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Recent Advances in Glycosylation Modifications in the Context of Therapeutic Glycoproteins

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1. Introduction

Glycosylation is one of the most complex post-translation modifications, commonly found in many cell surface and secreted eukaryotic proteins. 1-2% of the human transcriptome encodes proteins that link to glycosylation. Many protein-based biotherapeutics approved or in clinical trials are glycoproteins. The oligosaccharides covalently attached to therapeutic glycoproteins pose biological benefits as well as manufacturing challenges. The present chapter reviews the structure and function of glycosylation, glycoform patterns observed for the biotherapeutic proteins produced by various host systems, and analytic methods for the characterization of glycoforms. Recent advances in utilizing glycosylation as a strategy to improve biotherapeutics properties are also discussed.

2. Glycosylation as a major post-translational modification

Glycosylation has been studied intensively for the past two decades as the most common covalent protein modification in eukaryotic cells (Varki 2009). Sophisticated oligosaccharide analysis has revealed a remarkable complexity and diversity of this post-translational modification. About 1-2% of the human transcriptome (about 250-500 glycogenes) has been predicted to encode proteins that are involved in glycosylation processing (Campbell and Yarema 2005). Majority of proteins synthesized in the endoplasmic reticulum (ER) such as cell surface and extracellular eukaryotic proteins are glycoproteins. It has been estimated that more than 50% of proteins in human are glycosylated (Apweiler et al. 1999; Wong 2005).

Glycoproteins can be classified into four groups: N-linked, O-linked, glycosaminoglycans, and glycosylphosphatidylinositol-anchored proteins (Table 1). This chapter focuses only on N- and O-linked glycosylation. N-linked glycosylation is through the side chain amide nitrogen of a specific asparagine residue, while O-linked glycosylation is through the oxygen atom in the side chain of serine or threonine residues. The N-linked modification takes place in both ER and Golgi, while the O-linked glycosylation in higher eukaryotes occurs exclusively in the Golgi.

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Type	Consensus site	Sugar structures	Synthesis Location
(1) N-linked	N-X-T/S	High mannose Complex-type Hybrid-type	ER, Golgi
(2) O-linked	Ser/Thr	Mucin-type O-linked fucose O-linked glucose O-linked GlcNAc	Golgi
(3) Glycosaminoglycans	Asn/Ser/Thr	Glycosaminoglycans	ER, Golgi
(4) Glycosylphosphatidylinositol	phosphatidylinositol / phosphoethanolamine to protein carboxyl terminus	glycosylphosphatidylinositol	ER, Golgi

Table 1. Glycoproteins Categories

Comparing to other major molecular constituents of cells such as nucleic acids and proteins, the biological importance of glycans or carbohydrates in the post-translational modification has been much later appreciated (Varki 2009). There is no single theory explaining why cells go through such complex and highly conserved biosynthetic machineries. Though not all answers are known, it is now clear that glycosylation plays many key biological functions such as protein folding, stability, intracellular and inter-cellular trafficking, cell-cell and cell-matrix interaction (Varki 1993; Varki 2009).

It is therefore not surprising that congenital disorders with serious medical consequences have been identified linked to the defects in a number of genes in glycosylation pathway (Freeze 2006). Over 40 such disorders have been reported to be associated with glycogene mutations, and many more to be discovered. In addition, glycosylation profiles of specific proteins change as certain diseases progress, such as cancers and rheumatoid arthritis, and have been regarded as disease and diagnostic markers.

This chapter focuses on the biological structures and physiological roles of glycosylation modification in the context of biotherapeutics. Glycosylation differences in proteins produced by various host systems, and the potential impacts on biotherapeutics safety and side effects, are described. Various analytical characterization methods for glycoforms are also described. Lastly, several therapeutic examples with glycoengineering application are illustrated and discussed.

2.1 Structure and biosynthesis

N-linked glycosylation occurs in the sequon of Asn-X-Ser/Thr where X can be any amino acid except proline and aspartic acid (Helenius and Aebi 2004; Kornfeld and Kornfeld 1985). Glycosylation at Asn-Ala-Cys has also been reported (Stenflo and Fernlund 1982). Glycosylation efficiency of these Threonine, Serine, and Cysteine containing sequon is very different with an order of Thr>Ser>Cys (Bause and Legler 1981). N-linked oligosaccharides

are added to proteins *en bloc* in the lumen of ER as pre-synthesized core units of 14 saccharides ($Glc_3Man_9GlcNAc_2$) in virtually all eukaryotes. This core glycan is the product of a biosynthesis pathway in which monosaccharides are added to a lipid carrier (dolichol-pryophosphate) on both sides of the ER membrane by monosaccharyltransferases in the membrane. The sugar moiety is translocated from cytosolic side to the luminal side of the ER by an ATP-independent bidirectional flippase (Hirschberg and Snider 1987). The oligosaccharyltransferase then scans the emerging polypeptide from translocon complex for glycosylation sequon and adds the core glycan unit to the side chain nitrogen of the Asn residue by N-glycosidic bond. The oligosaccharides are added to the sequon when it is only 12-14 residues into the ER lumen, as the active site of the oligosaccharytransferase is no further than 5nm away from the exit of the protein translocon (Nilsson and von Heijne 1993).

After the core glycan is added to the growing nascent polypeptide chain, the oligosaccharide portion is modified by a series of glycosidases and glycosyl transferases. Various complex, hybrid, and high mannose types of N-linked oligosaccharides are generated. Glucosidase I and II located in the ER remove all three glucose residues from the core unit to produce a $Man_9GlcNAc_2$ high mannose structure. Hybrid and complex oligosaccharides can be produced from high mannose structures, from which α-mannosidases in the ER and the Golgi remove 4-6 mannoses. Then Golgi-bound glycosyl transferases add GlcNAc as well as galactoses and sialic acids to produce complex types of oligosaccharides. These modifications reflect a spectrum of functions related to glycoprotein folding, quality control, sorting, degradation, and secretion.

O-linked glycosylation normally takes place in the Golgi, most commonly initiated with a transfer of N-acetylgalactosamine (GalNAc) to a serine or threonine residue by an N-acetyl galactosaminyltransferase (Van den Steen et al. 1998). After the addition of the first GalNAc, a number of glycosyltransferases and enzymes in the Golgi can elongate the core structure and modify it with sialylation, fucosylation, sulphatation, methylation or acetylation(Van den Steen et al. 1998). O-linked glycosylation site is not readily predicted, any serine or threonine residue is a potential site and O-linked sugars are frequently clustered in short regions of peptide chain that contain repeating units of Serine, Threonine, and Proline. There are various types of O-linked sugars, including mucin-type O-glycans commonly found in many secreted and membrane-bound glycoproteins in higher eukaryotes, O-linked fucose and O-linked glucose found in the epidermal growth factor domains of different proteins, and O-linked GlcNAc on cytosolic and nuclear proteins. Yeast's O-linked oligomannose glycans take place in the ER utilizing dolichol-phosphate-mannose instead of a sugar nucleotide, which is similar to N-linked glycosylation occurred co-translationally (van den Steen et al, 1998).

2.2 Physiological function and roles

Protein folding and conformation stabilization function of N-linked glycans were first suggested by the early studies with tunicamycin, a glycosylation inhibitor (Olden et al. 1982). The sequential processing by glucosidases, mannosidases, and glycotranferases, of the core unit of 14 saccharides, provides recognition tags for lectins mediated folding pathway (Helenius and Aebi 2004). The content of oligosaccharides can regulate protein half-life. Large amount of sialic acids can increase plasma half-life while exposure of galactose and mannose can decrease half-life (Walsh and Jefferis 2006). N-glycans also play a critical role in intracellular trafficking with a well understood example of mannose-6-phosphate of

lysosomal enzymes (Kornfeld and Mellman 1989). For Antibodies, oligosaccharide moieties covalently attached at the highly conserved Asn297 at the CH2 domain of the Fc (crystallizable fragment) region, is critical to the activation of downstream effector mechanisms (Jefferis 2009; Natsume et al. 2009). Completely aglycosylated or deglycosylated IgGs do not bind effector receptors such as FcγRI, FcγRII, and FcγRIII (Leader et al. 1991; Leatherbarrow et al. 1985; Walker et al. 1989). Sialylated IgGs have a lower affinity to FcγRIIIA than non-sialylated IgGs, consequently a lower antibody-dependent cellular cytotoxicity (ADCC) activity (Kaneko et al. 2006; Scallon et al. 2007). Removal of terminal galactose residues from Fc glycans reduces complement-dependent cytotoxicity (CDC) activity (Boyd et al. 1995; Kumpel et al. 1995). Absence of a core α-1, 6 linked fucose from Fc glycans improves *in vitro* ADCC activity (Niwa et al. 2004; Shields et al. 2002).

O-linked glycosylation plays a role in maintaining secondary, tertiary, and quaternary structures of fully folded proteins. The examples are mucins and related molecules, in which peptide regions with O-linked sugar attachments assume a "bottle brush"-like structure (Carraway and Hull 1991; Gowda and Davidson 1994). Like N-glycans, O-glycans can modulate aggregation, maintain protein stability, confer protease and heat resistance. An example of O-linked sugars hindering protease cleavage is the modification at the hinge regions of IgA$_1$ and IgD (Field et al. 1994; Van den Steen et al. 1998). O-linked glycosylation is important for the expression and processing of particular proteins such as glycophorin A (Remaley et al. 1991) and IGF-II (Daughaday et al. 1993). They are also crucial for some glycoprotein-protein interaction, such as the interaction between P-selectin glycoprotein ligand-1 (PSGL-1) and P Selectin. Some O-linked oligosaccharides of PSGL-1 have a terminal sialyl-Lewis-x structure, which is important for its P-selectin receptor function (Hooper et al. 1996).

3. Glycoproteins as biotherapeutics

More than one-third of approved biotherapeutics and many in clinical trials are glycoproteins (Walsh and Jefferis 2006). The presence and nature of the oligosaccharides clearly affect these protein drugs' folding, stability, trafficking, immunogenicity as well as their primary activities.

3.1 Antibodies and Fc-fusion proteins

Therapeutic recombinant antibodies and fusion proteins of Fc region of immunoglobulin G$_1$ (IgG$_1$) represent a major class of biotherapeutics. An individual antibody molecule contains two light and two heavy polypeptide chains, forming two identical Fab (antigen-binding fragment) regions with a specific antigen-binding site, and a homodimeric IgG-Fc region. This Fc region is critical for phagocytosis, ADCC activity, CDC activity, and FcRn binding for recycling. As discussed above, the N-glycans attached to Asn297 in Fc region are critical to the activation of downstream effector mechanisms, while not affecting FcRn binding for catabolic half-life.

Besides the presence of core glycans at the Fc regions, about 30% of polyclonal human IgG molecules contain N-linked oligosaccharides within the IgG-Fab region (Jefferis 2009). The N-linked sites can be at the variable regions of either heavy chains or light chains or both. The licensed antibody therapeutics cetuximab has an N-linked glycan at Asn88 of the heavy chain variable region, and an unoccupied N-linked motif at Asn41 of the light chain variable region (Qian et al. 2007). Fab oligosaccharide is heterogeneous complex diantenary and

hybrid oligosaccharides with sialic acids and galactoses, which are very different from the Fc oligosaccharides with predominantly fucosylated non-galactosylated diantennary oligosaccharides. The difference may be due to the inaccessibility of Fc N-glycan for further modification, as the N-glycans at the Fc regions are integral to the IgG structure and has a defined confirmation (Jefferies, 2009). Many Fc-fusion therapeutics proteins, such as TNFRII-Fc, CD2-Fc, and CTLA4-Fc, contain glycosylation modifications in the fusion portions, in addition to their Fc glycans. The contents of these glycosylations are very similar to those of Fab oligosaccharides.

3.2 Non-immunoproteins

Many non-immunoproteins such as growth factors, cytokines, hormones, and therapeutic enzymes, are glycoproteins. Growth factors such as erythropoietin (EPO) have three N-linked and one O-linked sugar side chains. Removal of either two (Asn38 and Asn83) or all three sites results in poor product secretion (Egrie 1993). Cytokines such as interferon(IFN)-β and IFN-γ are glycoproteins (Pestka et al. 1987). Although glycosylation is not essential for INFs protein efficacy or safety, lack of glycosylation decreases their biological activity and circulatory half-life. Oligosaccharide structures of follicle-stimulating hormone heterodimer play an important role in its biosynthesis, secretion, metabolic fate, and functional potency (Ulloa-Aguirre et al. 1999). The glycans at each subunit seem to exhibit distinct roles, with those in α subunit critical for dimer assembly, signal transduction, and secretion, and those in β subunit more crucial for circulation clearance. In addition, many therapeutics enzymes such as recombinant human glucocerebrosidase for Gaucher disease (Van Patten et al. 2007) are glycoproteins and N-glycosylation is important for its targeting and functional activities.

3.3 Effects of glycosylation on therapeutic efficacy of glycoproteins

In comparison to small-molecule drugs, therapeutic proteins display a number of favorable therapeutic properties, such as higher target specificity, good pharmacological potencies, and lower side effects, but they also possess intrinsic limitations like poor physicochemical and pharmacological properties. Glycosylation of therapeutic glycoproteins can improve therapeutic efficacy through its positive impact on protein pharmacodynamics (PD) and pharmacokinetics (PK).

Pharmacodynamics refers to the potency of therapeutic proteins as enzymatic rates and receptor binding affinities. Pharmacokinetics exams the time dependency of drug action, which is influenced by drug absorption, distribution, excretion, initial response times and duration of effects. The parameters include circulatory half-life, volumes of distribution, clearance rates, and total bioavailability. Protein drugs' PK/PD are typically affected by adverse local adsorption in subcutaneous administration due to variable protein hydropathy surface, and by rapid elimination from body in intravenous administration, via proteolytic, renal, hepatic, and receptor mediated clearance mechanisms (Mahmood and Green 2005; Tang et al. 2004).

Glycosylation has multiple impacts on PK/PD properties of therapeutics glycoproteins. First, glycosylation can shield non-specific proteolytic degradation, as discussed above. Second, sialic acids at the terminus of glycan chains carry negative charge, which reduces renal clearance most likely due to repulsion from negatively charged polysaccharides on membranes in the glomerular filter (Chang et al. 1975; Venkatachalam and Rennke 1978). Third, size of glycans can increase protein molecular weight and hydrodynamic radius of

glycoprotein and therefore reduce glomerular filtration. Fourth, terminal sialic acids of glycan branches prevent the exposure of galactose, N-acetyl-glycosamine, or mannose that interacts with hepatic asialoglycoprotein receptor as well as other mammalian lectin-like receptors to be removed from circulation.

4. Glycosylation in various cell production systems

Glycosylation patterns of biotherapeutics are highly variable based on the production systems (Table 2) and their culture processes. Mammalian cells such as Chinese Hamster Ovary cells (CHO) and mouse myeloma cells (NS0, SP2/0) are the most commonly used systems. Alternative cell production systems are being developed and explored.

Host systems	Similarity to human glycans	Abnormal sugars
CHO	High	trace amount of α-Gal, NGNA
NS0/SP2/0	High	small amount of α-Gal, NGNA
Yeast	Low	high mannose
Plant	Low	bisecting β1,2 xylose, α1,3 fucose
Transgenic animals	Low	high mannose and NGNA

Table 2. Glycans comparison in various production systems

The glycoforms of CHO-produced IgGs are close to human IgGs, but having very little glycoform with the third N-acetylglucosamine bisecting arm, which makes up about 10% of human IgG glycoforms, and also very low amount of terminal N-acetyl neuraminic acid is generated. The glycosylation in mouse-derived cells such as NS0 and SP2/0 shows even more difference from human glycoforms. They produce small amounts of glycoforms with additional α-1,3-galactose (α-Gal) and a different predominant sialic acid, N-Glycolyl neuraminic acid (NGNA). NGNA is reported to be immunogenic in human (Sheeley et al. 1997), and in certain patient populations, α-Gal is associated with IgE-mediated anaphylactic responses, with the best known example of cetuximab (Chung et al. 2008). Detection of both α-Gal and NGNA in CHO-derived glycans is also reported, but only in trace amount (Hamilton and Gerngross 2007; van Bueren et al. 2011).

Yeast, insect cells, plants, and transgenic animals, are the alternative systems to the current mammalian hosts. They are being actively explored for biotherapeutics production because of their lower manufacture cost. However, restricted abilities to generate human-like glycoforms are their major limitations, as different glycosylation machinery yields immunogenic recombinant glycoproteins. For instance, complex type N-glycans are very different in plants and mammals. Plant N-glycans contain a bisecting β1,2 xylose in place of β mannose core, an α1,3 fucose instead of an α1,6 fucose, and are highly heterogeneous (Gomord et al. 2005), and allergenic. Glycans from yeast (Hamilton et al. 2006) and insect (Shi and Jarvis 2007) have a high mannose content, resulting a quick clearance through

binding to macrophage mannose receptor in the liver. IgGs produced in the milk of transgenic goats contain 50% NGNA and a higher level of mannose (Edmunds et al. 1998). Tremendous efforts have focused on "humanization" of the glycosylation pathways in these alternative systems to improve product consistency and pharmacokinetics, while decreasing the potential immunogenicity for product antibody response.

5. Analytic characterization of glycoforms

Various glycosylation analysis approaches (Table 3) have been developed and utilized for glycoform characterization. Glycans can be enzymatically or chemically released from glycoproteins, prior to electrophoretic, chromatographic or mass spectrometric analysis. Glycoproteins can also be treated with endoproteinases, followed by glycosylation analysis at the glycopeptides level.

Methods	Principles	Major advantages and shortcoming
(1) Electrophoresis		
SDS-PAGE	Size	General equipment, cheap, fast
		High-throughput possible,
IEF	Charge	Limited resolution
(2) Chromatography (HPLC)	Polarity	High resolution
		Separation of major glycoforms
		Risk of hydrolysis
(3) Mass spectrometry	Mass	Detailed information, fast
		High resolution, precise
		Expensive equipment
		Trained personnel

Table 3. Glyco-analytical methods

5.1 Electrophoresis
Sodium Docedyl sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and IsoElectrofocusing electrophoresis (IEF) are two methods that are routinely used for gross glycoprotein characterization. SDS-PAGE is for separation of mass variant due to the 2kDa mass addition of a single N-glycan. When treated with glycanase such as PNGase F and Endo-H, a migration shift can be detected. IEF is for separation of charge variants. The sialic acid content of glycans can increase negative charge of glycoproteins, while the PNGase F treatment generates a negatively charged aspartic acid instead of the neutral N-glycan linked asparagines.

5.2 Liquid chromatography
Normal phase high-performance liquid chromatography (NP-HPLC) is one of the most commonly used analytical methods to analyze oligosaccharides after enzymatic release and

fluorescent labeling. The glycans can be accurately quantified and detected in sub-picomolar levels (Guile et al. 1996). Different peaks in an NP-HPLC chromatogram can be isolated and submitted to off-line analysis by mass spectrometry or to sequential digestion with selective exoglycosidases (neuraminidase, α-galactosidase, β-galactosidase, β-hexoaminidase, α-fucosidase, α-mannosidase, β-mannosidase) for further biochemical confirmation. NP-HPLC can also be used for routine IgGs glycan finger printing for IgGs expressed in different cell lines.

5.3 Mass spectrometry

Mass spectrometry is a fast and powerful method to differentiate and estimate the relative proportion of different glycoforms. Glycans and glycopeptides are traditionally ionized by fast atom bombardment and laser desorption. In the past two decades, softer ionization techniques such as Electrospray Ionization-Time-of-Flight (ESI-TOF) and Matrix-assisted laser desorption ionization (MALDI) provide a much higher sensitivity and precision. It allows measuring intact glycoproteins and investigating non-symmetry of N-linked biantennary oligosaccharides between two heavy chains on intact antibodies (Beck et al. 2008).

6. Glycoengineering to improve protein therapeutics

It is obvious that selectively producing a certain type of glycoforms of biotherapeutics protein could be advantageous in terms of efficacy and safety. Residue screening with site-directed mutagenesis is widely used to introduce or eliminate N-glycosylation sites (Zhong et al. 2009). Though there is no "one-size-for-all" principle and guideline, the process has been aided by knowledge of the known structure and function of the target protein so that the changes can retain *in vitro* biological activity, stability, and high sugar occupancy rate. Cell line engineering to knock-out and knock-in glycogenes is another approach to enrich desired glycoforms. It is also possible to use in vitro glycoenzymes to modify glycoform profiles. The following are a few specific examples.

6.1 Half-life extension

One well known glycoengineering application is altering pharmacokinetic property of therapeutic proteins. Introducing new N-linked glycosylation site into target proteins to increase sialic acid containing carbohydrates can increase *in vivo* activity due to a longer half-life. This technology has been successfully applied to produce a hyperglycosylated analogue of recombinant human erythropoietin (Elliott et al. 2003). This glycoengineered protein contains two additional N-linked carbohydrates, which result in a threefold increase in serum half-life and a less frequent dosing for anemic patients (Sinclair and Elliott 2005). Sialic acid containing carbohydrates are highly hydrophilic and therefore increase protein solubility by shielding hydrophobic residues. Similar approach has been applied to a number of therapeutic proteins, including human growth hormone (Flintegaard et al. 2010), follicle stimulating hormone(Perlman et al. 2003), Leptin and Mpl ligand (Elliott et al. 2003). In case of human growth hormone, the terminal half-life in rats for the sialylated protein with three additional N-linked glycans was prolonged by 24-fold compared with that of wild type protein (Flintegaard et al. 2010). The correlation between half-life optimization and N-linked carbohydrate addition remain unclear.

6.2 Glycoengineered antibody for ADCC modulation

N-glycans in the Fc-region of IgG_1 play a critical role in ADCC activity. Absence of a core α-1, 6 linked fucose improves binding to FcγRIII and *in vitro* ADCC activity (Niwa et al. 2004; Shields et al. 2002). Addition of bisecting GlcNAc, which also results in the removal of core fucose, significantly enhances ADCC activity (Davies et al. 2001; Shinkawa et al. 2003; Umana et al. 1999). ADCC enhancement has also been shown for non-fucosylated IgG4 (Niwa et al. 2005), for Fc fusion proteins (TNFRII-Fc) (Shoji-Hosaka et al. 2006), for single chain-Fc and bispecific antibodies (Natsume et al. 2006). Several glycoengineered antibodies such as anti-GD3 (BioWa), anti-CD20 (Glycart-Roche), and anti-IL5R (BioWa/Medimmune) are currently being investigated in clinical trials.

Besides defucosylation, sialylation is also utilized for antibody and Fc engineering. Sialylated IgGs have been found to possess a lower ADCC activity than non-sialylated IgGs (Kaneko et al. 2006; Scallon et al. 2007). Overexpressing gal and sialic transferases in CHO results in sialylation increase of ≥ 90% of available glycan branches in Fc-fusion proteins (Weikert et al. 1999).

6.3 Mannose for target delivery

Engineered glycosylation has been employed for targeted delivery to disease affected tissues. One well established example is the treatment of lysosomal storage diseases. Recombinant human enzymes such as glucocerebrosidase can be digested with exoglycosidases to expose mannose or mannose-6-phosphate that can efficiently target the enzymes into the lysosomes of macrophages. The high mannose modified enzymes can also be produced by a glycosylation mutant such as *Lec1* mutant (Van Patten et al. 2007), or by treatment of chemical inhibitors (Zhou et al. 2008). Targeting the protein drugs to the desired site by glycoengineering have significantly increased therapeutic efficacy of a number of replacement enzymes, including α-glucosidase, α-galatosidase, and α-L-iduronidase (Sola and Griebenow 2010).

7. Conclusions and future directions

Glycosylation modification offers both an opportunity and a challenge to biotherapeutics glycoproteins. Complexity and heterogeneity of oligosaccharides present a considerable challenge to the biopharmaceutical industry to manufacture biotherapeutics with a reproducible and consistent glycoform profile. Meanwhile, a better understanding of the structure and function of glycosylation modification to glycoproteins can better facilitate the development of next-generation of biotherapeutics with optimized glycoforms and therapeutic utilities. Further humanization of glycosylation machinery in non-mammalian expression systems may represent a trend in lowering the manufacture cost for biotherapeutics such as antibodies and Fc-fusion proteins. With a full development of glycoanalytical techniques, an improved knowledge on glycoprotein activity *in vivo* will certainly help design a safer and more efficacious biotherapeutics drugs.

8. Acknowledgement

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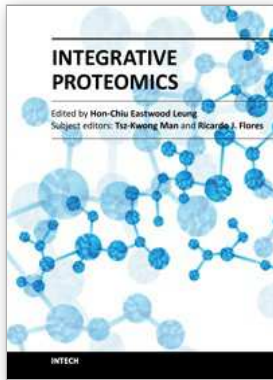
Integrative Proteomics

Edited by Dr. Hon-Chiu Leung

Proteomics was thought to be a natural extension after the field of genomics has deposited significant amount of data. However, simply taking a straight verbatim approach to catalog all proteins in all tissues of different organisms is not viable. Researchers may need to focus on the perspectives of proteomics that are essential to the functional outcome of the cells. In Integrative Proteomics, expert researchers contribute both historical perspectives, new developments in sample preparation, gel-based and non-gel-based protein separation and identification using mass spectrometry. Substantial chapters are describing studies of the sub-proteomes such as phosphoproteome or glycoproteomes which are directly related to functional outcomes of the cells. Structural proteomics related to pharmaceutics development is also a perspective of the essence. Bioinformatics tools that can mine proteomics data and lead to pathway analyses become an integral part of proteomics. Integrative proteomics covers both look-backs and look-outs of proteomics. It is an ideal reference for students, new researchers, and experienced scientists who want to get an overview or insights into new development of the proteomics field.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Xiaotian Zhong and Will Somers (2012). Recent Advances in Glycosylation Modifications in the Context of Therapeutic Glycoproteins, Integrative Proteomics, Dr. Hon-Chiu Leung (Ed.), ISBN: 978-953-51-0070-6, InTech, Available from: http://www.intechopen.com/books/integrative-proteomics/recent-advances-in-glycosylation-modifications-in-the-context-of-therapeutic-glycoproteins

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