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REVIEW

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Lectins as potential tools for cancer biomarker discovery from extracellular vesicles

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Abstract

Extracellular vesicles (EVs) have considerable potential as diagnostic, prognostic, and therapeutic agents, in large part because molecular patterns on the EV surface betray the cell of origin and may also be used to "target" EVs to specific cells. Cancer is associated with alterations to cellular and EV glycosylation patterns, and the surface of EVs is enriched with glycan moieties. Glycoconjugates of EVs play versatile roles in cancer including modulating immune response, affecting tumor cell behavior and site of metastasis and as such, paving the way for the development of innovative diagnostic tools and novel therapies. Entities that recognize specific glycans, such as lectins, may thus be powerful tools to discover and detect novel cancer biomarkers. Indeed, the past decade has seen a constant increase in the number of published articles on lectin-based strategies for the detection of EV glycans. This review explores the roles of EV glycosylation in cancer and cancer-related applications. Furthermore, this review summarizes the potential of lectins and lectin-based methods for screening, targeting, separation, and possible identification of improved biomarkers from the surface of EVs.

Keywords Extracellular vesicles, Glycosylation, Lectin, Glycan, Lectin assay, Cancer biomarker

Extracellular vesicles and glycosylation

Extracellular vesicles (EVs) are mostly sub-micron, lipid bilayer vesicles released by all cell types. EVs allow cells to dispose of unwanted materials and are thought to serve in intracellular communication by facilitating the exchange of genetic materials, proteins, and lipids [1–3]. EVs are greatly heterogeneous, overlapping in physiochemical properties including size. Most commonly, EVs are classified based on biogenesis, with "exosomes" derived from the endosomal system and "ectosomes" (or "microvesicles") from the plasma membrane. However, these definitions may be of limited value since subtypes of EVs are difficult to identify or separate after release from the cell. Perhaps more importantly, EVs display surface molecules and macromolecules that can potentially be used to identify their cellular source and influence interactions with recipient cells, and this is key to their theragnostic potential.

The surface of EVs is highly enriched with glycosylations [4–6]: the covalent attachment of one or more sugar residues to proteins or lipids (Fig. 1). Protein N-and O-glycosylation are the most abundant post-translational modifications in the extracellular milieu [7, 8].

In N-linked glycosylation, a glycan, built on a common core pentasaccharide, is attached to an asparagine residue´s nitrogen atom [9]. Based on the additional sugar moieties attached to the core, the N-glycans can be broadly classified as: a) a high-mannose oligosaccharides

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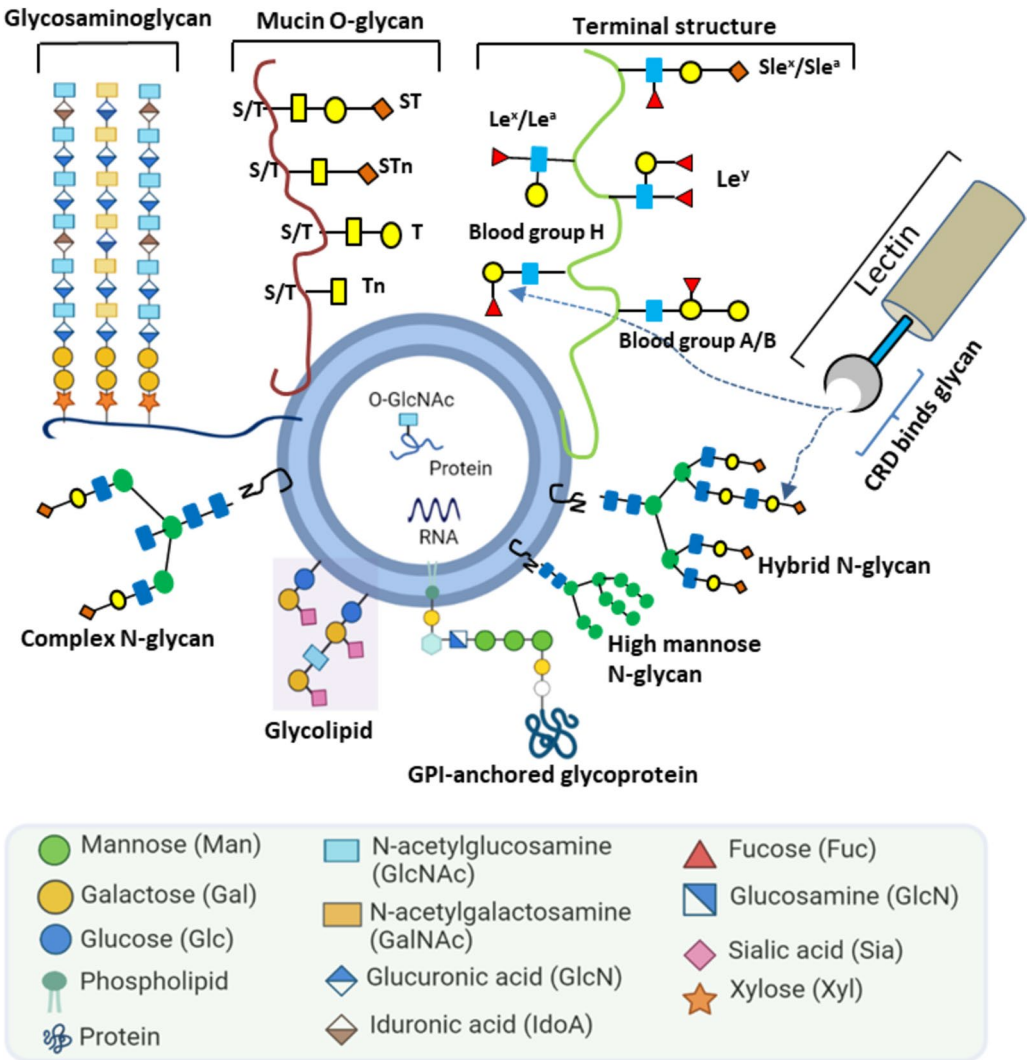

Fig. 1 Illustration of glycan structures on the surface of EVs. EVs display various glycoconjugates and alterations of glycan structures are commonly seen in cancers. The CRD (carbohydrate recognition domain) of lectin binds to specific glycans (Herein, GPI = glycosylphosphatidylinositol, sT = sialyl T, sTn = sialyl Tn, sLe^x = sialyl-Lewis x, and sLe^a = sialyl-Lewis a)

that contain mere unsubstituted terminal mannose residues attached to the core; b) hybrid glycans having both mannose and *N*-acetylglucosamine (GlcNAc) residues; c) complex oligosaccharides that lack additional mannoses but contain multiple other sugar types such as fucose, galactose and *N*-acetylneuraminic acid. In contrast, O-linked glycosylation involves glycan attachment to side-chain residues of serine, threonine, or hydroxyproline via oxygen. The most abundant proteins with O-linked glycosylation are mucins. These large multidomain proteins, produced by epithelial cells, are associated with cancer progression, proliferation, and metastasis [10]. Meanwhile, glycolipids contain different types of glycans compared with glycoproteins. Glycosphingolipid and glycoglycerolipid are two prominent glycolipids, typically conjugated to ceramide and diacylglycerol, respectively. Apart from the glycoproteins and glycolipids mentioned above, other types of glycosylation are also observed in mammalian cells, such as proteoglycans and glycosylphosphatidylinositol (GPI). Proteoglycans are heavily glycosylated and covalently attached to one or more glycosaminoglycan (GAG) chain(s). Each GAG consists of repeated two-sugar units (disaccharides) and has different degrees of sulfation. Based on their core disaccharide units, GAGs are primary classified into four groups including heparin/heparan sulfate, chondroitin sulfate/ dermatan sulfate, keratan sulfate, and hyaluronic acid [11]. GPIs anchor

proteins to the cell membrane. GPIs consist of two fatty acid tails that are inserted into the membrane and linked to a head group, a series of four saccharides, and a phosphoethanolamine residue that links to the carboxyl end of the protein.

Of the different types of EV glycosylation, glycoproteins are the most heavily studied. EV-glycoproteins include integrins (ITGs), mucins (MUC-1, -4, -16), epithelium cell adhesion molecules (EpCAM), carcinoembryonic antigen (CEA), carbohydrate antigen (CA 19–9), cell adhesion molecules such as CD24, and more [12–18]. In the current literature on EV glycosylation, only a limited number of studies have examined glycolipids and proteoglycans. In one EV glycolipid study, Llorente et al. identified several glycolipid candidates on PC3 prostate cancer cells by lipidomic analyses [19]. Proteoglycans are mostly studied in the context of EV biogenesis and cellular attachment [20–22].

Since cellular glycosylation changes can be reflected on released EVs [23, 24], quantitative or qualitative changes to EV glycosylation could form the basis for disease diagnosis [12, 25–28]. Various N-glycosylated EV proteins have been proposed as potential cancer biomarkers [29–31], among them CEA as a marker for colorectal cancer diagnosis and monitoring [32]. ADAM10 and CD109 are also heavily N-glycosylated, with high-mannose glycans that can be used for EV detection [29, 30]. Many other N-linked glycosylation profiling studies have been conducted for the discovery of glyco-specific cancer biomarkers on EVs surface [33, 34]. Similarly, O-linked glycosylations are also common on the EV surface [35, 36]. Glycoprofiling of serum EVs in pancreatic cancer showed a significant elevation of O-glycosylation [35]. Level of O-glycosylated proteins mucin 16 (CA125) was found to be significantly higher in serum EVs of ovarian cancer patients [13]. However, overexpression of mucin O-glycan along with high abundance of sialylated counterparts T, sialyl T, and sialyl Tn antigens is a common trend of glycosylation in cancers [37, 38]. For example, elevated expression of sialyl T, and sialyl Tn antigen could be used as biomarkers for ovarian and gastric cancers, respectively [37, 39]. Similarly, O-GlcNAcylation, *i.e.,* attachment of a single *N*-acetylglucosamine moiety, was found to be elevated on EVs associated with colorectal [36] and breast [40] cancers.

The methods highlighted for analyzing and characterizing EVs glycosylation are mass spectrometry (MS), liquid chromatography (LC), and lectin-based affinity approaches [30, 41–43]. The MS-based methods include matrix-assisted laser desorption-mass spectrometry (DALDI-MS), electrospray ionization-mass spectrometry (EIS-MS), and tandem- mass spectrometry (MS/MS) to detect the structure of a certain glycan. Similarly, chromatographic methods include high-performance liquid chromatography (HPLC) and gas chromatography (GC) for glycan analysis. By getting the advantages of LC–MS/MS and MALDI-TOF–MS techniques, several studies have revealed the detailed structure of glycoconjugates on EVs-derived from cell lines and human biofluids [44–47]. By using these MS and chromatographic methods researchers can gain valuable insights into the composition and structure of EV-glycans (reviewed in [6, 48, 49].

In several studies, after investigation of EV-glycan structures by LC–MS/MS and MALDI-TOF–MS and further validation was conducted by lectin-based techniques [37, 42, 43, 50]. In contrast to MS and LC, lectins can bind and recognize specific glycan motifs without the need for glycan release from samples, liberation or labeling of glycans [51]. Most of the published EV glycan studies have used lectins to detect glycans on the surface of EVs originating from either cell lines or biological fluids [18, 52]. Lectins are carbohydrate binding proteins that recognize specific glycans through their major carbohydrate-recognition domains (CRDs) (Fig. 1). As examples of studies that used lectins, Freitas et al. compared four separation techniques, reporting a diverse set of glycoconjugates on the resulting EVs [37]. They found that lectins E-PHA and L-PHA bind to cancer-related N-glycans, while lectin AAL binds to fucose glycans. In another comparative study, lectin/immune-transmission microscopy (TEM) and ion-exchange chromatography (IEC) were applied to detect differential surface display of sialylated and mannosylated glycan moieties on seminal prostasome EVs of normozoopermic versus oligozoospermic men [41]. Similarly, a study by Surman et al. showed that a panel of lectins can bind specific glycan epitopes on EVs compared to that of the parental cell membranes [53]. In this review, we describe different lectin families, including their structures and CRDs. We review the different sources of lectins, including plants, human and recombinant lectins (Table 2). Moreover, we delineate potential approaches whereby lectin-glycan interactions can be used for the separation of EVs and detection of EV glycans that may serve as diagnostic and prognostic biomarkers.

Cancer biomarker discovery from extracellular vesicles

Novel cancer biomarkers are urgently needed, not least as integrated components of precision and personalized medicine [54]. Several purposes of biomarkers can be envisioned: 1) diagnosis, or detection of cancers, from early to recurrent; 2) prognosis, to anticipate the likely course of disease; 3) personalization, to assign the right therapies to the individual patient; and 4) monitoring, to

assess progression of disease and/or response to therapies. Cancer biomarkers can be protein, DNA, RNA, lipids, carbohydrates, or metabolites which may be changed quantitatively and/or qualitatively during disease. Despite intensive research, only limited numbers of clinically useful biomarkers have been approved by the US Food and Drug Administration (FDA) due to poor sensitivity and specificity. EVs are currently being studied to overcome these limitations, possibly providing novel targets for biomarker discovery (Fig. 2).

EVs are known to occur in different biofluids including urine, blood, saliva, milk, semen, cerebrospinal fluid, and lymph [55]. Secretion of EVs has been found to be higher in cancer patients compared with normal conditions [56, 57]. Similarly, glycosylation patterns of EVs can be different in cancer and non-cancer sources [48]. Some examples of the association between EV glycans or glycoproteins moieties and cancers include: glypican-1(a proteoglycan) [58] and CD133 (prominin-1) [18] for pancreatic; a wide range of N-glycans (bisected, complex, and branched) for prostate, melanoma, and pancreatic [34, 53, 59, 60]; leucine-rich α-2, and α-2-HS-glycoprotein as well as MUC1 for non-small cell lung cancer [61–63]; and LGALS3BP, CD24 and EpCAM for ovarian cancer [15–17].

Starting several decades ago, FDA-approved cancer biomarkers, albeit small in number, have been successfully used in clinics for monitoring, diagnosis, and prognosis of different cancers. Most of these cancer protein markers are either N-or O-glycosylated proteins. Interestingly, the majority of these FDA-approved markers are also found on the surface of EVs derived from different cancers (Table 1).

Lectins and their potential role in EV research

Lectins: discovery and basic properties

Lectins are a diverse group of non-antibody glycan-binding proteins (GBPs) that are found abundantly in nature. (The other group of carbohydrate binding proteins, GAG-binding proteins [93], is not addressed here). Lectins were first discovered in plants by Peter Stillmark, who described them in his 1888 doctoral thesis at the University of Tartu, Estonia (reviewed in [94]. While working with extracts from castor bean seeds, he obtained a protein preparation that was capable of agglutinating animal red blood cells. Over time, seeds of many plants were found to contain such proteins, first dubbed "agglutinin", and later renamed as "lectin". The terminology 'lectin' came from the Latin term 'legere,' meaning to select or to choose [95]. Initially, it was thought that lectins were limited to plant agglutinins [96]. However, discoveries of lectins from animals and microorganisms including bacteria, algae, and fungi [97–99] have led to the understanding that lectins are present in all glycan-synthesizing organisms. All lectins are agreed to have exceptional sugar-binding abilities [100] and to play important roles in biological recognition phenomena at the cellular and molecular levels [101, 102]. Today, lectins are defined in that they "should have a carbohydrate recognition domain and should not modify the binding

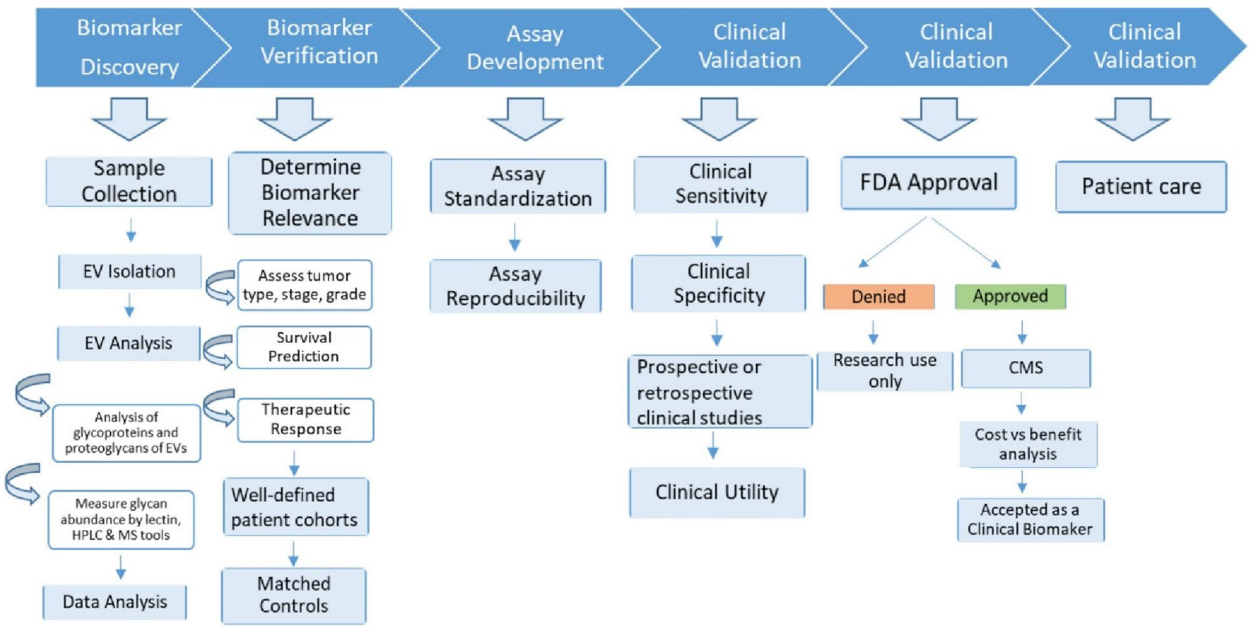

Fig. 2 Model flowchart of biomarker discovery using EVs derived from body fluids

Table 1 EV-associated FDA-approved glycoprotein biomarkers for cancer

FDA approved cancer biomarker used for clinical application						Same biomarker found on EVs			
Biomarker	**Cancer type**	**Sample**	**Glycosylation type**	**Clinical use**	**Year of FDA approval**	**Cancer type**	**EV sample type**	**Publication year**	**Reference**
Alpha-feto protein (AFP)	Testicular	Serum, plasma, amniotic fluid	N-glycosylated	Management of cancer	1992/2008	Melanoma, prostate	Cell lines	2015	[64–66]
CA 15–3 (MUC1)	Breast	Serum, plasma	N- and O-glycosylated	Monitor disease response to therapy	1997	Breast, prostate, bladder	Cell lines, milk, saliva, urine	2004/2007/2009	[67–69]
CA 19–9	Pancreatic	Serum, plasma	Glycan sialyl-Lewis[a]	Monitoring disease status	2002	Pancreatic	Plasma, serum	2017/2020	[70–72]
CA-125 (MUC16)	Ovarian	Serum, plasma	N- and O-glycosylated	Monitoring disease progression, response to therapy	1997/2011	Bladder, breast, colorectal, kidney, melanoma, ovarian	Cell lines, breast milk, serum, ascites, urothelial cells	2009/2011/ 2012/2013/ 2014/ 2016/2017	[73–75]
Carcinoembryonic antigen (CEA)	Colorectal and others	Serum, plasma	N-glycosylated	Aid in management and prognosis	1985	Colorectal, lung	Cell lines, ascites, plasma, urine	2002/2005/ 2009/2011/ 2012/2013/ 2016/2017	[76–78]
Circulating Tumor Cells (EpCAM, CD45, cytokeratins 8, 18+, 19+)	Breast	Whole blood	N-glycosylated	Prediction of cancer progression and survival	2005	Colorectal, ovarian, prostate, kidney, lung	Serum, ascites, urine, thymus, B & T cells, breast milk,	2001/2002/ 2005/2007/ 2009/2011/ 2013/2015/ 2017/2018	[79–82]
Estrogen receptor (ER)	Breast	FFPE Tissue	O-GlcNAc	Prognosis, response to therapy	1999	Breast	Urine	2013	[80]
Human epididymis protein 4 (HE4)	Ovarian	Serum	N-glycosylated	Monitoring recurrence or progression	2008	Colorectal, ovarian	Cell lines, seminal plasma, urine	2009/2013/ 2014/2015	[83–85]
HER2/NEU	Breast	FFPE Tissue	N-glycosylated	Assessment for therapy	1998	Breast, colorectal, ovarian, lung, prostate	Cell lines, ascites, breast milk, serum, urine	2002/2005/ 2009/2010/ 2012/2013/ 2016/2018	[75, 77, 86]
Prostate-specific antigen (PSA)	Prostate	Serum	N-glycosylated	Screening, diagnosis and monitoring	1986/1994/2012	Prostate, bladder	Seminal fluid, urine, colorectal cancer cells	2003/2008/ 2009/2012/ 2015/2018	[83, 87, 88]
Progesterone receptor (PR)	Breast	FFPE Tissue	No glycosylation	Prognosis, response to therapy	1999	Breast cancer	Mesenchymal stem cells	2009	[89]
Nuclear mitotic apparatus protein (NuMA, NMP22)	Bladder	Urine	O-linked glycan	Diagnosis and monitoring disease	1996	Bladder, colorectal, lung, ovarian, prostate	Thymus, urine, urothelial and mesenchymal stem cells	2008/2009/ 2011/2012/ 2014/2015/ 2017/2018	[74, 82, 90]
Thyroglobulin (Tg)	Thyroid	Serum, plasma	N-glycosylated	Monitoring disease	1997	Ovarian	T cells, cerebrospinal fluid, ovarian cancer cells	2013/2014/2018	[84, 91, 92]

carbohydrates" [103, 104]. Lectins are used as reagents for the detection, isolation, and structural studies of glycoproteins [94]. So far, more than 100 plant and mammalian lectins have been used for the detection, enrichment, or characterization of EVs (Supplementary Table 1).

Classification of lectins

Lectins are divided into several families based primarily on binding specificity for specific glycans [104–106], such as but not limited to GalNAc/*N*-acetylgalactosamine, GlcNAc, mannose, sialic acid, fucose, galactose, T antigen, and novel sialyl lewis antigen (Table 2). Specificity is in turn conferred by carbohydrate recognition domains (CRDs) [107], which can consist of either a single or multiple protein subunits. The number of domains attached to CRDs varies among the lectin families: R-, L-, P-, C-, I-, and S-types (Fig. 3).

Several lectins have already been identified on EVs according to the databases Vesiclepedia [108] and ExoCarta [109]. Endogenous tumor lectins are considered as a new class of tumor markers, as they are differentially expressed in tumor vs normal tissues [110]. Moreover, EV-associated lectins play important roles in identifying glycans during tumorigenesis and pathophysiological conditions [111, 112]. However, a wide range of lectins of these families has been used in EV glycosylation detection and profiling (Table 3). Therefore, we now focus on the formation, binding specificity, and biochemical properties of these lectin families below.

R-type lectins

R-type lectins contain an R-type carbohydrate recognition domain (CRD) that is structurally similar to the CRD in ricin. Ricin is known as the first lectin, discovered in *Ricinus communis* (castor bean) [94]. The R-type lectins usually recognize Gal/GalNAc moieties and occur in plants, bacteria, and animals [131]. Examples include *Ricinus communis* agglutinin (RCA-I & II), *Viscum album* agglutinin (VAA), *Sambucus sieboldiana* agglutinin (SSA), *Sambucus nigra* agglutinin (SNA), and *Maackia amurensis* agglutinin (MAA-I&II), which have been used in EV glycome studies [114, 124].

L-type lectins

The L-type lectins are typically found in the seeds of leguminous plants and require Ca^{2+} ions for ligand binding. This group of lectins is distinguished from other types by tertiary structure, consisting of antiparallel β sheets joined by short loops and β-bends and devoid of α -helices [132]. Examples of L-type lectins include concanavalin A (ConA) and phytohemagglutinin E (PHA-E), which are also potential tools for studying EV glycosylation [115, 128].

P-type lectins

There are two members of the P-type family: cation-dependent mannose 6-phosphate receptor (CD-MRP) and cation-independent mannose 6-phosphate receptor (CI-MRP), which can specifically recognize phosphorylated mannose residues [133]. Members of this group typically bind to mannose 6-phosphate (M6P), with the phosphate group being key to high carbohydrate binding affinity. The P-type lectins bring the M6P signal to lysosomes within the cells, resulting in the generation of functional lysosomes [134].

C-type lectins

C-type lectins are usually found on animal cells, including immune cells such as macrophages and dendritic cells. C-type lectins require Ca^{2+} ions for ligand binding and share primary and secondary structural homology in their CRDs. The CRD of C-type lectins is composed of a compact region of 110–130 amino acid residues with a double loop and two antiparallel β-sheets. Members have one or more characteristic C-type lectin-like domains (CTLDs) and have been subdivided into 17 subfamilies based on domain orientation and phylogeny [135]. Several subfamilies are again sub-classified based on common carbohydrate motifs. C-type lectins have a diverse range of functions, including cell-to-cell interaction, immune response to pathogens, and apoptosis [135]. Examples of these lectins include DC-SIGN, MGL, MMR, MBL, and selectins, which have been reported for the characterization of EVs in several studies [136–138]. Furthermore, some C-type lectins have been discovered in EVs [55], such as selectins (subtypes P, E, and L) and collectin (collectin-12) [55, 139–141].

I-type lectins

I-type lectins are members of the immunoglobulin superfamily (IgSF) that typically recognize sialic acids and other carbohydrate ligands. Most members are siglecs, which are type I transmembrane proteins. Siglecs are divided into two groups: one group includes siglec1 (CD22 molecule), siglec4 (MAG), and siglec15, and the other includes siglec3 (CD33 molecule), siglec5-11, and siglec14 [142, 143]. Siglecs are well known for their functional roles in cell–cell adhesion and cell signaling [144]. Because of their specificity for sialic acid, which is highly expressed on certain cancer EVs [15, 34], siglec lectins might be used to detect cancer glycosylations [145] and EV biodistribution [146–148].

S-type lectins (galectins)

S-type lectins specifically recognize β-galactosides with their CRDs and are thus also termed galectins. Galectins are anchored on cells through the interaction of CRDs and

Table 2 Classification of lectins based on glycan epitope

	GalNAc	GlcNAc	Mannose	Sialic acid/NeuAc	Fucose	Galactose	T antigen
Plant lectin	VVL	WGA	PSA	SNA	UEA-I	SBA	ABL
	WFA	PWM	GNA	MAA	TJA-II	RCA	ACA
	PTA	PHA-E	Con A	SSA	AAL	PNA	GS-I
	HPA	GSL-2	LCA	TJA-I	AOL	Jacalin	BS-II
	DBA	DSL	HHL		LTA	GSL-1B	
	BPL	ASA	PMA		PSA	GSL-A4	
	CFL	STA	Calsepa		LCA	GSL-B4	
		PAA	NPA		TxLC-I	ECA	
		RCA			EEA	ACG	
		LEL					
		UDA					
Human lectin		Ficolin 1	MBL	Dectin-1		Galectin 1	MGL
		Ficolin 2	DC-SIGN	Siglec 1		Galectin 3	
		Ficolin 3		Siglec 2		Galectin 5	
				Siglec 5		Galectin 7	
				Siglec 9		Galectin 8	
						Galectin 9	
						Galectin 10	
Recombinant lectin			RPL-αMan	RPL-Sia1	RPL-Fuc1	RPL-αGal	
			RPL-Man2	RPL-Sia2	RPL-Fuc2	RPL-Gal1	
				RPL-Sia3		RPL-Gal2	
						RPL-Gal3	
						RPL-Gal4	

cell-glycoconjugates. Galectins are found not only in mammals but also in birds, fishes, nematodes, sponges, and fungi. Galectins play important roles in immune response, inflammation, and tumor progression and metastasis [149–151]. Several galectin candidates have been identified in EVs derived from cancer cells and biological samples and have been extensively studied in EV research (Table 4). Galectin-1 and -3 have been found in EVs derived from bladder, ovarian, and colon cancer cells [67, 152–154]. Galectin-3 is also found in EVs of human semen [155] and has elsewhere been reported to be involved in polarized EV release [156]. Interestingly, galectin-3 is related to antimicrobial activity against bacteria and fungi [157–159]. Barres el al. reported that galectin-5 on rat reticulocyte EVs was essential for EV uptake by macrophages [112]. Galectin-9 was found on EVs from Epstein-Barr virus-infected nasopharyngeal carcinoma cells [160].

Application of lectins in biomarker discovery

Protein glycosylations can be highly useful in biomarker discovery, including in cancers. Glycosylation patterns may be a reliable signal of cancer [162], and several glycobiomarkers have been discovered that might be clinically useful [163]. Indeed, most cancer markers used in clinics are glycoproteins, and their glycan moieties may be structurally different in cancer [164]. Nevertheless, most FDA-approved cancer biomarkers are monitored at the protein level, i.e., antibodies of immunoassays used for the biomarker detection recognize merely protein-based epitopes. Biomarker detection with protein-specific antibodies can be hampered by moderate specificity and sensitivity, the presence of putative marker proteins in healthy and benign individuals, and even by the possibility that glycoconjugates mask protein epitopes in cancer [165]. As a result, lectins are an attractive option for the identification of altered glycosylation patterns in cancer [166]: they have often high sensitivity and relative specificity and are widely available [105]. Glycans of MUC1 (CA15-3), carbohydrate antigen (CA19-9) and alpha-fetoprotein (AFP) are monitored for management of breast cancer, pancreatic cancer, and hepatocellular carcinoma (HCC), respectively [167]. In HCC, elevated AFP is effectively detected by lectin LCA for early diagnosis [168]. Glycoconjugates on EVs may influence EV release, biodistribution, and uptake, and, as noted previously, can also be used for EV detection and separation with the help of lectins (reviewed in [48,

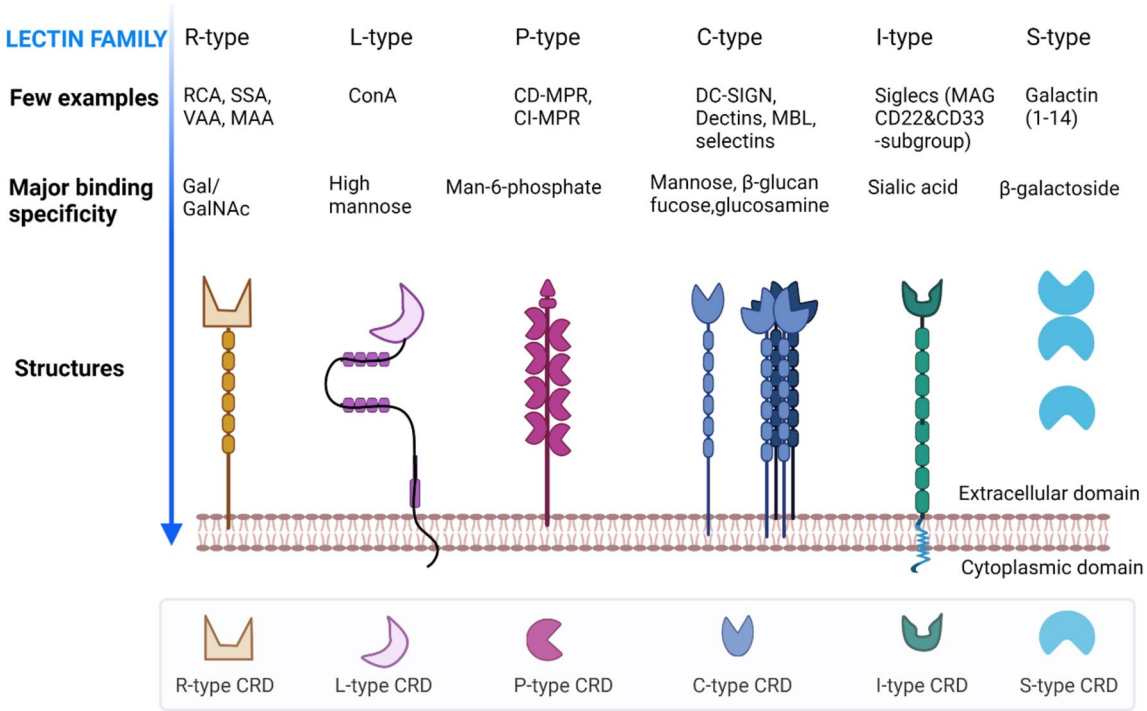

Fig. 3 Six lectin families with representative examples and corresponding glycan-binding sites, CRDs, and structures. Different color CRDs represent variation among six lectin families. The number of domains attached with CRD varies among the lectin members. Figure created using BioRender.com

49]. In sum, lectins are valuable tools for EV biomarker detection and discovery.

Lectin-based advanced methods for studying EVs

A wide variety of lectins and lectin-based assays have been used to profile EVs for biomarker discovery (Table 3). In this section, we address several lectin-based approaches, including lectin microarray [34, 117, 118, 169], immobilized lectin affinity chromatography [30, 51, 113, 170], lectin blotting [39, 114], lectin histochemistry [128], lectin-EV binding method [129, 136], and lectin-based immunoassays [12, 136, 171, 172]. In these methods, lectins target specific glycan moieties of EVs, lending insights into altered glycosylation and tumorigenesis and accelerating biomarker discovery (Fig. 4).

Lectin microarray for EV glycan profiling

Lectin microarray is a popular technology for medium- to high-throughput glycosylation analysis that was first proposed by Kuno et al. [169] in 2005. Lectin microarrays can be used for rapid and highly sensitive profiling of complex structures in both pure and crude glycoproteins [173], avoiding the liberation of glycans. They may have advantages over conventional methods like liquid chromatography (LC) and mass spectrometry (MS), where long branches and diverse structures of glycans create analytic challenges.

Several groups have applied lectin microarrays to EV studies, of which several variations are possible (graphically presented in Fig. 5). Basically, surface-immobilized lectins are used either to capture EVs (which are then detected using one or more labeling methods) or to detect specific glycosylations on EVs that have been captured e.g., by EV-surface protein binding antibodies. Recently, Feng et al. described the use of lectin-mediated in situ rolling circle amplification with an EV array for efficient multiplex detection of EV glycan structures [38]. The first report, in 2009 [118], used lectin microarrays to analyze surface glycans of intact HIV-1 virions and EVs from T-cells, finding a common glycome with enrichment and exclusion of specific glycans. A related study from the same laboratory [34] found that EVs have conserved glycan surface signatures, predominantly consisting of high mannose and complex N-linked glycans, polylactosamine, and α-2,6-sialic acids. Several studies have used lectin arrays to profile EVs from complex body fluids. As an example, Gerlach et al. [117] used a 43-lectin microarray to profile isolated urine (uEVs) and a non-vesicular protein fraction containing THP (Tamm-Horsefall protein). Surface glyco-patterns of uEVs were

Table 3 Lectin-based studies of EVs

Lectin used to detect glycan	Glycan on EV	EV source	Separation method	Glycan profiles/other readout	Profiling method	Ref
PNA, MPA, EEA, MAL-I, MAL-II, AIA, STA	Tn antigen and α2,3-linked sialic acids	Healthy donor urine (uEVs)	IA and UC	Lectin- and antibody-captured uEVs show variations in size and surface glycans	Lectin microarray	[113]
WGA, ECL, AAL, PHA-E, WFA, PNA, ConA, SNA, MAL	T-antigen, N-glycan with bisecting GlcNAc and LacdiNAc structure, and α2,3-linked sialic acids	OVMz ovarian cancer cells	UC	Identified specific glycosignature and blocking of those glycosignature has impact on EV composition	Lectin blotting	[39]
ConA, SNA, MAL	LGALS3BP, complex N-glycans of the di-, tri-, and tetraantennary type with fucose, mannose, and bisecting GlcNAc	SKOV3 ovarian cancer cells	UC	EV internalization by recipient cells	Lectin analysis/blotting	[114]
				Sialoglycoproteins & N-glycans found on the EV surface	Lectin blotting	[15]
PHA-M, ConA	Mannose, GlcNAc, GalNAc, and galactose	PCa cells and urine samples (uEVs)	Lectin agglutination	Lectin-induced agglutination of uEVs shows upregulation of PCa-associated mi-RNA	Lectin-immunoaffinity	[115]
WGA, LEL, STL, RCA, BPL, DSL, CAL, DBL, WFL	GlcNAc, and LacNAc oligomers	Healthy donor urine (uEVs)	IA and UC	lectin-based separation of uEVs shows a simple way of biomarker discovery	Lectin microarray	[51]
A panel of 47 lectins	N-glycans, and terminal sialic acids	Murine hepatic cell lines AML12 and MLP29	UC	Role of surface glycans in EV uptake	Lectin microarray	[116]
AIA, PNA, MPA, ABL, RCA-1	Core fucose and α2,3-linked sialic acids	Polycystic kidney disease (PKD) and healthy donor urine (uEVs)	UC and CF	uEVs glycoprofiling shows distinct patterns in PKD vs healthy individual	Lectin microarray	[117]
AAL, WFA, MAA, SNA	α2-3-linked sialic acids, and core fucosylated N-glycan	Cell lines of human HEK293 and glioma H4, and mouse glioma Tu-2449	UC and CF	structure elucidation and validation of N-glycan of EVs	Lectin blotting	[50]
Gal-1, DSL, ConA, AIA, GNA, NPA, PSA, UDA, HHL, CVN, SVN, GRFT, WGA	High-mannose, LacNAc, complex N-glycans, and fucose	H9, SupT1, Jurkat-Tat-CCR5 cells	UC	Glycomic profiling of HIV, cell membrane, and EVs derived from T-cells	Lectin microarray	[118]
A panel of 74 lectins	High mannose and complex N-linked glycans, polylactosamine, and α-2,6-sialic acids	SkMel-5, HT-29, HCT-15, H9, SupT1, Jurkat-Tat-CCR5, and breast milk	UC	Analyzing conserved glycan patterns of EVs	Lectin blotting and lectin microarray	[34]
PHA-M, ConA	Mannose, GlcNAc, and GalNAc	Blood, urine, ascites, and pleural liquids	UC and lectin agglutination	Lectin-aggregated EVs separation approach and characterized those EVs through proteomic studies	Lectin-induced agglutination, lectin blotting	[119]
GNA, DSA, PNA, SNA, MAA	Oligomannose	Astrocyte-enriched primary cultures (mouse)	UC	Synapsin is an oligomannose-binding lectin and releases from glial-derived EVs	ELISA, lectin staining	[120]

Table 3 (continued)

Lectin used to detect glycan	Glycan on EV	EV source	Separation method	Glycan profiles/other readout	Profiling method	Ref
A panel of 45 lectins	α2-3 and α-2,6-linked sialic acids, fucose, and mannose	ADSC cells	UC	Glycan profiling of EVs and analysis of their uptake in vivo Specific glycan biomarker discovery for osteogenic differentiation	Lectin microarray	[121, 122]
SSA	α-2,6-linked sialic acid	Melanoma cells	UC	Studied EV biogenesis and also identified membrane proteins on EVs as a membrane marker using a lectin	Lectin blotting	[123]
OAA	High mannose	Human glioblastoma, melanoma, colon and lung cancers	UC and lectin agglutination	A mannose binding lectin is used for affinity isolation of tumor derived EVs	Lectin-affinity approach	[30]
MAL-II, SNA, Jacalin, PNA, RCA120, GSA I-B4, DBA, SBA, Con A, sWGA, GSA-II, PSA, UEA-I, LTA	Galβ1, 3GalNAc, high mannose, and α-2,6-linked sialic acid	eAMCs	UC	Comparison of glycan composition on eAMCs and eAMCs-EVs	Lectin array	[124]
PHA-E, PHA-L, AAL	complex N-glycans with β1,6-branched tetraantennary and bisecting GlcNAc, and fucosylation linked (α1-3) or (α1-6)	MKN45 and glycoengineered MKN45 gastric cancer cell lines	UC, TEI, ODG, SEC	Different isolation approach yields distinct glycan associated EVs populations	Lectin blotting	[37]
SNA, ConA, Gal-3	Sialylated and mannosylated glycan structure	Seminal plasma from normozoopermic and oligozoospermic men	UC and gel filtration	Comparative analysis of EVs focusing their glycosylation	Lectin-TEM, and ion-exchange chromatography (IEC)	[41]
A panel of 45 lectins	Glycosylation of CD133 by sialic acids	Ascites samples and cell lines	TEI	Heavily glycosylated CD133 on EVs could be used as a potential biomarker for advanced pancreatic cancer	Lectin microarray	[18]
MAA, SNA, GNA, AAA, PHA-E and PHA-L	α-2,3 and α-2,6-sialic acids, fucoses, complex N-glycans with β1,6-branched tri/tetraantennary and bisecting GlcNAc	Primary WM115, WM793 and metastatic WM266-4, WM1205Lu melanoma cells	UC	EVs have specific glycan composition compared with their parental cell membrane fraction	Lectin-blotting	[53]
A panel of 48 lectins	High mannose, Galβ (1 → 3)-GalNAc, LacNAc, and GlcNAc	Adult helminth pathogen *F. hepatica*	UC	EVs released by parasite can interact with recipient host cells	Lectin microarray	[125]
SNA, Jacalin, PNA, HPA, UEA-I	Siaα-2,6Gal/GalNAc, fucose, T and Tn antigens	PANC-1 and HeLa cells	UC	Multiplexed detection of EV glycan pattern	Lectin array	[38]

Table 3 (continued)

Lectin used to detect glycan	Glycan on EV	EV source	Separation method	Glycan profiles/other readout	Profiling method	Ref
A panel of 96 lectins, lectin rBC2LCN assay specific to EVs	Fucα1-2 Galβ1-3 GalNAc, Siaα-2,6 and LacNAc	hiPSCs and non-hiPSCs	Magnetic bead-based IA approach	Glycan profiles of EVs derived from stem cells	Lectin microarray and sandwich assay	[126]
A panel of 45 lectins	Galα1-3 GalNAc (T-antigen), and Siaα-2,3 Galα1-3 GalNAc (sialyl T-antigen)	Pancreatic cancer patient serum	Magnetic bead-based IA approach	Differential glycomic profiles of EVs and recognition of O-glycosylated EVs for pancreatic cancer detection	Lectin microarray	[35]
DBA, ECA, PHA-E4, MAM, WGA, ConA	GalNAc, GlcNAc, high-mannose, and Siaα2-3Gal	MDAMB231luc—D3H1 &-D3H2LN, and BMD2a breast cancer cells	UC	Identified altered glycosylation patterns on EVs which has inhibitory effect on EVs uptake	Lectin blotting	[127]
PHA-E	N-glycans with bisecting GlcNAc	MCF10A, MCF7, SKBR3, and MDAMB23 cell lines	UC	Modification of bisecting GlcNAc level on EVs has significant effect on metastasis of recipient cells	Lectin histochemistry	[128]
ConA, WGA, RCA, SNA, MAA, PHA-E, PHA-L, LCA, PNA, VVL, DBL	High-mannose, Siaα2-3Gal, and GlcNAcβ1.4	Human urine (uEVs)	UC	uEVs isolation from THP-depleted urine and use those uEVs for lectin-EV assay	Lectin-EV binding assay	[129]
AAL, SNA, PHA-E, PHA-L	Bisected and branched N-glycans, Fucα1-6GlcNAc, and α2,6-linked sialic acid	B16F10, Pan02, 4T1, AsPC1, MADAMB4175 cell lines	Asymmetric flow field-flow fraction (AF4)	Identification of EVs and their subpopulation	Lectin blotting	[43]
A panel of 34 lectins	Fucα1-2Gal, GalNAc, GlcNAc, mannose, and sialic acid	PCa cell line and bladder cancer patient urine (uEVs)	UC	Integrin-lectin assay significantly discriminate bladder cancer patients compared to benign control	Lectin-nanoparticle assay	[12]
SNA, ConA, AAL	α2,6-linked sialic acid, Fucα1-6GlcNAc, and mannose	Pancreatic cell line and patient serum	UC	Lectin conjugated janus nanoparticles (JNPs) specifically binds to glycans on pancreatic cell derived EVs and shows diagnostic potential	JNPs assisted- lectin-EV assay	[130]
A panel of 50 lectins	High-mannose, GlcNAc, GalNAc, and sialic acid	Helminth parasite *F. hepatica*	UC	Parasite EVs has influence on host-immune response during infection and glycosylated proteins of these EVs play role in EVs internalization	Lectin microarray	[42]

UC ultracentrifugation, *IA* immunoaffinity, *CF* centrifugal filtration, *SEC* size exclusion chromatography, *TEI* total exosome isolation kit, *ODG* optiPrepTM density gradient, *ADSC* adipose derived mesenchymal stem cells, *eAMCs* Equine amniotic mesenchymal cells

GlcNAc = *N*-acetylglucosamine, GalNAc = *N*-acetylgalactosamine, Gal = galactose, Tn = T antigen, sTn = sialyl-Thomsen-nouveau antigen, LacNAc = *N*-acetyllactosamine

Table 4 Galectin-associated EV studies

Lectin	EV source	Separation method	Readout	Method	Ref
Galectin-3	Melanoma SK-Mel-5 cells	UC	Complex N-glycans mediate protein shorting to EVs	Lectin blotting	[161]
Galectin-5	Rat reticulocytes	UC	Galectin-5 has effect on EVs uptake by macrophages	Galectin blotting	[112]
Galectin-3	MDCK kidney cells	UC	Mechanism of galectin-3 delivery into ILVs for exosomes release	Galectin blotting	[156]
Galectin-3	Mice, *C. neoformans* strain, and patient serum	UC	Gal-3 inhibits fungal infection through direct antifungal effect	ELISA	[158]
Galectin-3	Mice, *P. brasiliensis* strain, and patient serum	UC	Gal-3 inhibits fungal growth through multiple mechanism	Galectin blotting	[159]
Galectin-3	Human semen	UC	Expression of gal-3 on human semen EVs	Galectin blotting	[155]
Galectin-9	Epstein-Barr virus (EBV)-infected NPC cells	UC and magnetic bead-based IA	EVs from EBV-infected tumor cells contain gal-9 protein	Galectin blotting	[160]

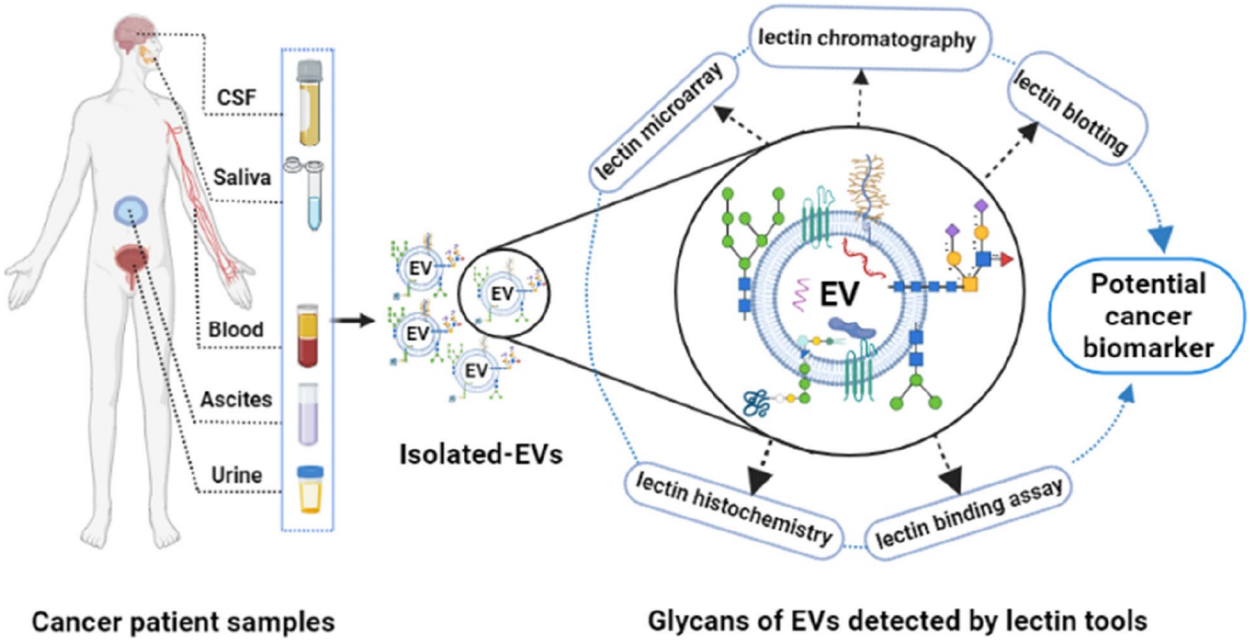

Fig. 4 Overview of lectin-based tools for studying surface glycans on various biofluids derived EVs

distinct compared with the THP fraction, and binding proficiency of lectins to the THP fraction was limited. Furthermore, uEVs from patients of autosomal dominant polycystic kidney disease (ADPKD) were compared with those of age-matched healthy subjects to seek biomarkers of ADPKD.

There are numerous additional examples of lectin microarrays being used for discovery of cell and disease markers In advanced pancreatic cancer, lectin microarray was used to reveal highly glycosylated CD133 as a prognostic marker on EVs from malignant versus non-malignant ascites [18]. In another study, using serum of pancreatic patients, A 45-lectin microarray identified six lectin candidates, including ABA and ACA, that differentiated O-glycosylated EVs of pancreatic cancer patients from those of the controls [35]. Importantly, ABA- and ACA-positive EVs were detectable in serum even when commercial pancreatic cancer serum marker CA19-9 was negative. Moreover, Bertokova et al. showed the potential of lectin fluorescent microarrays for the analysis of glycans of EVs from prostate cancer cells [174]. Saito

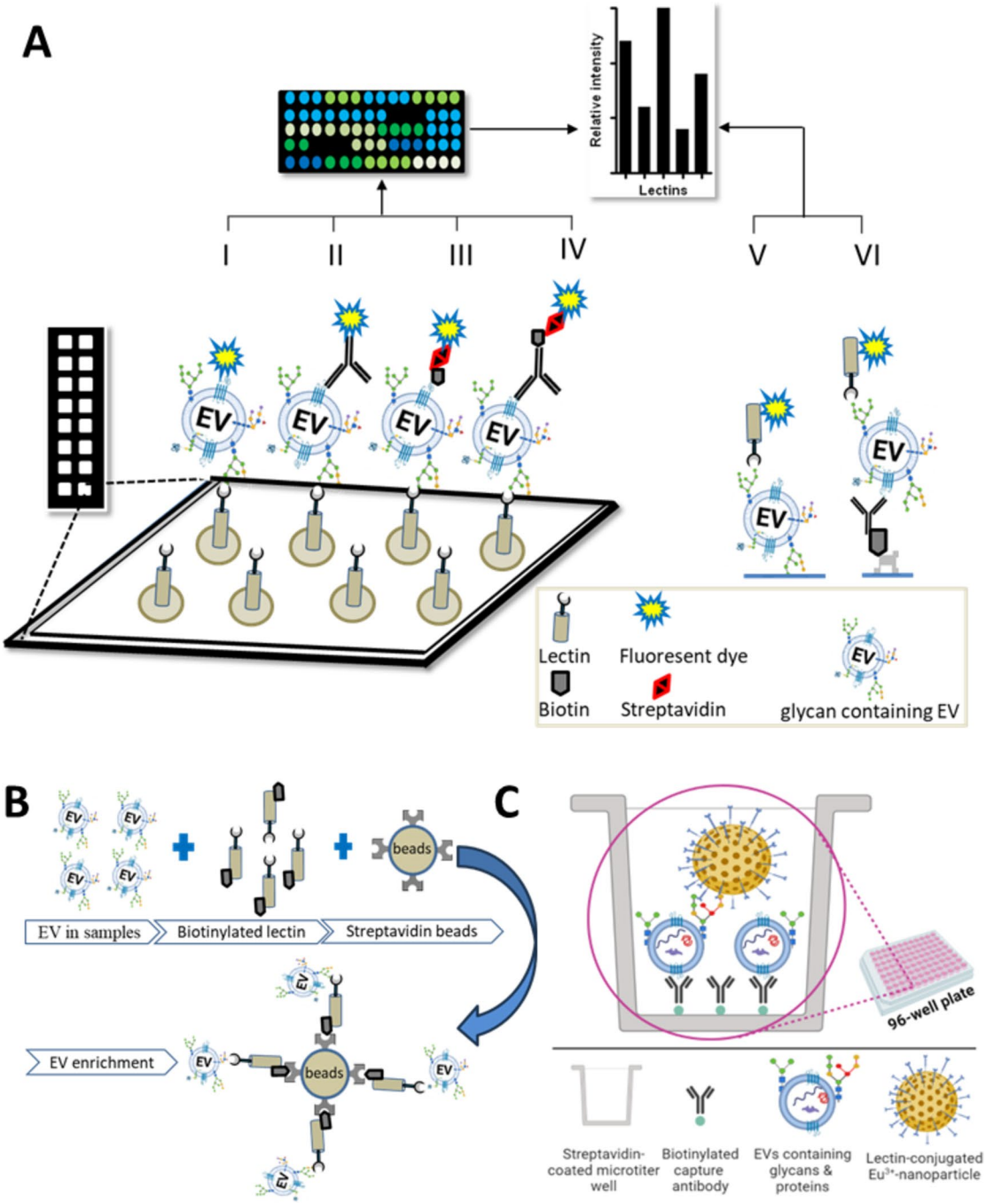

Fig. 5 Lectin-assisted approaches for studying EVs. **A** (I-VI) Schematic of lectin microarray approaches. In I-IV, lectins are immobilized onto a surface, e.g., a glass plate. For detection of captured EVs, labels might include I) a fluorescent dye that directly labels the EV, II) a fluorescently labeled antibody to an EV surface antigen, III) fluorescently labeled streptavidin that recognizes biotinylated EVs, and IV) a biotinylated antibody-fluorescent streptavidin system. Systems other than streptavidin–biotin could also be used. In V-VI, EVs are V) directly immobilized or VI) captured by immunoaffinity followed by detection with lectin(s). **B** Graphical representation: separating pure EVs using a lectin-bead technology. **C** Schematic representation of Eu^{3+}-nanoparticle based sandwich assay. Biotinylated antibody is immobilized on the surface of streptavidin-coated microtiter 96-well plates for capturing EVs. The captured EVs are detected with lectin-coated fluorescent Eu^{3+}-nanoparticle

Table 5 Advantages and disadvantages of lectin-based detection approaches in cancer biomarker discovery

Tools used	Advantages	Disadvantages
Lectin arrays	■ rapid and highly sensitive ■ allow high throughput screening ■ allow multiplexing ■ do not require sample preparation ■ use small amount of samples ■ unbiased binding to glycans	■ non-specific binding may happen ■ need extensive optimization
Lectin blotting	■ easy to use ■ reproducible ■ highly sensitive and specific ■ suitable for complex protein samples ■ visualize small amounts of antigens	■ membrane may affect chemical stability of lectins ■ non-specific binding may happen
Lectin affinity chromatography	■ produce pure analyte ■ high affinity ■ do not require sample preparation ■ do not need purified glycans	■ may co-elute other proteins ■ need large amounts of samples ■ non-specific binding may happen ■ time-consuming
Lectin-binding assay	■ simple and rapid ■ very cost effective ■ easy to perform ■ requires minute amounts of samples ■ high sensitivity and specificity	■ purified glycan may require as a standard ■ non-specific binding may happen

et al. found EV glycosylation patterns specific to human induced pluripotent stem cells (hiPSCs) using a panel of 96 lectins [126]. Lectin rBC2LCN bound to hiPSCs-EVs but not to EVs from control cells. Using rBC2LCN in combination with phosphatidylserine receptor Tim4 (rBC2LCN-Tim4), they developed a sandwich assay that was superior to a previous antibody-based assay (Tim4-CD63). Desantis et al. used 14 lectins to characterize amniotic mesenchymal cells and their EVs [124]. To identify markers of therapeutic EVs, Hayashi et al. used a 96-lectin array, reporting that fucose-specific TJA-II distinguished therapeutic MSC-EVs [175].

From lectin profiling to function: the case of cellular uptake Lectin arrays have been used to understand not only EV glycan profiles but also to probe EV biogenesis and uptake. Shimoda et al. isolated EVs from human adipose derived mesenchymal stem cells (ADSC) for glycan profiling using a 45-lectin evanescent-field fluorescent (EFF) array [121]. The greatest signal intensities were associated with polylactosamine-binding lectin (STL, UDA, and LEL), GlcNAc-binding lectin (WGA), and Gal-binding lectin (DSL). Specific siglecs (-1, -2, and -3) preferentially bound to ADSC-EVs (vs corresponding cell membrane) by recognizing sialic acid residues (a2-3, a2-6), suggesting the possibility of sialic acid involvement in biogenesis and cellular uptake. The EFF-lectin array method was also used to compare EVs of osteogenically differentiated and undifferentiated MSCs [122]. Lectins such as BPL, ECA, SBA, and WFA bound more strongly to EVs of differentiated cells, indicating that EV glycans may discriminate cellular differentiation and cancer stages. The same approach was used to establish that EV glycans define EV heterogeneity and influence biodistribution and cellular uptake efficacy [24]. The authors highlighted that glycoengineering of EV could be used to manipulate EV-cell interactions. Williams et al. added support for the role of glycans in EV uptake, comparing EV surface glycans from two murine hepatic cell lines using a 47-lectin microarray [116]. Clos-Sansalvador et al. used a 26-lectin array and PNGase-F treatment to investigate the importance of MSC-EV N-glycans in EV-endothelial cell interactions and EV uptake [176]. Together, these studies emphasize that EV glycans may have functional consequences, opening the door for glycoengineering of EVs and novel therapies.

EVs of non-human organisms have also been studied by lectin microarray. EVs were isolated from the helminth parasite *F. hepatica* [42, 125, 177] and studied with a 50-lectin microarray for the characterization of surface glycan topology. Among the 50 lectins, mannose-binding and complex type N-glycan-binding lectins showed the highest binding intensities. Furthermore, a total of 618 proteins were identified by proteomic analysis, among which 121 and 132 proteins contained putative N-and O-linked glycosylation sites, respectively [42]. These surface glycans thus hold potential for biomarker development in infectious diseases.

Lectins for EV separation

EV separation techniques remain a center of attention, with ongoing debate about how these techniques affect our conclusions about EV biogenesis, release, uptake,

and roles in disease development. Some traditional techniques for EV separation may be tedious, labor-intensive, and costly, and certain techniques may result in EV aggregation or damage or substantial presence of co-isolates that confound interpretation [178, 179]. For glycosylation studies, legacy separation methods do not yield subpopulations based on glycosylation [37]. To overcome these limitations, lectin-based affinity capture has gained popularity. Echevarria et al. first reported a lectin-based approach in 2014 to separate EVs from urine [51]. Of 62 screened lectins, STL, WGA, and LEL showed significant binding to uEVs, with STL having the highest affinity and also avoiding THP binding. A capture method was then devised, using a biotin-streptavidin and magnetic bead approach (graphically presented in Fig. 5B). A galectin-coupled magnetic bead approach was used to isolate pure EVs from human plasma for head and neck cancer biomarker discovery [180]. Interestingly, Gerlach and colleagues reported that lectin separated EVs had greater purity than EVs prepared by other methods [113]. Another study showed that EVs isolated by lectins (PHA-M and Con-A) from different biological fluids could be used to validate EV compositional studies [119]. Similarly, Ward et al. (mentioned in [113]) proposed uEV isolation by MAL-II, WGA, STA, and LEL lectins, while Samsonov et al. captured EVs with Con-A lectin and performed RNA analysis for prostate cancer diagnosis [115]. Taken together, lectin affinity capture is useful for enriching specific subsets of EVs, including in clinical applications.

Since high mannose-type glycans are highly enriched in tumor-derived EVs [34, 170], the Maruyama group developed a mannose-glycan-based isolation technique for tumor-derived EVs using a high mannose-type glycan-binding OAA lectin [30]. They showed that mannose-binding OAA lectin captures small EVs from different tumor cells, such as glioblastoma, melanoma, and colon and lung cancers. Their findings showed that N-linked glycans allow high-affinity capture of tumor-derived EVs.

Furthermore, Kanao et al. studied EV separation based on their surface glycans and revealed the difference of protein contents in EVs [181]. Interestingly, in their lectin-based EV separation method, apart from using a typical agarose gel, they used a sponge-like monolithic polymer (SPM) which has large flow-through pores that ensure high EV recovery.

Lectin blotting

Lectin blotting uses lectins to detect glycosylation on proteins or lipids that have been separated by gel electrophoresis (SDS-PAGE) and transferred to adsorbent membranes. The Costa group used lectin blotting to profile EVs and parent cellular extracts, finding EV enrichment with specific sialic acid and mannose-containing glycoproteins [114]. Sialoglycoproteins that were identified on EVs from ovarian carcinoma cells were subsequently confirmed by lectin blotting [15, 39]. Furthermore, N-glycans from cell lines derived EVs were analyzed by MALDI-TOF mass spectrometry and HPLC and subsequently validated by lectin blotting [50]. Zhang et al. applied asymmetric flow field-flow fraction (AF4) to obtain three size-separated extracellular particle populations including EVs, using lectin blotting to show that these populations displayed distinct N-glycan and sialylation patterns [43]. In another study, Nishida-aoki et al. found differential glycosylation patterns on EVs from breast cancer cell lines [127]. Using lectin blotting, they demonstrated that removal of O-and/or N-glycosylation from the surface of EVs has inhibitory effects on EV uptake. Similarly, alteration of complex N-glycans could control the recruitment of specific glycoproteins (e.g.-EWI-2) into EVs [161]. In another study, Tan et al. showed that modification of bisecting GlcNAc can suppress metastasis induced by EVs from breast cancer cells [128]. Using lectin blotting by PHA-E lectin that specifically binds to bisecting GlcNAc, they found that bisecting GlcNAc levels were significantly lower in human breast cancer cells compared to heathy controls [128, 182].

The presence of several cancer-associated glycoprotein biomarkers has been confirmed in various EVs subpopulations [48, 49]. Kondo et al. used lectin blotting by SSA and WGA to identify distinct patterns of N-glycosylation on small EVs of small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC) cells [183]. They also found a molecular link between lung cancer types and integrin N-glycosylation of small EVs. Recently, blotting with six lectins was done in a study that reported the presence of disease-associated glyco-epitopes in bladder cancer-derived EVs [184]. In another study, after 20-lectin microarray identified altered glycans on EVs from gastrointestinal cancer, findings were validated using lectin blotting [185].

Lectin-nanoparticle assays

It is already established that lectin can easily conjugate with nanoparticles, which can be used for targeted detection of biomolecules [186]. Based on this approach, Choi et al. developed nanoparticle assisted microfluidic device that can detect cancer-derived EVs following lectin-glycan interaction, which could discriminate pancreatic cancer EVs from those of the control sources [130]. Particularly, lectins with specific affinity, for sialic acid such as lectin SNA and fucose such as AAL, were attached to bifunctional Janus nanoparticles (JNPs), which facilitated binding to EVs in the microfluidic device. Moreover, lectin-conjugated JNPs

successfully captured EVs from pancreatic cells as well as serum samples with high affinities that were comparable to those of anti-CA19-9 antibody. This platform holds the possibility to achieve new biomarker discovery targeting glycan moieties of EVs derived from pancreatic cancer sources.

Our group developed a fluorescent europium nanoparticle (Eu^{3+}-NP)-assisted lectin approach for the detection of glycan on EVs. The lectins coated on polystyrene nanoparticles of ca. 100 in diameter and containing ca. 30000 Eu^{3+} ions per particle for time-resolved fluorescence-based detection [136]. Due to the presence of a number of lectins per nanoparticle, NP can give avidity effect in binding which helps to overcome potential issues related to the limited binding affinity of individual lectins [136, 187]. Unlike antigen–antibody binding affinities (dissociation constant, $K_d = 10^{-8}$-10^{-12} M), lectin-glycan affinities are much lower ($K_d = 10^{-4}$-10^{-7} M) [188]. Altogether, the NP-aided tool confers a highly sensitive time-resolved fluorescence-based detection in a simple two-step sandwich assay (schematic representation in Fig. 5C), and shows improved performance compared to conventional europium chelate labeled lectin reporters [136]. Furthermore, based on the developed assay platform, we have identified a glycovariant of ITGA3 on urine of bladder cancer (BlCa) patients which could be used for BlCa detection [189]. In a follow-up study, aiming to construct the further improved lectin assay for the specific detection of EVs, we have tested a panel of 34 lectins among which a fucose binding lectin UEA showed strong binding intensities toward EVs. This lectin-nanoparticle assay further was validated with a small cohort of clinical samples from urological malignancies, where we observed that lectin assay could discriminate bladder cancer patients compared to clinically challenging benign prostate hyperplasia and healthy individuals [12]. Following a similar approach, in another study, we tested 27 lectins for the detection of EVs from breast cancer cell culture medium. Among 27 lectins, fucose-binding lectin AAL and UEA showed strong binding to EVs from media of three breast cancer cell lines compared with one control cell culture medium [172].

Challenges and future directions

The EV field has grown rapidly in the last decade [190], and although significant progress has been made in EV-based cancer biomarker discovery [191, 192], translating these findings into clinical practice (including therapeutics) faces several challenges. These include the technical challenges of EV separation and detection and the need for more insights into molecular mechanisms governing EV release and EV uptake by target cells. In each of these areas, we submit that glycosylation holds a key to progress and that lectin-based approaches, with their specificity and ease of implementation, will unlock doors.

EV surface glycosylation patterns are a veritable hidden treasure for the discovery of disease biomarkers and disease drivers. Progression and development of cancers in particular are associated with aberrant glycosylation, and these altered patterns are also transmitted on cancer derived EVs [48]. In this review, we have examined the utility of lectin–based tools and strategies to identify EV glycovariant markers in cancer and beyond. A good number of innovative lectin-based methods have already been reported for studying glycosignatures of EVs (Table 3). We provide a comparative summary of advantages and disadvantages of lectin-based detection approaches (Table 5). Because of the wide variety of lectins that are available, there is also the possibility of using a combination of markers when one does not suffice [193].

Though lectins effectively recognize and bind the fine glycan structures, they cannot provide sufficient information regarding the glycan components and glycan types such as specific monosaccharides and linkages present in the glycan chain [194]. Some lectins have overlapping binding affinities and specificities towards multiple glycan structures which lead to difficulties in precisely identifying the glycan structures of interest [195, 196]. It is also known that the recognition of lectins to glycan structures depends on the appropriate orientation of glycans. Changes in the glycan conformation may also influence the lectin binding which leads to false-positive or false-negative results. Sometimes, glycan structures can be masked or hidden by other glycans or biomolecules which may prevent lectins from binding to the target epitopes [197]. Additionally, the binding of lectins towards glycans may be influenced by the surrounding microenvironment such as temperature, presence of ions, and pH [198]. This factor may affect the robustness and reproducibility of lectin-based assays. Despite these limitations, lectins and lectin-based methods remain valuable tools in glycan research. To overcome some of these challenges, scientists often use lectin tools in combination with other techniques such as MS and HPLC for a better understanding of detailed glycan structures and their applications. Moreover, advances in synthetic glycobiology and glycan array technology are constantly improving our ability to study glycan interactions and their functions in a systematic way.

To ensure accurate and reliable results from lectins and their carbohydrate interaction, it is essential to verify this interaction through a) competitive inhibition, b) through modification of the carbohydrate structures (i.e., by oxidation or enzymatic means) or c) by comparison with lectins which have been modified to disrupt their carbohydrate-binding domains [34, 117, 199]. This

combination approach is crucial when studying lectins and their biological roles, as it helps to avoid false positive or negative results and provides concrete evidence for carbohydrate-mediated binding [199, 200].

Several studies have reported that glycans found on EVs are to some extent different compared to their parental cell membrane-associated glycans [53, 118]. In another study, as expected, glycan profiles of plasma-derived EVs are distinct from donor-matched whole plasma [201]. However, we have found limited studies where comparisons between glycans on EVs vs tissue samples are addressed. In a recent study, glycan-associated proteins such as CD147, BGN, VCAN, and TNC were found to be enriched in tumor EVs compared to EVs secreting from non-tumor adjacent tissues, which can be potentially used as cancer EV biomarkers or even to identify the cancer origin [202]. In another study, using chemical staining on lectins ABA and ACA, authors showed that O-glycans expression is not only different on normal vs tumor tissues but also on the EVs surface [35]. In the same study, they demonstrated that ABA- and ACA-positive EVs were significantly increased in the serum of 117 pancreatic patients compared to 98 normal controls with area under curve (AUC) values 0.838 and 0.810, respectively of the ROC curve. Though this study warrants validation with a larger cohort of clinical samples, these specific lectins (ABA and/or ACA) have the potential to be developed into a diagnostic test for the early detection of pancreatic cancer. Similarly, we have developed ITGA3-UEA assay where fucose binding lectin UEA can detect the aberrant fucosylations of ITGA3 + EVs which could facilitate the detection of bladder cancer [12]. These findings are definite examples of detection of EV-glycans with lectins and their potential in diagnostics.

Several cancer markers that are based on glycoproteins are already approved and routinely used; since these proteins are also found in EVs (Table 1), it stands to reason that altered EV glycosylation could emerge as a powerful tool for the diagnosis of cancer. Hence, this review has highlighted that lectin could serve as an effective tool for screening glycan-specific EV cancer biomarkers. Moreover, lectin-based approaches will be instrumental in developing EV-based therapeutics by advancing our understanding of EV glycobiology in disease.

EVs are heterogenous populations of nano-sized membrane vesicles that display glycans on their surface, some of which may be changed quantitatively and/or qualitatively during disease. Lectins can be used to recognize EV glycans and monitor disease-related changes, e.g.—in cancers. To realize the benefits of lectins, we recommend further research to assemble versatile panels of lectins to identify specific and sensitive EV-based biomarkers, especially for cancers.

Abbreviations

ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
ADPKD	Autosomal dominant polycystic kidney disease
ADSC	Adipose-derived stem cells
CA 19-9	Carbohydrate antigen
CEA	Carcinoembryonic antigen
CD-MRP	Cation-dependent mannose 6-phosphate receptor
CI-MRP	Cation-independent mannose 6-phosphate receptor
CRDs	Carbohydrate-recognition domains
CTLDs	C-type lectin-like domains
DALDI-MS	Matrix-assisted laser desorption-mass spectrometry
EFF	Evanescent-field fluorescent
EIS-MS	Electrospray Ionization-mass spectrometry
EpCAM	Epithelium cell adhesion molecules
Eu^{3+}-NP	Europium nanoparticle
EVs	Extracellular vesicles
FDA	Food and Drug Administration
GAG	Glycosaminoglycan
GalNAc	*N*-acetylgalactosamine
GBPs	Glycan-binding proteins
GC	Gas chromatography
GlcNAc	*N*-acetylglucosamine
GPI	Glycosylphosphatidylinositol
HCC	Hepatocellular carcinoma
hiPSCs	Human induced pluripotent stem cells
HPLC	High-performance liquid chromatography
IEC	Ion-exchange chromatography
IgSF	Immunoglobulin superfamily
ITGs	Integrins
JNPs	Janus nanoparticles
LC	Liquid chromatography
MS	Mass spectrometry
MUC	Mucins
TEM	Transmission electron microscopy
SCLC	Small-cell lung carcinoma
THP	Tamm-Horsefall protein

Supplementary Information

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Additional file 1: Supplementary Table 1. Lectin used in EVs study and their major glycan specificities.

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Authors' contributions
M.K.I- searched the literature, conceptualized, and wrote the manuscript. M.K, K.G-revised the manuscript. K.W, U.L, and J.L-edited and critically revised the manuscript and finally approved the work. All authors read and approved the final manuscript.

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