the mechanisms by which glycosylation betters the molecular stability of protein pharmaceuticals. This is obtained by

conducting a survey of the different inconsistencies showed by protein pharmaceuticals, by finding how glycosylation can improve these inconsistencies, and also by discussing how glycans provoke these stabilization effects through possible mechanisms.

Since there is plenty of structural variability caused by physiological glycosylation, new strategies are recently being followed to make pharmaceutical glycoproteins, which are structurally homogeneous with humanely glycosylation types [23]. In this case, each of chemical, enzymatic, chemo-enzymatic in vitro methods and engineered glycoprotein expression systems illustrate various strategies. In order to figure out the how the protein physicochemical attributes are affected by glycosylation, relatively easier glycosylation strategies have been applied instead. These include using Maillard reaction for glycation of the lysine residues by decreasing sugars, using chemically activated glycans with simple structures for chemical glycosylation and deglycosylating the natural glycoproteins enzymatically. Despite probable undesirability of some of these glycosylation methods (e.g. glycation) for use in protein pharmaceuticals, for understanding the efficacy of glycosylation on protein stability, their substantial scientific avail should not be underestimated [24]. This is because of the fact that regardless of the method by which the structurally distinct glycans are connected to the protein surface they all may stimulate similar stabilization effects [25,26]. Thus, in this article, some characteristics of four principal kinds of therapeutic glycoproteins including Interferons (IFNs), monoclonal antibodies (mAbs), Erythropoietin (EPO) and tissue plasminogen activator (tPA) are outlined. Furthermore, some important proteomics features based on chemical (including proteolytic degradation, oxidation and chemical crosslinking) and structural inconsistencies (including precipitation, pH denaturation and aggregation) inconsistencies of therapeutic proteins are demonstrated to be improved by glycosylation. And finally we have discussed feasible mechanisms, which cause the above influences in glycans.

2. PARADIGMS OF IMPORTANT THERAPEUTIC GLYCOPROTEINS

2.1 Interferons (IFNs)

IFNs are a family of regulatory proteins and they generally have 166 amino acid residues. The molecular weight of each residues varies between 16-26 KDa. Although two important IFNs including IFN- β and IFN-γ[60-62] are glycosylated, the main human IFN-α subspecies are not glycosylated [27]. IFN-γ contains 143 amino acids with two N-linked glycosylation sites (Asn-25 and Asn-97) [28]. The sites contain approximately 10%, 40% and 50% glycosylated molecules at 0, 1 or 2 potential sites, respectively [29]. The active biological form of IFN-γ is a dimer. Approved recombinant IFN-γ for treating chronic granulomatous disease is called Actimmune® (Intermune, Brisbane, CA, USA). Actimmune® is produced in *Escherichia coli* and it is as a non-glycosylated therapeutic protein. When it was expressed in mammalian cells, the glycosylation pattern is similar to the native IFN-γ. Although glycocomponent of this protein has been involved in folding, dimerization, protein circulating half-life [30], providing protection against proteolytic inactivation [29], *in vitro* assay indicated the same specific antiviral activities of unglycosylated Actimmune® versus glycosylated form [31,32].

Glycan portions have a very different number of functions in biological systems, making them pertinent to biotherapeutics. Initiating with the co-translational attachment of glycan structure, Petrescu et al. [33] offered that glycan structures perform as nucleation sites for remote aromatic portions in an amino acid sequence in order to start the folding process. In this case, Interferon-β (IFN-β) is an example for indicating that glycan structures are often not necessary to sustain structure [34,35]. Human IFN-β as a glycoprotein is produced in response to viral infection or other biological menaces [36]. This contains a conserved Asn-80 glycan attachment site, which is marketed as a biotherapeutic for the treatment of multiple sclerosis. Avonex®, Rebif®, Betaseron® and Extavia® are IFN-β products which are available in market. The licensed drugs Avonex® and Rebif® are produced in Chinese hamster ovary (CHO) cells as fully glycosylated products while Betaseron® and Extavia® are produced in *E. coli* as glycosylated pharmaceutical [37]. In the comparison of Avonex® and Betaseron®, the glycosylated form had a 10-fold increased anti-viral assay activity [38]. Deglycosylation of Avonex® resulted in precipitating due to the formation of disulfide bonds between single molecules [26]. Also, further examination indicated that even when enzymatically deglycosylated, Avonex® still demonstrated increased heat stability (7℃ higher Tm) and anti-viral assay activity (3-fold

larger) when compared to Betaseron®, which reveals the significant role of glycan structure in the folding of the protein [39].

2.2 Monoclonal Antibodies (mAbs)

Monoclonal antibodies are recently the best-selling biotechnology products and are anticipated to constitute five of the top ten selling pharmaceutical products by 2016 (EvaluatePharma Ltd.). Recently, in order for the treatment of cancer and autoimmune disorders, over 30 mAbs are prescribed which are approved by FDA [40]. Also, it is assumed that the number of approved antibody therapeutics increases significantly in near future [41] with a total of 205 reported antibodies under trial [42]. In the late 19th century Paul Ehrlich introduced the concept of a 'magic bullet' that would go directly to the pursued target epitope without harming the healthy tissue [41]. The development of hybridoma cells by Kohler and Milstein [43] to produce antibodies of predefined specificity and the utilization of phage display libraries for antibody humanization by Greg Winter and Richard Lerner were the basis of Ehrlich's approach [44,45]. Antibodies are constructed by a pair of heavy chains and an additional pair of light-chains covalently bound together by disulphide bridges.

Hypervariable segments in the heavy and light chains are complementary determining regions (CDR). CDRs are the antigen-binding sites, which are liable for effector functions such as activating the complement cascade or binding to Fc receptors. Two basic aspects of biological action of IgG class antibodies are complement-dependent cellular cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). CDC means the high specificity of the CDR region of antibody for binding to Fc region of target cell. Antibody employs the C1q component of the complement cascade. The final result of the cascade is formation of the membrane attack complex, which disturbs the target cell's membrane and ultimately lyses it. In ADCC mechanism the antibody Fab binds to a target cell, and through the Fc, binds to the surface receptor (Fcγ) of neutrophils (FcγII-A) and natural killer cells (FcγRIII). As a result of this antibody-mediated interaction, the effector cells release cytokines and cytotoxic granules, by which the target cell is invaded and taken towards apoptosis. The biological *in vivo* mechanisms of antibodies are extremely related to the glycan moiety on the Cγ2 domains [46,47,48,49]. Since the Asn-297 glycans directly influence the three-dimensional structure of the Fc [50], they greatly affect the FcγRs affinity for the IgGFc [51,52]. The close vicinity of both Cγ2 domains in the Fc causes considerable steric hindrance, which restricts the oligosaccharide structures.

The host cell line determines particular features of the potential carbohydrate residues such as bi-secting GlcNAc residues or core fucosylation. With regard to the effect of bisecting structures present in antibody glycan moieties, it has been demonstrated that bisecting GlcNAc sugar residues cause increased ADCC [53]. Nonetheless, the present (or absence) of other monosaccharides have a more deep effect on *in vivo* modes of action. According to the former reports, it has been demonstrated that absence of core fucose can intensify ADCC up to 50-fold [54,55]. Following this finding, third generation mAbs have been largely developed and recently have been undergoing many promising clinical trials [56]. However, the affinity of the IgGFc for the C1q portion is enhanced significantly by galactose terminating structures in the Fc fragment of antibodies, (the most plentiful terminating saccharides on the Fc glycans of human polyclonal antibodies) [57]. Their removal leads to decreased complement activation [51].

Sialic acid terminating glycan moieties can also profoundly adjust the effectiveness of mAbs. Due to the special restrictions in the Cγ2 domain of IgG antibodies [58] only a small ratio of human IgG is naturally sialylated [59]. Sialic acid can be found in either α 2, 3- or α 2, 6-linkage to the galactose moieties of IgG [60], and these residues are essential for anti-inflammatory activity *in vivo*, where the α 2, 6-linkage has priority over the α 2, 3, even though they are not plentiful [61]. Actually, after removal of IgG containing α 2, 6-linked sialic acid on their Fc glycan moiety, anti-inflammatory activity has been nullified. Likewise, anti-inflammatory response at 10-fold reduced doses was provoked by a 10-fold enhancement in sialic acid containing IgGs. On the other hand, IgG with α 2, 3-linked sialic acid terminating glycan structures could not provoke anti-inflammatory response even at 4-fold higher doses, therefore showing the specificity of *in vivo* bioactivity not only to structural but also conformational distinctions in glycan moieties. Furthermore, glycan moieties can have an influence pharmacokinetics of biotherapeutic antibodies as well as adjusting the *in vivo* mechanism. Also high mannose

structures, which are oligosaccharides with five or more mannose residues, can raise plasma clearance and in consequence will decrease in *in vivo* half-life with a considerable negative impact on effectiveness of drug [62].

It is well known that the glycan moieties in Fc region [46] of therapeutic antibodies influence *in vivo* mechanism and pharmacokinetics significantly, but nevertheless the Fab region can be utilized to increase therapeutic antibodies features, as well. Estimation has been raised that in the Fab region, where a potential glycosylation site can be detected in both the heavy and the light chain, between 20-30% of antibodies carry a glycan structure, too [63]. Even though the role of variable region carbohydrates has not become obvious, it is offered that glycan moieties may effect on antigen affinity, specificity, antibody solubility and stability, thus restricting aggregation [64]. Injecting mice with humanized IgG with various glycan moieties at the Asn-56 position of the heavy chain variable region verified *in vivo* half-life modulation as another biological function of Fab region glycans [65]. It became evident from the outcomes that although sialic acid and galactose terminating glycan moieties had very restricted influence on clearance, exposed GlcNAc residues demonstrated a little faster clearance rates. It was suggested that the exposed GlcNAc residues are identified by Man/GlcNAc receptors and binding interactions may be strong enough to make greater clearance possible. It was later specified the tissue affected half-life where the antibodies accumulated according to their Fab region glycans and so it provides further possibility for improvement in targeting of mAb biotherapeutics.

By contrast a study has shown that Fab glycosylation has some negative effects in patients and patients treated with the commercial antibody Cetuximab, α-1, 3 galactose residues on the Fab glycans of this drug produced anti-α-1, 3-galactose IgE-induced anaphylaxis [66]. The relevance of Fab glycans for mAb safety is stressed by this impact. Notably, the complexity of the structure of glycoform on the Fab region is much more than the equivalent Fc glycoform including enhanced sialylation and occurrence of tri-antennary structures [63]. Not surprisingly, different glycoforms will appear at different positions due to changing accessibility, which is because the glycan tripeptide sequences will vary within a population of antibodies, and also glycan location in the sequence can shift [67]. It was demonstrated that the glycan moiety might vary from a high Man structure to a complex structure by shifting the tripeptide sequence in the variable region of a mAb. This would propose that meanwhile introducing the glycan precursor co-translationally to the protein backbone, the local conformation around the glycan might not permit for further enzymatic processing of the attached high Man structure in the Golgi apparatus. In addition, changing the position of glycan structure within the polypeptide chain can influence antigen affinity considerably by either contributing to it or by blocking it completely. Therefore, improving antigen affinity, antibody targeting and extending half-life is accessible through glycan engineering of antibody variable regions [39].

2.3 Erythropoietin (EPO)

EPO is a 165-amino acid, 36-KDa glycoprotein showing three N-linked and one O-linked sugar side chains that in overall account for almost 40% of the molecules mass. Innate human EPO is highly heterogeneous between individual chains and within each N-linked structure in the core structures at the sialic acid content, distribution and N-linked sites or both. N-linked glycosylation has important role in the stabilization, secretion and activity of the molecule [68]. Producer cells secrete poor product after removing either two (Asn-38 and Asn-83) or all three of the N-linked sites (Asn-24) by site-directed mutagenesis. EPO activity is slightly affected by removal of any N-linked chain, as measured *in vitro*. However, this removal considerably reduces its *in vivo* activity, for example, by stimulation of erythropoiesis in polycythemic mice. Apparently, the reduction of the molecule's plasma half-life mediated the latter impact largely. Highly branched EPO glycoforms and sialylated N-linked carbohydrates show longer half-lives in circulation compared to less branched or sialylated molecules, While the plasma half-life of recombinant human EPO given intravenously to rodent is 5-6 h; it is less than 2 min for desialylated EPO [69]. Removing sialic acid caps leads to exposure of side chain galactose residues, which are up taken by hepatic galactose-specific receptors, hence bringing about quick removal from circulation [70]. Through further experiments, diverse *in vivo* activity levels can be showed by expression of EPO preparations in different mammalian cell lines, which are probably a consequence of differential glycosylation [71]. As opposed to N-linked chains, the molecules *in vivo* biological

activity is slightly affected by removal of EPO's single O-linked side chain [72]. A glyco-engineered Darbepoetin was produced to increase protein half-life, enhance drug efficacy and thus, reduce dosing rates in patients [73]. By altering five amino acid residues through sit-directed mutagenesis, two further N-linked glycosylation were generated in Darbepoetin. The resulting biopharmaceutical commercially marketed as Aranesp® demonstrated three-fold lower plasma clearance rate and higher *in vivo* potency compared to epoetin with three N-linked glycan sites only [68,39]. Generally, it is essential to provide detailed biochemical feature of a glycocomponent, and also determine *in vitro* and *in vivo* activity in order to approve for general medical utilization [32].

2.4 Tissue Plasminogen Activator (tPA)

Tissue plasminogen activator is a glycosylated therapeutic protein synthesized mainly in vascular endothelial cells. Native tPA consists of 527 amino acids. Approved recombinant forms of human tPA are expressed in CHO cell lines (Activase as well as Methylase/ TNKase; Genetech, S. San Francisco, CA, USA). The tPA molecule has four possible glycosylation sites, three of which are ordinarily occupied (Asn-117, Asn-184 and Asn-448). Of these, Asn-117 and Asn-448 are always glycosylated, while Asn-184 shows changeable site occupancy in native and recombinant molecules. Thus, this changeable site glycosylation results in the production of two tPA variants: namely, type I (all three glycosylation sites indwelled) and type II (two glycosylation sites occupied) [74]. In spite of being biologically active, the former demonstrates slightly decreased fibrin binding and clot lysis activity [75], and glycosylation at Asn-184 can also affect plasma clearance rates [76]. These variations give significance of the monitoring of product batch-to-batch stability. This can be affected especially by cell culture conditions such as temperature, butyrate concentration and culture duration, which influence the degree of glycosylation [77,32].

3. GLYCOSYLATION ROLE IN RESTRAINING THE CHEMICAL INCONSTANCIES

In this context we describe oxidation, proteolytic degradation and chemical crosslinking. These are chemical inconstancies, which can be enhanced by glycosylation.

3.1 Proteolytic Degradation

Protease enzymes, which are present in digestive system, can chemically damage the therapeutic proteins. To avoid this it is mandatory to take this type of medicines intravenously. However, the proteolytic degradation of proteins given by other routes may occur due to the general expression of proteases [78]. Thus, the efficiency and constancy of protein pharmaceutics the *in vivo* are diligently depended on how stable they are against proteolytic degradation [79,78]. It has been discovered that glycosylation protects proteins against proteolytic degradation [80]. The existence of glycan mechanistically leads to a steric hindrance nearby the peptide backbone of the amino acids is caused by the presence of glycan in glycosylation site and leads to the proposed proteolytic stability [81,82,83]. Some instances are Granulocyte-colony stimulating factor (G-CSF) (Granocyte®, Chugai Pharma) [84], lipase (Merispase®; Meristem Therapeutics) [85], ribonuclease (Onconase®; Alfacell) [86], urokinase (Abbokinase®; ImaRx Therapeutics) [14], interferon-γ (Actimmune®; Intermune) [29], streptokinase [87], amylase [88], lysosomal integral membrane proteins, Lamp-1 and Lamp-2 [89], and peroxidase [90]. In addition, Holcenberg et al. [91] explained chemical glycosylation of asparaginase and Raju and Scallon [92] explained the enzymatic glycosylation of IgG-like antibodies as evidences of engineering of proteolytic stability. Based on findings of the last research, when IgG-like antibodies were exposed to papain digestion, *in vitro* proteolytic stability increased dramatically as a result of changing the glycan structures in end-terminal (e.g. sialic acid and N-acetylglucosamine) [92,26].

3.2 Oxidation

Owing to the oxidation of several amino acid side chains (Tyr, His, Cys, Met, Trp) the bioactivity of protein pharmaceuticals can be lost during production or storage process [93,6]. As a result of the combination of small amounts of transition metals, unprotected ultraviolet light facing, and atmospheric oxygen, active oxygen-based radicals are produced in protein formulations that lead to oxidation occurrence.

Up to the present time, erythropoietin (Epogen®, Procit®; Amgen, Ortho) is the only reported protein that can have influenced bioactivity by oxidation and glycosylation can improve this

chemical instability. For erythropoietin the level of oxidation in tryptophan in oxidizing conditions is cause the loss of bioactivity in this protein. Comparing the oxidative susceptibility in deglycosylated erythropoietin and naturally glycosylated one was disclosed that glycosylation decreased the inactivation and also the rate of oxidation in tryptophan [94]. Following these results it is suggested that using active oxygen radicals, glycosylation protection of the protein structure from damage is possible. However, more studies are still required to provide more information about the mechanisms of this stabilization and to discover the extent to which engineered glycosylation could hinder this type of instability. In addition, further research is needed to find out if stabilizing effect specifically happens when the glycans are attached to the protein surface or it depends on the radical scavenging proficiencies of the glycans [26].

3.3 Chemical Crosslinking

Polymerization triggered by both disulfide and non-disulfide crosslinking pathways can constitute covalent dimers and oligomers in protein therapeutics. It is consequential to avert the formation of these covalently linked species in therapeutic protein as these frequently result in loss of bioactivity. Moreover, for many proteins it has been realized that this type of instability, as well as protein unfolding, could bring about the formation of larger soluble and insoluble protein aggregates [8]. There are some reports to illustrate that glycosylation hinders the formation of these cross-linked species. For instance, according to Oh-eda et al. [95] the existence of the single glycan in human granulocyte colony-stimulating factor (G-CSF) (Granocyte®; Chugai Pharma) averted the polymerization-induced inactivation of the protein. Krishnan et al. [96] and Raso et al. [97] studied the mechanism G-CSF aggregation and figured out that it was due to disulfide crosslinking. Another example of a therapeutic protein is interferon beta (Rebif®, Pfizer/Serono; Avonex®, Biogen) whose inactivation is averted by glycosylation owing to disulfide crosslinking [38]. Furthermore, in case of non-disulfide protein, glycosylation has been reported to avoid crosslinking. For instance engineered chemical glycosylation of insulin, particularly at the PheB-1 amino group, averted the self-association of the protein into dimers and oligomeric species [98]. Because of a transamidation reaction between AsnA-21 and PheB-1, cross-linked insulin species form [99]. The resulted finding is consequential since it shows that this type of stabilization can also be engineered into proteins through logically designed glycosylation. These findings also offer that the mechanism by which this type of instability is obstructed is because of enhanced intermolecular steric repulsion between the crosslinking-prone protein species owing to the glycan's existence at the protein surface [26].

4. GLYCOSYLATION ROLE IN RESTRAINING THE STRUCTURAL INCONSTANCIES

The functional effectiveness of proteins essentially relies on the conformational stability of their intrinsically folded state [99]. In order to decrease the exposure of protein hydrophobic residues in aqueous solution, the majority of proteins use a tertiary structure by folding [100,101,102]. This forms a compact native state with a hydrophobic core that is additionally energetically stabilized by the existence of several kinds of atomic interactions within the protein core (e.g. electrostatic, and charge-charge interactions, hydrogen bonds, Van der Waals interactions) [91,92,101]. Unfortunately, because these forces are naturally non-covalent, thermodynamic and kinetic stability of this state which is resulted shows a tendency to be innately low [99] thus, small or large-scale protein structural changes will be resulted by any physical and chemical phenomena, which can interfere these forces. These conformational changed species are more likely to interact either with themselves or with the hydrophobic surfaces and interfaces present during protein manufacturing and storage, hence further structural instabilities such as adsorption, aggregation and precipitation [102,103]. Pharmaceutically-relevant phenomena can result in protein structural instability whose examples include being subject to extremes of temperature and pH; being subject to amphipathic interfaces (e.g. aqueous/organic solvent, aqueous/air), hydrophobic surfaces, and chemical denaturants; and formulation at extreme protein concentrations. In this context, we explain those physical protein instabilities, which reported to be bettered by glycosylation (including precipitation, pH denaturation and aggregation) [26].

4.1 Precipitation

In order to design a protein-based formulation, gaining the favorable therapeutic protein concentration in solution is one of the most basic challenges [104]. Indeed, this is because protein

solubility not only has negative correlation with the protein concentration but also has direct correlation with the solution's pH, temperature, ionic strength and excipient concentration [4,104,105]. Thus, by increasing the target concentration of the formulation (e.g.≥ 100 mg/mL) a more acute problem related to protein precipitation emerges [104]. It has been demonstrated that glycosylation enhance the solubility of many proteins [106], even though the generality of this influence has been questionable [107]. Some instances are interferon beta (Rebif®, Pfizer/Serono; Avonex®, Biogen) [108], alpha-galactosidase A (Replagal®, Shire) [109] and glucose oxidase [110]. In terms of the effect of glycosylation on peroxidase, it was identified that the solubility of the protein demonstrated a linear dependence with the glycosylation degree [111]. Regarding the fact that the glycans have a higher tendency for the aqueous solvent than the polypeptide chain, this enhanced solubility could be because of a greater hydration potential, but it is demonstrated that this is not the case, though [112]. Increased protein solubility cannot be caused by strengthened interactions with the aqueous solvent, but the mechanism caused by glycosylation [112]. Sola and Griebenow [25] conducted a comparative *in silico* structural and energetic analysis on a series of chemically glycosylated α-chymotrypsin conjugates with increasing levels of glycosylation that can be resulted in an alternative explanation. From these computer simulations it was discovered that there was a linear correlation between the whole molecular solvent accessible surface area (SASA) for the whole glycoprotein and the glycosylation degree which was in accord with the solubility findings [25,111,113]. Thus, the suggested mechanism for this increase protein solubility is that the presence of the glycans brings about an overall greater molecular solvent accessible surface area (SASA) which led to an increase in the number of possible interactions between the glycoprotein surface and the surrounding solvent molecules [26].

4.2 pH Denaturation

When protein is in extreme pH, it may lose its structure because of infraction of charge-charge interactions and also internal electrostatic forces. And when the pH value is much higher than isoelectric point, there will be enhances in unfolding propensity of proteins due to electrostatic repulsions between atoms with similar charge [99,114]. Moreover, in extreme pH the structural unfolding tendency of proteins can also be raised when the potency of salt bridge formation between the atoms with different charges is decreased [99]. Electrostatic free energy and local charge density of protein reduces due to this imperfect unfolding and so it causes the global unfolding [99,91]. According to several reports, in pH extremes we can maintain the conformational stability of proteins by glycosylation. GSCFS (Granocyte®; Chugai Pharma) [115], erythropoietin (Epogen®, Procit®; Amgen, Ortho) [116], amylase [88], bromelain [117], glucose oxidase [118] and tripeptidyl peptidase are some examples [119]. It is also possible to artificially engineer enhanced pH stability into proteins via the glycation of penicillin G acylase [120]. When contrasted to the non-glycated protein, at pH 3 and pH10, the half-life of glycated protein was enhanced 13-fold and 7-fold respectively [120]. Mechanistically as a result of glycosylation, an enhancement in the internal electrostatic interactions of the protein occurs which leads to this type of stabilization [25]. Sola and Griebenow [113] arranged some inductive *in silico* energetic and structural analysis. They increased the levels of glycosylation for a set of α-chymotrypsin conjugates, which were chemically glycosylated. The computer simulations revealed that the solvent accessible surface area (SASA) of glycoconjugates protein and number of glycans, which are bounded to surface, are negatively correlated [25,113]. Hence, the glycans as a molecular spacer increase the efficient distance between the solvent and protein electrostatics. The surrounding water molecules on protein leads to smaller dielectric screening effect and increases the stability of the internal electrostatic interactions. In Sola and Griebenow [113] in silico analyzes the coulombic energy parameter increased as the glycosylation degree was enhanced and generally it was reflected in larger negative values. This phenomenon also has the unique characteristic that it changes the entire conformational fluctuations in protein and makes it non-slaved. Physically, this phenomenon causes enhancement in conformational stability and reduction in structural dynamics for glycosylated proteins [25,26,113,121,122,123]. This phenomenon also has the unique characteristic that it changes the entire conformational fluctuations in protein and makes it non-slaved. Physically, this phenomenon causes enhancement in conformational stability and reduction in structural dynamics for glycosylated proteins [25,26,113,121,122, 123].

4.3 Aggregation

Proteins are colloids with large molecular measures and high intermolecular interaction potentials [10,12 and 124]. These characteristics of proteins result in the protein structure vulnerability to aggregation-prone phase transitions that are dependent on pH, temperature, and protein concentration. The increased production costs, which are due to additional protein recovery and refolding protocols, coupled with potential detrimental effects on patients result in undesirability of the aggregation of protein pharmaceuticals [8,9,10,11,12,13,125,126]. Several reports have demonstrated that glycosylation either diminishes or averts protein aggregation. Baudys et al. [98], showed that the physical stability of insulin may improve by chemically attaching small size glycan to it and decreasing the aggregation kinetics of the molecule. In this study, the decreased insulin aggregation was due to inhibiting transamidation crosslinking reaction offering a stabilizing mechanism including steric intermolecular repulsion phenomena [98]. According to Ioannou et al. [109] glycosylation at Asn-215 is needed for α-galactosidase A (REPLAGAL®; Shire) to divert the hydrophobic patch that simplifies the congestion of the protein to be exposed. Deglycosylation of thyroid-stimulating hormone (Thyrogen®; Genzyme) led to more susceptibility of the protein to aggregation. Endo et al. [127] reported same results for erythropoietin (Epogen®, Procit®; Amgen, Ortho). Also Hoiberg-Nielsen et al. [128] proved that colloidal stability will increase in the case of glycosylated phytase. The fore-mentioned studies on this protein suggested that the inhibition of aggregation possibly does not depend on their hydration-related features but on steric hindrance of the glycans in unfolded proteins. [112,128,129]. An accelerated aggregation study was performed by Sola et al. [130] that targeted at figuring out the systematic variation in the glycosylation parameters by which on-specific protein aggregation could be affected [25]. Findings revealed that under extreme conditions (temperature=60℃ and protein concentration= 20 mg/mL), aggregation inhibition was impossible by the smaller sized glycans regardless of the amount bound to the protein surface. Contrariwise, with two or more of the larger sized glycans, the aggregation process was completely prevented upon chemical glycosylation [25,130]. Regarding these results, this mechanism is suggested that the presence of the glycans on the surface of the protein leads to an increase in steric repulsions between aggregation-prone protein species, which then averts protein aggregation [26].

5. CONCLUSIONS

More than one-third of approved biotherapeutics and many clinical trials are glycoproteins [32] that are produced in eukaryotic systems (for example, CHO, NS0 or BHK) rather than prokaryotes. Protein drugs' folding, stability, trafficking, immunogenicity as well as their primary activities are influenced by the presence and nature of the oligosaccharides apparently [32,131]. The host selection for therapeutic protein expression and cell culture conditions has an important impact on glycosylation identity and heterogeneity of recombinant glycoproteins. Therefore, attached glycans forming the glycosylation profile can have some effect on biological activity and chemical effectiveness. It is evidently of high value and importance to precisely recognize glycan components and quantify various forms. One of the principal obstacles to accept alternate expression hosts is that the presence of nonhuman glycosylation may cause unwanted immunogenicity in humans. The use of glycoengineering to overcome the limitation of the improper glycosylation of these production systems is thus required for producing high quality and safe products for human use [132]. We predict that generic protein therapeutics will become more common as the patents related to the original proteins terminate. Glycoengineering monoclonal antibodies have a great potential to improve effector function, complement activation and use ADCC as a selective binding to FC receptors. Therefore, glycoengineering can increase the biological activity of therapeutic proteins and improve their pharmacokinetics. Glycoengineering, along with protein engineering, is very important in fine-tuning therapeutic protein to meet the therapeutic needs [133]. As mentioned in this article, a host of pharmaceutically relevant chemical and physical protein instabilities have been improved by glycosylation. The different glycosylation parameters (e.g. number of glycans attached and glycan molecular size) studied so far can seemingly affect stabilization of the protein differently. Although enhancing the glycosylation degree increases the internal non-covalent forces and strengthens protein structure, which apparently leads to stabilization of the protein native state, the protein unfolded-state seems to be destabilized by increasing the glycan molecular size. There are some areas in the

review, which require more basic knowledge to further describe the effects of glycosylation. For instance, more research is still need to be carried out in order to investigate the effect of glycosylation on the behavior of the unfolded state. Additionally, in order to understand the mechanisms by which glycans avert chemical instability occurrences further systematic studies are required. Glycosylation could also improve or avert other instabilities not explored so far (e.g. deamidation, β-elimination, racemization, adsorption to amphipathic interfaces and hydrophobic surfaces, which are important to mention and thus remains to be tested.

ACKNOWLEDGEMENTS

The authors are thankful to the Director of Branch of central Region of Iran, Agricultural Biotechnology Research Institute of Iran (ABRII), Isfahan, Dr. Mahmoud Otroshi and the Deputy of institute, Esmaeil Roohalamin.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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