



Review

# From Exosome Glycobiology to Exosome Glycotechnology, the Role of Natural Occurring Polysaccharides

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**Abstract:** Exosomes (EXOs) are nano-sized informative shuttles acting as endogenous mediators of cell-to-cell communication. Their innate ability to target specific cells and deliver functional cargo is recently claimed as a promising theranostic strategy. The glycan profile, actively involved in the EXO biogenesis, release, sorting and function, is highly cell type-specific and frequently altered in pathological conditions. Therefore, the modulation of EXO glyco-composition has recently been considered an attractive tool in the design of novel therapeutics. In addition to the available approaches involving conventional glyco-engineering, soft technology is becoming more and more attractive for better exploiting EXO glycan tasks and optimizing EXO delivery platforms. This review, first, explores the main functions of EXO glycans and associates the potential implications of the reported new findings across the nanomedicine applications. The state-of-the-art of the last decade concerning the role of natural polysaccharides—as targeting molecules and in 3D soft structure manufacture matrices—is then analysed and highlighted, as an advancing EXO biofunction toolkit. The promising results, integrating the biopolymers area to the EXO-based bio-nanofabrication and bio-nanotechnology field, lay the foundation for further investigation and offer a new perspective in drug delivery and personalized medicine progress.

**Keywords:** extracellular vesicle; exosome; nanocarrier; glycobiology; glycotechnology; polysaccharide-based hydrogel; targeted drug delivery; precision medicine; biomarker



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## 1. Introduction

Secreted and present in many biological fluids, collectively extracellular vesicles (EVs) represent a uniquely micro and nano volume-large surface area able to elegantly interact both with cells and with molecules in the extracellular microenvironment [1,2]. These cell-derived structures, enclosed by a lipid bilayer, originate from the endosomal network (EXOs) or from the plasma membrane (microparticles, oncosomes, or ectosomes, collectively referred to as microvesicles (MVs)) [3]. The innate informative activity of EVs is accomplished by their natural cargo, which includes nucleic acids (DNA, mRNA, miRNA, siRNA, etc.), lipids, proteins and peptides. The content, highly tissue- and organ-specific, may vary depending on the physiological or pathological condition of the parental cells [2,4–7]. EVs are actively involved both in the regulation of physiological processes, such as cell guidance, tissue repair, stem cell maintenance and in the dysregulation of pathological processes, including cancer, neurodegenerative disease, and response to injury and infection.

Their intrinsic role and distinctive features (including the high biocompatibility, the ability to cross physical barriers, cell targeting and interaction with intracellular trafficking pathways [8] paved the way to multiple applications in the biomedical field [3,9–12]. Besides more in-depth investigations on EV biogenesis and cargo sorting, EV subpopulations,

internalization and trafficking pathways, upscaling of the EV production and isolation process, as well as new guidelines for appropriate storage need also to be considered [8]. Within the heterogeneous subpopulations, EXOs have become the most widely studied EVs. The field of advanced drug delivery systems has promptly focused considerable research attention on the world of naturally occurring EXOs [13]. Although these vesicles have various potential advantages, their clinical application is associated with some inherent limitations. In a recent review, the authors have summarized their possible improvements by means of two main approaches: parental cell-based engineering methods (genetic engineering for loading therapeutic molecules into the lumen, or displaying them on the surface of EXOs) or post-isolation EXO engineering by means of chemical and mechanical methods including click chemistry, cloaking, bio-conjugation, sonication, extrusion, and electroporation [14]. Definitely, the flexibility of the EXO platform and the possibility to optimize carriers for different applications is a smart key. EXOs can be engineered to be taken up by antigen-presenting cells in cancer, to be retained at the injection site and to evade the reticulo-endothelial system. Commonly investigated in tissue regeneration, gene therapy, cancer, and immune-modulatory treatments, the present pandemic is also opening up new perspectives for EXO uses, developing a vaccine consisting of EXOs decorated with viral proteins on the surface [15]. By means of engineering improvements, more than ten clinical trials are in progress to evaluate the safety and efficacy of different EV-based therapies. Nonetheless, further efforts are required to achieve translational applications of EVs [8]. On the other hand, the development of basic research and clinical trials is hampered by the limited number of cell-secreted EVs [16]. This limitation has promoted the development of EV mimetic—semi-synthetic or fully synthetic—products, natural EXOs with pre- or post-isolation modifications, respectively. Top-down techniques (vesicles made of membrane fragments obtained from sliced or extruded cells), or bottom-up techniques based on supra-molecular chemistry (vesicles from individual molecules), represent the technology developed for those artificial EXOs [17–20]. However, the major challenge still regards the improvement of the therapeutic efficacy, while minimizing side effects and reducing dosages, maintaining biocompatibility and controlled biodegradability. Despite the great potential of artificial EVs, the design of the optimal functional system still requires some effort to turn from bench to bedside. There is no perfect technique, and, depending on the final purpose of artificial EVs, multidisciplinary teams and combinations of procedures would allow the definitive consolidation of these biomaterials in clinical practices [19]. As well, a deep understanding of EV molecular profile assumes equal importance. EXOs display well-known protein and lipid composition, while, though several components are heavily glycosylated, the characterization of EXO glycans remains understudied; nonetheless, glycosylation has a great influence on EXO biosynthesis and function [21], which is specifically classified into four distinct categories, involving (i) structural and modulatory role; (ii) extrinsic recognition; (iii) intrinsic recognition; (iv) molecular mimicry of host glycans [22].

Therefore, the modulation of EV glycosylation and their cargo composition has become a hot topic in the EXO-related area. There is a large body of genetic manipulation works that can be applied to EVs, counting the techniques of metabolic oligosaccharide engineering or the transfection of generating cells with exogenous glycan-processing enzymes [23]. Departing from the above-mentioned genetic engineering strategies, more properly, the present work aims to review those bio-structural and bio-functional soft technology approaches that can be applied to EXOs. Studies concerning the whole EV set will be described as methods potentially transferable to EXO implementation. To this aim, mainly taking into consideration the literature of the last decade, first, we discourse on the role of glycans concerning the structure, function and distribution of EXOs from various tissues, their biological features, and their potential for various clinical applications; accordingly, the different approaches developed to functionalize vesicle surface and the techniques to encapsulate, support and preserve EXOs-based carriers will be examined, fo-

cusing on the role of endogenous glycans and the role of natural exogenous polysaccharides as functional decoration molecules or 3D support systems.

## 2. Glycobiology of Exosomes

### 2.1. Glycans

Glycobiology generally refers to a wide branch of biology encompassing the studies of chemistry, the endogenous synthesis, the biological distribution and the function of the glycans and their derivatives in the complex biological system [24]. The International Union of Pure and Applied Chemistry (IUPAC) defines glycan as “synonymous of polysaccharides”, in which monosaccharides are the building blocks linked to each other through glycosidic bonds [25]. The term “glycan” is also used to describe the carbohydrate unit of glycoconjugates such as glycoproteins and glycolipids [26].

Glycosylation, as a post-translational modification, is widely recognized as a regulator of protein structure, localization, and function [27,28]. The reaction of glycosylation, established when a monosaccharide is activated to a high-energy donor form, results in a covalent link of glycosyl donor (nucleotide sugar, monosaccharides and oligosaccharides) with a functional group of a glycosyl acceptor (monosaccharide, oligosaccharide, protein, lipid, nucleic acid).

Among naturally occurring glycans, the proteoglycans (PGs) consist of a core protein and one or more covalently attached glycosaminoglycans (GAGs) chains (chondroitin sulfate, dermatan sulfate, keratan sulfate) or non covalently attached hyaluronan binding motifs. Specifically, GAGs are linear polysaccharides, whose disaccharide building blocks consist of an amino sugar (glucosamine that is *N*-acetylated or *N*-sulfated or *N*-acetylgalactosamine) and a uronic acid (glucuronic acid or iduronic acid) or galactose.

The extracellular matrix (ECM) PGs include small interstitial molecules (decorin, biglycan, fibromodulin), a PG form of type IX collagen, and one or more members of the aggrecan family (aggrecan, brevican, neurocan, or versican).

A given PG present in different cell types often shows structural variability (Box 1). These characteristics, distinctive of all PGs, create enormous diversity and biological variation in activity [29]. PGs and GAGs can be found in cytoplasm either on the surface of mammalian cells, or in the secreted ECM; the glycan distribution determines the involvement of glycoconjugates, in particular, in cell signaling and cell adhesion in physiological or in pathological conditions when their expression is altered [30]. Starting from PGs in cell biology (Box 2), from this point on (Sections 2.1.1 and 2.1.2), specifically, we discourse on the role of glycans in the structure and function of EXOs, and how the grade of glycosylation manages the endogenous EXOs distribution.

#### Box 1. Structural variation of proteoglycans [29].

**Substitution** with one or two types of GAG chains: i.e., glypicans contain heparan sulfate, whereas syndecan-1 contains both heparan sulfate and chondroitin sulfate chains.

**Number** of GAG chain: one chain (i.e., decorin), or more than 100 chains (i.e., aggrecan).

**Stoichiometry** of GAG chain substitution: i.e., syndecan-1 has five attachment sites for GAGs, but not all of the sites are used equally. Other PGs may exist with or without a GAG chain or with only a truncated oligosaccharide.

**Box 2.** Natural occurring proteoglycans (selected from those most investigated for EXO biology).

**Aggrin** is a widely expressed modular PG consisting of a core protein with two GAG side chains that carry heparan sulfate and/or chondroitin sulfate; it is generally present in the glomerular basement membrane, playing a role in the renal filtration and cell–matrix interactions, and it is involved in different developmental processes, like aggregation of acetylcholine receptors during synaptogenesis, and in the tumor microenvironment [31].

**Betaglycan**, also known as Transforming Growth Factor Beta Receptor III (TGFBR3), it is structured in a large extracellular binding domain, a single-pass transmembrane region, and a short intracellular binding domain. The extracellular binding domain contains two sites for GAG (heparan and chondroitin sulfate) attachment. Betaglycan is a co-receptor for the TGF $\beta$  superfamily, playing an important role in the trafficking of TGF $\beta$  superfamily receptors and in their signalling, with an active involvement also in cancer progression [32].

**Collagen XVIII** is a heparan sulphate PG, expressed ubiquitously in different basement membranes, throughout the body, which can be cleaved, in C-terminal fragment, to form endostatin. Collagen XVIII and its variants are involved in many physiological and pathological processes, like stability of basement membranes, cell proliferation, angiogenesis and tumor growth [33].

**Glypican** is a heparan sulfate PG proper of cell surface and anchored externally to membrane through glycosylphosphatidylinositol anchor. The core protein is composed of N-terminal secretory signal peptide, and a C-terminus hydrophobic domain for the GPI anchor and the heparan sulfate link. Glypican takes part in the regulation of many growth and survival factors [34].

**Lumican** is an ECM PG, member of the small leucine-rich family of keratin sulfate PG. It is composed of a negatively charged N-terminal sulphated domain, a leucine-rich repeats domain for binding to extracellular components, and a C-terminal domain with two conserved cysteine residues. It is involved in collagen fibril organization, cell migration, proliferation, and inflammatory responses [35].

**Serglycin**, also known as hematopoietic PG or secretory granule PG core protein, is a PG with a core protein coded by the SRGN gene in humans. It belongs to a family of small PG with serine-glycine dipeptide repeating units. The core protein links with GAG chains, like chondroitin-sulfate, in three functional domains. Intracellular PG can affect the retention and secretion of proteases, chemokines, or other cytokines; the extracellular PG form participates in metastasis mechanism [36].

**Syndecan** is a transmembrane PG. It is structured into three differentiated domains: the N-terminal ectodomain (where GAGs, like heparan sulfate and chondroitin sulfate, are attached), the transmembrane domain, and the C-terminal cytoplasmic domain. The different domains allow cell–cell or cell–matrix interaction, integrin and actin cytoskeleton interaction, and protein dimerization for cascade signal activation. Syndecan is actively involved in ECM remodelling, cell adhesion, cell migration and cytoskeleton organization [37].

**Versican** is a large ECM chondroitin sulfate PG, present in a variety of human tissues. It belongs to the family of hyaluronan-binding PG and consists in a N-terminal globular domain, for hyaluronan link, and in a C- type lectin terminal globular domain, adjacent to two epidermal growth factor (EGF) domains. The middle region of the core protein allows chondroitin sulfate attachment. The attached chondroitin sulfate chains differ in size and composition, depending upon native tissue or culture conditions. Versican plays a crucial role in cell adhesion, proliferation, migration and in extracellular matrix assembly [38].

### 2.1.1. Glycan Role in Exosome Biology

EXOs take part in cell communication, signaling and trafficking, travelling through physiological fluids (urine, blood, saliva, tumor effusions, breast milk, amniotic fluid and culture media of different cell types) [39] and transporting proteic, lipidic and genetic material. Constitutively, EXOs are nano-sized vesicles generated from late endosomes, after inward budding of the multivesicular body (MVB) membrane. After the EXO release, the phospholipid bilayer protects the inner message from extracellular proteases and nuclease action [40,41]. Their surface recalls the characteristic of parental cell [7]: EXO membrane exposes lipids (cholesterol, ceramide, phosphatidylserine and sphingomyelin), proteins (antigen presenter, adhesion and transmembrane proteins), and multiple relevant saccharide components (Table 1), like high mannose, poly N-acetylactosamine,  $\alpha$ -2,6 sialic acid, and complex N-linked glycan, signature common to different cell type-derived EXOs. Glycans are relevant in the sorting of glycoproteins to MVs and the glycan profile is annoverated in many different studies as the necessary equipment involved in the EXO composition, release, targeting and uptake [42–45]. N-linked glycans have been also

identified as determinants of protein trafficking into EXOs [46]. Overall, glycans of EVs are also important in their uptake by recipient cells, as disruption of native glycosylation alters uptake in a panel of different cell lines [47]. Recent findings improved the understanding of EXO transport and targeting in relation to glycans on exosomal surfaces, potentially enabling to standardize EXO for therapeutic application [48].

**Table 1.** Glycans in EXO biogenesis, layout and cell targeting.

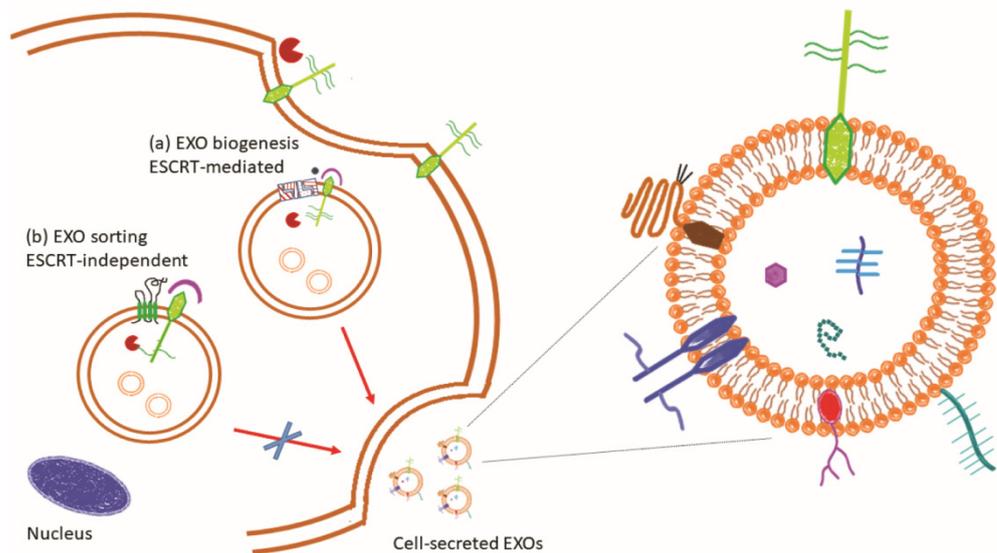
Glycan	Molecular Type	Process	Glycan Function in Cell Pathway	Application	Ref.
High mannose, poly <i>N</i> -acetylglucosamine, $\alpha$ -2,6 sialic acid, complex <i>N</i> -linked glycan	Mannose repeated residues, repeated Gal $\beta$ 1-4GlcNAc disaccharides, alpha-keto acid sugar, oligosaccharide linked to nitrogen atom of protein	EXO molecular composition	Determining protein and glycosylated protein cargo in EXOs	Signaling for cell targeting	[42]
C-terminal of Agrin	Heparan-Sulfate PG	Basement membrane and EXO molecular composition	Tumor-associated antigen released in circulation	Autoantibodies diagnostic biomarker	[49]
Glypican	Heparan-Sulfate PG	EXO molecular composition	Membrane EXO composition	Diagnostic and prognostic biomarker	[50–52]
C-terminal Collagen XVIII (endostatin)	Heparan-Sulfate PG	ECM integrity	Blocking macrophage inflammation and vascular endothelial cell migration	Antiangiogenic therapy	[53]
Syndecan-1	Heparan-Sulfate PG	EXO biogenesis	Molecular pathway activation in the complex syndecan-syntenin-ALIX-ESCRT	EXO release	[54,55]
Syndecan-1	Heparan-Sulfate PG	EXO molecular composition and miRNA cargo modulation	Cell proliferation, ECM shaping	Signaling for cell targeting and diagnostic and prognostic marker for cancer	[56]
Syndecan-4	Heparan-Sulfate PG	EXO biogenesis	Syndecan-4-tetraspanin-6 in regulation of EXO release ESCRT-independent	EXO release	[57]
Syndecan, Glypican	Heparan-Sulfate PG	EXO uptake	ERK1/2 cell signaling activation and cell migration	Drug delivery	[58]
Syndecan-1	Heparan-Sulfate PG	EXO uptake	Bind of syndecan-1 with PG of target cell through ECM fibronectin (FN)	Cell-cell communication	[59]

Table 1. Cont.

Glycan	Molecular Type	Process	Glycan Function in Cell Pathway	Application	Ref.
Syndecan-1	Heparan-Sulfate PG	EXO-hepatoma miRNA-122-5p regulates syndecan-1 expression in breast cancer cells	Activation of breast cancer cell motility and metastasis	Drug target for distant metastasis prevention	[60]
Betaglycan	Chondroitin-Heparan-Sulfate PG	TGF- $\beta$ binding in EXO surface	Fibroblast to myofibroblast differentiation	Therapy strategy targeting betaglycan in cancer-altered stroma	[61]
Serglycin	Chondroitin-Sulfate PG	EXO protein cargo modulation	Myeloma cells proliferation and macrophages migration	Cancer treatment	[62]
Versican	Chondroitin-Sulfate PG	Senescent endothelial cells - EXO versican localizes to the mitochondria of VSMCs	Alteration of mitochondrial membrane potential and vascular smooth muscle cells senescence/calcification	Therapeutics target in diabetic vascular damage	[63]
Lumican	Leucine-rich keratan sulfate PG	Aqueous humor-EXO-has-miR405b-5p regulates lumican expression	Organization of collagen fibrils in sclera	Studies for diagnosis, treatment and prognosis of myopia	[64]

*EXO (plus cargo) formation and release regulation.* The formation and the extracellular release of EXOs can be intermediated by the endosomal sorting complex required for transport (ESCRT) mediated mechanism, or by ESCRT-independent manner [65]. In the mechanism ESCRT-mediated, the four proteins of the complex work in symbiosis to support the MVBs, the budding process and the ubiquitinated protein cargo [66]. These types of EXOs are enriched, aside from ESCRT proteins, in functional protein necessary for EXO biogenesis process [67–69]. The fundamental molecular mechanism, of EXO biogenesis-ESCRT mediated, involves the heparan-sulfate PG syndecan-1, in the synergistic syndecan-1-syntetin-ALIX pathway. Syndecan-1, encoded by the SDC1 gene, consists of an internal domain, an integral membrane domain, and an extracellular domain, possibly substituted with heparan sulfate or chondroitin sulfate chain. The internalization of cell surface syndecan-1 allows syndecan-1–syntenin interaction, simultaneously connected to ALIX protein, and to ESCRT complex [54,55]. The syndecan-syntenin-ALIX-based EXO biogenesis is regulated by the heparanase activity [70], and its dysregulation in pathological conditions, such as hypoxia and acidosis (features of tumor microenvironment), promotes further EXOs formation [71]. This mechanism offers a possible research target in cancer treatment, and in other EXO-mediated disorders. Syndecan-1 is also engaged in the modulation of the genetic content of tumor cell-derived EXOs. In particular, it has been demonstrated that syndecan-1 lacking cells release EXOs enriched in specific miRNAs, and depleted of other ones, resulting in an enhanced tumorigenic power [56]. Syndecan interplay with tetraspanins is also relevant in EXO biogenesis. Tetraspanins are transmembrane 4 superfamily proteins (TM4SF), with four transmembrane elicits and two extracellular domains. They can interact with several receptors and proteins, on the cell surface, managing several molecular mechanisms, that allow the EXO genesis and sorting [72]. With this respect, many studies have revealed that several proteins of the tetraspanin family are responsible for the regulation of the ESCRT-independent endosomal sorting mecha-

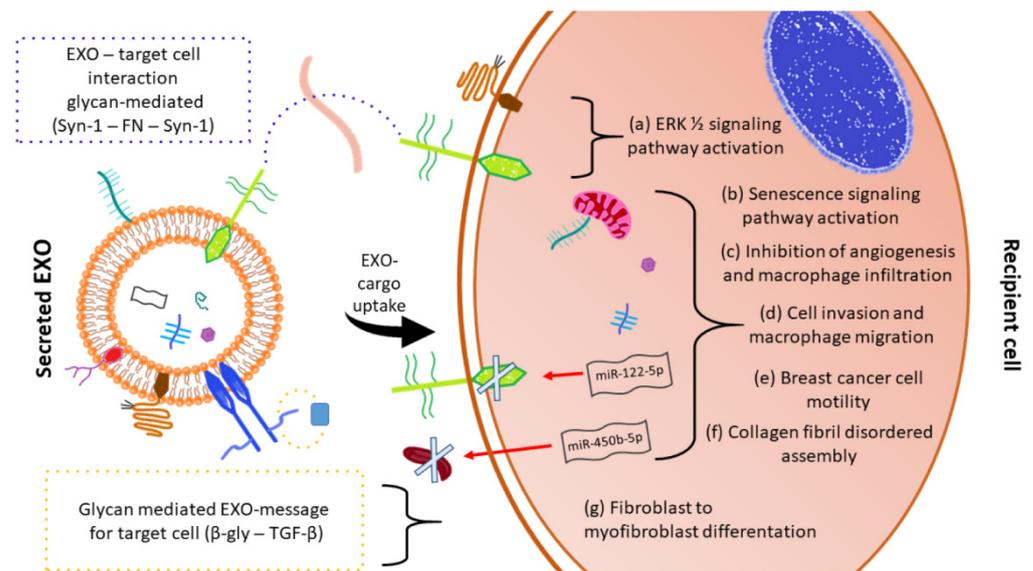
nism [65]. Specifically, it has been demonstrated that tetraspanin-6 interacts with syntenin, through the PDZ1 domain [73], and reduces the formation of syntenin/syndecan-4/CD63 EXO subclass. Tetraspanin-6 operates as a controller of syndecan-4–syntenin interaction, stabilizing EXOs secretion and lysosomal degradation [57] (Figure 1).



**Figure 1.** Graphical sketch of glycans involved in EXO biogenesis and release. Representative process of (a) EXO biogenesis and secretion (endosomal sorting complex required for transport (ESCRT)-mediated) with the participation of the cell surface syndecan-1, in the syndecan-1-syntenin-ALIX complex [54,55,65–71]; (b) syndecan-4–syntenin interaction in the balanced exosomal secretion under tetraspanin-6 negative control [57,72,73]. Mammalian cell EXO-magnification of the main investigated glycans (see Figure A1 in Appendix A for symbol interpretation).

As above-mentioned, syndecan-1 regulates EXO production and their miRNAs content; specifically, loss of syndecan-1 in cells induces an altered packaging of miRNA content, which can induce targeted cell proliferation thus promoting the cancer pathway [56]. Glycan’s role in the modulation of protein cargo was observed also investigating the chondroitin sulfate PG serglycin, proper of myeloma glycoalyx, and examined in depth in the mechanisms of cancer invasion and progression. Serglycin, synthesized by many different cell types and over-expressed by tumor cells, is able to interact with inflammatory molecules, promoting inflammatory processes in the tumor microenvironment and enhancing cancer progression, resistance and aggressiveness [62,74]. An extensive investigation has revealed that serglycin, which is present in the EXOs of myeloma cells line, promotes cell-invasive phenotype and macrophage migration [62].

*Injury and tumor microenvironment.* EXOs may mediate specific intercellular communication and prompt signaling pathways in cells they interact with. In many disorders, EXO content and secretion vary, resulting in molecular pathways alteration in the recipient cells. In cancer, much evidence suggests that they prompt tumorigenesis by modulating angiogenesis, immunity, and metastasis [75] (Figure 2). The specific glycan EXO payload (Table 1) is fundamental to carry out their role in pathological conditions and represents a possible marker for isolation as well for early diagnosis [42,76].



**Figure 2.** Glycans impact on EXO cell targeting and molecular pathway modulation. Graphical sketch of the reported glycans and miRNAs involved in: (a) EXO–target cell interaction mediated by fibronectin (FN) as a key syndecan-1-ligand [59], or by syndecan and glypican as internalizing receptors and ERK 1/2 signaling activators [58]; (b) the induction of cellular senescence due to versican targeting of recipient cell mitochondria [63]; (c) endostatin antiangiogenic and anti macrophage infiltration activity [53]; (d) increase of cell invasive phenotype and macrophages migration as the effect of EXO-serglycin uptake [62]; (e) induction of breast cancer cell motility by syndecan-1 down-regulation (EXO-miR-122-5p induced) [60]; (f) collagen fibril disordered assembly by ECM lumican down-regulation (EXO-miR-450b-5p induced) [64]; (g) TGF- $\beta$  delivery in recipient cell through EXO surface–betaglycan interaction, determining fibroblast differentiation into myofibroblast [61]. (Syn-1, syndecan-1;  $\beta$ -gly,  $\beta$ -glycan).

Heparan-sulfate PG and integrins mediate the binding of hepatic stellate cells (HSC)-derived EXOs to HSC (rather than to hepatocytes) as well as the delivery and intracellular action of the exosomal cargo, potentially suggesting them as carriers of therapeutic drugs to activated HSC in fibrotic livers [77].

Similarly, heparan-sulfate PG-dependent uptake by cancer cells is highly relevant for EXO biological activity, and thus represents a potential target for inhibition of EXO-mediated tumor development. Indeed, EXO uptake can be regulated (i) by enzymatic modification of cell-surface heparan-sulfate domain, (ii) by the presence of syndecan and glypican on receiving cell surface, and (iii) by unexpressed XYLT gene (which in humans encodes for the protein xylosyltransferase-1, an enzyme of GAG-biosynthesis for the PGs chains). Remarkably, when the EXOs uptake is performed, ERK 1/2 phosphorylation site and its signaling pathway activation promote cell migration [58].

Reporting on the involvement of heparan-sulfate PG in an interesting bridge mechanism, Sanderson’s group showed that heparan sulfate chains of syndecan-1 play a dual role in EXO–cell interaction; its moiety on myeloma patients EXOs captures fibronectin (FN), and, on the recipient cell membrane, it acts as a receptor for FN, thereby facilitating cellular uptake of EXOs [59]. Remarkably, it has been proposed that cells endocytose ECM molecules and re-secrete them on the exofacial surface of EXOs, such as in the case of FN–integrin complexes [78], playing an important role in cell migration. This aspect is critical for tumor microenvironment changing and tumor-EXO-mediated cancer invasion [59].

On a parallel route, the transmembrane PG betaglycan (chondroitin/heparan sulfate PG, known as transforming growth factor beta receptor III), expressed on EXO surface interacting with TGF- $\beta$ , operates as a messenger from cancer cells to stromal fibroblasts. The association of betaglycan with TGF- $\beta$  worsens the role of EXOs in cancer-altered stroma,

and represents a possible therapeutic target in a persistent altered microenvironment in cancer [61].

Considering the role of glycans in cellular restructuring, it has been stated that versican, a chondroitin sulfate ECM PG proper to the brain and large blood vessels, is over-expressed within restructuring ECM when growth or disordered circumstances occur. Specifically, in vascular aging, EXOs represent the way by which senescent endothelial cells communicate and induce vascular smooth muscle cells (VSMCs) calcification [79]. EXOs derived from senescent human umbilical vein endothelial cells (HUVECs), enriched in versican molecules (due to hyperglycemic condition), are able to induce mitochondrial dysfunction in VSMCs. It would seem that versican, transported through EXOs, reaches VSMCs mitochondria, upregulating membrane potential, and probably managing senescence/calcification. In all likelihood, versican represents a message molecule, between endothelial and smooth muscle cells, which is responsible for vascular damage in diabetic complications [63].

EXO surface proteins and glycans reflect originating cells and tissue features. This equipment is key in cell recognition and targeting, as well as in immune innate defense. Collagen XVIII is a heparan sulfate PG that guarantees ECM integrity for endothelial and epithelial cell support. When collagen XVIII is cleaved in endostatin (the C-terminal of collagen XVIII) by matrix metalloproteinase, it acquires an antiangiogenic activity. Actually, EXOs of retinal astroglial cells, endostatin-enriched, are able to block retinal vascular leakage and inhibit neovascularization in choroid, suppressing the macrophage infiltration, the release of inflammatory molecules in the wounded area, and the migration of vascular endothelial cells [53].

Glycans signature varies in function of pathophysiological condition, hence EXOs are particularly studied as circulating biomarkers in cancer development and progression. Glypican, an heparan sulfate PG, proper to cell surface, has been shown to be involved in signaling pathway, controlling tumor cell growth, motility and differentiation [80]. The presence of glypican-1 has been confirmed also investigating its content in EXOs isolated from the serum of patients affected by pancreatic cancer (PC) [50–52].

Also the tumor environment generally triggers an immune response in human host organisms, involving T and B lymphocytes, in which autoantibodies are produced against aberrant protein expression. These autoantibodies and their target have been considered, in the last decades, good candidates in early tumorigenesis identification and possible target in responsive immunotherapy [81]. In cell secretome, the residual of agrin (a secreted heparan-sulfate PG of basement membrane zone), enriches the EXO fraction in the plasma of patients with colorectal cancer, in the role of autoantigens. As a result of uncontrolled proteolytic processing of basement membranes in cancer, the C-terminal fragment of agrin can also be considered a promising circulating tumor-associated antigen in liquid biopsy, as a cancer diagnostic biomarker [49].

### 2.1.2. Glycan Expression in Exosome Targeted Cells

The function of circulating EXOs was already mentioned: they, through body fluids, transport different types of molecules, such as genetic materials (miRNA, lncRNA, etc.), protein, lipids and molecular complexes. miRNAs can be released from cells, under the protection of EXOs, to reach distant cells avoiding nucleic acid degradation. When they reach the target cell, they induce change in the microenvironment, with repercussion in cancer invasion, angiogenesis or drug resistance [82] (Figure 2). Although it is still primitive, it is also possible the involvement of EXOs in the regulation of glycans expression in human disorders (Table 1).

Syndecan-1 is involved in many cellular processes due to its cellular location, which makes it a good co-receptor or signaling activator for cell proliferation, differentiation, adhesion, or migration [83]. Promising results have evidenced the regulation of syndecan-1 expression in breast cancer cells, performed by hepatoma-derived EXOs, carriers of miR-122-5p. In particular, these miRNAs, whose hepatoma-EXOs are enriched, can reduce mRNA level of syndecan-1, interfering with its protein expression, and consequently,

the same EXOs from the liver are sufficient to induce breast cell cancer motility. Moreover, EXO miRNA content increases when hepatoma cells are treated with apoptotic drugs, demonstrating that EXOs from distant injured cells are able to cause metastasis of target cancer cells, downregulating syndecan-1 expression [60]. This possible mechanism is a key for the development of strategic drug therapy for the prevention of distant metastases.

Similarly, in a study on myopia disorder, EXOs of the aqueous humor are screened due to their implication in intercellular communication mediated by miRNA and protein content. As previously reported, the amount and the type of molecules transported are different in the case of pathological or normal condition; in particular, the affected cells release EXOs with myopia-specific miRNA profile (has-miR450b-5p) in aqueous humor. Bioinformatics has revealed that this miRNA can target lumican, a small leucine-rich (SLRP) family of keratan sulfate PGs, present in ECM for correct collagen fibril assembly; it is relevant for cell proliferation and migration, regulation of angiogenesis and immune response, and it is involved in myopia pathogenesis. This method is the basic tool for further studies of functional analysis of miRNA content for diagnosis, potential treatment and prognosis of many disorders EXO mediated [64].

### 3. Exosome Glycotechnology

#### 3.1. Exosome: A Tunable Endogenous Nano-Delivery System

EXO-based delivery systems are now a hotspot in nanomedicine for multiple advantages. In fact, EXO native structures and behavior may overcome the typical issues of drug delivery systems, as [12,84]:

- (i) the endogenous origin results in a low immune response;
- (ii) the surface ligands and receptors expressed on the lipid membrane permit to easily pass through biofilm barrier and penetrate into target cells;
- (iii) the membrane bilayer structure effectively protects cargo from rapid degradation, increasing its delivery efficiency and enhancing the stability in plasma;
- (iv) the natural targeting ability enables the EXOs to migrate specifically in the target tissue.

However, its applicability as a novel biotechnology tool and pharmacological treatment has to be deeply investigated, tested and upgraded. The delivery of a therapeutic dosage of EXOs to the target site via systemic injection has some disadvantages, mainly due to the rapid clearance from systemic circulation and the limited targeting capability [85–87]. These limits preclude the desired therapeutic effect at the target site and cause the accumulation in other tissues, leading to side effects.

Thus, the need to prolong the half-life of EXOs, by protecting and supporting carrier delivery steps, and to target selectively the site of action is crucial in order to develop EXO therapeutic agents. Different strategies have been proposed to design targeted and sustained EXO-based delivery systems.

Targeted modifications of EXO surface have been carried out both via genetic approach, inserting into the donor cells the gene encoding the targeting proteins, or via chemical functionalization techniques, to decorate the surface with small molecules, biomacromolecules and polymers. Sustained delivery systems have been designed and assessed by loading EXOs in biodegradable or highly porous hydrogels. The 3D hydrogels act as a supporting system by protecting the EXO carrier and preventing its clearance, and once placed in situ allow the local delivery of a more concentrated cargo dose. In the next paragraphs, the targeting strategies to functionalize EXO surface and the techniques to encapsulate, support and preserve EXO-based carriers will be examined focusing on the role of EXO endogenous glycans and the potential role of natural exogenous polysaccharides as functional decoration molecules or 3D support systems.

### 3.2. Saccharides in Exosome-Based Delivery Systems

The EXO *glycocalyx*, directly involved in the naturally occurring mechanism of intrinsic and extrinsic recognition, may be technologically exploited to mediate molecular recognition and interaction with a high degree of specificity.

Among the endogenous molecular players, heparan sulfate PGs have been shown to act as internalizing receptors of cancer cell-derived EXOs [58] and demonstrated to be involved in EXOs cargo delivery and EXO-regulated functions between hepatic stellate cells, including expression of fibrosis- or activation-associated genes and/or miR-214 target gene regulation [77]. Sugar recognition is involved in EXO uptake in different cell types [48,88] and, consequently, the disruption of native glycosylation has been shown to alter the uptake in a panel of different cell lines [47]. Glycans possess also a high affinity for chemokines due to electrostatic interactions. It has been demonstrated that the chemokine (C-C motif) receptor 8 expressed on tumor cells allows binding and entry of EVs via interaction of EV-bound glycans with the chemokine (C-C motif) ligand 18 [89]. Desialylation of extracellular vesicles also affects their biodistribution in vivo [90] and an important role of glycosylation is emerging for cargo recruitment [23]. Definitely, it is claimed that EXO *glycocalyx* is a key factor for efficient targeting, internalization and biodistribution.

The highly specific role of the surface molecular component has been only recently considered in the design strategies for therapeutic EXO targeting and delivery. The exogenous modification of surface glycan chemistry may be a valid alternative to the most commonly used endogenous techniques in engineering EXOs which are based on the genetic modulation or metabolic engineering of EXO donor cells. Genetic modulation has shown promising results, however different issues, including the poor control affecting carrier functional activity, have to be still overcome. Nonetheless, targeted surface modifications directly processed to the EXO membrane require proper protocols to avoid membrane disruption or surface protein denaturation. Temperature, pressure, solvent exposure and osmolarity need well-defined testings and vesicle aggregation and/or precipitation have to be prevented [91].

Molecular bioconjugation to the surface of EXOs can be accomplished using covalent or non-covalent chemistry [92]. Click chemistry is one of the ideal tools for covalent bioconjugation of small molecules and macromolecules, including polysaccharides, due to high efficiency and specificity, chemo-selectivity, fast reaction times and compatibility in aqueous buffers. The technique has been investigated to introduce functional moieties to EV surface for EXO targeted delivery to normal or cancer cells [93,94] or to the ischemic area [95] and for EXO in vivo tracking, by conjugating fluorescent, radioactive, and MRI agents [96].

Stable non-covalent modifications have been mainly obtained by molecular insertions into the lipid bilayer and electrostatic interactions. Self-assembling into exosomal membranes has been tested mainly for lipids via hydrophobic insertion or lipid nanoparticles fusion [92,97,98]. However, the lipid bilayer reconfiguration via membrane fusion of lipidic components together with hydrophilic polymer significantly increases EXO cellular uptake and enhances their in vivo circulation time [99,100].

Different studies, exploiting the already assessed advantages of the hydrophilic polyethylene glycol (PEG), have demonstrated, that PEG, possibly grafted with amino components, can both accumulate into EXO membrane, assemble within targeting motifs on EXO membrane and improve blood circulation time, resulting in a promising molecule in the design of drug carriers for targeted delivery [92,97,98,101].

Modification of surfaces by reversible physical interaction provides for weak bonding. Electrostatic interactions are commonly employed for functionalizing sites and coating surfaces. Carrier coating may provide a valid alternative to small molecule ligands, conveying advantageous properties, including an improvement of carrier stability and a lengthening of blood circulation time. Negative charge biological surfaces are typically remodeled with cationic polyelectrolytes. In the nanotechnology field, natural polymeric compounds have been widely used and characterized both in the synthesis of carrier systems for multiple

attractive advantages and in their functionalization as they are extremely amenable for modification.

In the design of EXO-based therapeutic agents, glycans and polysaccharides have been only recently employed to advance the strategies of targeting and internalization and to increase the carrier stability, biodistribution, improving pharmacokinetics and pharmacodynamic properties (Table 2).

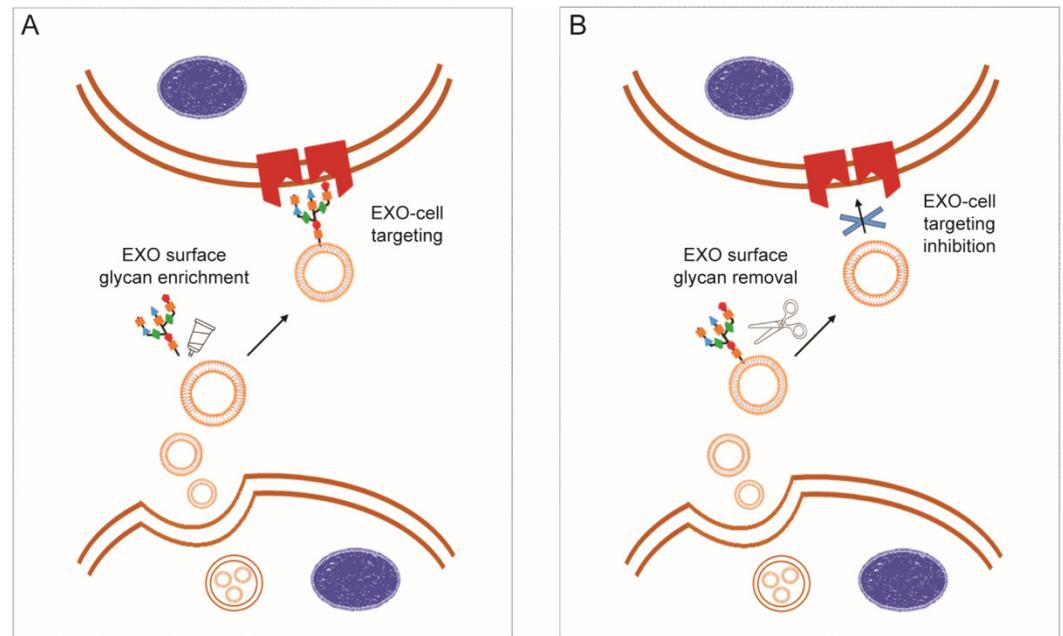
**Table 2.** Sugar-based decoration of EXO surface for targeted delivery and internalization, and for controlled biodistribution and pharmacokinetics.

Ligands	Methods	TargetCells/Tissues	Ref.
Sialic acid residues	Surface deglycosylation of mouse liver-derived EXOs with neuraminidase	Intravenous injection in mice	[90]
$\alpha$ -2,3- and $\alpha$ -2,6 linked sialic acid-capped complex, N-glycans and bi-antennary N-glycans	Removal of sialic acids Incorporation of dendritic cell-specific intercellular adhesion molecules for receptor-mediated glycan-dependent targeting	In vitro study on human glioblastoma and monocyte-derived dendritic cells	[102]
High-mannose glycans	Overexpression of high-mannose glycans on melanoma cell surface and on EVs derived after the induction of cell apoptosis	In vitro study on dendritic cells	[103]
$\alpha$ -D-mannose PEG	Surface modification with $\alpha$ -D-mannose and PEG via the incorporation of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine into the lipid layer of the EXOs	In vitro study on dendritic cells	[104]
HA3-(diethylamino)propylamine (HDEA)	Anchor of HA grafted with HDEA to EV membrane Doxorubicin loading	In vitro study on KB and HCT-116 tumor cells In vivo model of tumor-bearing mice	[105]
HA3-(diethylamino)propylamine (HDEA), monophosphoryl lipid A (MPLA), and mucin 1 peptide (MUC1)	Anchor of HA grafted with HDEA, MLA, MUC1	In vitro study on dendritic cells and CD8+ T-cell	[106]
HA pH-responsive 3-(diethylamino)propylamine (HDEA)	Anchor of HA grafted with HDEA to EV membrane Doxorubicin loading	In vitro study on BT-474 and SK-N-MC cells.	[107]
Lipidomimetic chain conjugated HA	Synthesis of HA derivative with octadecyl tails (lipHA) and insertion into the EVs membrane to generate lipHA-engineered EVs (lipHA-hEVs) Doxorubicin loading	In vitro study on drug resistant MCF7/ADR cells Preclinical multidrug tumor models	[108]
Heparin	Patching of doxorubicin-loaded heparin-based nanoparticles onto the surface of natural grapefruit EVs	Glioma tissue	[109]
Pullulan, Spermine	Pullulan cationization with spermine by an <i>N,N</i> 0-carbonyldiimidazole (CDI) activation method Mixing of MSC-derived EXOs with the cationized pullulan to incorporate the polysaccharide within the EXO membrane	In vitro study on HepG2 cells In vivo mouse model of liver injury	[110]
Azide containing sugars	Incorporation of tetra-acetylated N-azidoacetyl-D-mannosamine into glycans Bioorthogonal click reaction to label azido-containing EXOs with azadibenzylcyclooctyne-fluorescent dyes	In vitro tracking and in vivo biodistribution	[111]
Tetraacetylated N-azidoacetyl-D-mannosamine (ManNAz) azido sugar	Incorporation of ManNAz into EXOs Bioorthogonal click conjugation to modify and functionalize EXOs with a fluorescent dye Biotinylation	B16F10 cells	[112]

### 3.2.1. Exosome Glycocalyx for Targeted Delivery and Internalization

Glycan-based mechanisms may offer a promising strategy for advances in EXO-based targeted delivery systems. However, for the development of engineered vesicles with enhanced therapeutic delivery and outcome, a preliminary step to deeply understand the

appropriate moiety in relation to the target biological process is strongly required. Glycan enrichment or removal methods are, in fact, suggested to improve or limit recognition events at the cellular level. In relation to the research purposes, normal cell-derived vesicles are potential innate therapeutics with regenerative and immunomodulatory properties, whereas the pathological ones are targets to be modified or inhibited (Figure 3).



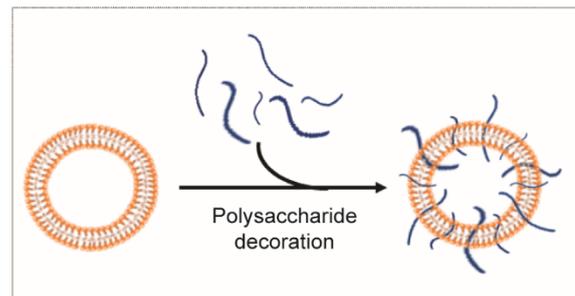
**Figure 3.** Sugar-based modulation of EXO surface for targeted delivery. Graphical sketch of EXO surface glycan enrichment or removal to improve panel (A) or limit panel (B) cellular recognition events [90,102–104].

Specific surface glycosylation can induce or avoid a host immunogenic response. Glycan motifs on glioblastoma-derived EXOs have been shown to be immunosuppressive; their removal in fact reduced immune inhibitory [102]. Conversely, specific glycosylation coating induces a host immunogenic response. Overexpression of high-mannose type glycans increased the uptake by monocyte-derived dendritic cells, resulting in enhanced priming of tumor-specific CD8+ T cells [103].  $\alpha$ -D-mannose onto the surface of bovine serum-derived EXOs has been also tested for a facile interaction with mannose receptors on dendritic cells (DCs) and for efficient delivery of immune stimulators to the DCs.

The effect of glycosyl structure impacts heterogeneously cellular uptake, depending on target cells. Removal of surface glycans from EXOs derived from human breast cancer cells stimulated their uptake by human umbilical vein endothelial cells [113]. Modification of the glycosylated complexes on the vesicle surface can also affect their distribution. Removal of sialic acid residues from mouse liver-derived EXOs has demonstrated that a higher amount of EVs reaches and accumulates in the lungs [90].

### 3.2.2. Polysaccharide Decoration for Controlled Biodistribution and Circulation Kinetics

The surface of EXOs has to be refined to accurately control the biodistribution and achieve active accumulation at the target site. Carrier pharmacokinetic evaluation is accomplished by conjugating onto EV surface bioluminescence molecules or radiotracers [114]. An effective approach to prolong EXO biological half-life and reduce immunogenic reactions provides the integration of hydrophilic polymers (Figure 4).



**Figure 4.** Polysaccharide decoration of EXO membrane. Graphical sketch illustrating the anchoring or integration of polysaccharides into EXO membrane for controlled biodistribution and circulation kinetics [105–110].

PEG is well known to prevent adsorption or aggregation of plasma proteins with nanoparticles, leading to an increase in particle circulation time [115]. Likewise, it has been hypothesized and investigated that these features would be beneficial also for EV-based drug delivery systems.

Different studies have confirmed that the integration of PEG into the lipid layer of EXOs inhibited nonspecific cellular uptake, which resulted in a long biological half-life and reduced immunogenicity.

Higher retention of EXOs containing mannose-conjugated PEG-1,2-distearoyl-sn-glycero-3-phosphoethanolamine has been observed in the lymph nodes after intradermal injection [104].

PEG-based modification onto EXO surfaces demonstrated to improve blood circulation time also in mice with pulmonary metastases and combinatorial incorporation of aminoethylanisamide-PEG specifically has been demonstrated to target the sigma receptor overexpressed by lung cancer cells [116]. The introduction of PEG-conjugated nanobodies onto EVs via post-insertion has been shown to affect both in vitro extracellular vesicle interactions with tumor cells and in vivo circulation time and tissue distribution in tumor-bearing mice. PEG chains have been employed to also favor the attachment of targeting ligands. Nanobodies against the epidermal growth factor receptor (EGFR) attached to the distal end of PEG chains have been shown to be higher recovered in EGFR-expressing cells [100].

Similar results have been obtained also with natural polysaccharides. EVs have been engineered by anchoring in the membrane the native polysaccharides HA together to a pH-responsive 3-(diethylamino)propylamine. These carriers containing HA, as a specific ligand against the CD44 tumor receptors, and a pH-responsive material against the acidic tumor pH have been proposed in different studies as novel drug carriers for efficient antitumor treatment [105–107].

Liu et al. anchored to an EV surface a lipidomimetic compound-modified hyaluronic acid, synthesized by grafting octadecylamine to HA molecules. Owing to CD44-mediated cancer-specific targeting the HA-functionalized EV remarkably promoted the intracellular doxorubicin accumulation in drug-resistant breast cancer cells. In preclinical MDR tumor models, the system deeply penetrated into tumor tissue and effectively transported the drug into the tumor site, finally inhibiting the growth [108].

Assembling into membrane vesicles has also been investigated inserting doxorubicin-loaded heparin-based nanoparticles. The engineered EV bypassed BBB/BBTB and penetrated into glioma tissues by receptor-mediated transcytosis and membrane fusion, greatly promoting cellular internalization and antiproliferation ability as well as extending circulation time [109].

Negative charge EXO surface has been fused with different cationic polyelectrolytes. Tamura et al. cationized pullulan with spermine to enhance the electrostatic interaction with the negatively charged EXO surface. Pullulan was selectively internalized by HepG2 cells and hepatocytes through an asialoglycoprotein receptor. EXOs modified with cation-

ized pullulan were efficiently and in higher content internalized by HepG2 cells and the enhanced accumulation and therapeutic effect were confirmed in vivo in a concanavalin A-induced liver injury model [110].

Glycan chemistry has been investigated also in EXO labeling strategies. Azido-sugars were efficiently incorporated on the surface of EXOs through metabolic glycan synthesis and then, the azide-sialic acid motifs were easily labeled by bioorthogonal click reaction [111,112]. The advantages of membrane surface engineering and biodistribution tracking have been demonstrated also by Antes et al. by coupling biotinylated fluorescent molecules to glycerol-phospholipid-PEG conjugates previously embedded into vesicle lipid bilayer membranes. Glycerol-phospholipid-PEG conjugates are a promising anchor to decorate vesicle membranes for targeted delivery with any biotinylated molecule (e.g., antibodies, tissue homing peptides) [117]. The metabolic EXO labeling strategy could be a promising tool in studying the biology, delivery, tracking and biodistribution of EXOs in vivo, optimizing EXO-based therapeutic approaches. Furthermore, based on this flexible chemistry, many other ligands could be anchored onto EXOs, improving and enhancing their bioactivity.

#### 4. Polysaccharide-Based Hydrogel for Sustained Exosome-Based Delivery System

The major limiting determinants influencing the in vivo application of EXO-based agents are the selectivity for targeted delivery and the stability for a controlled biodistribution and pharmacokinetics. Advanced biological and material designs have been introduced to improve precision therapies and therapeutic outcomes. The engineering strategies aim at optimizing biomaterial characteristics, investigating both small-scale barriers such as cell-specific targeting and intracellular trafficking and larger-scale issues such as stability and biodistribution.

An ideal combined approach finally improves therapeutic efficacy and safety. Therefore, the recent advances in the synthesis of EXO-based carriers aim to address both targeted and sustained delivery. Carriers have been both modified constitutionally via the incorporation of bio-responsive moieties or selective molecules or stabilized in a biomimetic microenvironment via controlled encapsulation and release. In the last approach, the active carrier is physically entrapped within a macromolecular network, able to improve blood circulation half-life, biodistribution and pharmacokinetics [118].

Hydrogels are hydrophilic 3D macromolecular networks, chemically or physically cross-linked, extensively used in drug delivery and tissue engineering for their biocompatibility, controllable biodegradability, tunable mechanical properties and porous structure. The structural and physico-mechanical properties generally may be precisely tailored to finally mimic the features of the native extracellular matrix. In fact, the hydrogel matrix has to contribute to system architecture by providing an organized 3D macromolecule network capable of mediating cell-cell or EXO-cell communication and promoting the exchange of growth and signaling factors. Hydrogel polymeric networks may physically trap active agents controlling their retain and their release. Different processes, including the structural and physico-chemical properties of the system, are involved in drug entrapment efficiency and loading and govern drug release kinetics via diffusion, dissolution, degradation, swelling [119].

The high versatility of natural polysaccharides permits the modulation of different properties to target specific biofunctions, finally improving precise applications in tissue engineering, regenerative medicine and pharmacology. The most widely used polysaccharides from natural sources are hyaluronic acid, chitosan, alginate, cellulose, starch, pectins, agar, xanthan, dextran, pullulan, gellan, carrageenan and GAGs. Polysaccharides have been applied in various forms, as matrices for biomedical applications, including injectable hydrogels, porous or fibrous scaffolds, and membranes. Polymeric matrix composites or interpenetrating polymer networks are additionally proposed by integrating natural or synthetic polymers and/or inorganic nanoparticles to improve system stiffness, strength, stability, conductivity or permeability.

Polysaccharide-based matrices have been recently investigated for a sustained delivery of EXOs and a controlled release of the cargo at the site of interest (Box 3).

**Box 3.** Polysaccharide sources in the design of sustained EXO-based delivery systems.

**Hyaluronic acid** is an endogenous linear non-sulphated GAG with alternating units of Dglucuronic acid and *N*-acetyl-*D*-glucosamine. It is a ubiquitous constituent of the extracellular matrix actively involved in the regulation of cell adhesion, migration, proliferation, and differentiation. Its peculiar physical and mechanical properties contribute to the maintenance of tissue's mechanical integrity, homeostasis and viscoelasticity.

**Heparin** is a heterogeneous highly sulphated GAG consisting of repeating disaccharide units of uronic acid residues (1-iduronic (IdoA) or Dglucuronic acid (GlcA)) and *N*-acetyl-*D*-glucosamine. Heparin is involved in various biological functions, including cell surface reception, differentiation, migration, proliferation, cancer metastasis. Heparin is also involved in anticoagulation of blood, binding antithrombin III (AT) which results in activation of AT [120,121].

**Chitosan** is a linear-chain copolymer composed of *D*-glucosamine and *N*-acetyl-*D*-glucosamine, obtained by the partial deacetylation of chitin. It is widely used in the biomedical field due to its biodegradability, biocompatibility, non-toxicity and non-antigenicity. It possesses high tensile strength and in acidic environments, it is an excellent viscosity-enhancing agent. Moreover, the presence of reactive amino and hydroxyl groups allows versatile physico-chemical modification and tailoring [122].

**Chitin** is a poly-(1-4)- $\beta$ -linked *N*-acetyl-*D*-glucosamine occurring in nature in three different crystalline allomorphs, which differ in the orientation of the micro-fibrils. It is the major structural component in the exoskeletons of the crustaceans, insects as well as the cell walls of fungi. Although it presents low solubility and chemical non-reactivity, it is widely used in biomedical applications for its structural integrity, biocompatibility, non-toxicity, ability to form films and biodegradability [123].

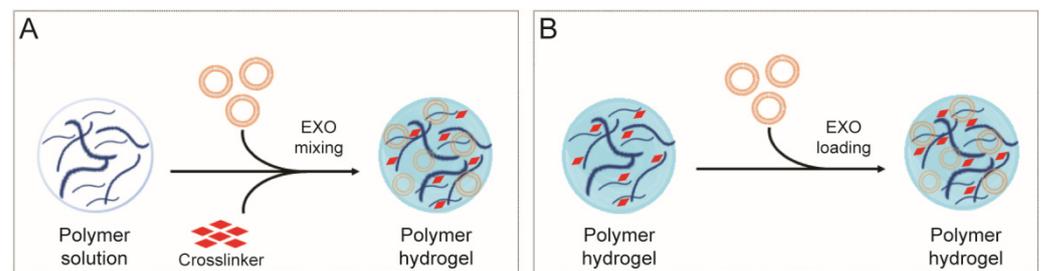
**Cellulose** is a natural polysaccharide composed of *D*-glucose subunits, linked via  $\beta$ -1-4-glycosidic bonds. The intra- and inter-chain hydrogen bonding give the cellulose fibrils high axial stiffness and tensile strength. It is used for numerous applications mainly for its good structural and mechanical properties [124].

**Sodium alginate** is a linear hydrophilic polysaccharide, mainly derived from marine brown algae and several bacteria strains, consisting of (1-4) linked  $\beta$ -Dmannuronic acid and  $\alpha$ -l-guluronic acid. The peculiar ability of sol-gel transition under mild conditions has been exploited in different fields. In biomedicine, alginate is typically used in the form of a hydrogel, as structural supporting biomaterials due to its tunable mechanical behavior, swelling properties and porosity [125].

**Pullulan** is a microbial polysaccharide composed of maltotriose units (three glucose units connected by  $\alpha$ -1,4 glycosidic bonds) connected to each other by an  $\alpha$ -1,6 glycosidic bond. This peculiar structure gives molecules high solubility in water and flexibility. It owns good adhesive properties and ability to form thin layers, films and fibers with considerable mechanical strength. The composites are thermally stable and have good elastic properties [126,127].

EXOs embedded in hydrogels may be considered as a nano-in-micro bioactive system. Different methods are proposed to entrap EXOs into polysaccharide-based matrices in relation to the physico-chemical characteristics of biomaterials and to the aims of the application. Two are the more common approaches, which mainly differ in the gelation step (Figure 5):

- (i) *in situ* gelation: cargo is mixed into the polysaccharide viscous solution and, subsequently, a cross-linking agent is added to gel the system. Gelation can be achieved by ionic exchange, pH modification, temperature variation or UV irradiation.
- (ii) pre-formed gels: cargo is loaded directly in the polysaccharide-based hydrogel.



**Figure 5.** Entrapment of EXOs into polysaccharide-based matrices. In panel (A), the EXOs are mixed into a polymeric solution, then gelled by the addition of a cross-linking agent [128–140]; in panel (B), the EXOs are directly loaded in a pre-formed gel [141–147].

#### 4.1. Polysaccharide-Based *In Situ* Gelling System, for a Sustained Delivery of Exosomes

EXOs are embedded within the polymer network followed by the gelation step induced by a cross-linking agent (Table 3). The procedure allows high tunability of hydrogel mechanical properties, however, poorly controllable gelation kinetics may limit system injectability.

Hyaluronic acid (HA) has been tested in different models. Since by nature HA does not form gels, chemical modifications are usually carried out by modulating functional groups before the gelling step via covalent crosslinking or gelling agents. Different studies have successfully integrated extracellular vesicles into a modified-HA-based hydrogel.

Bone marrow stem cell-derived EXOs have been incorporated in a network of thiolated HA, gelatin, and heparin and then crosslinked with polyethylene glycol diacrylate. It has been shown that EXOs released their cargo and stimulated osteogenic gene expression, osteoblast differentiation *in vitro* and bone regeneration *in vivo* [128]. A similar method was proposed for loading EXOs isolated from bone marrow-derived endothelial progenitor cells in an injectable HA hydrogel modified with adamantane and  $\beta$ -cyclodextrin. The system increased the therapeutic efficiency and efficacy of EV-mediated myocardial preservation by enhancing peri-infarct angiogenesis and myocardial haemodynamics in a rat model of myocardial infarction [129]. Liu et al. used O-nitrobenzyl alcohol moieties modified HA and gelatin to incorporate hiPSC-MSCs-derived EXOs. The system was gelled by irradiation with 395 nm LED light, which promotes a reaction between the novel aldehyde groups generated into modified HA with gelatin amino groups. The matrix efficiently retained stem cell-derived EXOs at the defect site and showed positive cellular regulation both *in vitro* and *in vivo* leading to the promotion of cartilage repair and regeneration [130].

Similarly, chitosan (CS) is commonly gelled after chemical modifications or by using cross-linking agents, as  $\beta$ -glycero phosphate or sodium hydroxide. Human placenta-derived MSC EXOs were incorporated in a CS solution, then gelled by adding  $\beta$ -glycero phosphate. The injectable EXO-based hydrogel showed enhanced angiogenesis and tissue regeneration in a murine model of hindlimb ischemia [131].

EXOs derived from miR-126-3p-overexpressing synovium mesenchymal stem cells (SMSCs) combined with CS were instead gelled in NaOH solution. *In vitro* results showed that EXOs derived from miR-126-3p-overexpressing SMSCs stimulated the proliferation of human dermal fibroblasts and human dermal microvascular endothelial cells (HMEC-1) in a dose-dependent manner. Furthermore, EXOs also promoted migration and tube formation of HMEC-1. The approach showed promising results also *in vivo*, accelerating re-epithelialization, activating angiogenesis, and promoting collagen maturity [132].

Polysaccharide combinations are frequently investigated to enhance physico-mechanical properties of the system. Wang et al. coupled CS together with modified cellulose, a polysaccharide possessing good mechanical strength. Definitely, placental mesenchymal stem cell-derived EXOs were incorporated in a solution of aldehyde-modified methylcellulose (MC-CHO) and then CS grafted poly(ethylene glycol) (CS-g-PEG) was added to form a homogeneous hydrogel. The system resulted in a highly efficient injectable self-healing hydrogel fabrication with excellent wound healing function for diabetic mice [133].

**Table 3.** Polysaccharide-based in situ gelling systems for sustained delivery of EXOs.

Material	Method	TargetCells/Tissues	Application	Reference
Thiol-Modified HA Gelatin Heparin	Bone marrow stem cell (BMSC)-derived EXOs entrapment in a matrix of thiolated HA, gelatin, and heparin Gel crosslinking with polyethylene glycol diacrylate	Human bone marrow stromal stem cell and osteoblast In vivo rat model of calvarial defects	Bone regeneration	[128]
Adamantane-modified HA $\beta$ -cyclodextrin-modified HA	EXOs isolated from bone marrow-derived endothelial progenitor cells entrapment in an injectable HA-based hydrogel	In vivo rat model of myocardial infarction	Cardiac regeneration	[129]
O-nitrobenzyl alcohol modified HA Gelatin	HiPSC-MSCs-derived EXOs entrapment in a matrix of O-nitrobenzyl alcohol moieties modified HA and gelatin Gel crosslinking with light irradiation	Chondrocytes and human bone marrow stromal stem cell In vivo rabbit model of articular cartilage defect	Cartilage regeneration	[130]
CS	Human placenta-derived MSC EXOs incorporation in a CS solution, then gelled by adding $\beta$ -glycero phosphate	In vivo murine model of hindlimb ischemia	Tissue regeneration Angiogenesis	[131]
CS	EXOs derived from microRNA-126-overexpressing synovium MSCs incorporation in a CS solution, then gelled	Human dermal fibroblasts and human dermal microvascular endothelial cells In vivo diabetic rat model	Wound healing	[132]
Aldehyde modified methylcellulose (MC-CHO) CS grafted poly(ethylene glycol) (CS-g-PEG)	Placental MSC-derived EXOs incorporation in a solution of MC-CHO Addition of CS-g-PEG and gelation	In vivo diabetic mouse model	Wound healing	[133]
CS Hydroxyapatite	EXOs derived from miR-126-3p overexpressed synovial MSC addition in a CS solution containing $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	Human dermal fibroblasts and human dermal microvascular endothelial cells In vivo diabetic rat model	Wound healing	[134]
SA	Platelet-rich plasma-derived EXOs incorporation in a solution of SA Gelation with $\text{CaCl}_2$	Endothelial cells and fibroblasts In vivo diabetic rat model	Wound healing	[135]
SA	Bone marrow MSC-derived small EVs addition in a solution of SA Gelation with $\text{CaCl}_2$	In vivo rat model of myocardial infarction	Tissue regeneration Angiogenesis	[136]
SA	Adipose-derived stem cells EVs entrapment in a solution of SA Gelation with $\text{CaCl}_2$	HUVECs In vivo rat model of full-thickness wound	Wound healing	[137]
SA Polyvinyl alcohol (PVA)	Human umbilical cord MSC-derived EXOs encapsulation in a solution of SA and PVA Gelation with $\text{CaCl}_2$ and ultrasonication	HUVECs In vivo diabetic rat model	Wound healing	[138]
SA	MSC-derived EVs alone or together with MSC entrapment in a solution of SA Gelation with $\text{CaCl}_2$	In vivo nude mouse model of subcutaneous bone formation	Bone regeneration Angiogenesis	[139]
Aldehyde-modified SA, HA-adipic dihydrazide Hydroxyapatite	Human umbilical cord MSC-derived EXOs integration in formulation containing aldehyde-modified SA, HA-adipic dihydrazide and hydroxyapatite before the gelation	Murine calvariae preosteoblast cell line In vivo rat model of calvarial bone defect	Bone regeneration	[140]

A different approach to improve mechanical properties of CS-based hydrogel provided for the formation of hydroxyapatite within the gel. Min Li et al. encapsulated EXOs derived from miR-126-3p overexpressed synovial mesenchymal stem cells (SMSCs) in

hydroxyapatite/chitosan (HAP-CS) composite hydrogels. EXO release stimulated the proliferation and the migration of human dermal fibroblasts and human dermal microvascular endothelial cells (HMEC-1). At the same time, the migration and capillary-network formation of HMEC-1 were promoted. In vivo tests demonstrated that the system successfully promoted wound surface re-epithelialization, accelerated angiogenesis, and promoted collagen maturity [134].

Conversely, sodium alginate (SA) is widely used for hydrogel preparation cause it easily gels in mild conditions in presence of divalent cations. EXOs derived from platelet-rich plasma (PRP) were mixed with a solution of SA and the resultant mixture cross-linked with  $\text{CaCl}_2$ . PRP EXOs effectively induced proliferation and migration of endothelial cells and fibroblasts, improving angiogenesis and re-epithelialization in chronic wounds. In vivo, the cutaneous healing process in chronic wounds was observed in a diabetic rat model [135]. Similarly, bone marrow mesenchymal stem cell (MSC)-derived EXOs have been encapsulated in SA, then cross-linked with  $\text{CaCl}_2$  and tested for myocardial infarction. Hydrogel system increased scar thickness and angiogenesis and reduced cardiac apoptosis and fibrosis, improving cardiac function if compared to the application of vesicles alone [136].

Shafei et al. followed an analogous approach by integrating EXOs isolated from the supernatant of cultured adipose-derived stem cells (ADSCs) in SA solution before the gelation with  $\text{CaCl}_2$ . The hydrogel was investigated to treat skin injuries. The EXOs loaded in the alginate-based hydrogel significantly improved wound closure, re-epithelization, collagen deposition, and vessel formation in the wound area [137].

SA has been also proposed in combination with polyvinyl alcohol (PVA). EXOs derived from human umbilical cord mesenchymal stem cells were encapsulated in PVA/SA nanohydrogel facilitating the proliferation, migration and angiogenesis of HUVECs. In vivo, EXO-based nanohydrogel significantly promoted diabetic wound healing by increasing the expression of molecules related to angiogenesis and activating ERK1/2 pathway [138]. A combined approach provides for the addition of cellular components to vesicles-modified scaffolds. It has been demonstrated that a SA-based hydrogel containing both mesenchymal stem cells (MSCs) and MSC-derived EVs has significant effects on bone regeneration in vivo. The bone volume and the bone volume to tissue volume in the MSCs + EV-modified scaffold group were increased compared with those containing only EV [139].

Formulations integrating different polysaccharide macromolecules and nanocomposites are mainly combined to improve peculiar characteristics. Yang et al., integrated human umbilical cord mesenchymal stem cells-derived EXOs in a formulation containing aldehyde-modified SA, HA-adipicdihydrazide and hydroxyapatite. The results showed that the combination of EXOs and injectable composite hydrogel effectively promoted the proliferation, migration, and osteogenic differentiation of a murine *calvariae* preosteoblast cell line in vitro and significantly enhanced bone regeneration in rats in vivo [140].

#### 4.2. Polysaccharide-Based Pre-Formed Hydrogels, for a Sustained Delivery of Exosomes

EXOs can be directly loaded into the hydrogel matrix after the formation of the gel (Table 4). Commonly solvent-dehydrated hydrogel is soaked into an aqueous solution containing EXOs causing EXO breathing-in.

HA- and CS-based hydrogels for sustained delivery of EXOs have been largely investigated in cargo loading after gel formation. Adipose-derived MSC EXOs were loaded in a hydrogel composed of Pluronic F127, oxidized HA and poly- $\epsilon$ -lysine, obtained by the Schiff base reaction and thermal-responsive sol-gel process. In vivo, the EXO-loaded hydrogel significantly enhanced the healing efficiency of diabetic full-thickness cutaneous wounds, characterized by enhanced wound closure rates, fast angiogenesis, re-epithelization and collagen deposition within the wound site [141]. Liming Li et al., implanted human placenta amniotic membrane mesenchymal stem cell (hMSC)-derived EXOs within a peptide-modified adhesive HA hydrogel. The hydrogel composition included aldehyde-modified HA (HA-CHO) and adipodihydrazide-modified HA (HA-ADH) and was modified with

the laminin-derived adhesive peptide PPFLMLLKGSTR. Topical transplantation of the EXO-based hydrogel provided an EXO-encapsulated extracellular matrix to the injured nerve tissue elicited significant nerve recovery and urinary tissue preservation by effectively mitigating inflammation and oxidation [142].

**Table 4.** Polysaccharide-based pre-formed hydrogels for sustained delivery of EXOs.

Material	Method	Target Cells/Tissues	Application	Reference
Oxidized HA Pluronic F127 Poly- $\epsilon$ -lysine	Adipose-derived MSC EXOs loading in a hydrogel composed of Pluronic F127, oxidized HA and poly- $\epsilon$ -lysine, obtained by the Schiff base reaction and thermal-responsive sol-gel process	In vivo diabetic mouse model	Wound healing	[141]
Aldehyde-modified hyaluronic acid (HA-CHO) Adipodihydrazide-modified hyaluronic acid (HA-ADH)	Human placenta amniotic membrane MSC-derived EXOs encapsulation within a hydrogel composed of HA-CHO and HA-ADH, modified with the laminin-derived adhesive peptide PPFLMLLKGSTR	In vivo spinal cord injury rat model	Spinal cord regeneration	[142]
CS Silk fibroin	EXOs derived from gingival MSCs loading in a hydrogel sponge composed of CS and silk fibroin prepared by freeze-drying method	In vivo diabetic mouse model	Wound healing	[143]
CS Silk fibroin	Platelet-rich plasma EXOs loading in a hydrogel sponge composed of CS and silk fibroin	In vivo diabetic rat model	Wound healing	[144]
Pullulan	EXOs derived from MSCs loading into a scaffold fabricated by the reversible Schiff base reaction between Pluronic F127 grafting polyethylenimine and aldehyde pullulan	In vivo diabetic mouse model	Wound healing	[145]
Bacterial cellulose	Human umbilical cord MSCs-derived EXOs loading into a bacterial cellulose membrane	In vivo laminectomy rabbit model	Epidural fibrosis prevention	[146]
Chitin	EXOs from gingival MSCs combination with biodegradable chitin conduits	In vivo sciatic nerve defect rat model	Peripheral nerve regeneration	[147]

EXOs derived from gingival mesenchymal stem cells (GMSCs) were instead added to CS/silk-based hydrogel sponge previously prepared by using the freeze-drying method. The combination of GMSC-derived EXOs and hydrogel effectively promoted skin wound healing in diabetic rats by promoting the re-epithelialization, deposition and remodeling of collagen and by enhancing angiogenesis and neuronal ingrowth [143].

Likewise, platelet-rich plasma EXOs loaded in CS/silk sponge significantly accelerated wound contraction, re-epithelialization, collagen synthesis and deposition, along with dermal angiogenesis in diabetic rats, thus resulting in faster healing of diabetic wounds [144].

Among natural polysaccharides, pullulan a linear polysaccharide produced from starch fermentation has reached considerable attention because it forms a crosslinked network via the formation of intermolecular phosphate bonds. Wang et al., proposed a

scaffold fabricated by the reversible Schiff base reaction between Pluronic F127 grafting polyethylenimine and aldehyde pullulan to load EXOs derived from mesenchymal stem cells. In an *in vivo* model of diabetic mice, the system accelerated wound healing by stimulating angiogenesis. The fast healing with less scar tissue was obtained thanks to enhanced cell proliferation, granulation tissue formation, collagen deposition, remodeling and re-epithelization [145].

The versatility of polysaccharides allows their applicability in different formulations. Bacterial cellulose has been employed to prepare an anti-adhesion membrane, a three-dimensional network structure with attractive mechanical and physico-chemical properties. The membrane loaded with EXOs from human umbilical cord mesenchymal stem cells inhibited epidural fibrosis and peridural adhesions in laminectomy rabbit models [146].

Biodegradable chitin conduits combined with EXOs from gingival mesenchymal stem cells (GMSCs) instead repaired peripheral nerve defects in rats. Specifically, chitin conduit combined with EXOs from GMSCs significantly increased the number and diameter of nerve fibers and promoted myelin formation. In addition, muscle function, nerve conduction function, and motor function were recovered [147].

## 5. Discussion

Considering exosome biology molecular interplay with a focus on glycans, we performed a comparative analyses of the last decade's developments in polysaccharide exploitation (versus other strategies) to preserve and ameliorate exosome-based biomedical approaches.

EXO-based delivery systems effectively provide an endogenous opportunity to advance novel therapeutics. The recent intriguing investigations on the role of glycome in EXO biology and function motivate current research efforts aiming at exploiting sugar-based components in the development and upgrade of the carrier.

Natural polysaccharides have been already largely employed to modulate nanocomposite structures and properties, and recently polymeric carrier systems have moved to the clinical phase. Likewise, the promising results obtained integrating the biopolymer area to the EXO-based nanotechnology field paved the way for further investigation on this new emerging and promising topic, offering a new frontier in drug delivery.

The challenge for clinical studies still remains the establishment and the characterization of an effective delivery strategy. The methods to functionalize EXO surface and the techniques to encapsulate, support and preserve EXO-based carriers, may be deeply investigated in relation to both the role of EXO endogenous glycans and the potential role of natural exogenous polysaccharides as functional decoration molecules and 3D support systems. Promising glycan-based targeting strategies have already been shown to improve carrier selectivity and bio-functional activity in the drug delivery field, while 3D polysaccharide-based matrices have achieved interesting results as sustained delivery platforms in the tissue engineering area.

The biocompatible three-dimensional system is, in fact, able to effectively integrate, retain, deliver and release signaling or bioactive agents and target and bridge within the tissue site, without impair biologic integrity. EXO-hydrogel hybrid complex synergistically integrates the properties of two distinct constituents in one formulation with peculiar bio-functional and physico-chemico and mechanical properties that cannot be achieved independently by a single component. The combined system has shown higher performances in modulating carrier release kinetics, assisting site-specific drug targeting and enhancing biodistribution.

Although in its infancy, the topic reveals considerable potential and attracts research attention. Advanced nanoparticle-hydrogel systems activities may be implemented focusing on (1) passively controlled release, (2) stimuli-responsive delivery, (3) site-specific delivery.

The contribution of polysaccharides may boost biomaterial-mediated therapies. Thus, biomaterial design to implement EXO-based delivery systems may still improve, taking into account material structural configuration, mechanical and biological properties.

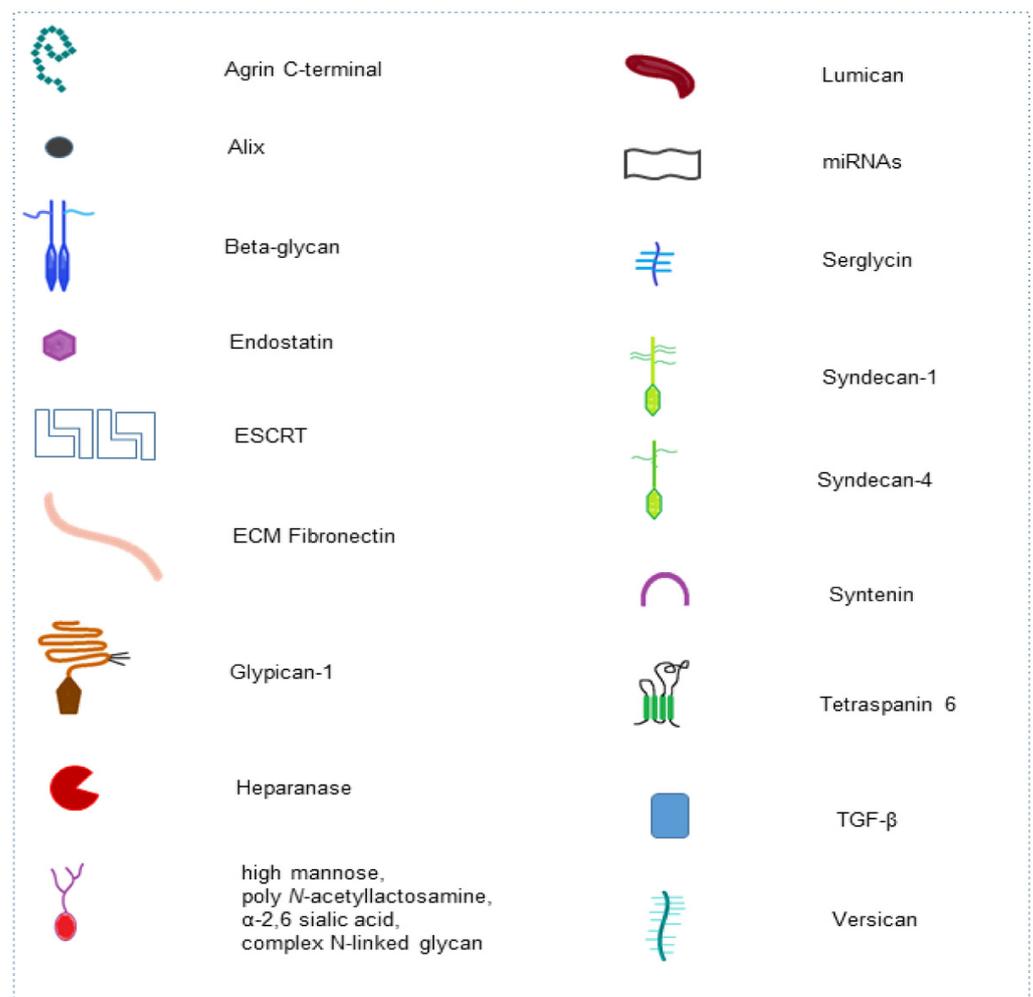
In conclusion, natural smart polysaccharides may offer selective decorations and a dynamic and responsive matrix to EXO-based carriers, providing effective targeting strategies and multipurpose 3D platforms with high application potential in drug delivery and tissue engineering fields.

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## Appendix A



**Figure A1.** Map legend of glycans and other molecules of EXO cargo, for good interpretation of Figures 1 and 2. Agrin C-terminal, Alix, Betaglycan, Endostatin, ESCRT, ECM Fibronectin, Glypican-1, Heparanase, polysaccharides (high mannose, polylactosamine,  $\alpha$ -2,6 sialic acid, complex *N*-linked glycan), Lumican, miRNAs, Serglycin, Sindecyan-1, Sindecyan-4, Syntenin, Tetraspanin-6, TGF- $\beta$ , Versican.

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