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Effects of Glycosylation on the Stability of Protein Pharmaceuticals

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Abstract

In recent decades, protein-based therapeutics have substantially expanded the field of molecular pharmacology due to their outstanding potential for the treatment of disease. Unfortunately, protein pharmaceuticals display a series of intrinsic physical and chemical instability problems during their production, purification, storage, and delivery that can adversely impact their final therapeutic efficacies. This has prompted an intense search for generalized strategies to engineer the long-term stability of proteins during their pharmaceutical employment. Due to the well known effect that glycans have in increasing the overall stability of glycoproteins, rational manipulation of the glycosylation parameters through glycoengineering could become a promising approach to improve both the *in vitro* and *in vivo* stability of protein pharmaceuticals. The intent of this review is therefore to further the field of protein glycoengineering by increasing the general understanding of the mechanisms by which glycosylation improves the molecular stability of protein pharmaceuticals. This is achieved by presenting a survey of the different instabilities displayed by protein pharmaceuticals, by addressing which of these instabilities can be improved by glycosylation, and by discussing the possible mechanisms by which glycans induce these stabilization effects.

Keywords

biopharmaceutics; biophysical models; chemical stability; glycosylation; molecular modeling; physical stability; physicochemical properties; proteins; stabilization; thermodynamics

INTRODUCTION

The employment of proteins as pharmaceutical agents has greatly expanded the field of molecular pharmacology as these generally display therapeutically favorable properties, such as, higher target specificity and pharmacological potency when compared to traditional small molecule drugs.[1,2] Unfortunately, the structural instability issues generally displayed by this class of molecules still remain one of the biggest challenges to their pharmaceutical employment, as these can negatively impact their final therapeutic efficacies (Tab. 1).[2–50] In contrast to traditional small molecule drugs whose physicochemical properties and structural stabilities are often much simpler to predict and control, the structural complexity and diversity arising due to the macromolecular nature of proteins has hampered the development of predictive methods and generalized strategies concerning their chemical as well as their physical stabilizations.[51,52] While the protein primary structure is subject to

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the same chemical instability issues as traditional small molecule therapeutics (e.g. acid-base and redox chemistry, chemical fragmentation, etc), the higher levels of protein structure (e.g., secondary, tertiary) often necessary for therapeutic efficacy can also result in additional physical instability issues (e.g., irreversible conformational changes, local and global unfolding) due to their non-covalent nature.[2,15,53-55] The innate propensity of proteins to undergo structural changes coupled with the fact that there is only a marginal difference in thermodynamic stability between their folded and unfolded states provides a significant hurdle for the long-term stabilization of protein pharmaceuticals. This is due to the fact that a thermodynamically stabilized protein could still inactivate kinetically even at the relatively low temperatures used during storage.[2,53,55-59] Additionally, as a result of their colloidal nature, proteins are prone to pH, temperature, and concentration dependant precipitation, surface adsorption, and non-native supramolecular aggregation.[11,14,20,47,60-65] These instability issues are further compounded by the fact that the various levels of protein structure can become perturbed differently depending on the physicochemical environment to which the protein is exposed.[2] This is of special relevance in a pharmaceutical production setting where proteins can be simultaneously exposed to several destabilizing environments during their production, purification, storage, and delivery (Tab. 1).

Due to these stability problems much emphasis has been given to the development of strategies for the effective long-term stabilization of protein pharmaceuticals.[2,4,11,61,66-77] These include external stabilization by influencing the properties of the surrounding solvent through the use of stabilizing excipients (e.g., amino acids, sugars, polyols) and internal stabilization by altering the structural characteristics of the protein through chemical modifications (e.g. mutations, glycosylation, pegylation).[2,53,58] While many protein pharmaceuticals have been successfully formulated by employing stabilizing mutations, excipients, and pegylation, their use can sometimes be problematic due to limitations, such as, predicting the stabilizing nature of amino acid substitutions, the occurrence of protein and excipient dependant non-generalized stabilization effects, protein / excipient phase separation upon freezing, cross-reactions between some excipients and the multiple chemical functionalities present in proteins, acceleration of certain chemical (e.g. aspartate isomerization) and physical (e.g. aggregation) instabilities by some excipients (e.g., sorbitol, glycerol, sucrose), detection interferences caused by some sugar excipients during various protein analysis methods, and safety concerns regarding the long-term use of pegylated proteins *in vivo* due to possible PEG induced immunogenecity and chronic accumulation toxicity resulting from its reduced degradation and clearance rates.[2,4,33,48,66,78-95]

Due to these limitations, there is still a need for further development of additional strategies of protein stabilization.[2] Amongst the chemical modification methods, glycosylation represents one of the most promising approaches as it is generally perceived that through manipulation of key glycosylation parameters (e.g. glycosylation degree, glycan size and glycan structural composition) the protein's molecular stability could be engineered as desired.[2,66,96-105] In this context, it is important to highlight the fact that glycosylation has been reported to simultaneously stabilize a variety of proteins against almost all of the major physicochemical instabilities encountered during their pharmaceutical employment (Tab. 2), suggesting the generality of these effects.

Even though a vast amount of studies have evidenced the fact that glycosylation can lead to enhanced molecular stabilities and therapeutic efficacies for protein pharmaceuticals (Tab. 3), an encompassing perspective on this subject is still missing due to the lack of a comprehensive review of the literature. The intent of this article is therefore to further the field of protein glycoengineering by increasing the general understanding of the mechanisms

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by which glycosylation improves the molecular stability of protein pharmaceuticals. This is achieved by presenting a survey of the different instabilities displayed by protein pharmaceuticals, by addressing which of these instabilities can be improved by glycosylation, and by discussing the possible mechanisms by which glycans induce these stabilization effects.

PROTEIN GLYCOSYLATION

Protein glycosylation is one of the most common structural modifications employed by biological systems to expand proteome diversity.106-108 Evolutionarily, glycosylation is widespread found to occur in proteins through the main domains of life (archaea, eubacteria, bacteria and eukarya).109,110 The prevalence of glycosylation is such that it has been estimated that 50% of all proteins are glycosylated.111 Functionally, glycosylation has been shown to influence a variety of critical biological processes at both the cellular (e.g. intracellular targeting) and protein levels (e.g. protein-protein binding, protein molecular stability).103 It should therefore not come as a surprise that a substantial fraction of the currently approved protein pharmaceuticals need to be properly glycosylated to exhibit optimal therapeutic efficacy.100,112

Structurally, glycosylation is highly complex due to the fact that there can be heterogeneity with respect to the site of glycan attachment (macroheterogeneity) and with respect to the glycan's structure (microheterogeneity). Although many protein residues have been found to be glycosylated with a variety of glycans (for a detailed discussion see review by Sears and Wong), in humans the most prevalent glycosylation sites occur at asparagine residues (N-linked glycosylation through Asn-X-Thr/Ser recognition sequence) and at serine or threonine residues (O-linked glycosylation) with the following monosaccharides: fucose, galactose, mannose (Man), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine, and sialic acid (N-acetylneuraminic acid).109,113-115 Since all of the potential glycosylation sites are not simultaneously occupied this leads to the formation of glycoforms with differences in the number of attached glycans. Further structural complexity can occur due to variability in the glycan's monosaccharide sequence order, branching pattern, and length. In humans N-linked glycan structures are classified in three principal categories according to their monosaccharide content and structure: high mannose type ($Man_{2-6}Man_3GlcNAc_2$), mixed type ($GlcNAc_2Man_3GlcNAc_2$), and hybrid type ($Man_3GlcNAcMan_3GlcNAc_2$).113 The terminal ends of these glycans are often further functionalized with chemically charged groups (e.g., phosphates, sulfates, carboxylic acids) in human glycoproteins, leading to even greater structural diversity. These charged glycans most probably impact to some degree the overall stability of glycoproteins since they can alter their isoelectric point (pI).116,117 Some of these charged terminal glycans (e.g., sialic acid) have also been found to be critical in regulating the circulatory half-life of glycoproteins. This has led to the development of glycosylation as a novel strategy to improve the therapeutic efficacies of protein pharmaceuticals by engineering their pharmacokinetic profiles (for a detailed discussion see the recent review by Sinclair and Elliot).100

Due to the high degree of structural variability arising from physiological (natural) glycosylation, novel strategies are currently being pursued to create structurally homogeneous pharmaceutical glycoproteins with humanized glycosylation patterns.118 These include engineered glycoprotein expression systems (e.g., yeast, plant, and mammalian cells) as well as enzymatic, chemical, and chemo-enzymatic *in vitro* glycosylation remodeling methods. Alternatively, to understand the mechanisms by which glycosylation influences protein physicochemical properties researchers have employed comparatively simpler glycosylation strategies. These include enzymatic deglycosylation of natural glycoproteins, chemical glycosylation via the use of structurally simple chemically-

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activated glycans, and glycation of the lysine residues with reducing sugars via the Maillard reaction. Although some of these glycosylation methods (e.g., glycation) may be undesired for use in protein pharmaceuticals their fundamental scientific value for the understanding the effects of glycosylation on protein stability cannot be ignored.119 This is due to the fact that independently of the method by which the structurally different glycans are attached to the protein surface (e.g. enzymatic and chemical glycosylation, or reductive glycation) they all seem to induce similar stabilization effects.103 In the next sections, we thus focus on discussing which pharmaceutically-relevant chemical and physical protein instabilities have been reported to be ameliorated by glycosylation and discuss possible mechanisms by which glycans achieve such effects.

EFFECTS OF GLYCOSYLATION ON PROTEIN STABILITY CHEMICAL INSTABILITIES PREVENTED BY GLYCOSYLATION

The presence of multiple reactive chemical functionalities in the amino acids side chains of proteins makes them particularly sensitive to several chemical degradation processes. These can include: glutamine (Gln) and asparagine (Asn) deamidation; histidine (His), methionine (Met), cysteine (Cys), tryptophan (Trp), and tyrosine (Tyr) oxidation; serine (Ser), threonine (Thr), phenylalanine (Phe), lysine (Lys), and Cys β-elimination; disulfide fragmentation, exchange, and crosslinking; backbone peptide hydrolysis caused either by proteases or by pH sensitive backbone sequences (e.g., aspartic acid-proline (Asp-X)); transamidation; racemization; and chemically-triggered non-specific crosslinking (Tab. 1). 2,6,8,15,54,55,112 For further detailed discussions on the general mechanisms which trigger these chemical instabilities the reader is referred to several excellent reviews on the subject. 2,6,8,9,12,55 In the next section, we focus on those chemical instabilities which have been reported to be improved by glycosylation (e.g., proteolytic degradation, oxidation, and chemical crosslinking) (Tab. 2).

Proteolytic Degradation

Protein pharmaceuticals are typically administered intravenously and not via the oral route due to their chemical degradation by the proteases of the digestive system.120 However, the systemic expression of proteases also makes proteins administered by other routes highly susceptibly to proteolytic degradation.120 Therefore, the *in vivo* molecular stability and therapeutic efficacy of protein pharmaceuticals is intimately related to their stability towards proteolytic degradation.2,6,100,120 In general, glycosylation has been found to protect proteins against proteolytic degradation.96,121-123 Some examples include granulocyte colony stimulating factor (G-CSF) (GRANOCYTE®, Chugai Pharma),124,125 lipase (MERISPASE®; Meristem Therapeutics),126 protein C (XIGRIS®; Eli Lilly),127 ribonuclease (ONCONASE®; Alfacell),128,129 thyroid-stimulating hormone (THYROGEN®; Genzyme),130 urokinase (ABBOKINASE®; ImaRx Therapeutics),131 interferon-γ(ACTIMMUNE®; Intermune),132 streptokinase,133 cellulose,134 ovomucoid, 135 amylase,136,137 lysosomal integral membrane proteins Lamp-1 and Lamp-2,138 peroxidase,139 and catalase.140 There is also evidence that this proteolytic stability can be engineered into proteins as was described by Holcenberg *et al.* upon chemical glycosylation of asparaginase and by Raju and Scallon upon enzymatic glycosylation of IgG-like antibodies.141,142 Particularly, in this last study it was found that altering the end-terminal glycan structures (e.g., N-acetylglucosamine, galactose, and sialic acid) led to increasingly greater *in vitro* proteolytic stability when subjected to papain digestion.142 Mechanistically, it has been proposed that this proteolytic stability arises due to the fact that the glycan's presence provides a steric hindrance around the peptide backbone of the amino acids adjacent to the glycosylation site.114,115,143 This prevents the contact between the glycoprotein's surface and the cleaving protease's active site.

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Oxidation

Protein pharmaceuticals can potentially lose their bioactivity during their manufacture and storage due to the oxidation of several of their amino acid side chains (His, Met, Cys, Trp, and Tyr).2,6,9,22,55,144 These oxidation events have been mainly attributed to the production of active oxygen-based radicals in protein formulations due to the combination of trace amounts of transition metals, atmospheric oxygen, and exposure to ultraviolet light.2,6 Thus far, erythropoietin (EPOGEN®, PROCIT®; Amgen, Ortho) is the sole reported case of a protein whose bioactivity can be impacted by oxidation and where glycosylation has been found to ameliorate this chemical instability.145 The loss of bioactivity for this protein was found to correlate with the levels of tryptophan oxidation when exposed to oxidizing conditions.145 Comparison of the oxidative susceptibility for the naturally glycosylated erythropoietin with that of its deglycosylated form revealed that glycosylation diminished the tryptophan oxidation rates and the inactivation of this protein.145 These results suggest that glycosylation can protect the protein structure from damage by active oxygen radicals although more studies are still needed to shed some light on the mechanisms of this stabilization and to determine the extent to which engineered glycosylation could prevent this type of instability. Also, whether this stabilizing effect is specific to when the glycans are chemically attached to the protein surface or non-specific having to do more with the radical scavenging capabilities of the glycans remains to be established.70

Chemical Crosslinking

Protein therapeutics can form covalent dimers and oligomers due to polymerization triggered by both disulfide and non-disulfide crosslinking pathways.2,6 Preventing the formation of these covalently linked species in protein pharmaceuticals is important as these frequently lead to loss of bioactivity.2,6 Additionally, for many proteins it has been found that this type of instability, in addition to protein unfolding, could trigger the formation of larger soluble and insoluble protein aggregates.2,6,11 There are several reports in the literature were it has been found that glycosylation prevents the formation of these crosslinked species. For example, Oh-eda *et al.* reported that the presence of the single glycan in human granulocyte colony-stimulating factor (G-CSF) (GRANOCYTE®; Chugai Pharma) prevented the polymerization-induced inactivation of the protein.146 The mechanism by which G-CSF polymerizes was studied by Krishnan *et al.* and Raso *et al.* and found to be due to disulfide crosslinking.147,148 Interferon beta (REBIF®, Pfizer / Serono; AVONEX®, Biogen) is another example of a therapeutically relevant protein where glycosylation prevents its inactivation due to disulfide crosslinking.149 Glycosylation has been also reported to prevent non-disulfide protein crosslinking. For example, Baudys *et al.* reported that engineered chemical glycosylation of insulin, especially at the Phe^{B-1} amino group, suppressed the self-association of the protein into dimers and oligomeric species.97 The formation of these crosslinked insulin species occurs due to a transamidation reaction between Asn^{A-21} and Phe^{B-1}.2 This finding is highly significant since it demonstrates that this type of stabilization can also be engineered into proteins via rationally designed glycosylation. These results additionally suggest that the mechanism by which this type of instability is prevented is due to increased intermolecular steric repulsion between the crosslinking-prone protein species due to the glycan's presence at the protein surface.

PHYSICAL INSTABILITIES PREVENTED BY GLYCOSYLATION

The functional efficacy of proteins critically depends on the conformational stability of their natively folded state.2 Most proteins adopt a tertiary structure by folding as to minimize the exposure of their hydrophobic residues in aqueous solution.56,150-152 This creates a compact native state with a hydrophobic core that is additionally energetically stabilized by the presence of several types of atomic interactions within the protein core (e.g. electrostatic

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and charge-charge interactions, hydrogen bonds, Van der Waals interactions).151¯154 Unfortunately, the resulting thermodynamic and kinetic stability of this state tends to be intrinsically low due to the non-covalent nature of these forces.2,53 Therefore, any physical or chemical phenomena which can disrupt these forces will trigger either small or large scale protein structural changes. These conformationally altered species are more prone to interact either with themselves or with the hydrophobic surfaces and interfaces present during protein manufacturing and storage leading to additionally physical instabilities, such as, adsorption, aggregation, and precipitation.2,42 Examples of pharmaceutically-relevant phenomena that can lead to protein physical instability include exposure to extremes of temperature and pH; exposure to amphipatic interfaces (e.g., aqueous/organic solvent, aqueous/air), hydrophobic surfaces, and chemical denaturants; and formulation at extreme protein concentrations (Tab. 1). For further detailed discussions on the general mechanisms which trigger these physical instabilities the reader is again referred to a series of excellent reviews on the subject.2,6,8,9,11,12,14,42,61,63,64 In the next section, we focus on those physical protein instabilities which have been reported to be improved by glycosylation (e.g., precipitation; pH, chemical, and thermal denaturation; and aggregation) (Tab. 2).

Precipitation

One of the most fundamental challenges when designing a protein-based formulation involves achieving the desired therapeutic protein concentration in solution.2,63 This is due to the fact that protein solubility is not only inversely proportional to the protein concentration but also dependant on the solution's pH, temperature, ionic strength, and excipient concentration.2,52,63,155,156 Therefore, as the target concentration of the formulation is increased (e.g. ≥ 100 mg/mL) protein precipitation becomes a more critical problem.63 Glycosylation has been shown to increase the solubility of many proteins,99,157 although the generality of this effect has been questioned.158 Some examples include interferon beta (REBIF®, Pfizer / Serono; AVONEX®, Biogen),159,160 alpha-galactosidase A (REPLAGAL®, Shire),161 glucose oxidase,162 and invertase.163 While studying the effects of glycosylation on peroxidase, Tams *et al.* determined that the solubility of the protein showed a linear dependence with the glycosylation degree.164 Although one could logically consider that this increased solubility is due to a greater hydration potential since the glycans have a higher affinity for the aqueous solvent than the polypeptide chain, Bagger *et al.* recently showed that this is not the case.165 From this study it was concluded that it is unlikely that strengthened interactions with the aqueous solvent are the mechanism for increased protein solubility due to glycosylation.165 Analternative explanation can be provided from a comparative *in silico* structural and energetic analysis recently performed by Solá and Griebenow on a series of chemically glycosylated α-chymotrypsin conjugates with increasing levels of glycosylation (Fig. 1).103,166 From these computer simulations it was found that the overall molecular solvent accessible surface area (SASA) for the whole glycoprotein increased linearly as the glycosylation degree was increased (Fig. 2A).103,166 The linear dependence of these results are agreement with the solubility findings of Tams *et al.*.164 These results therefore suggest the mechanism by which glycosylation increases protein solubility is due to an increase in the number of possible interactions between the glycoprotein surface and the surrounding solvent molecules due to an overall greater molecular solvent accessible surface area (SASA) caused by the presence of the glycans.

pH Denaturation

Exposure of proteins to extremes of pH can result in loss of structure by disruption of both internal electrostatic forces and charge-charge interactions.2 At extreme pH values, far from the isoelectric point (pI), the unfolding propensity of proteins increases as a result of electrostatic repulsions between similarly charged atoms.2,151,167,168 Additionally, the diminished capability of salt bridge formation between differently charged atoms at

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extremes of pH can also increase the structural unfolding propensity of proteins.2 This partial unfolding leads to a reduction in local charge density which can further decrease the electrostatic free energy of the protein leading to global unfolding.2,169

There are several reports were glycosylation is essential in maintaining the conformational stability of proteins against pH denaturation. Some examples include GCSF (GRANOCYTE®; Chugai Pharma),124,170 erythropoietin (EPOGEN®, PROCIT®; Amgen, Ortho),171 acid phosphatase,172 amylase,137 bromelain,173 fibronectin,174 cathepsin E, 175 glucose oxidase,176 and tripeptidyl peptidase.177 Increased pH stability can be also artificially engineered into proteins as was demonstrated by Masárová through the glycation of penicillin G acylase.178 The half-life for the glycated version of this protein was increased 13-fold at pH 3 and 7-fold at pH 10 when compared to the non-glycated protein. 178

Mechanistically this type of stabilization occurs due to an increase in the internal electrostatic interactions of the protein as a result of glycosylation.103 Support for this mechanism was recently provided by the comparative *in silico* structural and energetic analysis conducted by Solá and Griebenow on a series of chemically glycosylated αchymotrypsin conjugates with increasing levels of glycosylation (Fig. 1).103,166 From these computer simulations it was found that the solvent accessible surface area (SASA) for the protein portion of the glycoconjugates decreased linearly as the number of surface bound glycans was increased (Fig. 2B).103,166 The presence of the glycans thus increases the effective distance between the protein electrostatics and the solvent electrostatics by acting as a molecular spacer. This should lead to an increase in the strength of the internal electrostatic interactions for the protein due to a smaller dielectric screening effect on the protein by the surrounding water molecules.103,166 The observed increase in the coulombic energy parameter (reflected in larger negative values) as the glycosylation degree was increased for the *in silico* glycoconjugates analyzed by Solá and Griebenow provide support for the occurrence of this phenomena (Fig. 2C). This phenomenon also has the peculiarity that it transforms the overall conformational fluctuations of the protein from being solvent slaved to non-slaved (slaved refers to molecular phenomena influenced by the solvent electric dipole moment fluctuations).103,166,179,180 Physically this transduces into the generally observed decrease in structural dynamics and increase in conformational stability for glycosylated proteins (Fig. 3 and 4).102,103,166

Chemical Denaturation

In addition to electrostatic interactions, the native state of proteins is also conformationally stabilized by other non-covalent forces, such as, hydrophobic interactions and hydrogen bonds. The strength of these forces is often probed indirectly by exposing the protein to chemical denaturants that can selectively disrupt them, such as, guadinidium hydrochloride (GdnHCl), urea, and sodium dodecyl sulfate (SDS).2 Multiple studies have shown that glycosylation can increase the conformational stability of proteins against chemically induced denaturation. Some examples include alpha-1 antitrypsin (PROLASTIN®; Talecris Biotherapeutics),181 erythropoietin (EPOGEN®, PROCIT®; Amgen, Ortho)171 lecithin cholesterol acyltransferase,182 acid phosphatase,172 bromelain,183 lysozyme,184 amylase, 185 and peroxidase.186,187 Evidence that this type of stability can also be engineered into proteins was recently provided by Sundaram through the chemical glycosylation of α-chymotrypsin and by Srivastava through the chemical glycosylation of amylase.136,188 In the α-chymotrypsin studies it was found that the protein could be stabilized against both urea and SDS denaturation by glycosylation.188 These results therefore suggest that the mechanism by which glycosylation increases the chemical denaturation stability of proteins must involve an increase in the strength of their hydrogen bonding and hydrophobic interactions. The increase in Van der Waals (VdW) energy as a function of increased

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glycosylation degree observed by Solá and Griebenow during the *in silico* structural energetic analysis recently conducted on this protein provides further support to this argument (Fig. 2D).103,166 While increased hydrogen bonding strengths can be explained by the reduced water dielectric screening (H-bonds are treated as pure electrostatic interactions in current protein computational forcefields), increased hydrophobic interaction strengths can be explained by the increased structural compactness and rigidification of the protein core upon glycosylation.103,166

Thermal Denaturation

Proteins can also denature due to exposure to extremes of temperature since all of the forces that stabilize their native-state structure are sensitive to thermal changes.53,56-58 Therefore, it is no surprise that the principal stability indicator used to establish if a formulation strategy stabilizes a protein involves the determination of its thermal denaturation susceptibility.2,6,53,58 Coincidently, this is one of the most fundamental biophysical properties which becomes altered for proteins upon their glycosylation.99,101-103 The number of proteins whose thermal stability has been reported to be increased by glycosylation is extensive. Some pharmaceutically-relevant examples include erythropoietin (EPOGEN®, PROCIT®; Amgen, Ortho),171 alpha 1-antitrypsin (PROLASTIN®; Talecris Biotherapeutics),181 G-CSF (GRANOCYTE®; Chugai Pharma),124,146,170 interferon-beta (REBIF®, Pfizer/EMD Serono; AVONEX®, Biogen),149,159 RNAse (ONCONASE®; Alfacell),129,189,190 follicle-stimulating hormone (GONAL-F®; EMD Serono),191 urokinase (ABBOKINASE®; ImaRx Therapeutics),192 α-glucosidase (MYOZYME®; Shire),193 α-chymotrypsin (MOBE MUGOS®; Marlyn Nutraceuticals),101-103,188 lecithin cholesterol acyltransferase,182 and IgG-like antibodies.194,195 It is important to note that thermodynamic theory also predicts that all proteins will also be susceptible to cold denaturation at ambient pressures.154,196,197 This creates a significant problem during the production of protein-based pharmaceuticals as their handling often requires repeated freeze-thawing cycles.2,10,47,55,198-200 In this context, it was recently reported by Jiang *et al*. that glycosylation increases the conformational stability of cystatin during freezing.201

Multiple mechanistic studies have been conducted to try to determine the molecular mechanisms involved in protein thermodynamic stabilization by glycosylation. For example, Dwek and coworkers related the increased thermostability of glycosylated RNAse to a decrease in its overall structural dynamics through H/D exchange NMR studies.128,190 Gervais *et al*. came to the same conclusion upon examination of the structural dynamics of glycosylated G-CSF by NMR.202 It is interesting to note that from the studies conducted by Dwek and coworkers it was found that the reduction in structural mobility due to glycosylation occurred in regions as far as 30Å away from the glycosylation site suggesting that these local effects could be transferred throughout the whole protein structure.203 Additionally, in both of these studies it was found that the glycans interacted weakly with the protein surface suggesting that the glycans extend into the solution, away from the protein surface.128,190,202

Wang *et al*. performed a systematic study on several natural glycoproteins (invertase, fetuin, glucoamylase, ovotransferrin, and avidin) to determine the generality of these stabilizing effects by glycosylation.98 In this study, the naturally glycosylated proteins were deglycosylated enzymatically and the changes in their stability studied through calorimetric analysis.98 For all these proteins, a decrease in T_m was found after enzymatic deglycosylation with the most glycosylated proteins displaying the greatest changes in T_m. Curiously, the magnitude of this change was found to be independent of the linkage (N- or O-linked) and branching (mono- or multi-branched) of the glycans but dependant on the carbohydrate content of the structurally different glycoproteins.98 Subsequent comparative calorimetric studies between the glycosylated isoform of ovomucoid and its non-

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glycosylated isoform led DeKoster and Robertson to conclude that the increase in thermodynamic stability of glycoproteins was mainly of an entropic nature due to the lack of change in the enthalpy of unfolding (ΔH_m) between these homologous proteins.204 Another study that provided some additional fundamental insights into the increased thermodynamic stability for glycoproteins was performed by Kwon and Yu in 1997 by studying the effects of glycosylation on the unfolding and refolding rates of human alpha 1-antitrypsin (PROLASTIN®; Talecris Biotherapeutics).181 It was found that glycosylation slows the protein unfolding process without affecting the refolding rates significantly. From these results it was proposed that the increase in thermodynamic stability caused by glycosylation could be due to stabilization of the native state and not due to destabilization of the unfolded state.181

Through the use of glycation with small sized glycans (e.g. glucose, fructose) De Jongh and collaborators recently reported that β-lactoglobulin thermostability could be artificially enhanced by increasing the degree of glycosylation, reinforcing the generality of these effects.205 From this work, it was proposed that glycans achieved such effects by lowering the protein's change in heat capacity of unfolding (ΔCp).119 It is important to note that in theory ΔCp can be lowered by both stabilizing the native state as well as by destabilizing the unfolded state ($\Delta Cp = Cp(unfolded) - Cp(native)$). To determine the influence that the glycosylation parameters had on increasing the thermodynamic stability of proteins and to further the mechanistic understanding of these effects by glycans, Solá *et al*. recently performed a detailed experimental thermodynamic analysis on a series of chemically glycosylated α-chymotrypsin conjugates by differential scanning calorimetry (DSC).101-103 In this study, both the amount of surface bound glycans (glycosylation degree) and the size of the attached glycans were systematically varied. It was found that increases in the glycosylation degree shifted the T_m linearly to higher temperature values independently of the glycan's molecular size (Fig. 3A).101-103 It is important to note that although the thermostabilizing effects of both glycation and chemical glycosylation could be caused by a decrease in the protein's isoelectric point (pI) due to alteration of the surface lysine charges, this is not the case. Evidence of this comes from the fact that acetylation of α-chymotrypsin lysine residues which is chemically analogous to glycosylation at the lysine residues and leads to a similar decrease in pI, leads to a decrease in protein stability.206 Interestingly, increasing the pI of proteins by making them more positively-charged through guanidination increases thermostability.206,207 Since the observed increase in thermal stability upon chemical glycosylation occurred only up to a certain maximum temperature and could be statistically correlated with an overall structural rigidification of the protein, from data determined by H/D exchange FTIR experiments (Fig. 4), this suggests that the protein core has reached its maximum compactness.101-103 Therefore the magnitude of thermal stabilization achieved by increasing the glycosylation degree should be specific to each different protein and reflects the maximum amount of native state stabilization that the protein can obtain (it is important to note that additional overall stabilization can be brought about by destabilizing the unfolded state). An additional effect that was observed in this study was that increasing the glycosylation degree led to a decrease in ΔCp although here it was found that increases in glycan size led to a more pronounced lowering of ΔCp, reaching even negative values for the most glycosylated conjugates which is rare for protein unfolding (Fig. 3B).101-103 Since the decrease in ΔCp as a result of increased glycosylation degree could be also related to native-state stabilization through a decrease in protein structural dynamics this result suggests that increasing the glycan's size could possibly destabilize the unfolded state.101,103 This is due to the fact that a negative ΔCp implies a lower Cp for the unfolded state than for the folded state ($\Delta Cp = Cp(unfolded) - Cp(native)$). This conclusion is further supported by the fact that the Gibbs free energy of unfolding ($\Delta G_U(25°C)$) which is indicative of overall protein stability increased with increases in the glycosylation degree and to an even larger extent with increases in the glycan size (Fig. 3C).

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101-103 Comparison of the magnitude of maximum gains in overall conformational stability ($\Delta\Delta G_U(25°C)$) induced by chemical glycosylation of αchymotrypsin ($\Delta\Delta G_U(25°C) \sim 9$ kcal/mol) with those induced by the traditionally employed carbohydrate excipients in liquid formulations (e.g. trehalose, sucrose, fructose) ($\Delta\Delta G_U(25°C) \sim 3$ kcal/mol) reveals the potentially greater stabilization effect by the covalent attachment of the glycans to the protein surface at a greatly reduced effective molar glycan concentration (~ 0.1 mM for surface bound glycans vs. 1M for solution free glycans).101,103,208,209 Furthermore, examination of the literature reveals that the average thermodynamic stabilization afforded per glycan unit attached to the protein surface is ~ 1−2 kcal/mol.103,183,210,211 Mechanistically all of these results suggest that the glycosylation parameters play different roles in the overall thermodynamic stabilization of the protein.103 For example, while the glycosylation degree mainly influences protein thermal stability by stabilizing the native state through increased internal non-covalent forces and decreased structural dynamics, the glycan size can further influence the overall thermodynamic stability of proteins by destabilizing the unfolded state.103

Kinetic Inactivation

The long-term storage times to which protein-based pharmaceuticals are usually exposed provide an additional challenge for the preservation of their structural intactness. This is due to the fact that many of the aforementioned physicochemical instabilities could still occur kinetically for a thermodynamically stabilized protein.2,55,59 Several studies conducted under accelerated degradation conditions suggest that glycosylation can increase the long-term stability of proteins. For example, early reports by Dellacherie *et al*., Lenders and Crichton, and Srivastava on glycated hemoglobin and amylase evidenced an increase in the functional lifetimes of these proteins when exposed to extremely high temperatures. 136,212,213 In subsequent studies, it was found that deglycosylation of catalase, human interleukin 5, erythropoietin, G-CSF, and the chemokine CCL2 led to a decrease in their kinetic stabilities.146,214-217 While studying the effects of the natural glycans of phytase on its overall stability Hoiberg-Nielsen *et al.* recently found that their presence significantly increased the kinetic stability of the protein by reducing the rate of aggregation while leaving the equilibrium melting temperature relatively unaltered.218 More recently Solá *et al.* studied the effects of the glycosylation degree and glycan size on the kinetic stability of α-chymotrypsin.101,103 It was found that both the degree of glycosylation and the glycan size increased the protein's inactivation half-lifes but with significantly greater magnitude of kinetic stabilization brought about at increasing glycan size.101,103 In agreement with these results, Tams and Welinder also found a correlation between increased glycosylation amount and increased kinetic stability for peroxidase relating these effects to a dampening of both native and unfolded state backbone fluctuations.186 These results again suggest that both the glycosylation degree and glycan size can play different roles in the kinetic stabilization of proteins with the glycan's size leading to a larger stabilization effect by possibly destabilizing the unfolded state. These results are also intriguing since they highlight the fact that protein samples with similar thermal stabilities (T_m values) will not necessarily display similar kinetic and overall stabilities ($\Delta G_U(25°C)$) which is often an assumption during protein stability studies.2

Aggregation

Proteins behave as colloids due to their large molecular sizes coupled with their high intermolecular interaction potentials.20,60,219 This makes the protein structure susceptible to aggregation-prone phase transitions that are dependant on pH, temperature, and protein concentration. Aggregation of protein pharmaceuticals is undesirable due to the potential harmful effects of these on the patient and on the increased production costs due to additional protein recovery and refolding protocols.11,14,20,47,60-65,220,221 There are

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several reports where glycosylation has been shown to either reduce or prevent protein aggregation. For example, Baudys *et al.* reported that the physical stability of insulin could be improved by reducing its aggregation kinetics through the chemical attachment of small sized glycans.97 Reduced insulin aggregation was related in this work to prevention of a transamidation crosslinking reaction which suggests a stabilizing mechanism involving steric intermolecular repulsion phenomena.97 Ioannou *et al.* found that for α-galactosidase A (REPLAGAL®; Shire) glycosylation at Asn215 is required to prevent the exposure of a surface hydrophobic patch that facilitates the aggregation of the protein.161 Weintraub *et al*. reported that deglycosylation of thyroid-stimulating hormone (THYROGEN®; Genzyme) made the protein more prone to aggregation.130 Similar results were found for erythropoietin (EPOGEN®, PROCIT®; Amgen, Ortho) by Endo *et al*..222 Hoiberg-Nielsen *et al.* also reported increased colloidal stability for the glycosylated form of phytase.218 From their studies on this protein it was proposed that the inhibition of aggregation was likely dependant on steric hindrance of the glycans in the unfolded protein state and not on their hydration-related properties.165,218,223 More recently Solá *et al.* conducted an accelerated aggregation study directed at understanding the mechanisms by which systematic changes in the glycosylation parameters could impact non-specific protein aggregation.101,103 It was found that under extreme conditions (temperature = 60°C and protein concentration = 20 mg/mL), aggregation could not be prevented by the smaller sized glycans irrespective of the amount bound to the protein surface. In contrast, the aggregation process was completely inhibited upon chemical glycosylation with two or more of the larger sized glycans.101,103 All of these results therefore suggest a mechanism in which protein aggregation is prevented due to an increase in steric repulsions between aggregation-prone protein species due to the presence of the glycans on the protein surface.

SUMMARY

Design of successful protein-based therapeutics requires the simultaneous optimization of both *in vitro* and *in vivo* molecular stability as well as improved pharmacokinetics and pharmacodynamics. Glycosylation could provide ample opportunities in this respect since in principle all of these could be simultaneously optimized through glycoengineering.100 While the pharmaceutical application of glycosylation still suffers from some technical challenges due to the intrinsically complex nature of glycoprotein structure and the difficulties related to glycoprotein production in host-expression systems (e.g., low glycoprotein expression yields, glycosylation macro- and micro-heterogeneity), further advancements in the understanding of chemical- and enzyme-based glycan remodeling strategies being currently pursued by glycoengineering companies (e.g., Neose Technologies, GlycoFi, GlycArt Biotechnology, GlycoForm), will allow for the rational design of targeted glycoprotein structures.

As discussed in this review, glycosylation has been shown to ameliorate a multitude of pharmaceutically-relevant chemical and physical protein instabilities. Mechanistically, the different glycosylation parameters (e.g., number of glycans attached and glycan molecular size) studied so far can apparently impart different stabilization effects on the protein. While increasing the glycosylation degree apparently stabilizes the protein native state by increasing the internal non-covalent forces and rigidifying the protein structure, increasing the glycan molecular size appears to destabilize the protein unfolded state. The review also points out areas in which a more fundamental knowledge is necessary to further decipher the effects of glycosylation. For example, the impact of glycosylation on the behavior of the unfolded state still needs further investigation. Furthermore, more systematic studies are needed to understand the mechanisms by which glycans prevent chemical instability events. It is important to note the possibility that other instabilities not explored so far (e.g. deamidation, β-elimination, racemization, adsorption to amphipatic interfaces and

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hydrophobic surfaces) could be also ameliorated or prevented by glycosylation; this therefore remains to be tested. Nevertheless, the significant potential that glycosylation engineering holds towards improving the physicochemical properties of protein pharmaceuticals should lead to further research towards the understanding of the fundamental effects that glycans have on proteins.

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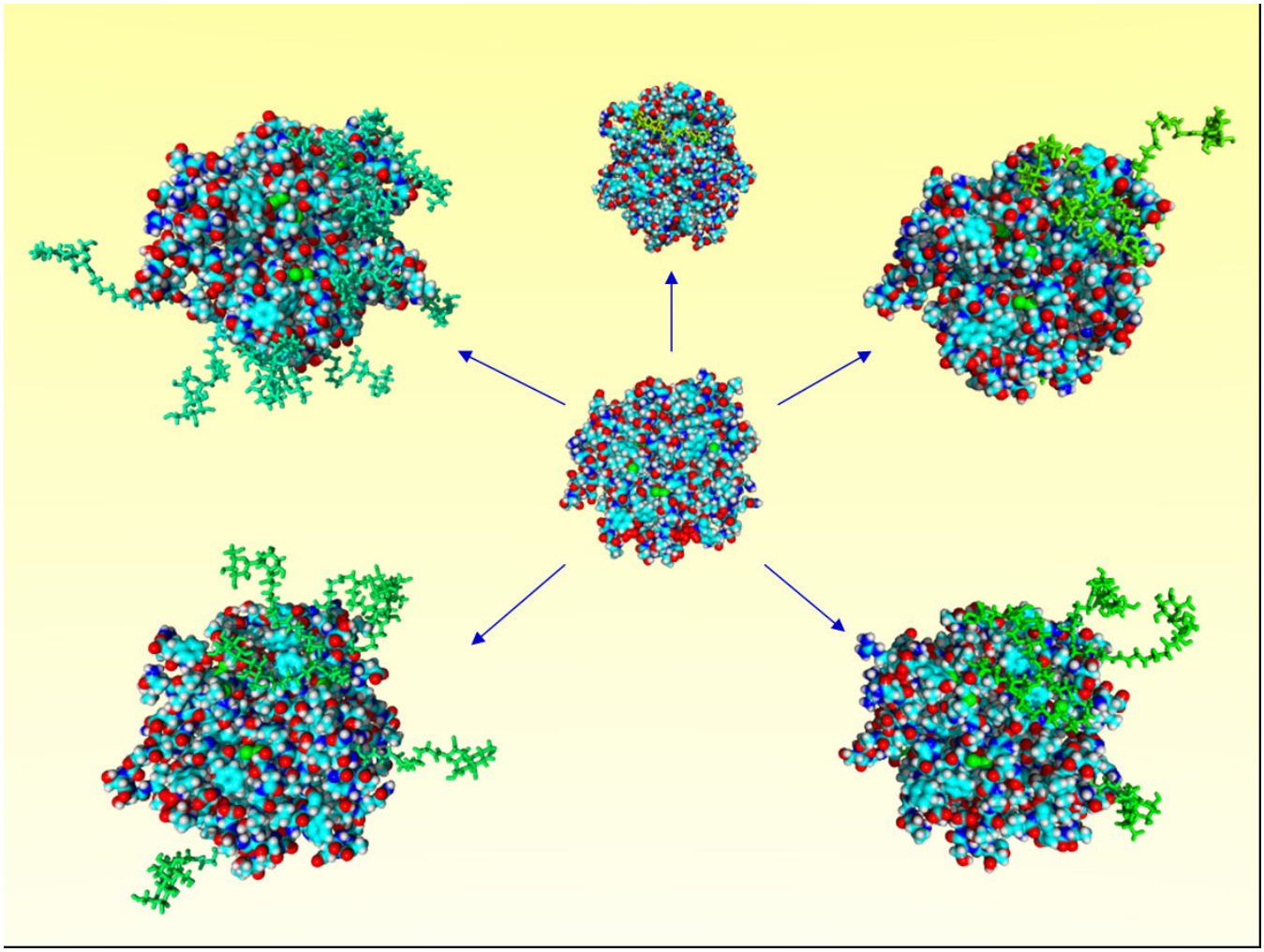

Figure 1.
Molecular models for the α-chymotrypsin (α-CT) glycoconjugates engineered through chemical glycosylation. α-CT at center and α-CT glycoconjugates with glycosylation degree increasing clockwise from top (Lac_n-α-CT with varied n: 1, 3, 5, 7, and 14). Protein represented in CPK style with standard atom coloring, glycans represented in stick style with green coloring. Reproduced with permission of Springer, from Solá *et al*.103

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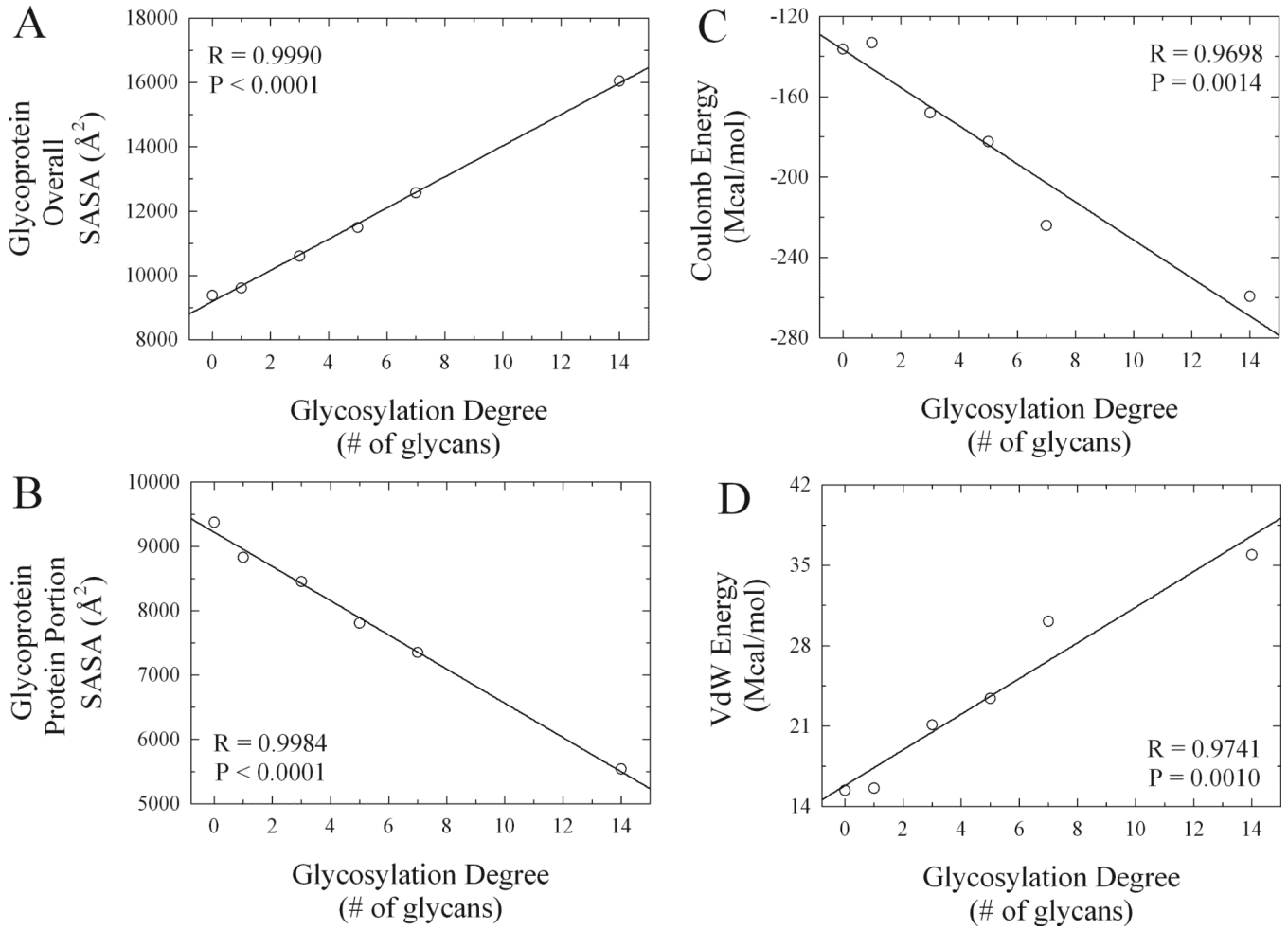

Figure 2.

Changes in energetic parameters and in solvent accessible surface area (SASA) for the overall glycoprotein and for the protein portion of the glycoprotein as a function of glycosylation degree. Results were derived from calculations performed on the molecular models constructed for the various α-CT glycoconjugates engineered through chemical glycosylation (see Fig. 1). Glycosylation degree is equal to the number of glycan molecules chemically attached to the protein surface. Adapted with permission of Wiley-Blackwell, from Solá and Griebenow.166

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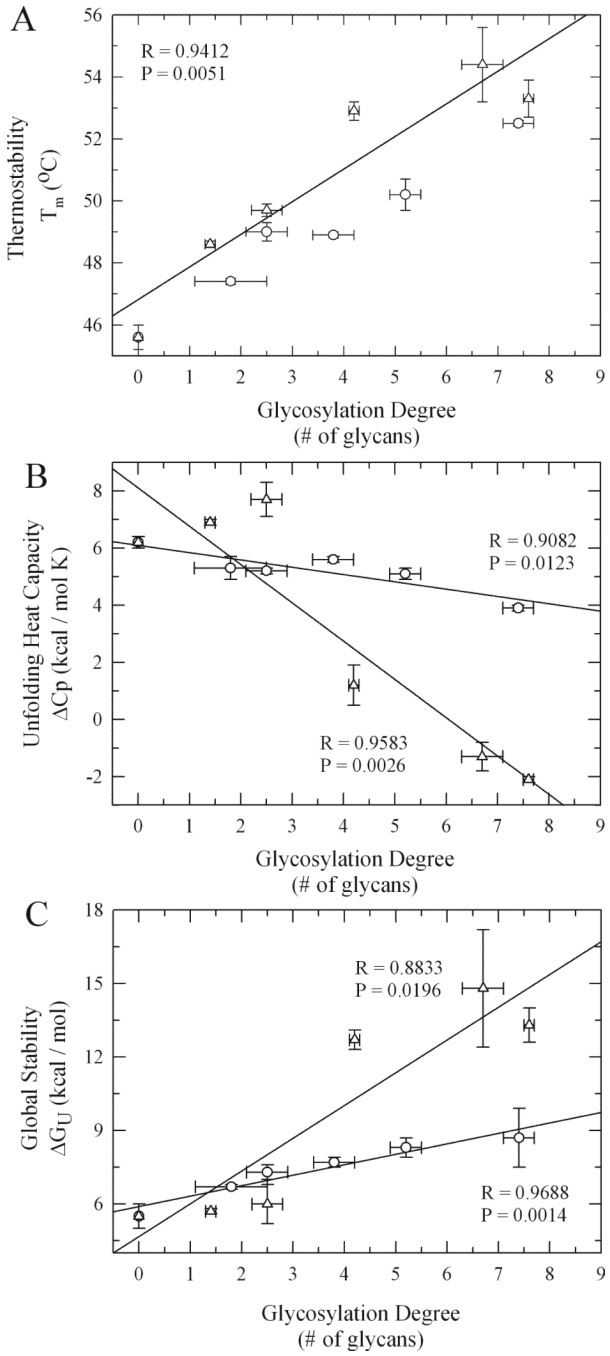

Figure 3.

Changes in thermodynamic unfolding parameters as a function of glycosylation degree and glycan size (lactose (○) and dextran (Δ)) for the various α-CT glycoconjugates engineered through chemical glycosylation. Reproduced with permission of Springer, from Solá *et al*. 103

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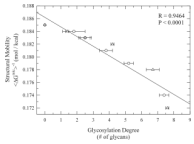

Figure 4.

Changes in protein structural mobility ($\langle \Delta G^{mic} \rangle^{-1}$) as a function of glycosylation degree and glycan size (lactose (○) and dextran (Δ)) for the various α-CT glycoconjugates engineered through chemical glycosylation. Reproduced with permission of Springer, from Solá *et al*. 103

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Table 1

Chemical and Physical Instabilities Encountered by Protein-based Pharmaceuticals and Typical Countermeasures[a]

Process	Main Stress Factors	Main Degradation Pathways	Typical Countermeasures	References[b]
Purification	Proteases, contaminations,[c] extremes of pH, high pressures, temperature,[f] chemical denaturants, high salt and protein concentrations, amphipatic interfaces, hydrophobic surfaces[i]	Proteolytic and chemical hydrolysis, fragmentations, crosslinking, oxidation, deamidation,[e] denaturation, adsorption, aggregation,[k] inactivation	Protease inhibitors, control of pH and temperature, chelating agents,[d] antioxidants, addition of surface active[g] and stabilizing excipients[h]	2, 5, 6, 10, 19–22, 68–72
Liquid storage	Contaminations,[c] extremes of pH, temperature,[f] chemical denaturants, high protein concentrations, freeze thawing, amphipatic interfaces, hydrophobic surfaces[i]	Fragmentations, chemical hydrolysis, oxidation, crosslinking, β-elimination, racemization, deamidation,[e] denaturation, adsorption, aggregation,[k] inactivation	Control of pH and temperature, chelating agents,[d] antioxidants, addition of surface active[g] and stabilizing excipients[h]	2, 5–12, 19–22, 47, 49, 50, 68–72
Lyophilization	Ice-water interface, pH changes, dehydration, phase separation	Aggregation,[k] inactivation	Co-lyophilization with surface active[g] and stabilizing excipients[h,j]	4, 18, 23–29, 48, 73
Solid-phase storage	Contaminations,[c] protein-protein contacts, moisture[k]	Aggregation,[k] fragmentation, oxidation, deamidation, inactivation	Similar to lyophilization	4, 16–18, 30
Spray-drying, Spray-freeze drying	Liquid-air interface, dehydration	Similar to lyophilization	Similar to lyophilization, precipitation[l]	31–38, 74
Sustained-release formulations[m]	Liquid-organic solvent interface, hydrophobic surfaces,[i] mechanical stress	Aggregation,[k] inactivation	Addition of surface active[g] and stabilizing excipients,[h] avoidance of water/organic interfaces[n]	39–44, 77

[a]Covalent modification as countermeasures are excluded in the table because they are discussed in the paper and in table 2 for glycosylated proteins;

[b]the references cited include many reviews to which the interested reader is referred to for details;

[c]i.e , contaminating (transition) metal ions and proteases can catalyze fragmentations;22

[d]to remove metal ions;2

[e]other prominent chemical instabilities are oxidations and disulfide scrambling;2

[f]control of temperature can be non-trivial when ultrasonication is being used because of local heating events;

[g]mild detergents at low concentration can prevent detrimental interactions of proteins with hydrophobic surfaces/interfaces;42

[h]such excipients include sugars, polyols, and amino acids that stabilize protein structure by so-called preferential exclusion;2,75

[i]the potentially most harmful surfaces are hydrophobic, e.g., Teflon;45

[j]the mechanism of stabilization is believed to be a combination of hydrogen-bond forming propensity and increase in the glass transition temperature in the solid;23

[k]a prominent pathway to aggregation is by so-called sulfide-disulfide interchange;11

[l]precipitation prior to the procedure afforded stabilization;

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[m] the sole FDA approved formulation thus far consists in the encapsulation of the protein in microspheres comprised of poly(lactic-co-glycolic) acid;

[n] stabilization is mostly achieved by keeping the protein away from denaturing interfaces or by simply avoiding such interfaces altogether.

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Table 2

Protein Instabilities Improved by Glycosylation

Instability	References	Instability	References
Proteolytic degradation	96, 121-141	Heating denaturation	98, 101-103, 119, 124, 128, 129, 146, 149, 159, 170, 171, 181, 182, 188-195, 202, 204, 205
Oxidation	145	Freezing denaturation	201
Chemical crosslinking	97, 146, 149	Precipitation	159-165
pH denaturation	124, 137, 171-178	Kinetic inactivation	101, 103, 136, 146, 186, 212-218
Chemical denaturation	136, 164, 171, 172, 181-185, 187, 188	Aggregation	97, 101, 103, 130, 218, 222

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Table 3

Partial List of Approved Protein-based Pharmaceutical Products Stabilized by Glycosylation

INN*	Brand Name (Company)	Indication	Effects of Glycosylation	Glycan (#)	References
Agalsidase alfa (galactosidase)	Replagal® (Shire)	Treatment of Fabry disease	Protects against aggregation and precipitation	3	161
Alglucosidase alfa (α-glucosidase)	Myozyme® (Shire)	Treatment of Pompe disease	Protects against thermal denaturation	6	193
Alpha 1-antitrypsin (α1AT)	Prolastin® (Talecris Biotherapeutics)	Treatment of congenital α1AT deficiency with emphysema	Protects against chemical and thermal denaturation	3	181
Bucelipase alfa (cholesterol esterase)	Merispase® (Meristem therapeutics)	Treatment of lipid malabsorption related to exocrine pancreatic insufficiency	Protects against proteolytic degradation	11	126
Chymotrypsin	Wobe Mugos® (Marlyn Nutraceuticals)	Adjunct therapy for multiple myeloma	Protects against thermal, chemical, and kinetic denaturation and aggregation	**	101-103, 188
Corifollitropin alfa (FSH)	Gonal-F® (EMD Serono)	Treatment of infertility	Protects against thermal denaturation	10	191
Drotrecogin alfa (CF-XIV, Protein C)	Xigris® (Eli Lilly)	Treatment of severe sepsis	Protects against proteolytic degradation	4	127
Epoetin alfa	Epogen® (Amgen)	Treatment of anemia associated with chronic renal failure (CRF)	Protects against oxidation, thermal, chemical, and pH denaturation, kinetic	3	145, 171, 216, 221
	Procrit® (Ortho Biotech)		inactivation, and aggregation		
IgG-like antibodies	***	Multiple indications	Protects against proteolysis and thermal denaturation	2	142, 194, 195
Insulin	***	Treatment of diabetes	Protects against non-disulfide crosslinking and aggregation	**	97
	Avonex® (Biogen)				
Interferon beta-1a (rHuInf-β1[a])	Rebif® (Pfizer / EMD Serono)	Treatment of multiple sclerosis	Protects against disulfide crosslinking, precipitation, thermal denaturation, and aggregation	1	149, 159, 160
Interferon gamma-1b	Actimmune® (Intermune)	Treatment of chronic granulomatous disease	Protects against proteolytic degradation	2	132
Lenograstim (G-CSF)	Granocyte® (Chugai Pharma)	Treatment of chemotherapy induced neutropenia	Protects against disulfide crosslinking, proteolytic degradation, thermal and pH denaturation, and kinetic inactivation	1	124, 125, 146, 170
Ranpirnase (RNAse)	Onconase® (Alfacell Corp.)	Treatment of malignant mesothelioma	Protects against proteolytic degradation and thermal denaturation	**	128, 129, 189, 190
Sargramostin (G-CSF)	Leukin® (Bayer Healthcare)	Treatment after induction chemotherapy with acute myelogenus leukemia	Protects against disulfide crosslinking, proteolytic degradation, thermal and pH denaturation, and kinetic inactivation	8	124, 125, 146, 170
Thyrotropin alfa (TSH)	Thyrogen® (Genzyme)	Detection of thyroid cancer and hypothyroidism	Protects against proteolytic degradation and aggregation	3	130

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INN*	Brand Name (Company)	Indication	Effects of Glycosylation	Glycan (#)	References
Urokinase alfa	Abbokinase® (ImaRx Therapeutics)	Treatment of acute massive pulmonary emboli	Protects against proteolytic degradation and thermal denaturation	2	131, 192

Information was obtained from the Prescribing Information (PI) for each product.

*INN: International nonproprietary name.

**Commercially available protein is not glycosylated.

***Multiple approved products. Further information available at www.fda.gov and www.biopharma.com.

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