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Minireview

Extracellular vesicles in a maze of glycomic complexity

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Summary. The carbohydrate portion of proteins and lipids mediates a variety of biological processes. Revealing their underlying principles is a challenging task that could contribute to a better understanding of many patho/physiological conditions. On the other hand, the interest in extracellular vesicles (EVs) has increased in recent years due to their involvement in intercellular communication leading to an array of functional and structural changes in recipient cells. Their characterization uncovered an exceptional diversity in size, morphology, as well as in membrane and cargo content. Monitoring/analysis of surface glycosylation of EVs originating from the prostate, termed prostasomes, revealed their substantial contribution to the complexity of seminal plasma (SP) glycome. Heterogeneity of surface glycans confirm the existence of several prostasome subpopulations. Presentation of surface glycans on prostasomal membrane is strongly affected by co-localized membrane-associated glycoproteins and tetraspanins. They appear to be organized in established/regular distribution patterns on membrane domains. Surface glycans are a component of EVs membrane that affects its functionality and potentially a distinction marker of prostasome subpopulations. Further understanding of the complex composition of glycans on EVs might explain the relation of their structure with functional alterations in distinct patho/physiological conditions.

Keywords: extracellular vesicle, glycans, prostasomes, seminal plasma, tetraspanin.

GLYCOME

Complex carbohydrates (glycans) attached to proteins and lipids are enrolled in vital biological functions that have been investigated for more than a hundred years (Taylor and Drickamer 2011). After the key principles were established in this regard, the emphasis was on the importance of sugars for cell-cell adhesion and signaling, protein stability and secretion, as well as on innate and adaptive immunity (Varki 2017). A set of all glycans of an organism, the composition of which reflects its current state, is termed glycome (Taylor and Drickamer 2011). In a process marked as glycosylation, glycans are covalently attached to proteins (Mechref and Muddiman 2017). In contrast to the protein synthesis, this process is not defined at the genetic level i.e. is non-template driven (Spiro 2002; Varki et al. 2015). It is orchestrated by two types of enzymes glycosyltransferases and glycosidases (Singh et al. 2012; Vliegenthart 2017) and could be achieved through several biosynthetic pathways (Taylor and Drickamer 2011; Varki et al. 2015).

Glycosylation of proteins is a ubiquitous co- and post-translational modification since more than 70% of the total cellular proteome is glycosylated (Apweiler et al. 1999; Hortin et al. 2008; Mechref and Muddiman 2017; Gagneux et al. 2022). Glycan part and protein can be linked through five types of glycopeptide bonds. In N-glycosylation and glypiation, through different mechanisms, the previously formed glycan part is *en-bloc* added to the protein. In the other three

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types, O-glycosylation, C-mannosylation, and phosphorylation, after the single monosaccharide is transferred directly to a specific amino acid residue, the rest of the glycan component is made by sequential enzymatic addition of carbohydrate units (Spiro 2002).

The two most common ways of glycan attachment to the amino acid are through the nitrogen of asparagine (N-glycosylation) or oxygen of serine or threonine subsets (O-glycosylation). All N-glycans share a common core of two N-acetyl glucosamines (GlcNAc) and three mannoses (Man), GlcNAc2Man3 to which are added other monosaccharides like galactose (Gal), fucose (Fuc), or sialic acid (Neu5Ac). There are three classes of N-glycans, depending on the monosaccharides attached to the core. High-mannose N-glycans exhibit only two GlcNAc with a variable number of Man and can contain glucose (Glc) residues; in complex type, GlcNAc, Gal, Fuc, sialic acid and GalNAc residues are added to the pentasaccharide core while hybrid type is structurally a combination of high-mannose and complex type N-glycans (Bieberich 2014; Varki et al. 2015). In O-glycosylation, after the addition of N-acetylgalactosamine (GalNAc) residue to serine or threonine (Tn antigen), the glycan chain is elongated with the addition of Gal, GlcNAc, fucose, or sialic acid. This results in four common O-GalNAc glycan core structures (cores 1 to 4), and an additional four structures (cores 5 to 8). All these structures are predominantly present in mucins (Varki et al. 2015). More than one type of glycopeptide bonds can be found in the same protein or on the same glycosylation site, depending on the protein conformation and available enzymes. Macroheterogeneity reflects variations in glycosylation site occupancy while microheterogeneity refers to variations of glycan structures at the same site (Čaval et al. 2021). Heterogeneity of glycans originates from the structural characteristics of sugar residues, their number and type, stereoisomerism, type of anomeric linkage, and their position in branching glycan component (Spiro 2002).

Glycans are abundantly present in all cell compartments (Roth 2002). Synthesis of the majority of glycoproteins occurs in the endoplasmic reticulum (ER) and Golgi apparatus while certain types of O-linked glycosylation take place in the cytoplasm and nucleus (Roth 2002; Guzman-Aranguez and Argüeso 2010). Afterward, glycoproteins with a role in intercellular communication are expelled to the surface of the plasma membrane or secreted as a part of the secretory vesicle (Roth, 2002). Glycoproteins recycling occurs in lysosomes and emerging monosaccharides are expelled to the cytoplasm to be reused (Varki et al. 2015).

Glycans modulate the function of proteins and lipids to which they are attached, affecting the cell function in major biological processes like cell signaling, growth, adhesion, differentiation, and survival (Fukuda 2002; Zhao et al. 2008; Varki 2017). They are involved in interspecies recognition and molecular mimicry in pathogen – host interactions (Gagneux et al. 2022).

EVS IN SEMINAL PLASMA (SP) GLYCOME

Exosomes

EVs are nano-sized vesicles enclosed by a lipid bilayer (Théry et al. 2009; Pegtel and Gould 2019). They are released from the cell as a mechanism of intercellular communication (Théry 2011; Mathieu et al. 2019). According to their biogenesis, there are three types of EVs: apoptotic bodies, microvesicles, and exosomes. Apoptotic bodies are the largest type of EVs (up to 5μm) released from the cell under apoptosis while two other types are secreted by living cells (Battistelli and Falcieri 2020). Microvesicles are larger (100-1000 nm) than exosomes (40-100 nm) and they bud directly from the surface of the plasma membrane (Cocucci and Meldolesi 2015; Hartjes et al. 2019).

Exosome biogenesis involves the formation of late endosome through invagination of the plasma membrane, followed by inward budding of endosome outer membrane (formation of multivesicular bodies, MVB) to encompass and import cytosol and membrane components inside the newly formed vesicle (Sahu et al. 2011; Zhang et al. 2019). These exosome precursors (intraluminal vesicles) inside MVB, containing distinct membrane microdomains and densely packed cargo, are further discharged from the cell by exocytosis (Bobrie et al. 2011). Exosomes are made by nearly all cell types so their internal cargos and membrane composition greatly differ between cell types, reflecting the physiological condition of the cell in which they are formed (Raposo and Stoorvogel 2013). The internal content (cargo) of exosomes includes proteins, lipids, metabolites, DNA, and different types of ribonucleic acids (RNA) (Wei et al. 2021). Exosome membrane contains different kinds of proteins. Tetraspanins (CD9, CD63, CD81) are mostly used as EVs markers (Kleijmeer et al. 1998; Hemler 2001; Yoshioka et al. 2013). Lipid-anchored proteins (Rab proteins) are in charge of membrane fusion and transport, while inner peripheral scaffold factors (ezrin-radixin-moesin (ERM), syntenin, Alix) are involved in membrane organization and biogenesis (Pegtel and Gould, 2019). Prostasome membrane also contains Wnt proteins, extracellular matrix proteins (ECM), cytokines, and GPI-anchored molecules (CD55 and CD59) (Clayton et al. 2003; Izquierdo-Useros et al. 2009; Mulcahy et al. 2014; Shelke et al. 2019).

Distinct exosome populations are observed regarding the size and RNA composition (Willms et al. 2016) and phosphoprotein and glycoproteins content (Zheng et al. 2020). Besides the cargo content differences, membrane composi-

tion can represent a distinction mark between prostasomal populations. Variations in exosome cargo and membrane composition are found between different cell types but also within the same cell type (Smith et al. 2015). Their number and composition change in pathological conditions (Soung et al. 2017; Elashiry et al. 2020). Exosomes can be collected from different body fluids in the non-invasive procedure known as "liquid biopsy". This method detects and analyzes different biological matrices, like circulating tumor cells, cell free nucleic acids and EVs from body fluids, instead of using tissue fragments in classical biopsy (Zhou et al. 2020). This feature makes EVs a valuable prognostic and diagnostic tool in cancers (Soung et al. 2017), autoimmune (Anel et al. 2019), metabolic (Lee et al. 2016) and degenerative diseases (Elashiry et al. 2020).

Prostasomes

Cells produce exosomes in both *in vivo* and *in vitro* conditions (Lv et al. 2012; Marton et al. 2012) and release them in all body fluids (blood, urine, seminal liquid, amniotic fluid, etc.) (Cappello et al. 2017). This includes SP as a liquid component of semen, which is produced by male accessory sex glands (Zaneveld and Chatterton 1982). It enables key fertilization processes through constant interaction with spermatozoa based on the exchange of different organic compounds (proteins, glycoproteins, lipids) and inorganic ions (Juyena et al. 2013; Alberts et al. 2014; Machtinger et al. 2016). Glycoproteins of SP are involved in the maintenance of spermatozoa functionality and structure, including maturation, capacitation, acrosome reaction, and interaction between gametes (Diekman 2003). SP consists of three classes of N-glycans (high mannose and two complex types of bi-, tri-, tetraantennary N-glycans, one terminated with Lewis sequences and one with sialic acid) and O-glycans (core 1 and core 2) (Pang et al. 2009). Most SP glycoproteins are either sialylated or fucosylated or both (Saraswat et al. 2016). Core and antennary fucosylated glycoproteins abundantly participate in SP glycoproteome mediating direct sperm binding to the zona pellucida (Olejnik et al. 2015). High levels of Lewis^{x} and Lewis^{y} carbohydrate antigens/epitopes on some SP glycoproteins enroll them in immune homeostasis maintenance (Pang et al. 2011). Recent studies emphasize protein glycosylation as one of the main underlying causes of certain pathological events in the male reproductive tract like conditions of abnormal semen parameters (decreased sperm cells count (oligozoospermia), abnormal morphology (teratozoospermia), or lowered motility (asthenozoospermia)) and leukocytospermia with accompanying inflamation (Janiszewska and Kratz 2020; Lan et al. 2020). Alterations in glycan/oligosaccharide branching, *O*-glycosylation, and sialylation (Kratz et al. 2015) and increased level of fucose residues on glycans affect semen quality and result in impared fertility (Olejnik et al. 2015).

SP contains EVs predominately originating from prostate epithelial cells, termed prostasomes (Poliakov et al. 2009; Alberts et al. 2014). They are released in SP through the prostate fluid in high concentrations (Aalberts et al. 2014) and enclosed by a very rigid cholesterol-rich membrane enriched with lipid rafts and this composition makes prostasomes very stable and steady structures (Dubois et al. 2015). Prostasomal lipid content resembles that of other exosome types and includes high levels of sphingomyelin, cholesterol, and glycosphingolipids (Brouwers et al. 2013). Human prostasomes contain chromosomal deoxyribonucleic acid (DNA) and various classes of RNA (Ronquist et al. 2009; Chevillet et al. 2014; Vojtech et al. 2014) while protein content includes 1282 proteins (Garcia-Rodriguez et al. 2018). Prostasomes are a heterogeneous group with structural and functional features. The main distinction parameters of prostasomal subpopulations are size, protein profile, lipid composition, and surface protein glycosylation (Brouwers et al. 2013; Chiasserini et al. 2015; Garcia-Rodriguez et al. 2018; Milutinović et al. 2019). Prostasomes fuse with the sperm plasma membrane and induce capacitation and acrosomal reaction (Cross and Mahasreshti 1997; Visconti et al. 1999). They are also involved in sperm motility and liquefaction process and exhibit antioxidant, antimicrobial, and immunosuppressive properties (Garcia-Rodriguez et al. 2018).

SURFACE GLYCANS AS A DISTINCTION PARAMETER OF PROSTASOMAL SUBPOPULATIONS

Prostasomes cargo and membrane molecular composition reflect the status of the parent cell and/or tissue (Simpson et al. 2009) and can be modified under some pathological (benign and malignant prostate disease) (Sahlén et al, 2002; Nilsson et al, 2006) and physiological conditions (liquefaction of semen, spermatozoa motility, antibacterial activity and immunological functions) (Arienti et al, 2004; Yáñez-Mó et al. 2015). As a component of prostasomal membrane, surface glycans are a crucial component of SP plasma glycome (Milutinović et al. 2016), predominantly involved in cell-cell and cell–molecule interactions (Williams et al. 2019). Taking these premises into account, differences in SP samples of normozoospermic (N) and oligozoospermic (O) (decreased sperm count) men were established to investigate if surface glycans composition also reflects alterations in prostate functions as a vesicle cargo content (Sahlén et al. 2002; García-Rodríguez et al. 2018). The starting point was ion-exchange chromatography IEC to obtain prostasomes (Fig. 1) (Milutinović et al. 2019). They were eluted in the

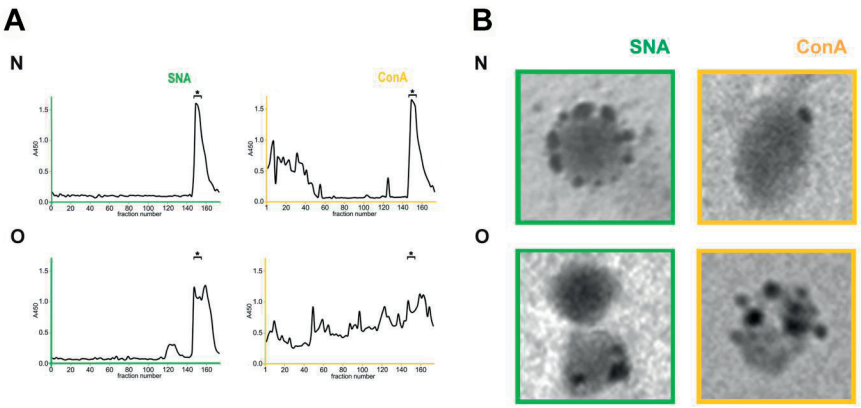

Fig. 1. Surface glycosylation of seminal prostasomes. (a) Ion-exchange chromatography elution was monitored by *Sambucus nigra* agglutinin (SNA) and concanavalin A agglutinin (ConA) binding reactivity. Asterisk denotes fractions with prostasomes (CD63-positive fractions); (b) Lectin-transmission electron microscopy show characteristic pattern of SNA-reactivity and ConA-reactivity of the vesicles in each sample group. (N = seminal prostasomes from normozoospermic men; O = seminal prostasomes from oligozoospermic men).

highest charge IEC fraction confirming their net negative surface charge. This was followed by monitoring of surface glycosylation using *Sambucus nigra agglutinin* (SNA) (binds sialylated structures) and *Concanavalin A* (ConA) (binds mannosylated structures) lectins (Fig. 1A) (Milutinović et al. 2019). This indicated the existence of one reactive population in N and at least two subpopulations in O. ConA binding pattern was more heterogeneous in both groups, reflecting the presence of several prostasomal subpopulations (Fig. 1A) (Milutinović et al. 2019). This finding was further supported by the distinct presentation of surface-associated glycans on native/unseparated prostasomes detected using lectin transmission electron microscopy (lectin-TEM) (Fig. 1B) (Milutinović et al. 2019). In N, SNA bound to all vesicles showing a characteristic rosette-like appearance, while ConA stained only some vesicles. Most of the vesicles were ConA reactive, while the minority of them stained with SNA in O (Fig. 1B).

Towards further characterization of prostasome subpopulation and their contribution to the complexity of seminal plasma glycome, N-glycan distribution and their co-localization with membrane-associated proteins was investigated. For this purpose, two approaches were applied: analysis of intact prostasome subpopulation and investigation of membrane domains obtained after treatment of prostasomes with non-ionic detergent Triton X-100 (Janković et al. 2020, 2021).

Lectin affinity chromatography (LAC) was applied to separate different prostasomal subpopulations. Lectins of choice were ConA and *Wheat germ agglutinin* (WGA) (binds syalic acid/GlcNAc). Four fractions were obtained, WGA-bound, WGA-non bound, ConA-bound, and ConA-non bound. These fractions were further characterized regarding co-distribution with surface-associated markers of EVs (CD63) and activity of glycoprotein enzymes gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) as well as total protein composition (Janković et al. 2020). The pattern of distribution of examined markers was similar in both N and O. In WGA separated populations, GGT distribution overlapped the distribution of CD63. They were marked as prostasomal subpopulations considering CD63 immunoreactivity and the presence of specific protein bands at the region of 150-90 kDa. On the other hand, the population enriched in ConA column was exhibiting ALP activity and lacked a specific prostasomal signature. The difference between N and O, was noticed in GGT activity of prostasomal populations and surface glycan presentation in related populations tracked by ALP activity (Janković et al. 2020).

Considering the role of the tetraspanin web (CD63, CD9 and CD81) and galectin-3 in the shaping of prostasome surface by the assembly at different macromolecular complexes, their re-distribution with selected N-glycans and GGT, were obtained analyzing detergent-treated prostasomes (Janković et al. 2021).

Two distribution patterns were established in both groups consisting of overlapping WGA-reactive glycoproteins with CD9 and gal-3 and ConA-reactive glycoproteins with CD63 and GGT (Fig. 2) (Janković et al. 2021). Redistribution of N-glycans and GGT was similar between groups, while differences were observed in the redistribution of TS and galectin-3. In O, gal-3 and tetraspanin CD9 were perturbated in means of their segregation on different microdomains as a result of their engagement with high molecular mass complexes.

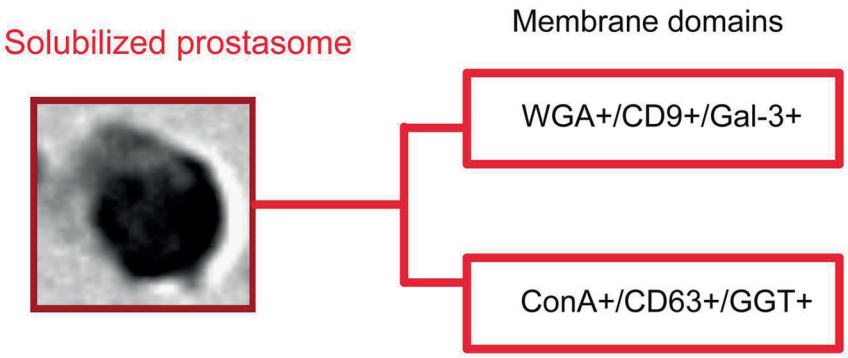

Fig. 2. Scheme illustrates patterns of surface-associated prostasomal proteins on detergent-sensitive/resistant membrane domains of prostasomes that are obtained under normal physiology and conditions of low sperm count. The two patterns consist of overlapped WGA-reactive glycoproteins/CD9/gal3 and Con A-reactive glycoproteins/CD63/GGT.

CONCLUSION AND FUTURE PROSPECTIVES

At this point, overall comprehension of glycans is considerable but some of their features remain to be elucidated. Our modest contribution to this subject enlightens the role of selected N-glycans and their spatial distribution on prostasomal membrane, indicating that their profile may reflect alterations in EVs membrane structure and functionality in different pathological conditions.

Investigations on EVs function and structure are gaining momentum and the EVs field is developing rapidly due to their promising potential as non-invasive biomarkers. This will be facilitated after successful standardization of methods for EVs purification and separation and will enable its production to meet clinical demands. Implementation of EVs in clinical studies could result in significant improvement of early diagnosis setup and particularly of prognosis assessment and disease surveillance. Moreover, current knowledge about glycans in the EVs 'puzzle' holds them a key factors in the tuning of EVs uptake and a possible distinction marker for better defining of EVs types.

Changes in glycosylation constitute a hallmark of variety of disease conditions and profound glycan characterization of EVs could contribute to their application as an efficient tool for diagnosis, prognosis and therapy development.

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