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Mini review

Glycans in health and disease

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Summary. If a cell would need to produce one specific protein for each biological function, there would probably be over a million different proteins. However, the actual number is much lower. To save energy, cells use different methods to modify proteins, thus repurposing them and enabling them to perform different functions. The most common modification is glycosylation, a process in which one or more (the same or different) monosaccharide units are enzymatically linked to a protein (or another biomolecule). Glycan synthesis is a complex and incompletely understood process in which different forms of various types of glycans are formed. A set of glycans present in one cell or an organism represents their glycome and is a reflection of the cellular conditions in a specific environment. Glycomes are very flexible entities that constantly change in response to physiological circumstances, thus reflecting the habits of an organism as well as the process of biological ageing. Even more interestingly, glycome also changes under specific and pathological conditions. Understanding how and why the glycome changes will help in design of new medical treatments for such pathological processes.

Keywords: carbohydrate, glycosylation, monosaccharide, N-glycan.

INTRODUCTION

The discovery of nucleic acids by Swiss biochemist Friedrich Miescher took place in 1869 (Lamm et al. 2020), although in the years to come the glory was attributed to Watson and Crick. In their article published in the year 1953, the two proposed a three dimensional structure of the DNA double helix, that rocketed them to eternal fame after their crowning with the Nobel Prize in 1963. Contributions from many other scientists who worked so hard on this problem, including those whose work paved the way and made it all possible (e.g. Friedrich Miescher, Phoebeus Levene, Erwin Chargaff, and many others), were somewhat unfairly ignored (Pray 2008). The cherry on top of this story, called the "secret of life", came decades later, when the code of the human genome was finally broken. Its deciphering took years and was finally finished in the year 2000, with different improvements done over the following years (Nurk et al. 2022). The closure of this circle took several decades, and one could say it suitably reflected the astonishing complexity and importance of this biomolecule. On the other hand, other types of biomolecules, far less complex, are not even close to being fully understood, and the story of the "sugars of life" has yet to be written. Thus, this review is a short overview of the glycomics field; and in the following pages, more complex relatives of carbohydrates - the glycans, will be introduced.

MAMMALIAN GLYCANS

After their discovery, glycans were assumed to be only for decorative purpose, and thus their essential function in some vital processes such as protein folding, cell adhesion,

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biomolecular interactions and immunity remained under the radar. Their presence, as well as their structure, reflects the conditions of a cell and organism. Their structure, together with their position, is not template-driven, as in the case of other biomolecules, but rather relies on the genetic coding of a variety of enzymes involved in their synthesis and processing.

Glycan diversity can be attributed to the characteristics of their building blocks - monosaccharides, as these units can be attached in a linear manner, but can also branch, due to several positions at which monosaccharide units can interact. For example, even in the case of just two monosaccharides, eight combinations of possible interactions can occur. Considering the complexity of monosaccharide units, the Symbol Nomenclature for Glycans was adopted to improve their understanding and provide clarity and uniformity. Based on this system each glycan is assigned a specific shape, color and filling (Fig. 1). In this way, complex structures can easily be respresented even on a small piece of paper - and be less "horrifying" to junior glycoscientists! Glycan synthesis is driven by more than 200 enzymes, sugar phosphate intermediates and nucleotide sugars (Schjoldager et al. 2020), whose availability is affected by the cell surroundings and can be easily modulated by various environmental factors. Acti-

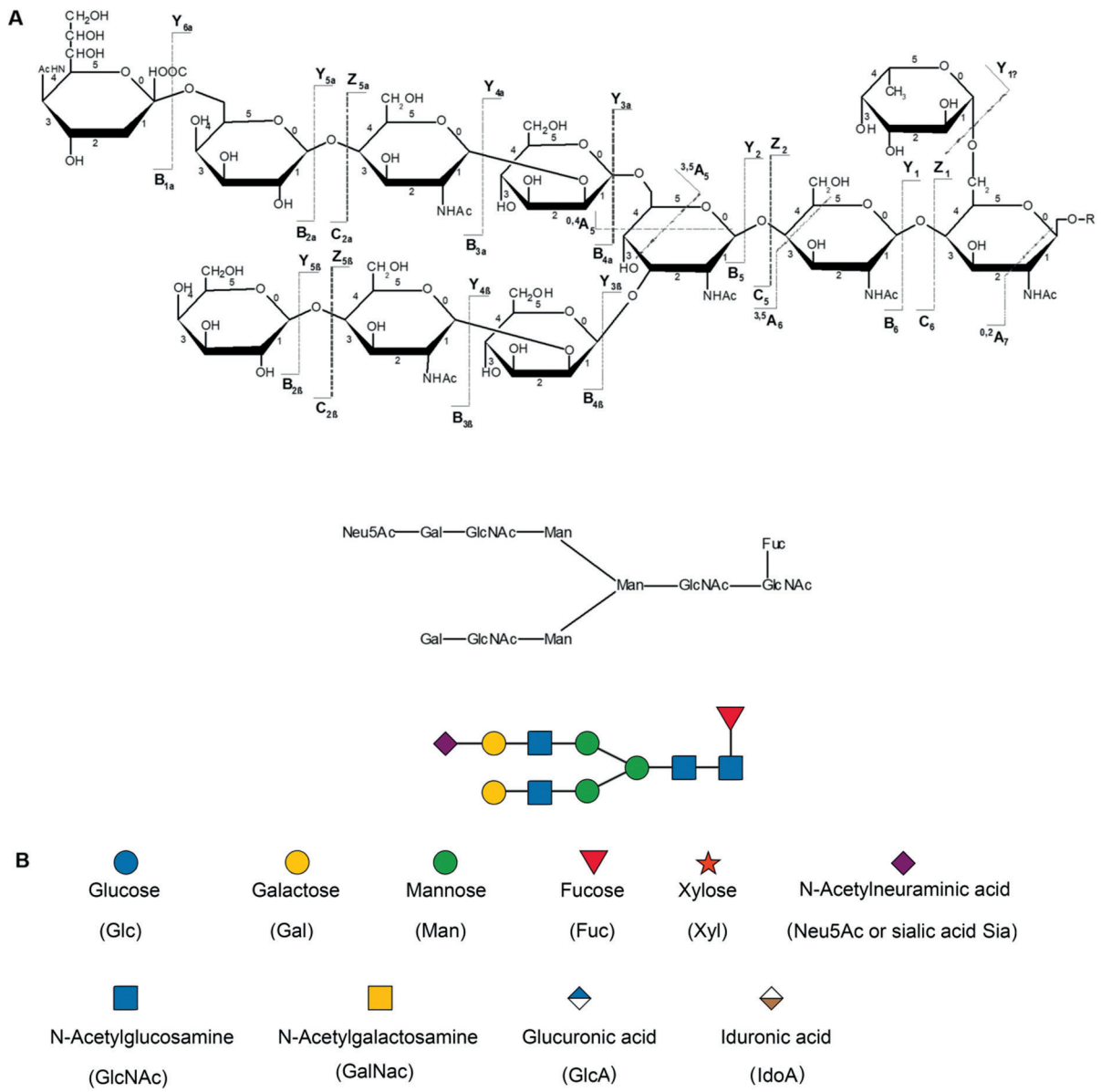

Fig. 1. Interaction of monosaccharides presented using the Haworth representation and the Symbol Nomenclature for Glycans. Modified from Paton et al. (2021).

vated sugar donors are formed after binding of nucleotides to sugars, either hexoses (synthesized from glucose) or pentoses (synthesized from fructose). This process is not as random as it might seem. For example, guanosine diphosphate (GDP) is a carrier for Man and Fuc, uridine diphosphate (UDP) for Glc, Gal, GlcNAc and GalNAc and cytidine monophosphate (CMP) for Neu5Ac. With the exception of Neu5Ac which is activated in the nucleus, the formation of activated sugar donors occurs in the cytosol. The process of glycosylation is a dramatic event and its elucidation will help in better understanding of disease progression and, consequently, will enable new approaches for disease treatment, including glycan therapeutics as potential candidate drugs. Mammalian glycans can be classified into four groups: glycosaminoglycans, O-GlcNAc-glycans, O-glycans, and N-glycans.

GLYCOSAMYNOGLYCANS (GAGS)

GAGs are composed of negatively charged repeating disaccharide units that are present in the extracellular matrix. Depending on their structure, modification and/or sulfation, GAGs can be classified as hyaluronic acid, heparin/heparan sulfate, chondroitin/dermatan sulfate and keratan sulfate (Casale et al. 2023). Their synthesis starts in the cytoplasm with the formation of activated sugar components in the form of UDP-glucuronic acid/GlcNAc/xylose/galactose/GalNAc and continues in the Golgi apparatus, where further modification and sulfation occurs. This synthetic pathway is followed by all types of GAGs, except for hyaluronic acids whose UDP-sugars (glucuronic acid and GlcNAc) are transported to plasma membrane and then processed (Ghiselli 2017). After sulfation in the Golgi, GAGs are covalently bound to proteoglycans *via* a tetrasaccharide linker that is connected to Ser residues on the protein core. This linkage is formed differently in the case of keratan sulfate: where complex glycan binding to Asn, GalNac binding to Ser/Thr or Man binding to Ser/Thr, all depend on the type of keratan sulfate (Prydz 2015). GAGs can be constructed from more than 40 disaccharide pairs. They form glycocalix and are involved in cell hydration, growth and proliferation, adhesion, coagulation, wound healing, carcinogenesis and metastasis, etc. Among all of the above mentioned, probably the most famous is hyaluronic acid, due to its usage in cosmetic products, owing to its effect on skin properties, such as elasticity and hydration (Bukhari et al. 2018). Altered levels of different GAGs, as well as their involvement in the development and progression of atherosclerosis, diabetes mellitus, neurodegenerative diseases and various forms of cancers, has been documented (Wang and Chi 2022).

O-N-ACETYLGLUCOSAMINE GLYCANS

O-GlcNAc glycans are formed by attachment of GlcNAc via a β-linkage to hydroxyl groups in Ser or Thr residues, in a process that is in competition with amino acid phosphorylation. This type of glycosylation, reserved for intracellular proteins, is driven by O-GlcNAc transferase, an enzyme that transfers activated monosaccharides in the form of UDP-GlcNAc, and O-GlcNAcase, an enzyme that removes GlcNAc, and can be seen as a mode of protein labeling that serves as nutrient and a stress sensor (Chang et al. 2020). O-GlcNAcylation is more simple compared to other glycosylation types, as well as more dynamic. It helps determine the fate of protein cellular localization, stability and interactions with other proteins. O-GlcNAcylation promotes T and B cell development, proliferation and activation, and regulates microphage response. Changes in protein O-GlcNAcylation are found in different disorders, such as diabetes, neurodegenerative diseases and cancer (Chang et al. 2020).

O-GLYCANS

O-glycosylation is another type of glycosylation where hydroxyl groups of Ser or Thr residues of a protein are modified. O-glycosylated proteins are present extracellularly and can be secreted. Common sugar moieties in this type of glycosylation include GalNAc, Fuc, Glc and Man. Mucin-type O-glycans, where the sugar is GalNAc (also known as the Tn antigen), serve as a physical barrier and for cell protection against environmental stress and microbes, as well as for self-recognition by the immune system. Probably the best-known O-glycans, which are often given as an example, are the blood group or ABO(H)-associated glycan epitopes. Type O blood groups express H antigen (Fig. 2A), while in type A blood groups α1,3GalNAc is attached to H antigen and in type B blood groups the GalNAc of an A antigen is altered with α1,3GlcNAc (Reily et al. 2019; Scheper et al. 2023).

N-GLYCANS

Most secreted proteins are **N-glycosylated**, with the conserved pentasaccharide core consisting of three Man and two GlcNAc ($Man_3GlcNAc_2$) linked to an amino group of Asn in the position Asn-X-Ser/Thr, where X can be any amino acid except Pro. The number and type of monosaccharide units that are used to further elongate this conserved core can vary dramatically. Therefore, based on the composition of monosaccharide units these glycans can broadly be classified as high-mannose (with varying branches containing only Man units), complex (with varying branches containing different monosaccharide units) and hybrid (with antennae corresponding to high-mannose and antennae

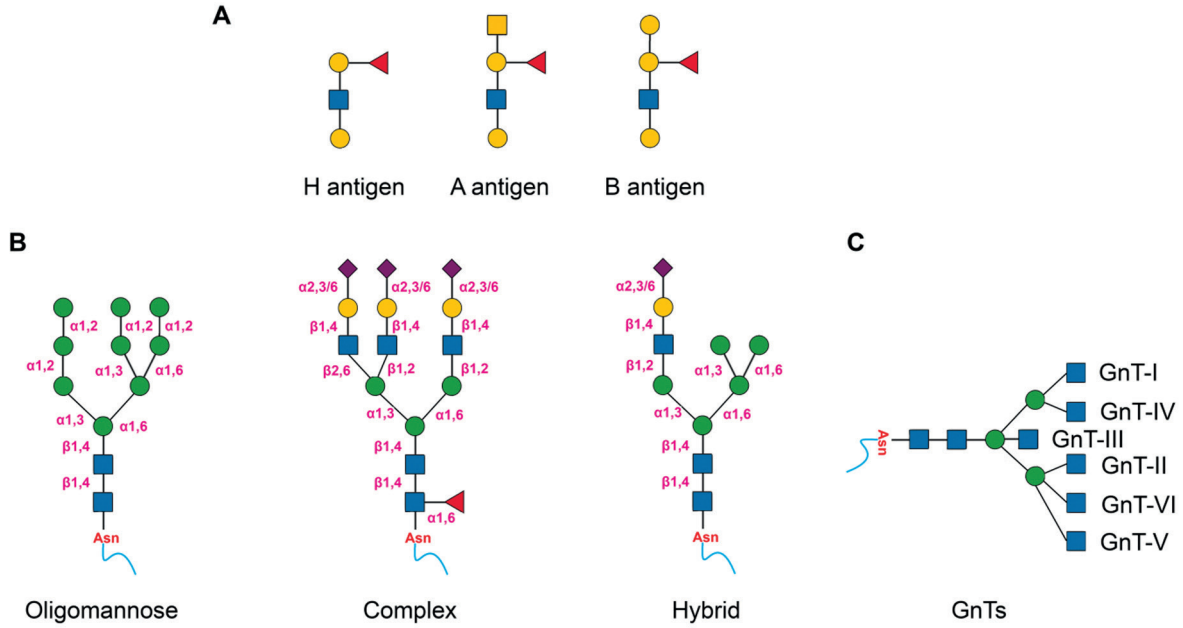

Fig. 2. A, O-glycans determining blood type; **B**, types of N-glycans; **C**, action of different N-Acetylglucosaminyltransferas (GnT).

corresponding to a complex types) (Fig. 2B). N-glycans are synthesized in two steps: *en bloc* transfer of a lipid-glycan precursor ($Glc_3Man_9GlcNAc_2$-dolichol-phosphate) to an Asn residue by oligosaccharyltransferase complex in the endoplasmic reticulum (ER) and downstream processing (both trimming and elongation by different glycosyltransferases and glycosidases) in the Golgi apparatus (Reily et al. 2019).

The process of N-glycan synthesis is not completely understood, and is given in a simplified form. On the cytosolic side of the ER, GlcNAc is first transferred from UDP-GlcNAc to dolichol-phosphate by dolichol-phosphate N-acetylglucosamine-phosphotransferase 1 (Fig. 3). After addition of a second GlcNAc and three Man units (also mediated by glycosyl-transferases), the resulting structure is flipped to the lumen of the ER for further elongation. Mannosyltransferases orchestrate the transfer of four additional Man units, while glucosyltransferases regulate the transfer of three Glc units (from membrane-embedded dolichol-phosphate-sugar donors) until a $Glc_3Man_9GlcNAc_2$ structure is formed. This N-glycan is in whole or *en bloc* transferred by an oligosaccharyltransferase complex from its lipid carrier to the amino group of a targeted Asn. If the resulting structure passes quality control, the terminal Glc residues are removed one-by-one by α-glucosidase I and II, acting as a signal for α-mannosidase I to further remove Man from the central arm. The formed $Man_8GlcNAc_2$ structure is then transferred to the *cis*-Golgi for additional trimming of Man by α-mannosidase I resulting in the formation of $Man_5GlcNAc_2$. In *medial*-Golgi GlcNAc is added by the action of N-acetylglucosaminyltransferase 1 (GnT-I), resulting in the formation of $GlcNAcMan_5GlcNAc_2$. By the action of α-mannosidase II, two Man residues are trimmed enabling the GnT-II to add another GlcNAc, resulting in formation of a bi-antennary structure. Further branching of $GlcNAc_2Man_3GlcNAc_2$s is driven by the action of GnT-IV or GnT-V, resulting in the formation of more branched types of complex N-glycans (tri- and tetra-antennary). In contrast with the mentioned GnTs, GnT-III enables formation of a glycan structure with bisecting or trimannosyl core β1,4-bound GlcNAc (Fig. 2C), while at the same time inhibiting the activity of other mentioned GnTs, and hence further branching (Chen et al. 2020). The enzymatic action of α1,6-fucosyltransferase results in the modification of inner-core GlcNAc, which is bound to Asn, in a process that is usually reserved for biantennary glycans. In the *trans*-Golgi, glycan branches are further elongated by the action of galactosaminyltransferase and the addition of Gal. Final processing of N-glycans involves capping and (optional) decoration with GlcNAc, Gal, Fuc and Neu5Ac, driven by the action of the relevant transferases (Reily et al. 2019; Scheper et al. 2023).

INVESTIGATION OF GLYCANS

The relevance of glycobiology was confirmed in the year 2022, when the Nobel Prize in chemistry was awarded to Carolyn Bertozzi, Morten Meldal and Carl Sharpless for their work on the development and application of click chemistry

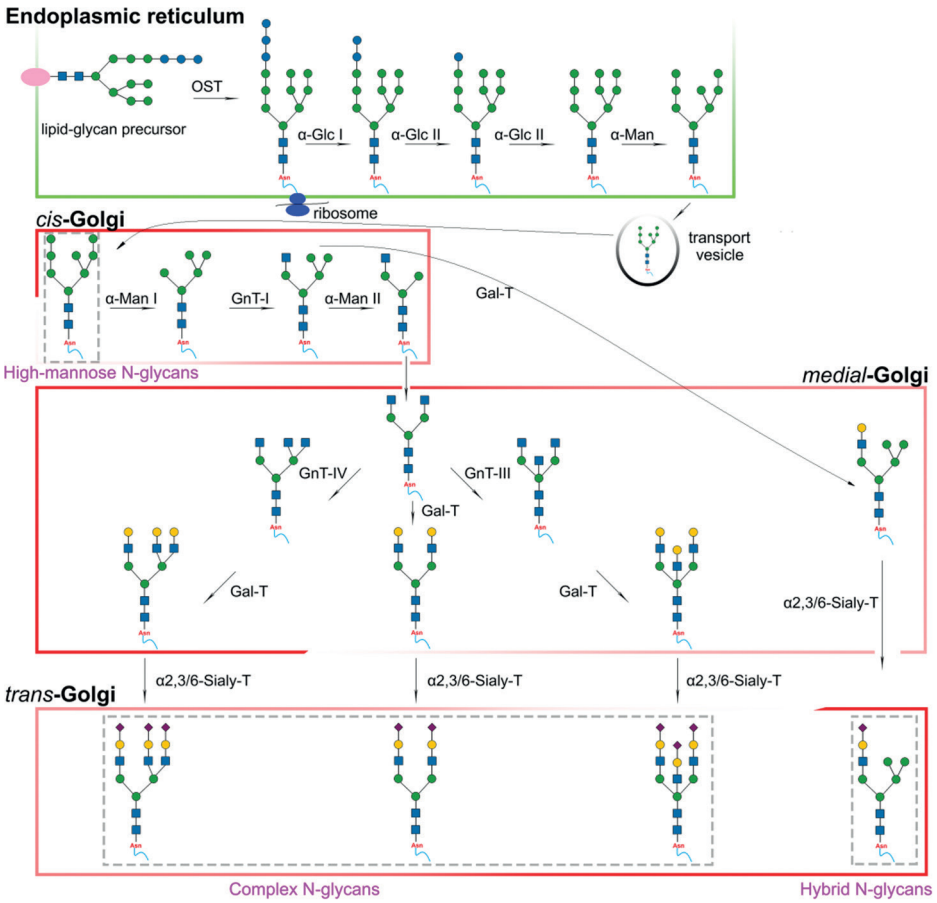

Fig. 3. Biosynthesis of N-glycans.

and bio-ortogonal chemistry, and their application in studying the glycobiology of different diseases. A search for the term "glycan" in the Scopus scientific database results in over 31000 different documents, with a significant increase since the beginning of the 2000s. There are also more than 1000 book chapters and more than 24000 research articles on this subject, with the first one dating from 1961. Over this period of six decades, the world leader in the field is the USA with over 10000 documents, followed by Japan with 5000. Serbia is in 47th place with 51 documents from different fields; still, the number of publications has remained constant over the past 15 years. This number is probably even lower, since the search was based only on the term and not on the exact number of researchers or papers dealing with the investigation of a glyco-process or carbohydrate synthesis.

Two facts regarding glycan determination are important: i) the exact site where the modification exists on a biomolecule and its surrounding (e.g. amino acid position in the polypeptide chain) and ii) the exact structure of the glycan. Therefore, glycans can be studied in the form of conjugates bound to biomolecules, but they need to be released from their binding partners in order to determine their structure in detail. Glycan release can be done either chemically or enzymatically. Processes like hydrazinolysis and β-elimination can be employed. In the first case, both N- and O-type glycans can be released from the binding protein, whereas the use of a strong base will induce cleavage of O-glycans only. Employed conditions need to be harsh for some groups (e.g. sulfation and O-acetylation) and they can lead to loss of the sugar reducing terminal or reaction with the reducing group of hydrazine, or degradation of the glycan-binding partner (e.g. protein). To avoid these problems, different enzymes are more readily used, since they require milder and less destructive conditions. Various endoglycosidases can cleave diverse N-glycan types: PNGase F cleaves the bond between the inner GlcNAc and amino group of Asn, PNGase F1 cleaves the bond between the two GlcNAc molecules of the pentasaccharide core of oligomannose N-glycans, and PNGase H cleaves the same bond as the PNGase F1 but preferentially in oligomannose and hybrid type of N-glycans (Kayili et al. 2022). In contrast to endoglycosidases, exoglycosidases cleave the bond between terminal and penultimate mono-

saccharides, and are classified as neuraminidases, galactosidases and fucosidases, etc. In addition, they can be specific for only one type of linkage, such as α2,6-neuraminidase or α2-3-neuraminidase etc. Cleavage will cause a reduction in the molecular weight of the remaining molecule and, thus, will influence the retention time. To make their detection easier, glycans can be tagged, or covalently labeled with a fluorescent probe (Fig. 4). By employing the reaction of reductive amination, for example, the primary amine group of the probe can be linked to the reducing end of the glycan, i.e. the aldehyde group. Considering that the reactions are carried out in a 1:1 ratio, in which one molecule of the tag reacts with one sugar residue, the information obtained is also quantitative.

Decoding glycans and discovering their exact function and purpose in a specific environmental context is truly tedious work that requires consolidation of different research fields and the combination of different (more or less) sophisticated techniques (Griffin and Hsieh-Wilson 2022). Widely applied and affordable techniques are based on lectin recognition and can be performed either in the context of the native surroundings of the investigated glycan: lectin histochemistry, lectin blotting, lectin-based microarray, or with the aid of glycan labeling and/or derivatization: liquid chromatography coupled with fluorescence detection (LC-FLR), capillary electrophoresis (CE), matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), LC-MS, DNA sequencer-aided fluorophore-assisted carbohydrate electrophoresis (DSA-FACE), ion mobility and NMR (Paton et al. 2021). The choice of the technique depends on the nature of the tested sample, the number of tested samples as well as the final information that one wants to obtain.

A new front in glycan discovery opened with the application of bio-orthogonal click chemistry (Fig. 5). One of the specificities of glycosyltransferases is their ability to use different sugar donors. Sugar donors, on the other hand, can be labeled with a small nontoxic group, that can later react with another small ligand, thus, enabling selective and fast reactions in living organisms. A combination of chemoenzymatic glycan labeling with bio-orthhogonal reactions enabled investigation of glycan expression in live cells, crude cell lysates and tissue samples (Aguilar et al. 2017).

GLYCANS FOR LIFE

As previously mentioned, glycans determine protein conformation and hence their function, affecting protein-ligand interactions, cell-cell interactions, adhesion and migration; and are involved in numerous physiological pro-

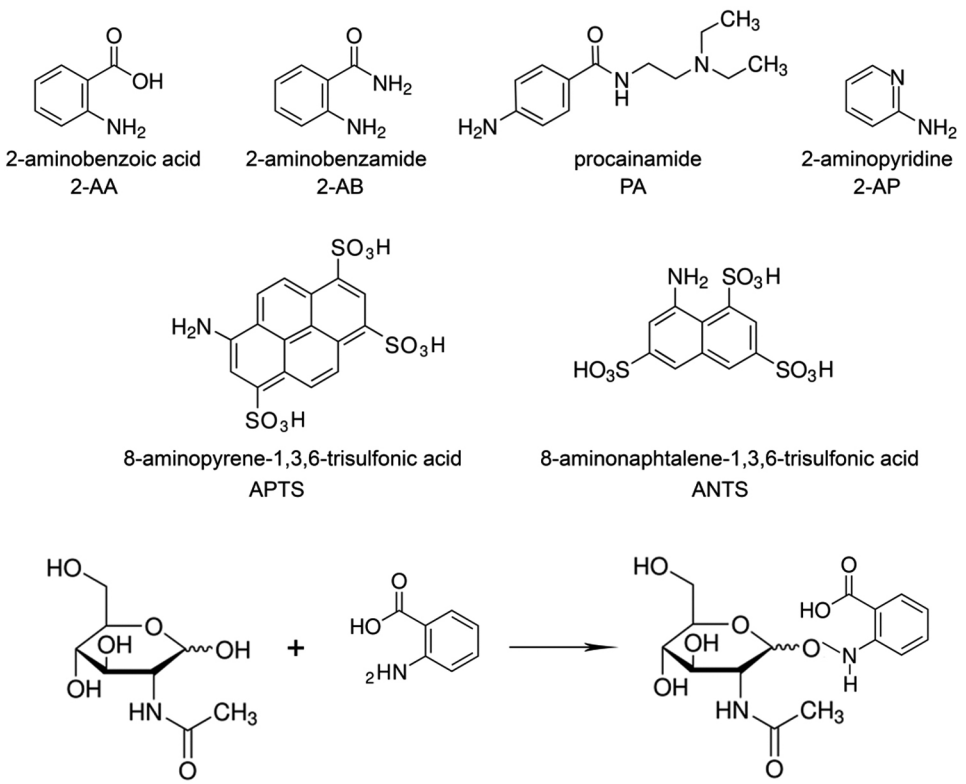

Fig. 4. Common fluorophores used for glycan labeling.

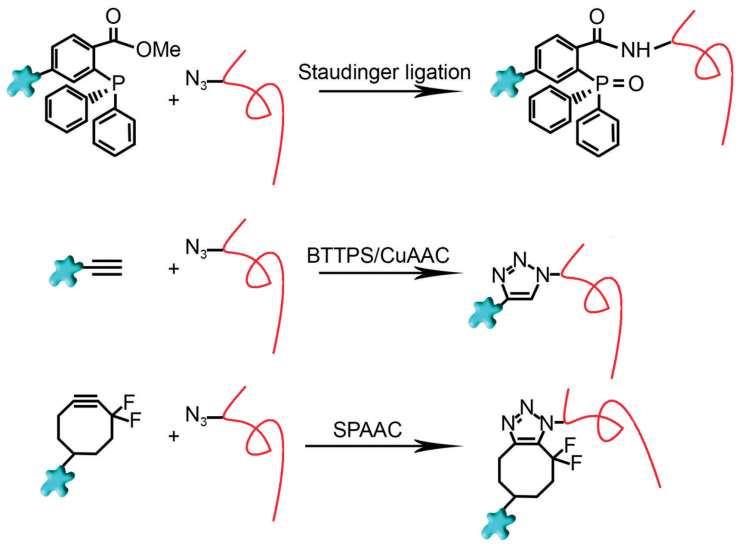

Fig. 5. Examples of bioorthogonal reactions: Staudinger ligation, ligand-accelerated copper-catalyzed azide-alkyne cycloaddition (CuACC), Cu-free cycloaddition between azides and cyclooctynes known as strain-promoted alkyne-azide cycloadditions (SPAAC).

cesses, such as immune response and fertilization, as well as pathological events, including neurodegenerative diseases, diabetes and cancer. Congenital disorders of glycosylation (CDGs) can lead to impaired neurological and muscular development, and can be lethal. In type 1 CDGs, the formation of oligosaccharide structure on the glycolipid precursor is disturbed, whereas the type 2 CDGs reflect disorders in the control of glycan branching on the native protein (Lipiński and Tylki-Szymańska 2021). Understanding and deciphering each step of glycan metabolism and identifying all of the participants will also help in treating these disorders.

Probably the most studied protein, in terms of its glycosylation and impact of this modification on different physiological events, is IgG. IgG is involved in various immune processes, and is abundantly present in human serum, making it easily accessible for isolation and further investigation. Its function is mostly regulated by glycosylation and the presence or absence of some monosaccharides that alter the proteins' potential between anti-inflammatory and pro-inflammatory (Dekkers et al. 2017). Glycans occupying Asn297 of the Fc domain affect the conformation and stability of IgG (Le et al. 2016). Although present, N-glycosylation of the Fab region is much less explored. However, Gal, Sia and bisecting GlcNAc are more frequent in the Fab region, in contrast with core fucosylation, which is more frequent in the Fc region (Holland et al. 2006; Bondt et al. 2014). IgG glycans regulate affinity towards its receptor. The affinity of afucosylated IgG towards FcγRIIIa/b is higher compared to the affinity of the fucosylated form (Shields et al. 2002). This process, initiated by the disruption of FUT8 alleles, induces antibody-dependent cell-mediated cytotoxicity and is considered pro-inflammatory. Similar effects are observed with the bisecting of GlcNAc, although to a lower extent (Davies et al. 2001). The opposite was seen in the presence of sialylation, as lower affinity to FcγRIIIa was detected, probably leading to the reduction of antibody-dependent cell-mediated cytotoxicity (Scallon et al. 2007). The impairment of complement-dependent cytotoxicity also contributes to the anti-inflammatory effect of sialylated IgG (Quast et al. 2015). Galactosylated IgG inhibits C5a-dependant inflammation, whereas the agalactosylated form can activate the complement system through lectin and other pathways (Banda et al. 2008; Haddad et al. 2021). Variations in IgG glycosylation in healthy individuals are quite small, while high heterogeneity is present in pathological conditions (Štambuk et al. 2020), which additionally complicates the already complex biology of IgG. IgG glycans are proposed as diagnostic biomarkers (with AUC>0.900) for rheumatoid arthritis, osteoarthritis, hypertension, type 2 DM, colorectal cancer, breast cancer, ovarian cancer, pancreatic cancer, urothelial carcinoma and are a predictive marker of Parkinsons disease (as elaborated in an extraordinary and extensive review by Shkunnikova et al. 2023).

By investigating glycan alterations in the chronic kidney disease patients, we found that glycans decorating proteins involved in the metabolism of iron and coagulation in patients on dialysis (transferrin and fibrinogen, respectively), are drastically altered compared to healthy controls (Baralić et al. 2020, 2023; Miljuš et al. 2024). The content of biantennary bigalactosylated glycan with bisecting GlcNAc was increased while the content of fucosylated biantennary glycan is decreased. These patients receive different glucose solutions as a mode of therapy that can disturb glycan metabolism, since there are more Glc molecules for the production of other monosaccharides. Patients receiving glucose polymer had higher content of multi-antennary N-glycans

(Baralić et al. 2020). Additionally, higher content of paucimannosidic/highly mannosidic glycan structures on fibrinogen correlated positively with death rate (Baralić et al. 2023).

By investigating glycosylation of placental membrane proteins, we identified more than 60 N-glycan structures, with the most abundant belonging to biantennary glycans with bisecting GlcNAc, fucosylated glycans and mannosidic structures in the form of immature glycans (paucimmanosidic structures) and high-mannose type N-glycans (Robajac et al. 2019). The major MS peaks originated from: 1) biantennary complex type N-glycan with a bisecting GlcNAc residue and 2) core-Fuc paucimannosidic and high mannose type structures M3-M9. The age of mothers and the stage of placental development affected general N-glycome as well as glycans of IR and IGFRs (Robajac et al. 2014, 2016, 2019).

Differences in the glycosylation of serum proteins in healthy people of different age was also documented (Šunderić et al. 2019), as well as the changes in patients with colorectal cancer (Šunderić et al. 2016; Kianičková et al. 2024) or breast cancer (Baričevič et al. 2010). Furthermore, receptors of the IGF system in tumor tissue of patients with colorectal carcinoma express higher levels of fucosylation and branched mannose structures (Robajac et al. 2020). Our data confirmed that glycosylation is both tissue and protein specific, as glycosylation of isolated proteins was not always in accordance with the N-glycome of whole tissue.

CONCLUSION

Glycans are astonishingly versatile molecules that interact with and modify all other biomacromolecules, hence determining their function, localization and fate. The most studied is glycan interactions with proteins as small changes that can have enormous impact on the life of a protein, and effect the whole organism. Although it took decades for this ugly duck called glycan to transform into a beautiful swan, we are certain that the future belongs to the "sugars of life".

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