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INTEGRATION OF SYSTEMS GLYCOBIOLOGY WITH BIOINFORMATICS TOOLBOXES, GLYCOINFORMATICS RESOURCES AND GLYCOPROTEOMICS DATA

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Abstract

The glycome constitutes the entire complement of free carbohydrates and glycoconjugates expressed on whole cells or tissues. 'Systems Glycobiology' is an emerging discipline that aims to quantitatively describe and analyse the glycome. Here, instead of developing a detailed understanding of single biochemical processes, a combination of computational and experimental tools are used to seek an integrated or 'systems-level' view. This can explain how multiple biochemical reactions and transport processes interact with each other to control glycome biosynthesis and function. Computational methods in this field commonly build *in silico* reaction network models to describe experimental data derived from structural studies that measure cell-surface glycan distribution. While considerable progress has been made, several challenges remain due to the complex and heterogeneous nature of this post-translational modification. First, for the *in silico* models to be standardized and shared among laboratories, it is necessary to integrate glycan structure information and glycosylation-related enzyme definitions into the mathematical models. Second, as glycoinformatics resources grow, it would be attractive to utilize 'Big Data' stored in these repositories for model construction and validation. Third, while the technology for profiling the glycome at the whole-cell level has been standardized, there is a need to integrate mass spectrometry derived site-specific glycosylation data into the models. The current review discusses progress that is being made to resolve the above bottlenecks. The focus is on how computational models can bridge the gap between 'data' generated in wet-laboratory studies with 'knowledge' that can enhance our understanding of the glycome.

INTRODUCTION

Glycosylation is one of the most ubiquitous and complex post-translational modifications in nature [1–3]. A majority of secreted and cell-surface mammalian proteins bear at least one attached glycan (or carbohydrate structure). These macromolecules either absolutely control or finely-tune a very large number of diverse cellular processes in higher organisms [4]. Studies of the glycome (collection of all glycans in a system) are also more complex compared to investigations of the genome and proteome since the number of natural monosaccharides and its variants across different species far exceeds the limited repertoire of naturally occurring nucleotides and amino acids. Due to this diversity and the branched

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nature of glycans, a bewilderingly diverse set of carbohydrate structures can be synthesized by a single cell. To add to this complexity, glycan expression on a single protein is heterogeneous both in terms of whether a particular peptide site is glycosylated (macroheterogeneity) and also in terms of the distribution of different glycans at a single site (microheterogeneity). This heterogeneity is in part due to diversity in the available metabolites, glycosyltransferases (glycoTs)footnote 1 and glycosidases which vary across cell-types and also cell growth conditions. Finally, the same glycan attached to different protein scaffolds may have different functions, and this highlights the need to consider context and site-specific glycosylation in functional assays. Due to these complexities, the application of computational tools in addition to high-throughput experiments may provide a more complete, quantitative understanding of the cellular glycosylation process and the glycome.

Systems Glycobiology is an emerging research theme that aims to examine glycosylation from a 'systems perspective' [1]. Instead of traditional biochemical methods that focus on developing a detailed understanding of single biochemical processes, this field aims to develop experimental and computational tools that can provide insight into how different biochemical reaction and transport processes interact with each other to condition the 'emergent properties' of the system (Figure 1). While mathematical modeling is not a necessary criterion for the development of systems based approaches, computational frameworks and data analysis tools can aid the interpretation of complex experimental data. Such quantitative approaches allow the mathematical testing of different hypotheses, and quantification of the input-output response of the system in the face of perturbation. In general, systems glycobiology may study all aspects of the carbohydrate life-cycle including (Fig. 1): i. The biosynthesis reactions which occur in the cytoplasm and nuclear compartments to make various sugar-nucleotide donors (e.g. UDP-Galactose), ii. glycosylation reactions that occur in the endoplasmic reticulum and Golgi cisternae to guide the biosynthesis of a variety of glycoconjugates, iii. transport processes that result in the sub-cellular localization of glycoconjugates in their functional compartments, and iv. salvage pathways that aid the recycling of glycoconjugates back to their basic building blocks.

In terms of a 'grand challenge' for the field of Systems Glycobiology, one would ideally like to develop a computational framework where the glycoprotein and glycolipid profiles of an arbitrary cell could be quantitatively predicted based on limited experimental data at the gene, protein and/or functional level. Ideally, then, we would also like to predict *a priori* the outcome of specific cell/organism level perturbations without the need to perform corresponding wet-lab measurements. While this goal of collating data, information and knowledge is far from being achieved at the current time, efforts are being made to bridge the gap. This review article explains the basis for the biological complexity of the glycosylation machinery, summarizes areas were progress has been made, highlights current

[1]Glycosyltransferases (GlycoTs) are a family of ~250 enzymes that catalyze the biosynthesis of cellular glycoconjugates by transferring monosaccharides from nucleotide-sugar donors to carbohydrate/protein/lipid acceptors. Glycosidases are glycoside hydrolases that assist with the breakdown of glycosidic bonds. These enzymes typically remove monosaccharides from specific glycoconjugates.

bottlenecks for the field, and describe directions that scientists are currently undertaking to address these shortcomings.

GLYCAN COMPLEXITY IN STRUCTURE AND FUNCTION

Glycans regulate a variety of fundamental biological processes. This includes structural features that control protein stability and conformation, and molecular recognition processes that control cellular binding. Such interactions are termed 'intrinsic' if they occur within or between cells of a single organism and 'extrinsic' if the molecular recognition involves external pathogenic microbes, agglutinins or toxins [5]. For example, in the area of protein therapeutics, glycans attached to proteins have a dramatic effects on their half-life in circulation as exemplified by the case of erythropoietin (EPO) [6] and tissue plasminogen activator (tPA) [7]. In these molecules, increasing the level of sialylation improves serum half-life by reducing clearance via the liver's Ashwell-Morell receptor. In the case of IgG antibodies, the N-glycan structure at Asn-297 also regulates their efficacy, not in terms of altering antibody-antigen binding affinity but in terms of their binding to the Fcγ receptor expressed on immune cells and molecules of the complement pathway. Due to this, changes in glycan structures profoundly influence the efficacy of both antibody-dependent cellular toxicity (ADCC) and complement dependent cytotoxicity (CDC) [8]. In the field of infectious diseases, glycosylation regulates host-virus interactions for a range of pathogens such as SARS-CoV, influenza, West Nile and Hendra [9]. Here, the glycans regulate multiple steps during viral immune evasion and virulence including entry into host cell, proteolytic processing and protein trafficking. During immunity, glycans affect a number of steps including selectin dependent cell adhesion during immune cell trafficking, T-cell and B-cell receptor mediated signaling, lymphocyte development and a plethora of other signaling cascades [10]. In the last example, cancer is accompanied by changes in cell-surface and secreted protein glycoconjugate structures. Due to such alterations, the unique cellular carbohydrate profiles of cancer cells serve not only as biomarkers or prognostic indicators of the disease, but it can also have functional impact on cell signaling, survival and metastasis [11, 12].

There is considerable structural diversity among the glycans expressed by mammals (Figure 2) [13]. These macromolecules are principally divided into five categories: i. N-linked glycans, ii. O-linked or O-GalNAc (N-acetylgalactosamine) type glycans, iii. O-GlcNAc (N-acetylglucosamine) type glycans, iv. Proteoglycans, and v. glycosphingolipids (please see ref. [5, 14] for details). In this regard, most mammalian secreted and cell membrane proteins are commonly decorated by N- glycans that are attached to Asn residues in the sequon Asn-X-Ser/Thr (X ≠ Pro) and O-GalNAc linked glycans which appear at Ser and Thr. Here, the N-glycans are classified to be 'oligomannose' if all the antennae have terminal mannose (Fig. 2A). They are termed 'complex' if all antennae have GlcNAc extended chains, and 'hybrid' if they contain a mixture of terminal mannose and extended GlcNAc chains. The O-glycans appear in eight different core structures and these can be either extended/linear or branched. Fig 2B shows two examples of an extended core-1 glycan and a branched core-2 glycan containing the tetrasaccharide sialyl Lewis-X. Besides glycosylation on secreted proteins, the O-GlcNAc modification is one of the most abundant post-translational modifications in the nuclear and cytoplasmic compartments of metazoan cells, with more

than 600 O-GlcNAcylated proteins identified to date [15] (Fig 2C). This is a terminal modification which is not further elaborated. In the case of proteoglycans, glycosaminoglycans (GAGs) containing repeating disaccharide units are attached to proteins via Xyl-O-Ser linkages (Fig. 2D). In the final category, glycosphingolipids or glycolipids are elaborated on lipid moieties called ceramides, a family of molecules containing long chain sphingosines in amide linkage with fatty acids. The first sugar linked to ceramide in higher animals is typically β-linked galactose in the case of the GalCer glycolipids and glucose for the GlcCers (Fig. 2E). In addition to the classical endoplasmic reticulum (ER)/Golgi derived O-glycans that are initiated by GalNAc, other important modifications have also been reported though these are less prevalent [3, 16]. Notably, whereas fucose is typically observed at the terminal ends of glycans, more recently it has been observed to be O-linked to Ser/Thr on the peptide backbone (Fig. 2F). Such O-fucose modifications decorate and have functional impact on the EGF-like repeat domains of the Notch signaling receptor and thrombospondin (TSR) domains of various coagulation related proteins including members of the ADAMTS family. Coagulation factors like factor VII and factor IX, and also the EGF-like repeats of Notch are additionally decorated by O-glucose type chains (Fig. 2G). Additional unusual modifications include the O-mannose initiated chains on Ser/Thr that are common on the protein α-dystroglycan (Fig. 2H) and c-mannosylation on Trp residues of RNase 2 (Fig. 2I).

Systems glycobiology aims to resolve the above complexity in structure and diversity in function with the goal of identifying key rate-limiting steps that regulate glycosylation pattern changes during the transition from the normal condition to disease states. Such critical steps may represent druggable targets for a range of pathologies.

CURRENT BOTTLENECKS FOR SYSTEMS GLYCOBIOLOGY

Whereas systems-based analysis have progressed relatively smoothly in studies involving signaling networks [17], metabolic processes [18] and physiological modeling [19], they are still gaining traction in the nascent field of Systems Glycobiology. This is potentially due to critical bottlenecks, both from the computational and analytical side. In particular:

- **There is no accepted standard for model building**: A number of systems based models that simulate glycan biosynthesis have been developed over the last decade (reviewed in the next section). However, systematic model building has been lacking in this field since it is difficult to incorporate glycan structures and glycosylation-related enzyme specificity data into mathematical models. Further, few of the existing models are available in Systems Biology Markup Language (SBML) format [20]. This limits the extent to which such computational models can be developed, shared and validated.
- **Glycoinformatics databases are under developed**: A number of glycoscience-related databases have appeared in recent years. However, most of these repositories store glycan structure and taxonomy data, with only a limited amount of functional information. The development of systems-based models in the future will likely have greater reliance on these databases. Thus, the ability to relate

carbohydrate structure with the specific enzymes that synthesize them, the rates of their synthesis and also their function will be key to future model building.

- **Insufficient quantitative data from glycoproteomics experiments:** Two approaches are commonly used to measure the glycome. The first uses either enzymes or mild hydrolysis to separate the glycans from the peptide backbone. Following per-methylation of glycans, MS (mass spectrometry) analysis is performed to obtain semi-quantitative information regarding the composition and relative abundance of the carbohydrate structures [21]. With the goal of gaining site-specific glycosylation information, which is lost in the above approach, the second method aims to analyze intact glycopeptides by adopting the conventional LC-MS^n workflow that is common to the field of proteomics [22]. The problem here is the lack of well-developed software and the complexity involved with the size of the search space since a single peptide may have many different sites of glycosylation and each site can bear a multitude of glycans. More sophisticated computational tools for glycoproteomics data analysis can accelerate systems-based model building and validation.

Below we provide a summary of ongoing developments to address each of the above challenges.

AN ENTITY-BASED MODELING FRAMEWORK

Several mathematical models of glycosylation have appeared in recent years (summarized in Table 1, detailed review in [23]). Most of these focus on N-linked glycosylation pathways [24–31] with one of them also analyzing O-glycosylation [32]. The various models of N-glycosylation were specifically developed to handle different aspects of the glycan biosynthesis process including the mechanism of N-glycosylation initiation [24], glycan branching via the action of different N-acetylglucosaminyl (GlcNAc)-transferases or GnT enzymes [25, 26], and chain extension by the action of galactosyltransferases, fucosyltransferases and sialyltransferases [27, 30, 31]. While the primary focus of the N-glycosylation models is on protein production in the context of biotechnology, the model of O-glycosylation attempts to identify rate regulating steps controlling the kinetics of leukocyte adhesion to selectins in the context of human inflammation [32].

While the above approaches are valuable, unfortunately, most of these models are not written using SBML [20], the *de facto* standard for representing computational models in systems biology. Additionally, many of the previous manuscripts use rule-based synthesis of glycosylation networks that only partially account for detailed enzymatic specificity [27, 29]. Thus, while useful for specific applications, the models cannot be readily shared among laboratories and developed further by the community. The reason for this is because SBML does not specifically handle glycan structure information, and it does not have facilities to define enzymes. Additionally, a streamlined strategy for the automated construction of glycosylation pathways has only recently been described with the development of the MATLAB based toolbox called the “Glycosylation Network Analysis Toolbox (GNAT)” [33, 34]. Such model reconstruction is based on the object-oriented definitions of various ‘entities’ that participate in glycosylation reaction networks. These entities include

glycans, enzymes, reactions, compartments and additional elements that are depicted in the UML diagram (Figure 3).

The *GlycanSpecies* class

Linear, graphical and machine-readable formats have been utilized to describe glycans in literature (Figure 4). Among these, the LINUCS [35] (Fig. 4A), IUPAC (Fig. 4B) and Linear Code [36] nomenclatures are common methodologies for the linear representation of glycan structures. Two-dimensional graphical visualization allows the intuitive understanding of the branching pattern in carbohydrate structures. Thus, this approach has been used by the CarbBank, IUPAC and also the Consortium for Functional Glycomics (CFG) to render glycans (Fig. 4C–E). Finally, efforts have been undertaken to create machine readable descriptions for glycans using the Glyde-II [37] and GlycoCT [38] XML (eXtensible Markup Language) standards (Fig. 4F). In these last cases, the glycan structures contain descriptors for carbohydrate ring type, anomeric carbon position and stereoisomer configuration ("RingType", "StereoConfig" and "Anomer"). Additional details are also provided regarding the "GlycanBond" and "GlycanLinkage" that join different monosaccharide residues (Fig. 3, bottom). These data are also included in the *GlycanStruct* class of GNAT, similar to the *Glycan* class in GlycanBuilder [39]. This class utilizes a tree data structure to construct the branched sequence of a glycan. The final *GlycanSpecies* class in this modeling approach stores the entire *GlycanStruct* sequence, species short name, relative abundance and other data for the purpose of model building. To facilitate integration of glycosylation specific knowledge into SBML format files, *GlycanStruct* information is stored in the annotation field of the species-element definition (**Box 1**).

The *Enz*, *GTEnz* and *GHEnz* classes

The "*Enz*" class describes various enzymes participating in glycosylation reactions within GNAT (**Box 2**). A number of facilities are available in this program to automatically populate the fields of the *Enz* class by directly querying the International Union of Biochemistry and Molecular Biology (IUBMB) enzyme database (Box 2). These fields include the Enzyme Commission (EC) number, enzyme names (including family, systematic and other names) and reaction description.

GTEnz and *GHEnz* are children classes of *"Enz"* that are used to describe the detailed specificity of the glycoTs and glycosidases. Besides the basic properties inherited from their parent *Enz* class, these structures contain additional features that fine tune the enzyme properties by either including or excluding specific types of acceptor substrates. Among these, *resfuncgroup* (or Residue 1, Box 2) and *linkFG* (or Link1) describe the monosaccharide and glycosidic bond that are either coupled by the *GTEnz* or that is released by the *GHEnz. ResAtt2FG* (or Residue 2) and *linkAtt2FG* (Link 2) define the substrate residue and glycosidic bond adjacent to the newly formed or removed linkage as depicted in the schematic in Box 2. This example presents the addition of GlcNAc (N-acetylglucosamine, Residue 1) to mannose (Residue 2) on N-linked glycan using the GTEnz β1,2-N-acetylglucosaminyltransferase or GnT II. Here, the 'Residue' and 'Link' described above represent the minimal fields necessary to describe the enzymatic activity. The additional properties of GnT II that constrain the feasibility of the reaction are also described

in Box 2. In this regard, *substNABranch* describes specified sub-structures the presence of which prevents enzyme activity. All these properties can be viewed using an enzyme viewer ('enzViewer') provided in GNAT [33].

Automated *Pathway* object construction and simulation

The above systematic *Enz* definition rules enable the rapid construction of the *Pathway* object. Here, '*Pathway*' depicts the entire glycosylation network, including the full complement of biochemical reactions ('*Rxn'* object), species (*GlycanSpecies*) and reactor compartments ('*Compt'* object, Fig. 3). During such *Pathway* construction, graph data structures are employed to represent the highly connected biochemical reactions, with each node representing a "*GlycanSpecies*" and edges corresponding to "*Rxn*" objects. Such data structures enable efficient calculation of the general properties of the network, e.g. the set of biosynthetic pathways connecting any two nodes or the identification of nodes with the highest degree of connections. Here, the kinetic rate law for the individual biochemical reactions can be mathematically expressed as elemental rate equations, reversible/ irreversible Michaelis-Menten reactions, various type of Bi-Bi reactions (Ping-pong, random and sequential mechanisms) or transport equations. The compartments can be described as ideal continuous stirred tank reactors (CSTR), plug flow reactors (PFR) or batch reactors.

Various facilities are available in GNAT to automate the synthesis of *Pathway* objects including (Figure 5): i) 'Forward network inference': Here, starting with one or a small set of starting glycans, a product inference algorithm is applied in order to infer the potential reactions and products emanating from this starting material based on the enzymes available in the system. All newly generated glycans and reactions are then consolidated into a list by removing repeated elements. This process is then repeated until no additional new product(s) are formed in a given cycle. All unique structures at this point are incorporated into the *Pathway* object. ii) 'Reverse network inference': Here, the network is inferred given a set of final products and enzymes. This is similar to the 'forward network inference' algorithm, only it starts with the network products and applies backward substrate-inference to determine the starting material. iii) 'Connection network inference': This is applied to identify the intermediate glycans when a limited set of substrates and products are known. This algorithm considers pairs of glycans, one set to be the input/substrate and the second being the output/product based on the enzymes present in the system. For this substrate-product pair, starting with the substrate, a step-wise method is applied to identify all possible reaction pathways that link the substrate-product pair. This methodology is applied repeatedly until all substrate-product pairs are exhausted, with the final list of species and reactions being consolidated in the *Pathway* object.

Once the *Pathway* object is constructed, computer simulations can be performed as appropriate for the system. The *Pathway* structure and simulation results are stored in the *GlycanNetModel* (Top of Fig. 3). Figure 6 depicts a sample glycosylation reaction network model visualized using the GNAT's *GlycanNetViewer* (Fig. 6A) and corresponding simulation results (Fig. 6B).

GLYCOINFORMATICS TO LINK GLYCAN STRUCTURE WITH FUNCTION

Over the years, a number of Glycoinformatics databases and related tools have appeared to serve as repositories for glycan structures (reviewed by [40–42]). Many of these databases simply collate glycan structure, taxonomy and bibliography information. As there is considerable overlap among these resources, GlycomeDB (www.glycome-db.org) has undertaken the effort of collating at a single site, the data from multiple sources including CarbBank (Complex Carbohydrate Structure Database), GLYCOSCIENCES.de, KEGG (Kyoto Encyclopedia of Genes and Genomes) GLYCAN, CFG, JCGGDB (Japan Consortium for Glycobiology and Glycotechnology Database), BCSDB (Bacterial Carbohydrate Structure Database), GlycoBase, EuroCarbDB, and more recently also the Protein Data Bank [43]. Many of the inconsistencies between the databases were resolved in the new databases. In all, >100,000 datasets from these different repositories were unified into 33,000 unique sequences and these are presented in the GlycoCT XML format [38].

With the goal of developing links between glycan structure and function, recent years have witnessed the growth of relational databases. While independent research laboratories have participated in this effort, there have also been notable contributions from international consortia formed in the USA (CFG, www.functionalglycomics.org) [44], Europe (EuroCarbDB, glycomics.ccrc.uga.edu/eurocarb/) [45] and Japan (JCGGDB, jcggdb.jp/index_en.html) (Table 3). Many of these repositories are at their formative stages currently, with data being collected only for a limited set of organisms. The array of experimental techniques employed in each of these efforts is also limited, and thus there remains considerable opportunity to expand these glycoinformatics resources. While some of the data stored here have to be downloaded manually, in many instances the query process can be automated. The following text summarizes these glycoinformatics activities with a focus on data that can feed into systems based model building:

CFG—The CFG is largely focused on human and murine systems. This repository collates results from four complementary experimental methods: i. glycan array, ii. glycan profiling, iii. glyco-gene microarray and iv. mouse phenotyping. In the glycan array experiments, hundreds of glycans were printed on substrates and these were probed using an assortment of antibodies, serum samples, animal glycan-binding proteins, plant and microbial lectins and pathogens to measure binding specificity. In the glycan profiling studies, MALDI-TOF was applied to determine the N- and O-glycan profiles of a range of individual cells and also tissue samples from humans and murine knockouts. In some cases, MALDI-TOF-TOF-MS/MS and ESI-MS/MS were applied to distinguish between isomeric glycan structures. The gene expression data stored at the CFG database are based on custom Affymetrix glyco-gene microarray chips that focus on mouse and human glycosyltransferases, glycan-binding proteins, signaling and adhesion molecules, and other glycosylation related proteins that are relevant to cell communication. These studies measured gene expression data for both cell and tissue samples. Finally, the mouse phenotyping core stores histology, hematology, basic immunology and metabolism data that characterize a set of transgenic animals.

EUROCarbDB—The EUROCarbDB was designed to provide freely accessible informatics tools and databases to support glycomics research. With a focus on glycan sequence, this database was designed to collate experimental evidence for the existence and function of glycans based on high-performance liquid chromatography (HPLC), MS and nuclear magnetic resonance (NMR). This database contains 13,964 unique glycan sequences, 64 MS and 1261 NMR analysis. The GlycoBase database stores HPLC data [46].

Extending the informatics standards set out in EUROCarbDB, recently, UnicarbKB introduced a glycoproteomics knowledge base that includes data originally from GlycoSuiteDB [47] along with additional new entries [48]. The detailed information about biological source, methods used and primary citations for these site-specific glycosylation data are also provided when available. The first release of this database includes 3740 glycan structure entries, 400 glycoproteins, and 598 protein glycosylation sites. These are largely annotated with experimental confirmation from over 890 literature references.

JCGGDB—This is a web portal for the storage of carbohydrate related data developed by different Japanese laboratories. Principally this database includes the following: i. 'Lectin Frontier Database (LfDB)' quantifies the binding affinity (K_a) of a few hundred lectins to a panel of pyridylaminated glycans using frontal affinity chromatography, and the 'GlycoEpitope' repository stores binding specificity information for ~613 glycosylation related antibodies to ~174 glycoepitopes; ii. 'Glycogene Database' includes glycosylation-related enzyme specificity and tissue-specific expression data for ~150 enzymes; iii. 'Glycan Mass Spectral Database' includes MS^n spectra of O- and N-glycan standards in CID (collision induced dissociation) fragmentation mode; and iv. 'Galaxy' contains the elution profile of 500 pyridylamino-glycans separated by HPLC using three different columns.

Besides the above consortium driven efforts, there are several additional noteworthy databases that are relevant to systems glycobiology. In this regard, KEGG GLYCAN is an integrated knowledge base of pathway networks, genomic information, and chemical information [49]. CAZY (Carbohydrate-Active enZYmes Database) [50] provides access to genomic, 3D-structural and biochemical data for glycosylation related enzymes including glycosyltransferases, glycosidases and polysaccharide lyases, carbohydrate esterase and auxiliary redox enzymes. The specific enzyme nomenclature defined by The IUBMB can be accessed through ExplorEnz database using the enzyme commission number [51]. BRENDA is a twenty-five year old, rich enzyme database with 77,000 enzymes annotated from 135,000 references [52]. This knowledgebase provides enzyme-disease relations, organism specific expression data, protein sequence, kinetic rate constants, catalytic reaction descriptions and genome annotations. Finally, the CBS prediction server (http://www.cbs.dtu.dk/services/) contains several resources for the prediction of N- and O-glycosylation sites, including the NetOGlc server which uses experimental data and neural networks to predict the location of mucin type GalNAc O-glycosylation sites in mammalian proteins [53].

While the above resources are all valuable, validation of the accuracy of data collated in these databases remains a challenge. In addition, data exchange between the resources is not straightforward due to the unique features of each database that do not allow a uniform

machine-readable interface for easy extraction and cross-referencing of stored information. To address this, a new Resource Description Framework (RDF) called GlycoRDF is being implemented across several of the above databases [54]. Once completed, this may speed-up the dynamic and automated construction of models for systems glycobiology using glycoinformatics databases.

GLYCOPROTEOMICS ANALYSIS ALGORITHMS AND SOFTWARE

The same glycan expressed on different protein scaffolds can have distinct functions. Thus, beyond glycomics profiling, which yields the overall distribution of carbohydrate structures on the cell surface, site-specific glycoproteomics data are necessary for the development of both glycoinformatics databases and systems glycobiology models. For such data collection, MS is the tool of choice due to recent technology breakthroughs and the high-throughput nature of such experiments (reviewed in [55]). However, data interpretation remains a major challenge even for experienced users. Due to this necessity to collect quantitative glycoproteomics data for model building, we review many of glycoproteomics analysis algorithms available currently.

An ideal software for glycoproteomics analysis should have the following features. It should: i) Provide a complete set of functions for the analysis of every step in the experimental procedure, such as *in silico* protease digestion to create a list of all theoretically possible glycopeptides in the sample, facilities for MS^1 precursor mass matching, and statistical scoring of MS/MS and MS^n spectra to identify the most feasible structure; ii) Support MS data inputs in standard open formats such as mzXML [56], thus allowing usage with a variety of MS hardware; iii) Support multiple fragmentation modes, especially CID, HCD (High-energy collision dissociation) and ETD (electron transfer dissociation). In this regard, each of these collision modes fragment glycopeptides in a distinct manner. CID and HCD primarily fragment the glycan while leaving the peptide backbone largely intact, with HCD causing more intense fragmentation. ETD, on the other hand, prefers to fragment the peptide backbone while leaving the glycan intact. These different fragmentation modes thus provide complementary information regarding the underlying glycopeptide; iv) Provide facilities for both N- and O-linked glycopeptide analysis, ideally in a high-throughput manner, possibly with parallel computing facilities; v) Beyond being freely available, the ideal code should also be open-source so that it can be developed by the community for different applications. To date no software satisfies all the above criteria.

Programs for glycoproteomics analysis can be broken down into software that either: i. Focus on matching only the unfragmented glycopeptide mass; ii. Handle MS/MS fragmentation spectra but that only handle one or a limited number of spectra at a time; or iii. Process high throughput MS/MS data (Table 3).

Among the programs that are based on matching either the mass of the precursor ion or glycopeptide alone, GlycoMod [57] is popular since it was among the first to be developed, and also since it is hosted as part of the ExPASy Bioinformatics Resource Portal. This program is specifically designed to identify the glycan composition of glycopeptides by

matching the experimentally measured mass to theoretical masses generated by varying the number and type of monosaccharide units attached to the peptide backbone. Prior knowledge of biochemistry regarding naturally occurring glycans is not necessary. Since the scope of the initial search in GlycoMod can be large, additional programs have appeared like GlycoSpectrumScan [58], GlycoX [59] and GlycoPep DB [60] that use a more focussed database.

While matching MS1 mass alone is useful, particularly if the data are from high-resolution MS instruments, MS/MS spectra analysis is necessary for assignment confirmation and also for glycan structure determination. Sweet Substitute [61] is the simplest example of the second class of programs and it is specifically designed to generate theoretical MS/MS spectra of tryptic digested peptides following the fragmentation of N-linked glycosidic bonds. GlycoPep Grader [62], Glycopeptide Evaluator [63] and Peptoonist [64] are additional programs that can perform CID or ETD fragmentation mode data analysis on either a single or a small set of MS/MS spectra.

The above programs that focus on limited MS/MS analysis perform relatively simple calculations and have few input and output fields. Due to their simplicity they are well suited for web-based interfaces. Increasing the complexity of the calculations and the handling of larger amounts of high-throughput data typically necessitates more computer resources and the development of stand-alone applications. To address this need, several programs focused on high-throughput data analysis have been developed either based on the application of principles akin to *de novo* sequencing, or algorithms that first generate a list of potential/candidate glycopeptides before scoring the tandem-MS data.

Glycominer [65] and Medicel [66] are two programs that utilize algorithms that do not create explicit glycopeptide databases. Among these, Glycominer is a GUI based software based on the premise that the CID mode fragmentation of glycopeptides results in the release of glycan oxonium ions [65]. Thus, this program identifies spectra corresponding to glycopeptides by scoring the oxonium ions. Once a match is identified, the program discovers the underlying peptide sequence by attempting to identify the specific spectral peak that corresponds to the deglycosylated peptide. Once this is done, attempts are made to match the molecular weight of the putative peptide with databases, for example the FASTA database. Medicel utilizes a similar approach to identify glycopeptide spectra based on the appearance of carbohydrate oxonium ions and the glycan ladder pattern that is common to N-glycan. SweetSEQer facilitates the interpret of the ladder pattern seen in the MS/MS spectra by using a graph based approach [67]. Sweet-Heart is another program that utilizes supervised machine learning to identify patterns in a training dataset prior to using this knowledge to identify N-glycopeptides [68].

Among the programs that match MS/MS spectra with respect to a database containing potential glycans and peptides, GlycoProteinSearch (GPS) identifies N-linked glycopeptides using CID MS/MS spectra data [69]. Here, the peptide sequence is determined by using the MS^1 precursor mass, glycan-loss ladder pattern in the MS/MS spectra and a library of potential peptides containing the Asn-X-Ser/Thr motif. Based on the overall glycan mass and fragments, the glycan is then conjectured by searching the GlycomeDB database. GlycoMasterDB is another program in this class that is specifically designed to handle high

throughput HCD and HCD-ETD tandem MS data [70]. The program uses the HCD spectra to identify spectra containing glycopeptides and potential glycans. Once this is done, peptide identification is done by either utilizing ETD data if this is available or alternatively simply matching the peptide molecular mass against a list of theoretically possible peptides. Neither of the above programs has a well-developed statistical scoring algorithm. Mayampurath *et al*. have developed GlycoFragWork [71] to handle CID, ETD and HCD fragmentation mode data. In the GlycoFragWork workflow, the oxonium ions in HCD mode are used to identify glycopeptide spectra, MS^1 precursor ion matching is then applied to determine candidate glycopeptides that may explain the spectra, subsequently ETD mode data are used to confirm peptide identity and in the final step CID fragment spectra scoring is performed for a representative glycopeptide. Byonic is a software with a comprehensive scoring algorithm and a user-friendly GUI [72], but it is not freely available or open-source.

Conclusion

The human glycome is far more complex compared to the genome and proteome, both from the structural and regulatory perspective. While a study of such carbohydrates was complicated in the past, the development of new analytical technologies is beginning to provide large scale qualitative and quantitative ‘data’ for this field. This is then opening new possibilities since these data are now starting to be collated into glycoinformatics relational databases, which derive ‘information’ on the relation between glycan structure and function. More advanced analysis of these data and information is possible using systems based modeling since standardized tools for computational model synthesis and sharing have become available. Together, such analyses can lead to new experimentally testable hypotheses, for the iterative refinement of ‘knowledge’ in the field of systems glycobiology.

While the focus of the current review is on how quantitative protein and carbohydrate structure data can be used for such systems-based model building and analyses, data at the metabalome [26, 30], transcriptome [28] and genome [73] levels can also provide additional complementary information. For example, Lau *et al.* [26] studied the effect of feeding N-acetyl glucosamine (GlcNAc) to cells with a focus on understanding how this perturbation alters the pattern of N-glycan branching. These authors used mathematical models to fit MS based N-glycan profiling data following perturbation while simultaneously performing metabolome analysis to index the status of the cells. Bennun *et al.* [28] also fit experimental MS data of prostate cancer cells with computer models, while using gene microarray experiments to rationalize cellular enzymatic activities. Similarly, in the case of O-linked glycans, Liu *et al*. [32, 74] used enzymology based glycosyltransferase activity measurements to guide computer models that fit the distribution of O-glycans on specific human myeloid cell proteins. In the final example, recently, Agrawal *et al*. [75] compared lectin binding data for 55 cell lines taken from the NCI-60 (National Cancer Institute) panel with corresponding miRNA expression data using singular value decomposition. Their analysis reveals the critical miRNA controlling biosynthetic pathways that construct high mannose, fucose and βGalNAc bearing glycans. Thus, beyond simply fitting experimental data on glycan distribution, these efforts attempt to link glycan structure data to additional levels of biosynthetic control within cells. Such large-scale experimental strategies, along with a number of system-perturbation methods that use RNA interference [21, 76], miRNA

perturbations [75], small molecule inhibitors [77] or genome editing [21, 53], may be applied in order to interrogate and further refine the computational models. Together, these approaches are likely to provide a more holistic view of the cellular glycosylation process. This may also lead to new exciting translational opportunities for this burgeoning field.

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Further Reading/Resources

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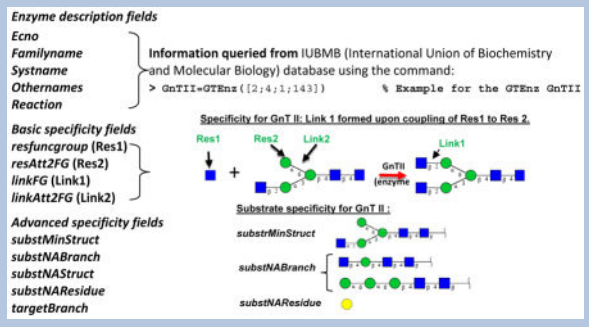
How can glycan structure data be incorporated into SBML files?

Glycan structures have been described using at least two XML standards, Glyde-II [37] and GlyoCT [38]. Inter-conversion between these two standards and also additional formats described in Figure 4 is possible using GNAT [33, 34] and also other programs [39]. These glycan XML structures can be incorporated into SBML format files within the species-annotation field as illustrated below. The *addGlycanAnnotation* command of GNAT can be used to automate the incorporation of multiple glycan structures rapidly into SBML files.

```
<sbml xmlns="http://www.sbml.org/sbml/level2" level="2" version="1">
<model id="demo">
....
<listOfSpecies>
<species id="S1".../>
<annotation>
<glycoct xmlns="http://www.eurocarbdb.org/">
<sugar version="1.0">
<residues>
....
</residues>
<linkages>
....
</linkages>
</sugar>
</glycoct>
</annotation>
</species>
....
</listOfSpecies>
....
</model>
</sbml>
```

How can glycosyltransferase specificity be described *in silico*?

It is desirable to have machine-readable definitions for the glycosyltransferases (glycoTs). GNAT captures this using the *GTEnz* class which contains various fields, some of which are shown below. Here, the enzyme description fields are automatically populated by querying the IUBMB database with the EC number. The 'basic' and 'advanced' specificity fields capture the absolute, group, linkage and stereochemical specificity of the glycoTs. Here, substrMinStruct presents the minimal substrate necessary for enzyme activity. substNABranch and substNAResidue describe structures and residues the presence of which prevent enzyme activity.



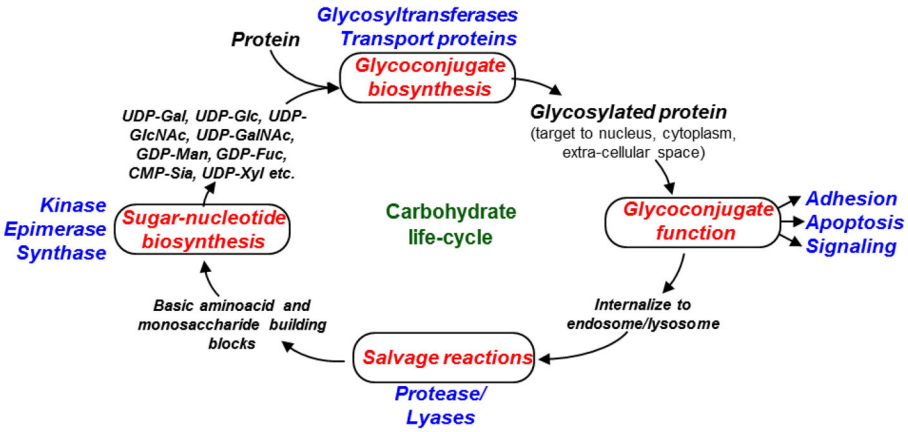


Figure 1. Carbohydrate life-cycle

Carbohydrates are processed through various biosynthetic and degradative transformations in cells.

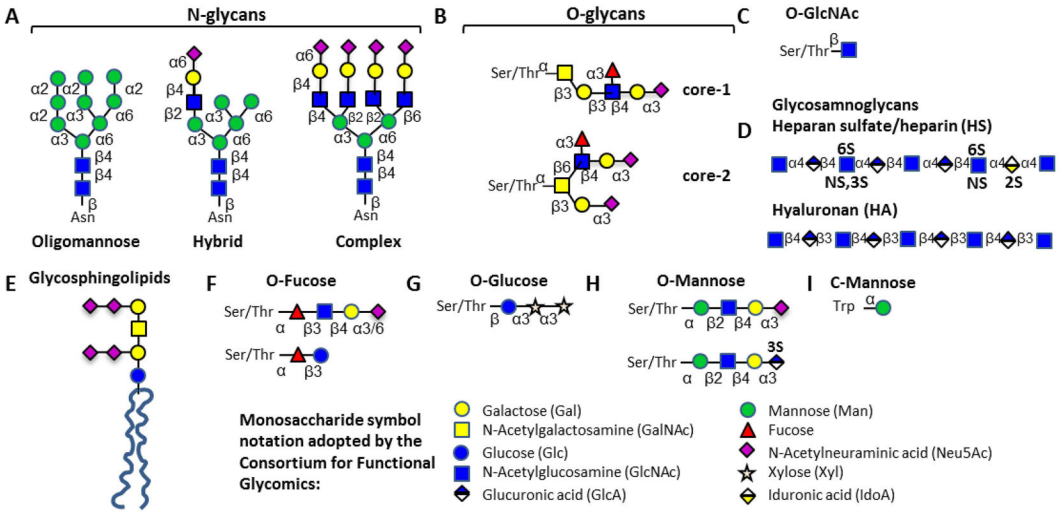


Figure 2. Glycan repertoire

A variety of glycans with diverse structures are found in mammals. These include: **A.** N-linked glycans; **B.** O-GalNAc type O-linked glycans; **C.** O-GlcNAcylated glycans; **D.** Glycosaminoglycans; **E.** Glycosphingolipids; **F**–**H.** O-linked glycans initiated by Fucose (panel F), Glucose (G) or Mannose (H); and **I.** C-Mannosylated glycans attached to Trp. Note that only selected examples of each glycan class are shown.

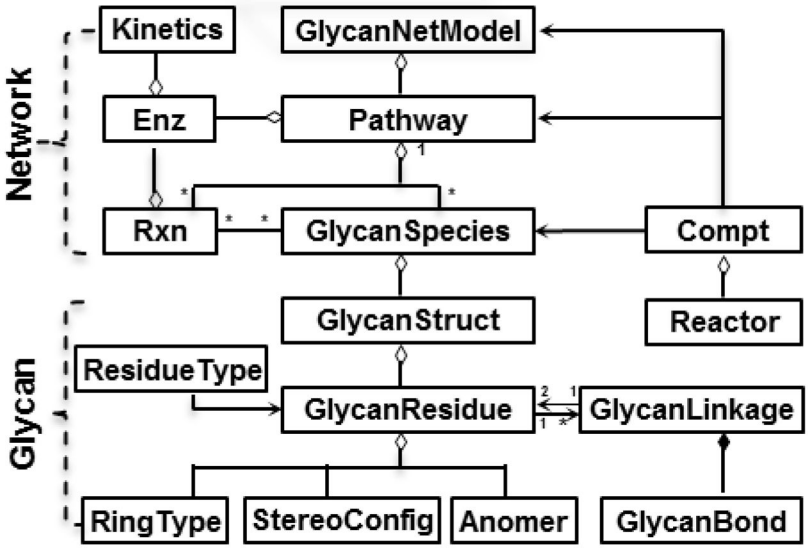
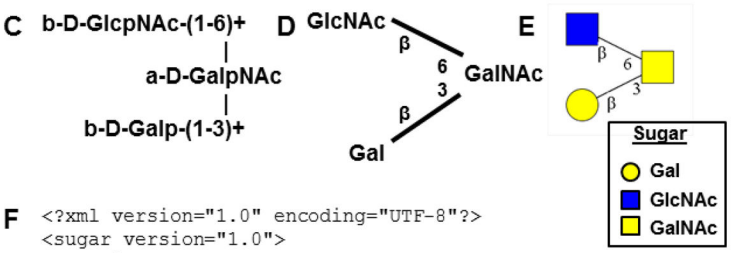


Figure 3. Entity-based modeling framework

UML diagram of the classes used for the construction of glycosylation reaction networks in GNAT (http://sourceforge.net/projects/gnatmatlab/). (adapted from ref. [34] with permission)

A []][a-D-GalpNAc]{[(3+1)][b-D-Galp]{}[(6+1)][b-D-GlcpNAc]{}}

B Gal b1-3(GlNAcb1-6)GalNAca



F

```
<?xml version="1.0" encoding="UTF-8"?>
<sugar version="1.0">
  <residues>
    <basetype id="1" anomer="a" superclass="hex" ringStart="1"
ringEnd="5" name="a-dgal-HEX-1:5">
      <stemtype id="1" type="dgal" />
    </basetype>
    <substituent id="2" name="n-acetyl" />
     …
  </residues>
  <linkages>
    <connection id="1" parent="1" child="2">
      <linkage id="1" parentType="d" childType="n">
        <parent pos="2" />
        <child pos="1" />
      </linkage>
    </connection>
    …
    </linkages>
</sugar>
```

Figure 4. Representation of glycan structures

Glycan representation in linear, graphical (2-dimensional) and data exchangeable formats: **A.** LINUCS; **B.** IUPAC; **C.** CarbBank 2-D representation; **D.** IUPAC graphics; **E.** CFG recommended graphics; and **F.** GlycoCT XML format.

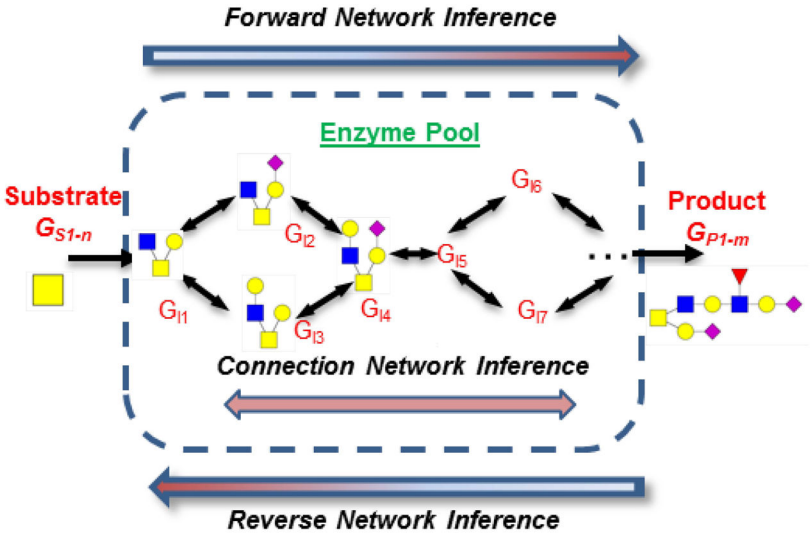


Figure 5. Automated *Pathway* construction

Three algorithms have been implemented in GNAT to automate network synthesis. These include the forward, reverse and connection network inference algorithms. (adapted from ref. [33] with permission)

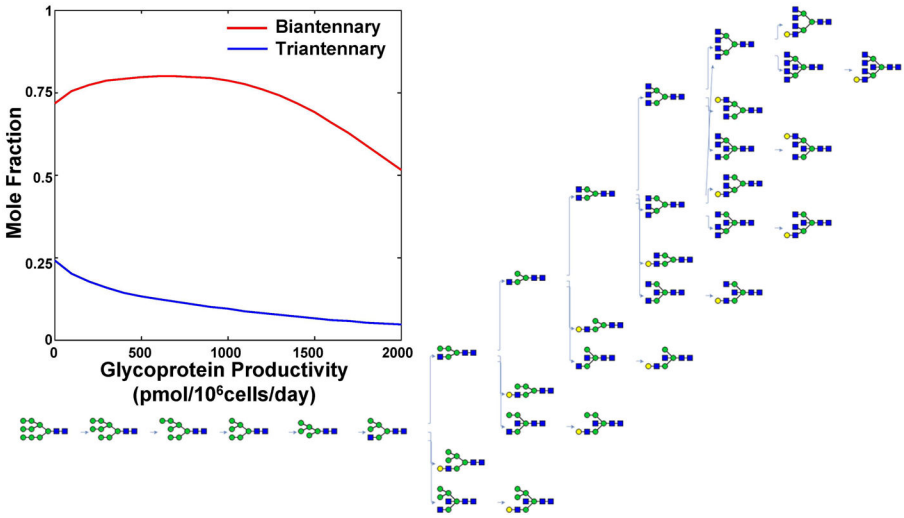


Figure 6. Visualization and modeling of glycosylation reaction networks

Synthesis of the N-linked glycosylation pathway described by Umana and Bailey [25]. Glycosylation reaction network presented here was visualized using the *GlycanNetViewer* function of GNAT (main figure). The network can be exported into an SBML file that contains all glycan sequences. The computational simulation of this network using MATLAB predicts that the extent of N-linked glycosylation decreases upon increasing protein productivity (i.e. macroheterogeneity increases, see plot at top-left). This affects the relative concentration of bi- and tri-antennary N-glycans.

Table 1

Network models of glycosylation

Model description	Key outcome or conclusion	Reference
Model of N-glycosylation initiation	N-glycan macroheterogeneity is dependent on the relative rates of protein translation versus enzyme activity of oligosaccharyltransferase (OST) complex.	24
Model of N-glycan branching	Predicted distribution of complex-galactosylated glycoforms with varying numbers of antennae. Simulation results were consistent with recombinant protein biosynthesis data	25
Ultrasensitivity in the N-glycosylation branching pathway	Demonstrated ultrasensitivity in the N-glycan branching pathway due to: i) A sequential increase in KM among the N-glycan branching enzymes, and ii) Removal of intermediate products in this reaction pathway.	26
Modeling of N-glycan terminal fucosylation and sialylation	Compared model based heterogeneity predictions with experimental data for thrombopoietin expressed in CHO cells	27
Integration of the transcriptome and glycome for N-glycan modeling	Demonstrated that information from multiple datasets can be combined to better understand complex cellular processes	28
N-glycosylation models in Continuous Stirred Tank Reactors (CSTRs) and Plug Flow Reactors (PFRs)	Suggested that vesicular transport can be modeled as a series of CSTRs while Golgi maturation resembles a single plug-flow reactor (PFR) or a series of PFRs in series.	29
N-glycosylation Quality by design (QbD) simulations	Prediction of N-glycan microheterogeneity in a kinetic model that includes sugar-nucleotide biosynthesis/transport	30, 31
Model of O-linked glycosylation	Simulated glycan distribution on the protein PSGL-1 (P-selectin glycoprotein ligand-1) and matched it with experimental results	32

Table 2

Glycoscience related databases *

Data source	Gene and enzyme level data	Glycomics data	Glycan–protein binding data	Glycoproteomics data	Pathway data	Mouse phenotyping data
CFG	Custom Glycogene microarray data for human and murine cells/tissue	Profiling of N- and O-glycans from cells and tissue using MALDI-TOF	Glycan microarrays quantify binding to various glycan binding proteins		CFG Molecular Pages contains data on glycan structures, glycosyltransfera ses and glycan- binding proteins	Hematology, Immunology & histology phenotyping of transgenic mice
EuroCarbDB & affiliated laboratories		GlycoBase, UniCarb-DB: LC, LC- MS/MS repository for N- & O-glycans.		UniCarb-KB: knowledgebase contains site-specific glycosylation data, built on the EUROCarbDB framework.		
JCGGDB	Tissue specific expression and enzyme specificity data for ~150 enzymes	MS^n analysis of glycan standards	LfDB: frontal chromatography data for lectin binding; GlycoEpitope: knowledgebase with antibody specificity data			
Other data sources	ExplorEnz: gateway for IUBMB enzyme nomenclature; BRENDA: kinetic data for enzymes collated from literature			CBS Predictor server: NetCGlyc, NetNGlyc, NetOGlyc & YinOYang for prediction of sites of c-mannosylation, N-glycosylation, O-glycosylation and O-GlcNAcylation, resp.	KEGG GLYCAN: Glycosylation pathway data	

* All repositories do not contain all types of experimental data. Thus, there are several empty fields in the Table.

Table 3

Some computer programs for glycoproteomics data analysis

Software Name	mzXML/ mzML input	High-throughput Processing	Parallel Computing	Decoy Database Generation	False Discovery Calculation	GlycoPeptide Database	MS^1 match	N- and O-linked	MS^2 Scoring	Fragmentation Mode	Command Line/GUI/Web (Open-source)	New Features	Reference
i. Programs specializing in analysis of unfragmented glycopeptide (MS^1 only)													
GlycoMod	No	No	No	No	No	Yes	Yes	N- and O-	No	-	Web application (Non-open-source)	part of ExPASy suite	57
GlycoSpectru mScan	No	No	No	No	No	Yes	Yes	N- and O-	No	-	Web application (Non-open-source)	Use glycan composition as inputs to generate glycopeptide database	58
GP(finder) GlycoX	No	No	No	No	No	No	Yes	N- and O-	No	-	Command Line (Available upon request)	Isotope filter/Diagnostic ion	59
GlycoPep DB	No	No	No	No	No	Limited	No	N	No	CID	Web application (Non-open-source)	Glycopeptide database	60
ii. MS/MS data analysis programs with limited ability to handle high-throughput data													
Sweet substitute	No	No	No	No	No	No	No	N-	No	-	GUI (Non-open-source)	*In silico* Fragmentation of glycopeptide	61
GlycoPep Grader	No (csv)	No	No	Yes	Yes	Yes	Yes	N-linked	Yes	CID	Web application (Non-open-source)	Glycan-type-dependent fragmentation rules	62
GlycoPep Evaluator	No	No	No	Yes	Yes	No	No	N-linked	Yes	ETD	Java-based GUI (Non-open-source)	FDR Prediction for small data sets	63
Peptoonist	No	No	No	No	No	Yes	Yes	N-	Yes	CID	GUI (Non-open-source)	Use cartoonist glycan database to create	64
iii. High-throughput algorithms/software													
***a.* Scoring without explicit glycopeptide database creation**													
GlycoMiner	No	Yes	No	No	No	No	No	N-	Yes	CID	GUI (Non-open-source)	Spectra and glycan quality score	65
Medicel N-glycopeptide library	No	Yes	No	Yes	Yes	No	Yes	N-linked	Yes	CID	GUI (Propriety Software)	Branch and Bound algorithm for glycan identification	66
SweetSEQer	No (mgf file)	Yes	No	No	Yes	No	No	N-linked	No	HCD/CID	Command Line (Open-source)	De novo sequencing and annotation	67
Sweet-Heart	Yes	Yes	No	No	No	No	Yes	N-linked	Yes	CID	Command Line (Non-open-source)	Machine learning algorithm/MS^3 Score	68
***b.* Scoring based on glycan/glycopeptide database creation**													
GlycoPeptide Search	Yes	Yes	No	No	Yes	Yes	Yes	N-linked	No	CID	Command Line/GUI for inputs	Diagnostic Ion Filtering	69
GlycoMaster DB	No (mgf file)	Yes	No	No	No	No	Yes	N-linked	Yes	HCD/ETD	Web application (Non-open-source)	mixed HCD/ETD fragmentation	70
GlycoFrag- Work	Yes	Yes	No	Yes	Yes	Yes	Yes	N-linked	Yes	CID/ETD/HCD	Command Line (Non-open-source)	Mixed ETD-CID, mixed CID- HCD	71
Byonic	Yes	Yes	Yes	Yes	Yes	Yes	Yes	N- and O-	Yes	CID/ETD/HCD	GUI (Propriety)	Commercial software	72