REVIEWS

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. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 98:1223–1245, 2009

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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

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Table 1. Chemical and Physical Instabilities Encountered by Protein-Based Pharmaceuticals and Typical Countermeasures*

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

*Covalent modification as countermeasures are excluded in the table because they are discussed in the paper and in Table 2 for glycosylated proteins.
[a]The references cited include many reviews to which the interested reader is referred to for details.
[b]The sole FDA approved formulation thus far consists in the encapsulation of the protein in microspheres comprised of poly(lactic-co-glycolic) acid.
[c]Contaminating (transition) metal ions and proteases can catalyze fragmentations.[22]
[d]Control of temperature can be nontrivial when ultrasonication is being used because of local heating events.
[e]The potentially most harmful surfaces are hydrophobic, e.g., Teflon.[45]
[f]A prominent pathway to aggregation is by so-called sulfide–disulfide interchange.[11]
[g]Other prominent chemical instabilities are oxidations and disulfide scrambling.[2]
[h]To remove metal ions.[2]
[i]Mild detergents at low concentration can prevent detrimental interactions of proteins with hydrophobic surfaces/interfaces.[42]
[j]Such excipients include sugars, polyols, and amino acids that stabilize protein structure by so-called preferential exclusion.[2,75]
[k]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]
[l]Precipitation prior to the procedure afforded stabilization.
[m]Stabilization is mostly achieved by keeping the protein away from denaturing interfaces or by simply avoiding such interfaces altogether.[39,42,46,76]

generalized strategies concerning their chemical as well as their physical stabilizations.[51,52] While the protein primary structure is subject to 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 noncovalent 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 nonnative 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 nongeneralized 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 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

Table 2. Protein Instabilities Improved by Glycosylation

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

domains of life (archaea, eubacteria, 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 ($Man_{2\text{-}6}Man_3GlcNAc_2$), mixed type ($GlcNAc_2$-$Man_3GlcNAc_2$), and hybrid type ($Man_3GlcNAc$-$Man_3GlcNAc_2$).[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 (p*I*).[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 activated glycans, and glycation of the lysine residues with reducing sugars via the

Table 3. Partial List of Approved Protein-Based Pharmaceutical Products Stabilized by Glycosylation

INN	Brand Name (Company)	Indication	Effects of Glycosylation	Glycan (#)	Refs.
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 (α1-AT)	Prolastin® (Talecris Biotherapeutics)	Treatment of congenital α1-AT 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	[b]	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), Procrit® (Ortho Biotech)	Treatment of anemia associated with chronic renal failure (CRF)	Protects against oxidation, thermal, chemical, and pH denaturation, kinetic inactivation, and aggregation	3	145,171,216,221
IgG-like antibodies	[a]	Multiple indications	Protects against proteolysis and thermal denaturation	2	142,194,195
Insulin	[a]	Treatment of diabetes	Protects against nondisulfide crosslinking and aggregation	[b]	97
Interferon beta-1a (rHuInf-β1)	Avonex® (Biogen), 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

(*Continued*)

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