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Chapter

Extracellular Vesicles and Their Interplay with Biological Membranes

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Abstract

Most cells secrete vesicles into the extracellular environment to interact with other cells. These extracellular vesicles (EVs), have undergone a paradigm shift upon the discovery that they also transport important material including proteins, lipids and nucleic acids. As natural cargo carriers, EVs are not recognised by the immune system as foreign substances, and consequently evade removal by immune cells. These intrinsic biological properties of EVs have led to further research on utilising EVs as potential diagnostic biomarkers and drug delivery systems (DDSs). However, the internalisation of EVs by target cells is still not fully understood. Moreover, it is unclear whether EVs can cross certain biological membranes like the blood-brain barrier (BBB) naturally, or require genetic modifications to do so. Hence, this review aims to evaluate the relationship between the composition of EVs and their association with different biological membranes they encounter before successfully releasing their cargo into target cells. This review identifies specific biomarkers detected in various EVs and important biological barriers present in the gastrointestinal, placental, immunological, neurological, lymphatic, pulmonary, renal and intracellular environments, and provides a recommendation on how to engineer EVs as potential drug carriers based on key proteins and lipids involved in crossing these barriers.

Keywords: biological barriers, diagnostic biomarkers, drug delivery, engineering, extracellular vesicles, bioengineering

1. Introduction

Extracellular vesicles (EVs), phospholipid bilayer-enclosed vesicles consisting of proteins, lipids and nucleic acids, were once thought of as merely how cells may discard their waste materials and debris. However, recent discoveries have proven them to be indispensable to cells even in normal physiological functions and as diagnostic biomarkers for various diseases [1]. EVs are secreted by various cells and can be isolated from diverse biological sources like saliva, breast milk and blood serum [2].

Over the years, EVs have been researched as promising diagnostic biomarkers for pathological conditions. This is because their concentration and composition correlate with disease progression, a unique characteristic that sets them apart from other types of paracrine secretions [3, 4]. EVs have also been explored as possible carriers for

drug delivery. Recent studies have shown promising results regarding the utilisation of EVs as drug delivery systems (DDSs) to treat various conditions, such as cardio-vascular diseases [2, 5], osteoporosis [2, 6] and brain tumours [2, 7]. In light of this, EVs are seen as a more desirable strategy for drug delivery compared to other conventional nanoparticles like liposomes, micelles and polymeric nanoparticles [8, 9]. Conventional DDSs have been extensively used for their ability to protect drugs from inactivation in the external environment. However, plasma proteins risk adsorbing onto the surfaces of these non-EV nanoparticles upon injection into the body, making them an easy target of immune cells and decreasing their uptake by their target cells [10]. Although these nanoparticles may undergo modification to avoid immune cell removal, they still lack biocompatibility due to their non-biological origins. EVs, on the other hand, can evade phagocytosis by immune cells naturally, in addition to being highly selective for designated target sites, due to their biological origins and cell-specific surface properties inherited from the parent cells that secrete them.

Although EVs are promising in their diagnostic and therapeutic applications, it is still unclear whether they can cross membranes like the blood-brain barrier (BBB) naturally or when genetically modified, or only when the membranes become more permeable in certain conditions like injury [11, 12]. Furthermore, the uptake of EVs by target cells is still not fully understood at a microscopic level, be it *via* endocytosis, membrane fusion or other mechanisms [3]. The ability to pass through biological membranes is an important factor to consider when engineering EVs to deliver drugs to specific cells. As there remains a lack of understanding on how EVs can cross significant biological membranes before reaching their target sites, this review aims to identify potential key proteins and lipids that play a dominant role in the functions of EVs, and evaluate the relationship of these key components on EVs with different biological membranes, so that a recommendation can be given on how to best engineer EVs as potential drug carriers.

2. EVs—classification and key components

Classified by their biogenesis, size, morphology and function, there are three main EV categories—exosomes, microvesicles and apoptotic bodies (**Figure 1**) [16–18].

Although exosomes, microvesicles and apoptotic bodies are distinct from one another, there is a partial overlap among their respective size range and composition. Although many different methods have been previously deployed to isolate EVs from their sample sources (a notable example being ultracentrifugation in isolating and purifying exosomes and microvesicles [19–22]), these methods are unable to provide an accurate attribution of unique characteristics to each EV category. This is due to the complex nature of EVs, such that different size ranges can be derived from the same EV source depending on the isolation technique used [23]. As such, this review will mainly elaborate on EVs in general, unless otherwise stated.

Apart from biogenesis, size and morphology, each EV category possesses its own unique set of key proteins, lipids and nucleic acids (**Table 1**). Being able to differentiate EV categories based on their key components is vital in understanding their specific roles in both normal and pathological conditions. In general, all EVs possess cell adhesion proteins [13, 14, 17, 18, 24–28], heat-shock proteins [13, 14, 18, 25, 28–30], biogenesis-associated proteins [13, 14, 17, 18, 24, 25, 28], fusion proteins [13, 14, 18, 25], cell-type specific proteins [13, 14, 18, 27, 28], cytoskeletal proteins [13, 18], signalling molecules [13, 14, 28, 31], enzymes [13, 25, 28], messenger ribonucleic acid (mRNA), micro ribonucleic acid (miRNA), non-coding ribonucleic
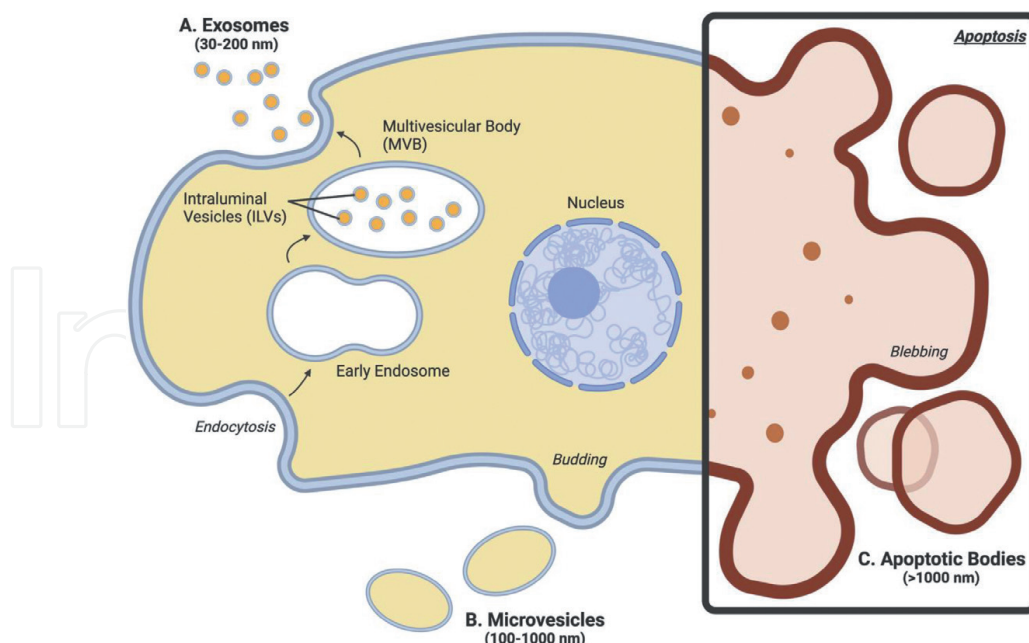
Figure 1.
Biogenesis, size, morphology and function of exosomes, microvesicles and apoptotic bodies. (A) **Exosomes** *(spheroid shape, 30–200 nm) are mainly involved in regulating intercellular communication. Their formation begins when the plasma membrane undergoes endocytosis to generate an early endosome. Intraluminal vesicles (ILVs) within the endosome are then formed from the inward budding of the endosomal membrane, resulting in a multivesicular body (MVB). This process is facilitated by either endosomal sorting complex required for transport (ESCRT)-dependent or -independent mechanisms [1, 13]. The MVB finally fuses with the plasma membrane to release the ILVs as exosomes. (B)* **Microvesicles** *(irregular shape, 100–1000 nm), like exosomes, also regulate intercellular communication. They are formed via budding from the plasma membrane directly without going through endocytic processes. (C)* **Apoptotic bodies** *(variable shape, usually >1000 nm but can be as small as 50 nm [14, 15]) are formed only during cell apoptosis, during which the post-apoptotic cell bulges outwards to form vesicles for easier removal by macrophages (created with BioRender.com).*

acid (RNA), phosphatidylethanolamine, sphingolipids and higher levels of phosphatidylserine (PS) than the cell plasma membrane [24, 25, 28, 35].

The distinct protein, lipid and nucleic acid profiles of each category might be correlated with its formation processes and functions. Both exosomes and microvesicles consist of key protein components which are responsible for cell-to-cell communication [18], such as glycoproteins [18, 28], membrane signalling receptors, growth factors and cytokines [18, 25], while apoptotic bodies do not. This is most likely because exosomes and microvesicles are meant to reach target cells, while apoptotic bodies are merely the means for discarding dead cells. Microvesicles and apoptotic bodies consist of other cytoplasmic proteins which seem to be less prominent in exosomes [13]. This might be due to the similar "budding/bulging" nature of the biogenesis of microvesicles and apoptotic bodies from the cytoplasmic membrane directly, a characteristic that differs from the endocytic-driven biogenesis of exosomes. Unlike exosomes and microvesicles, apoptotic bodies are composed of chromosomal deoxyribonucleic acid (DNA) fragments, chromatin remnants, cytosol portions, degraded proteins and cell organelles from dead cells [25, 35], indicative of their role in removing dead cells.

EVs also possess additional key features according to the specific cell line they originate from (**Table 2**). In general, cancer cells consist of higher levels of sphingolipids, glycerophospholipids, sterol lipids, ceramide, phosphatidic acid and matrix metalloproteinases like a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), while non-cancer cells consist of higher levels of prenol lipids, glycerolipids and fatty acids [24, 83].

Components	Exosomes	Microvesicles	Apoptotic bodies	Reference(s)
Proteins				
Tetraspanins	CD9, CD63, CD81, CD37, CD82, CD53, TSPAN 6, TSPAN 8, TSPAN 29, TSPAN 30	CD40 ligands, CD82	CD40 ligands, CD82	[13, 14, 24, 25]
Cell adhesion proteins	Integrins (integrin-alpha, integrin-beta), selectins (P-selectin), lactadherin, ICAM	integrins, selectins (P-selectin), fibronectin, PECAM-1	integrins, fibronectin, PECAM-1	[13, 14, 17, 18, 24–28]
Heat shock proteins	Hsc70, Hsp20, Hsp27, Hsp60, Hsp70, Hsp90	Hsp70, Hsp90	Hsp70, Hsp90	[13, 14, 18, 25, 28–30]
Biogenesis-associated proteins	ESCRT proteins (Alix, Tsg101), VPS4, clathrin, ubiquitin, syntenin, VPS32, PLD	ESCRT proteins (Alix, Tsg101), VPS4, ERK, PLD	VPS4, ERK, PLD	[13, 14, 17, 18, 24, 25, 28]
Fusion proteins	Flotillin 1 and 2, annexins, GTPases, Rab GTPases, dynamin, syntaxin	Flotilin-2, Rab GTPases, annexins	Rab GTPases, annexins (Annexin V)	[13, 14, 18, 25]
Cell-type specific proteins	MHC class I, MHC class II, APP, PMEL, TCR, CXCR4, HSPG, CD86, PrP, WNT	MHC class I, MHC class II, LFA1, CD14	MHC class I, LFA1, CD14	[13, 14, 17, 18, 27, 28]
	Actin, tubulin, cofilin	Actin, tubulin	Actin, tubulin	[13, 18]
Cytoskeletal proteins	Protein kinases, beta-catenin, 14-3-3, G proteins	ARF6, Rab11, ROCK	ARF6, Rab11, ROCK	[13, 14, 28, 31]
Signalling molecules	PLA2, peroxidases, pyruvate kinase, enolase, GADPH, ATPases	GADPH	GADPH	[13, 25, 28]
Other enzymes	Glycoproteins • e.g. beta-galactosidase, O-linked glycans, N-linked glycans	Glycoproteins • e.g. Glycoprotein Ib		[18, 28]
Additional proteins	Growth-factors and cytokines • e.g. TNF-α, TGF-β, TNF-related apoptosis-inducing ligand	Growth factors and cytokines		[18, 25]
	Membrane signalling receptors • e.g. FasL, TNF receptor, TfR	Membrane signalling receptors		
	Phosphoproteins	High phosphoproteins		[28, 32]
	Ribosomal proteins	GTP-binding protein ARF6		[28, 33]
	Lysosomal proteins • e.g. Lamp2b	Chemokines		[25]

Components	Exosomes	Microvesicles	Apoptotic bodies	Reference(s)
Lipids	High phosphatidylserine	High phosphatidylserine		[14, 25]
	Phosphatidylethanolamine	Phosphatidylethanolamine		[24, 25, 28, 32]
	Sphingolipids • e.g. sphingomyelin, gangliosides	Sphingolipids		[18]
	High cholesterol			[14, 28, 34]
	High diacylglycerol			[14]
	Ceramides			[13, 24, 28]
	Phosphatidylcholine			[28]
	Phosphatidylinositol			[18]
	LBPA			[13]
Nucleic acids	mRNA		mRNA	[25, 35]
	miRNA		miRNA	
	Non-coding RNA • e.g. small nucleolar RNAs, microRNAs, piwi-interacting RNAs, other long non-coding RNAs		Non-coding RNA • e.g. small nucleolar RNAs, microRNAs, piwi-interacting RNAs, other long non-coding RNAs	
	DNA with histones		Chromosomal DNA fragments with histones, chromatin remnants, cytosol portions, degraded proteins, cell organelles	

Abbreviations: ADP: adenosine diphosphate, APP: amyloid-beta precursor protein, ARF: ADP ribosylation factor, CXCR: CXC chemokine receptor, DNA: deoxyribonucleic acid, ERK: extracellular signal-regulated kinase, ESCRT: endosomal sorting complex required for transport, FasL: Fas ligand, GTP: guanosine triphosphate, HSPG: heparan sulphate proteoglycan, ICAM: intercellular adhesion molecule, Lamp: lysosome-associated membrane protein, LFA: lymphocyte function-associated antigen, MHC: major histocompatibility complex, PECAM: platelet endothelial cell adhesion molecule, piwi: P-element induced wimpy testis, PLA2: phospholipase A2, PLD: phospholipase D, PMEL: premelanosome protein, PrP: prion protein, Rab: Ras-associated binding, TCR: T-cell receptor, RNA: ribonucleic acid, ROCK: Rho-associated protein kinase, TSPAN: tetraspanin, Tsg: tumour suppressor gene, VPS: vacuolar protein sorting-associated protein, WNT: wingless/integrated, GADPH: glyceraldehyde 3-phosphate dehydrogenase, TDP: transactive response DNA-binding protein, TfR: transferrin receptor, TGF: transforming growth factor, TNF: tumour necrosis factor.

Table 1.
Classification of key components of EVs by their main categories.

EV source	EV source subtype	Component(s)	Reference(s)
Bacteria	Gram-positive	ABC transporters, mobility-related proteins (FliC, PilQ), multidrug efflux pumps, porins (Omps, OprF, PorA, PorB)	[24, 36]
	Gram-negative	Beta-lactamase, coagulation factor, penicillin-binding protein	[24, 37–39]
	Myxobacteria	Chaperonin GroEL1, GroEL2, hydrolase, peptidase	[24, 40, 41]
Blood cells	Platelets	CD31, CD41, CD42a, CD62, C-type lectin, CXCR4, GPIIb/IIIa, PF4, SDF-1α	[24, 42–44]
	Erythrocytes	Glycophorin A, stomatin	[24, 34]
	Reticulocytes	Galectin-5	[42, 45]
Bone cells	Osteoblasts	Cadherin-11	[42, 46]
Cancer cell lines	Breast cancer cells (MM231, MM231LN)	Rab-5b, actin, integrin beta 1, cavolin-1	[47]
	Breast cancer cells (MCF7)	Actin, Rab-5b	[47]
	Breast cancer cells (MCF10A)	Integrin beta 1	[47]
	Cervical cancer cells (HeLa)	EGF	[42, 48, 49]
	Colon cancer cells (LIM1863—EpCAM apical exosomes)	CD44, CD46, CD59, CLDN7, EpCAM, HMGB2, HMGB3, Muc-13, sucrase isomaltase	[50]
	Colon cancer cells (LIM1863—A33 basolateral exosomes)	ADP-ribosylation factor, AP1G1, AP1M1, AP1M2, AP3B1, CLSTN1, CLTA, CLTB, COPB2, EEA1, GPA33, HLA-A, HLA-B, HLA-C, HLA-E, HLA-A29.1, Rab-13, REEP6	[50]
	Colorectal cancer cells (CRC line SW403, CRC28462)	Carcinoembryonic antigen, class I HLA	[51]
	Hepatoblastoma cancer cells (HepG2, K562)	TfR1, TfR2	[42, 52]
	Hepatocellular cancer cells (HKCI-C3, HKCI-8, MHCC97L, MIHA)	ADAM10, ARHGEF18, BROX, CAV1, CAV2, CD44, CDC42, CLDN3, EDIL3, EIF4A3, GNA11, GNA13, GNAQ, GNAS, GRB2, MET, RHOG, RRAS, SNTA1, TNFRSF21, TNFAIP2	[53]
	Myeloma cancer cells (RPMI-8226, CAG)	Fibronectin	[54]
	Nasopharyngeal cancer cells (C15)	Galectin-9, LMP1	[55]
	Nasopharyngeal cancer cells (C17)	Galectin-9	[55]
	Ovarian cancer cells (IGROV1, OVCAR-3)	Beta-actin, EpCAM, hnRNPA1, hnRNPK	[56]
	Prostate cancer cells (PC3)	Rab-5b, integrin beta 1, cavolin-1	[47]
	Prostate cancer cells (PC-3 M-luc)	Rab-5b, actin, Integrin beta 1	[47]
	Prostate cancer cells (22Rv1)	Rab-5b, actin	[47]
	Prostate cancer cells (PNT2)	Actin, integrin beta 1	[47]

EV source	EV source subtype	Component(s)	Reference(s)
Endothelial and epithelial cells		C-type lectin, galectin-3, Muc-1	[42]
Immune cells	B-cells	A2,3-linked sialic acid, CD169	[42, 57]
	T-cells	CXCR4, SDF-1α	[42, 48, 58, 59]
	Dendritic cells	FLOT1, galectins, Lamp-1, MFG-E8, MHC class I and II, TNFR1, TNFR2	[24, 42, 60–62]
	Macrophages	C-type lectin, LFA-1	[42, 63, 64]
	Natural killer cells	Granzyme B, perforin	[65]
Mesenchymal stem cells		Alternative splicing and Golgi apparatus component mRNA encoding transcription factors, CD54, CD73, CD86, CD90, CD105, CD166, MHC class I and II, sialic acids	[24, 28, 42, 66–75]
Milk cells	Bovine milk cells	β-casein, β-lactoglobulin mRNA, CD59, MFG-E8, miR-30a, miR-92a, miR-223, Rab-1b, Rab-11a	[24, 76–80]
	Human breast milk cells	miR-17, miR-181a	[81]
Nervous cells	Astrocytes	MCP-1, MMP3, MMP9, TIMP-1	[65]
	Microglia	CD13, CD107a, CD107b	[65]
Placental cells		MHC class I chain-related proteins A and B, placental alkaline phosphatase, placental leucine aminopeptidase, pregnancy specific glycoprotein 3, RAET1 proteins/ULBP1–5, TGFβ1, TRAIL, trophoblast glycoprotein 5 T4	[82]

Abbreviations: ABC: adenosine triphosphate-binding cassette, ADAM: A disintegrin and metalloproteinase domain-containing protein, ADP: adenosine diphosphate, AP: adaptor related protein complex, ARFGEF: Rho/Rac guanine nucleotide exchange factor, BROX: BRO1 domain and CAAX motif containing, CA: carbohydrate antigen, CAV: caveolin, CLDN: claudin, CLSTN: calsyntenin, CLT: clathrin light chain, COP: coatomer protein complex, CXCR: CXC chemokine receptor, EDIL: EGF like repeats and discoidin domains, EEA: early endosome antigen, EGFR: epidermal growth factor receptor, EGF: Epidermal growth factor, EpCAM: epithelial cell adhesion molecule, FLOT: flotillin, GN: guanine nucleotide-binding protein, GP: glycoprotein, GRB: growth factor receptor-bound protein, HLA: human leukocyte antigen, HMG: high-mobility group, HNRNP: heterogeneous nuclear ribonucleoprotein, Lamp: lysosome-associated membrane protein, LDLR: low-density lipoprotein receptor, LDL: low-density lipoprotein, LFA: lymphocyte function-associated antigen, LMP1: Epstein-Barr virus latent membrane protein 1, MAPK: mitogen-activated protein kinase, MCP: membrane cofactor protein, MET: mesenchymal-epithelial transition facror, MFG-E: milk fat globule-EGF factor, MHC: major histocompatibility complex, miRNA: microribonucleic acid, MMP: matrix metalloproteinase, Muc: mucin, PF: platelet factor, RAET: retinoic acid early transcript, Rab: Ras-associated binding, REEP: receptor expression-enhancing protein, RHOG: Ras homolog family member G, RRAS: RAS-related protein R-Ras, SDF: stromal cell-derived factor, SNT: syntrophin, TfR: transferrin receptor, TNF: tumour necrosis factor, TNFR: tumour necrosis factor receptor, TNFRSF: TNF receptor superfamily, TNFAIP: TNF alpha-induced protein, TRAIL: tumour necrosis factor-related apoptosis-inducing ligand, TYRP: tyrosinase-related protein, ULBP: UL16 binding protein.

Table 2.
Classification of additional key components of EVs by their specific cell lines.

3. The EV journey—overcoming biological barriers

To reach their target sites, EVs need to overcome various biological barriers (**Figure 2**). Complementing these barriers are blood vessels (capillaries in particular). EVs can enter and extravasate from these vessels *via* diffusion, due to their lipidic nature which enables them to pass through the highly-lipidic capillary endothelium and their small size that enables them to pass or squeeze through fenestrations in the capillary wall [84].
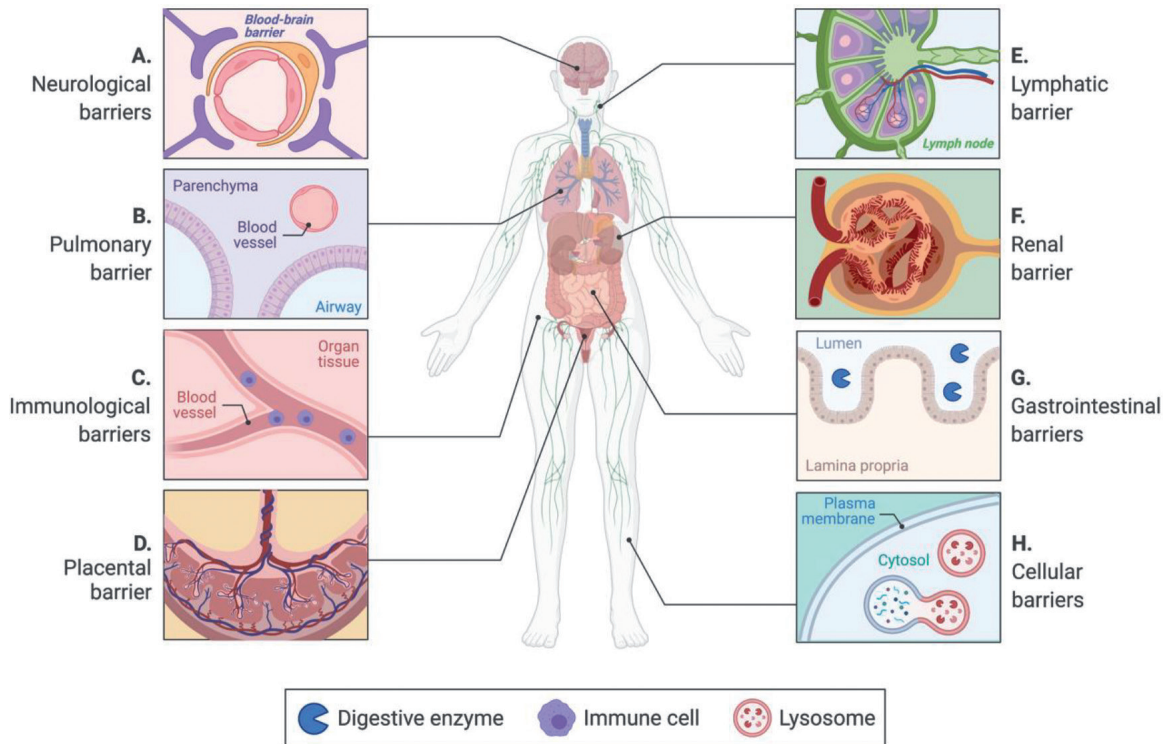
Figure 2.
*Biological barriers encountered by extracellular vesicles (EVs). (A) **Neurological barriers** include the blood-brain barrier (BBB), blood-labyrinth barrier (BLaB) and blood-retinal barrier (BRB). (B) **The pulmonary barrier**, or blood-air barrier (BAB), guards against the invasion of pathogens in the lungs via its immune cell-rich lung mucosa, lung epithelial cells and ciliary action. (C) **Immunological barriers** eliminate pathogens and perceived foreign substances from the body via the mononuclear phagocyte system and the adaptive immune system. (D) **The placental barrier** consists of an inner blood-vessel-rich layer with the syncytiotrophoblast facing the bloodstream and an outer layer of trophoblasts. (E) **The lymphatic barrier**, or blood-lymph barrier (BLyB), is regulated by various mechanisms including extravasation, overcoming the interstitium, diffusion, and passage through the mucosal barrier. The collagen reticular network (RN) also hinders soluble substances from passing through. (F) **The renal barrier**, or glomerular filtration barrier, composes of the fenestrated endothelium, glomerular basement membrane and glomerular epithelium, and this hinders the passage of large molecules across the barrier. (G) **Gastrointestinal barriers** are associated with digestive enzyme degradation, harsh stomach acidic conditions and the small intestinal barrier. (H) **Cellular barriers** of the target cell include the plasma membrane, endosomal membrane and lysosomal membrane. EVs internalised by cells via endocytosis are packaged into endosomes which may risk fusing with lysosomes to undergo degradation (created with BioRender.com).*

3.1 Gastrointestinal barriers

EVs administered orally need to overcome digestive enzymatic degradation, harsh stomach acidic conditions and the small intestinal barrier before entering the bloodstream for systemic absorption. As milk and plant-derived EVs are delivered into the body naturally *via* oral consumption, they might provide key insights into how EVs can be used and/or engineered for oral administration. *In vivo* evidence in rodents showed that unmodified bovine milk-derived EVs naturally containing immune-active proteins were able to cross the intestinal barrier *via* endocytosis to treat inflammatory bowel disease (IBD) [85], and were distributed significantly in the bloodstream 24 h post-oral consumption [86]. EVs can pass through the intestinal barrier *via* intestinal epithelial cell (IEC) mediated transendocytosis, a process that requires surface glycoproteins on both EVs and target cells, based on *in vitro* findings of skimmed bovine milk-derived EVs being internalised by human colon carcinoma Caco-2 cells and rodent small intestinal IEC-6 cells [87, 88]. Paracellular translocation is another possible mechanism by which EVs may cross the intestinal epithelium,

through tight junctions between adjacent epithelial cells [76, 87, 89]. Although *in vivo* evidence is lacking, it is possible that EVs might cross the intestinal epithelium para-cellularly to a greater extent in pathological conditions like IBD as the tight junctions would be disrupted [90], making the intestinal epithelium more penetrable.

Milk-derived EVs have been shown to withstand acidic and enzymatic condi-tions [87, 91]. However, their ability to do so might be dependent on the milk source, as EVs from processed milk would have undergone more damage than those from unprocessed milk and hence possess less integrity [87, 92–97]. Although bovine milk-derived EV surface proteins CD9 and CD81 were found to be partially degraded by acidification at pH 4.6 in one study [98], these findings did not demonstrate whether these EVs can survive stomach acidic conditions, which are usually characterised by a much lower pH. Moreover, the study was focused on evaluating the effectiveness of acidification in ultracentrifugation to isolate EVs. Thus, these conditions would have differed vastly from true gastrointestinal conditions. Although the underlying mechanism is unclear, the ability of both processed and unprocessed milk-derived EVs to withstand harsh conditions might be correlated with milk calcium content [87]. This could be due to the adhering of milk calcium to the surface of EVs, which might strengthen their membrane integrity against acidic and enzymatic degrada-tion. Another hypothesis is that calcium might influence milk-derived EV biogenesis pathways in alveoli cells to increase the expression of certain proteins or transporters in secreted EVs that enable them to withstand gastrointestinal conditions.

Fruit and vegetable-derived EVs have been shown to withstand gastrointestinal conditions and eventually be internalised by rodent intestinal tissue *in vivo*, though their passage across the intestinal barrier into the bloodstream cannot be concluded in some studies [77, 99–102]. Grape EVs derived *via* cold-pressing have been discovered to enter rodent IECs *via* macropinocytosis [100], while a previous analysis of grape-fruit EVs derived *via* homogenization revealed their internalisation by intestinal mac-rophages *via* macropinocytosis and clathrin-mediated endocytosis [101]. Watermelon EVs were also observed to be taken up by human IECs in an *in vitro* experiment *via* clathrin-mediated endocytosis, causing the cells to multiply rapidly and their basal secretome to change [103]. Ginger EVs were found to accumulate in rodent liver tissue 12 h post-oral consumption, implying that the EVs were able to withstand gastrointes-tinal conditions and cross the intestinal barrier into the bloodstream while remaining intact [104]. Though unconfirmed, the uptake of plant-derived EVs *via* clathrin-mediated endocytosis and macropinocytosis probably indicates that these EVs possess receptor tyrosine kinases, G protein-coupled receptors (GPCRs) and transferrin receptors [105], while passage across the intestinal barriers into the bloodstream might imply that these plant-derived EVs undergo transendocytosis like milk-derived EVs, a mechanism which requires EVs to possess surface glycoproteins [87, 88]. The ability of milk and plant-derived EVs to withstand and overcome gastrointestinal conditions and barriers makes them highly suitable as DDSs *via* the oral route as a non-invasive alternative to intravenous DDSs.

3.2 Placental barrier

The placenta supports foetal growth and development while secreting female hormones [106–111]. The placental barrier (PB) is suggested to be selectively penetra-ble, given that drugs administered to pregnant women can either cause adverse side effects in both the mother and the fetus or not penetrate the PB at all. It consists of an inner blood-vessel-rich layer with the syncytiotrophoblast facing the bloodstream

and an outer layer of trophoblasts [106, 112–114]. Occurring in large amounts during pregnancy [115, 116], placental exosomes exert their functions during foetal growth and development, being involved in processes like angiogenesis regulation and cell migration [106, 116–126]. This implies that they can overcome the PB, though the underlying mechanism is unclear. Placental exosomes have also been tested as diagnostic biomarkers for foetal development [106, 115] and gestational diabetes [106, 127].

Although placental EVs may be used to pass through the PB, the use of non-placental EVs to deliver drugs across the PB is a potential area for exploration. The placenta can respond to signals from immune cells and exert an inflammatory response during infection. An *in vitro* study revealed that THP-1 monocyte-derived exosomes were internalised by human placental trophoblast cells *via* clathrin-mediated endocytosis, exerting a pro-inflammatory effect that caused the cells to release cytokines [128]. Provided that this mechanism can be proven *in vivo*, packaging drugs in EVs derived from immune cells might be one way to deliver drugs across the PB. Another possible method to deliver drug-containing EVs across the PB might be *via* administering EVs that target IECs instead of placental cells, as IECs can communicate with the placenta [103]. IECs that internalise watermelon EVs can secrete watermelon EV contents *via* the formation of intestinal exosomes, which are shown to be taken up by placental cells *via* clathrin-mediated endocytosis [103]. This concept, however, is deduced from a few *in vitro* studies and has yet to be proven in a single *in vivo* experiment. Nevertheless, being able to deploy non-placenta-derived EVs to treat placental pathological conditions like chorioamnionitis may offer some flexibility in EV engineering, as researchers would not need to adhere strictly to using placental EVs.

3.3 Immunological barriers

The body is heavily guarded by immune cells responsible for eliminating pathogens and perceived foreign substances. As such, nanoparticles injected into the bloodstream risk being removed by phagocytes of the mononuclear phagocyte system (including those in the liver and spleen), or the adaptive immune system *via* antibody production [8]. Thus, in conventional non-EV drug therapy, immune cells can potentially hinder the therapeutic effects of nanoparticles by decreasing their systemic circulation half-life [10]. EVs, on the other hand, can evade removal by immune cells naturally. CD47 is a prominent component found on EVs that binds to signal regulatory protein alpha (SIRPα) on dendritic cells and macrophages, which inhibits phagocytosis *via* a "don't eat me" signal [129–131]. Other EV components found on both cancer and non-cancer cell-derived EVs like CD24, CD31 and PD-L1 have been associated with exerting a similar "don't eat me" signal, with PD-L1 also inhibiting T-cell activation [130, 132–134].

A recent study on an *in vivo* rodent tumour model seems to suggest that it may be possible for EVs to be phagocytosed by Kupffer cells in the liver and eliminated *via* biliary excretion, given that the fluorescent markers tagged to the U937 human myeloid leukaemia EVs used in the study were found to accumulate in the liver and eventually detected in the faeces [10]. However, these fluorescent markers were also predominantly detected in CT26 mouse colon adenocarcinoma cells targeted by the EVs, probably because the EVs might have already undergone disintegration in the cells, and the florescent marker component might have been excreted *via* exocytosis before being transported to the liver *via* systemic circulation. In other words, the accumulation of dyes associated with EVs in the liver is not synonymous with a

definite uptake of EVs by Kupffer cells. Nevertheless, EVs may still be removed by immune cells, as shown in another study where melanoma, myoblast, fibroblast, aortic endothelial and macrophage-like cell exosomes from rodents were eliminated by rodent liver macrophages *in vivo*, most likely due to the presence of PS on EVs which is recognised by macrophages [135]. As to whether these EVs possess high amounts of CD47, CD24, CD31 or PD-L1, the study did not include such findings.

3.4 Neurological barriers

The blood-brain barrier (BBB) is characterized by an innermost layer of endothelial cells (which prevents blood and extracellular fluid from mixing), pericytes surrounding the endothelial cells and astrocyte end-feet acting as a sheath in the outermost layer. Though the movement of substances across the BBB is tightly regulated [136], different EVs have been observed to cross the BBB. One study demonstrated the ability of exosomes to carry miR-193b-3p across the BBB to exert an anti-inflammatory effect on rodent brain cells with subarachnoid haemorrhage [137], although the mechanism of crossing was unclear. Other studies involving the BBB in zebrafish showcased the ability of various human breast cancer cell EVs and brain endothelial cell EVs to cross the BBB *via* clathrin-mediated endocytosis [7, 138] and macropinocytosis [138], a notable surface protein that enabled clathrin-mediated endocytosis being CD63 [7]. Another study conducted on rodent BBB showed that human and rodent EVs derived from both cancer and non-cancer cells were able to cross the BBB *via* adsorptive-mediated transcytosis, which correlated with the presence of CD46 on the surface of EVs [11].

Modifications have also been made to EVs to enhance their ability to cross the BBB. In one experiment, after overexpressing the rabies virus glycoprotein (RVG) peptide on their surface, dendritic exosomes became significantly localized in rodents' brain cells [139]. Mouse L929 fibroblastic cell exosomes loaded with methotrexate and functionalized with LDL peptide in another experiment showed enhanced BBB exosome extravasation in rodents [140]. When miR-210-loaded mesenchymal stromal cell-derived exosomes were coupled with c(RGDyK) peptide in another experiment, they displayed enhanced targeting of rodent ischaemic brain cells, indicating greater angiogenesis and improving animal survival significantly [141]. Another experiment showed that RGE-Exo EVs demonstrated greater accumulation and duration of accumulation in murine glioma tumour cells than free exosomes [142].

Apart from surface components, the size of EVs might also be a crucial factor in determining whether EVs can cross the BBB, as deduced from another study where intranasal administration of exosomes to rodent microglial cells *via* the extra-neuronal pathway showed rapid translocation of exosomes to target cells, in contrast to larger microparticles of at least 500 nm in diameter which did not reach these target cells [143]. However, surface components of EVs might be a more vital factor than the size of EVs in enabling passage across the BBB, as proven by how larger brain endothelial EVs can penetrate the BBB better than smaller EVs of the same cell source, due to the higher levels of CD63 in the larger EVs [7, 106].

The blood-labyrinth barrier (BLaB) and blood-retinal barrier (BRB) are two other neurological barriers pertaining to the ear and eye, respectively. The BLaB consists of five layers, namely, the blood-endolymph barrier, blood-perilymph barrier, cerebrospinal-fluid-perilymph barrier, middle-ear-labyrinth barrier and endolymph-perilymph barrier [106, 144]. The BRB consists of the retinal vascular endothelium and the retinal pigment epithelium (RPE) [106]. These two barriers share similarities

with each other and the BBB, though the number of EV studies on these two barriers is smaller than that involving passage across the BBB [106]. Nevertheless, the utilization of EVs as potential drug carriers targeting the ear and eye with negligible side effects is worth further research, especially when current drug treatments have resulted in adverse side effects [106]. EVs from RPE cells are involved in the progression of age-related macular degeneration *via* regulating the production of pigment granule and lipid balance in RPE cells [106, 145]. They also promote vascular leakage *via* miR-105 which interferes with the tight cellular junctions of barriers [106, 146]. It is hoped that these seemingly destructive EV mechanisms can be manipulated to enable drug delivery across the BLaB and BRB, by modifying these EVs in a way that does not harm the barriers yet still permits their passage across them.

3.5 Lymphatic barrier

The process of crossing the blood-lymph barrier (BLyB) is regulated by various mechanisms including extravasation, overcoming of the interstitium, diffusion and passage through the mucosal barrier [106, 147]. In addition, the collagen reticular network (RN) hinders soluble substances from passing through [106, 148–153]. However, EVs possess certain characteristics that enable them to cross the BLyB. For instance, human ovarian cancer cell exosomes were found to be able to travel from the periphery to the lymph node in just a matter of minutes in rodents due to their small size [106, 154], and their lipidic rather than soluble nature seemed to enable them to cross the RN [106, 155].

Although EVs already possess intrinsic advantages that enable them to cross the BLyB, methods like microfluidic surface engineering have been conducted on EVs to modify them further as potential drug carriers for lymphoma treatment or other diseases related to the lymphatic system [106, 156, 157]. A recent study explored the modification of exosomes derived from bovine serum. α-D-mannose was added to the exosomes containing immune stimulators to enable them to interact with the mannose receptors on dendritic cells for uptake, and the exosomes were PEGylated. This has been found to enhance the internalisation of the exosomes by murine dendritic cells and to increase their localisation in the lymph nodes, paving the way for efficient delivery of immune stimulators *via* EVs *in vivo* as a potential form of drug therapy [158].

3.6 Pulmonary barrier

Located in the lungs, the blood-air barrier (BAB) possesses characteristics that enable it to guard against pathogenic invasion. For instance, the lung mucosa is a rich source of immune cells [106, 159], and lung epithelial cells can sense a wide range of bacteria and viruses *via* a broad array of membrane-bound, endosomal and cytosolic pattern-recognition receptors (PRR) [106, 160]. In response to the presence of pathogens, the BAB regulates paracellular flow, cell-to-cell communication, synthesis of mucus and the composition of periciliary fluid [160], which complements the removal of foreign substances *via* ciliary action [106, 161]. While the passage of EVs across the BAB is still largely unexplored [106], exosomes derived from bronchoalveolar lavage fluid (BALF) have been discovered to possess a similar protein profile to mesenchymal derived dendritic cells, given that they carry CD54, CD63, CD86 and in particular, MHC classes I and II [162, 163], implying their involvement in triggering

an immune response against pathogenic invasion in the BAB. In light of this, BALF exosomes might potentially be used as diagnostic biomarkers for pathogenic detection, in addition to engineering them as personalised medicine for effective drug delivery across the BAB.

3.7 Renal barrier

The nephron's ability to efficiently filter out waste materials from the blood into the urine is attributed to the high pressure in the glomerulus due to high blood flow, as well as the presence of the glomerular filtration barrier consisting of three layers—fenestrated endothelium, glomerular basement membrane and glomerular epithelium [66, 164–166]. Despite the tiny pores (2.5–2.8 nm) of the glomerular basement membrane [166, 167] which are smaller than the smallest EVs (30 nm [16–18]), and the presence of filter proteins lining the slits in the glomerular epithelium [165, 166, 168], the urine is surprisingly a rich source of EVs from both renal and non-renal sources. While the majority of EVs found in urine originate from the kidney, urinary bladder, testis, prostate, epididymis and seminal vesicle [169–172], studies have also identified EVs from outside the urinary tract, such as those carrying biomarkers of acute myocardial infarction [173]. EVs injected into the bloodstream of rodents in one study were found to accumulate in the kidneys, and eventually the urine, without undergoing degradation, as indicated by their ability to be internalized by HEK293 cells after being retrieved from the urine and introduced to the cells [166]. The presence of EVs in urine might imply that EVs can squeeze through the tiny pores of the glomerular filtration barrier due to their fluid membranes, or undergo mechanisms like transcytosis to reach the glomerular filtrate. It is also logical to deduce that EVs can cross the glomerular filtration barrier better in pathological conditions like diabetic nephropathy when the glomerular filtration barrier becomes more porous due to injury [166].

3.8 Cellular barriers

EVs that eventually reach the target cell has to overcome the plasma membrane, escape the endosome and evade lysosomal degradation to release their cargo into the cytosol (**Figure 3**).

The plasma membrane is an intricate structure consisting of various domains formed *via* different mechanisms. Some of these mechanisms include the formation of plasma membrane protein fences to reduce lateral diffusion in the plasma membrane, the arrangement of plasma membrane proteins into a scaffold that interacts with certain plasma membrane lipids, and protein-lipid interaction to form lipid rafts [174]. These domains represent the first barrier that a freshly-secreted EV needs to cross and determine the way the target cell internalizes EVs. Environmental factors also influence the interaction of EVs with the plasma membrane. For instance, the uptake of EVs *via* fusion with the cell membrane is observed to occur at a higher rate under acidic conditions [175], while endocytosis is shown to be hindered by neutral pH or high cholesterol levels [14, 176].

EVs can be internalised by target cells *via* endocytosis, be it caveolae-dependent endocytosis, flotillin-dependent endocytosis, ARF6-dependent endocytosis or other forms of endocytosis [105]. Clathrin-mediated endocytosis (CME), or "receptor-mediated endocytosis", plays an especially prominent role in the uptake of small EVs [105],
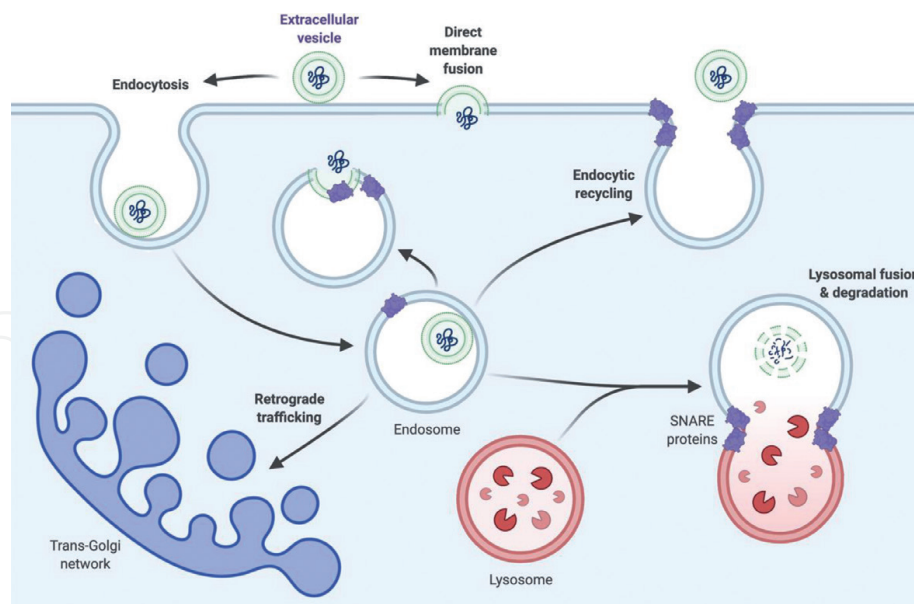
Figure 3.
Internalisation, lysosomal degradation and lysosomal escape mechanisms of extracellular vesicles (EVs) in target cells. Upon reaching the target cell, EVs may be internalised by the cell via endocytosis or direct fusion with the plasma membrane. EVs that are internalised via endocytosis are packaged into endosomes, which may fuse with lysosomes to degrade the EVs. To escape lysosomal degradation, endocytosed EVs may undergo retrograde trafficking to the trans-Golgi network, endocytic recycling to be secreted out of the cell, or another mechanism altogether. Endocytosed EVs that do not undergo lysosomal degradation fuse with the endosomal membrane via the mediation of soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins to release their cargo into the cytosol. EVs that fuse directly with the plasma membrane to release their cargo into the cytosol evade endosomal and lysosomal activity completely (created with BioRender.com).

as supported by recent studies on the uptake of human epidermoid carcinoma EVs [177] and rat pheochromocytoma EVs [178] by human cervical carcinoma (HeLa) cells and rat bone marrow-derived mesenchymal stromal cells respectively. During CME, a temporary membrane scaffold forms as a result of membrane binding of Bin/amphiphysin/Rvs (BAR) domain-containing proteins which recruit clathrin. Clathrin then binds to the cytoplasmic tails of membrane proteins with the help of adaptor proteins, resulting in a clathrin-coated pit that internalises the EV [105]. EVs internalised *via* endocytosis are packaged into endosomes. These EVs then proceed to release their cargo into the cytoplasm by fusion of their membranes with the endosomal membrane, a process mediated by soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins which join the cytosolic sides of the EV and endosomal membranes together [3].

In general, the CME of EVs is initiated through the mediation of lectins, tetraspanins, cell adhesion proteins and other receptor-ligand interactions [18, 179]. For instance, exosomes from macrophages possess C-type lectin, which interacts with the C-type lectin receptor found on dendritic and brain endothelial cells [179, 180]. Galectin-5 on red blood cell (RBC) EVs enables them to be internalised by macrophages [45, 179]. Integrins on tumour EVs have been associated with the uptake of these EVs by lung fibroblasts and liver macrophages [42, 179]. Exosomes and target cells can exploit the interaction between intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen 1 (LFA-1) in exosomal uptake [179–181]. Heparan sulphate proteoglycans on target cells bind to EV fibronectin to facilitate uptake of EVs [54, 179, 182]. The high levels of outward-facing PS on the surface of exosomes also enable the recognition and

uptake of these exosomes by antigen-presenting cells *via* T-cell immunoglobulin and mucin domain (TIM) receptors located on the antigen-presenting cells' surface [63, 179, 183].

EVs internalised *via* endocytosis might risk being degraded by lysosomes in the cytosol [3]. The fusion of the membrane of endosomes containing the endocytosed EV with the lysosomal membrane is mediated by SNARE proteins and involves the active transport of vesicles along the cytoskeleton [18]. EV size may play a role in determining the fate of EVs upon uptake *via* endocytosis, as EVs larger than 100 nm may require macropinocytosis for their uptake, which tends towards lysosomal degradation more than other internalisation mechanisms accessible to smaller EVs [177, 184, 185]. Endocytosed EVs might escape lysosomes *via* pathways similar to those of viruses, like the CD81 positive lysosome-associated membrane protein 1 (Lamp-1) negative route in dendritic cells which resembles that of HIV-1 uptake [186]. A study showed that HEK293 exosomes internalised by human fibroblastic, hepatic and renal cells were transported to the endoplasmic reticulum where they released their cargo, a pathway that might be a potential escape route from lysosomal degradation [187]. EVs may also evade lysosomal degradation *via* endocytic recycling out of the cell [188] or retrograde trafficking from the endosomal pathway to the trans-Golgi network [189].

EVs have also been reported to deliver their cargo into target cells *via* direct membrane fusion with the cell membrane, with EV surface proteins syncytin-1 and syncytin-2 seemingly playing a significant role in this [190–192]. Originally found on the plasma membranes of placental trophoblast cells [190, 191], gamete cells [190, 193] and various cancerous and non-cancerous cells known to fuse directly with other cells [190, 194–197], these proteins have also been detected on EVs secreted from these cells [190, 192]. In light of this, incorporating these surface proteins into EVs to increase their uptake *via* direct membrane fusion might be a possible way to evade endocytosis and lysosomal degradation completely.

4. Recommendation—EVs as drug carriers

In providing a recommendation to engineer EVs as DDSs, EV engineering methods to overcome specific barriers can be deployed. Natural evasion of immune cells is a highly favourable quality and should mark all engineered EVs regardless of the barrier(s) they target. CD47, CD24, CD31 and PD-L1 are prominent surface proteins that achieve this quality and should be incorporated into engineered EVs in high amounts if not originally present [129–134]. Fusing EVs with liposomes to create hybrid DDSs can also increase their drug loading capacity without risking cargo aggregation [198, 199].

The choice of the source of EVs depends on its availability and the ability of its EVs to overcome respective biological barriers associated with the disease. Milk and plant-derived EVs, which are highly available in nature and able to overcome gastrointestinal barriers [76, 77, 85–105], can be engineered for drug delivery *via* the oral route to treat IBD. The patient's own EVs might also be used as a form of personalised medicine. EVs from the patient may be chosen based on whether their cell of origin matches the target cell for better selectivity, but there are exceptions. For instance, immune and intestinal cell-derived EVs can be internalized by placental cells [103, 128]. Breast milk might also be a possible EV drug carrier source to treat

both gastrointestinal and neurological conditions, as milk-derived EVs can cross gastrointestinal barriers [76, 85–90] and the BBB [87, 96] respectively. Human Type O RBC EVs loaded with antisense oligonucleotides were also found to target human leukaemia and breast cancer cells *in vitro* and *in vivo*. This is advantageous as RBCs are widely accessible from blood banks and lack DNA, which ensures that no oncogenic material is transferred from EVs to target cells [200]. This offers diverse compatible EV sources to choose from for a single ailment, enhancing the flexibility of the engineering process.

A summary of the recommendation is shown in **Figure 4**.
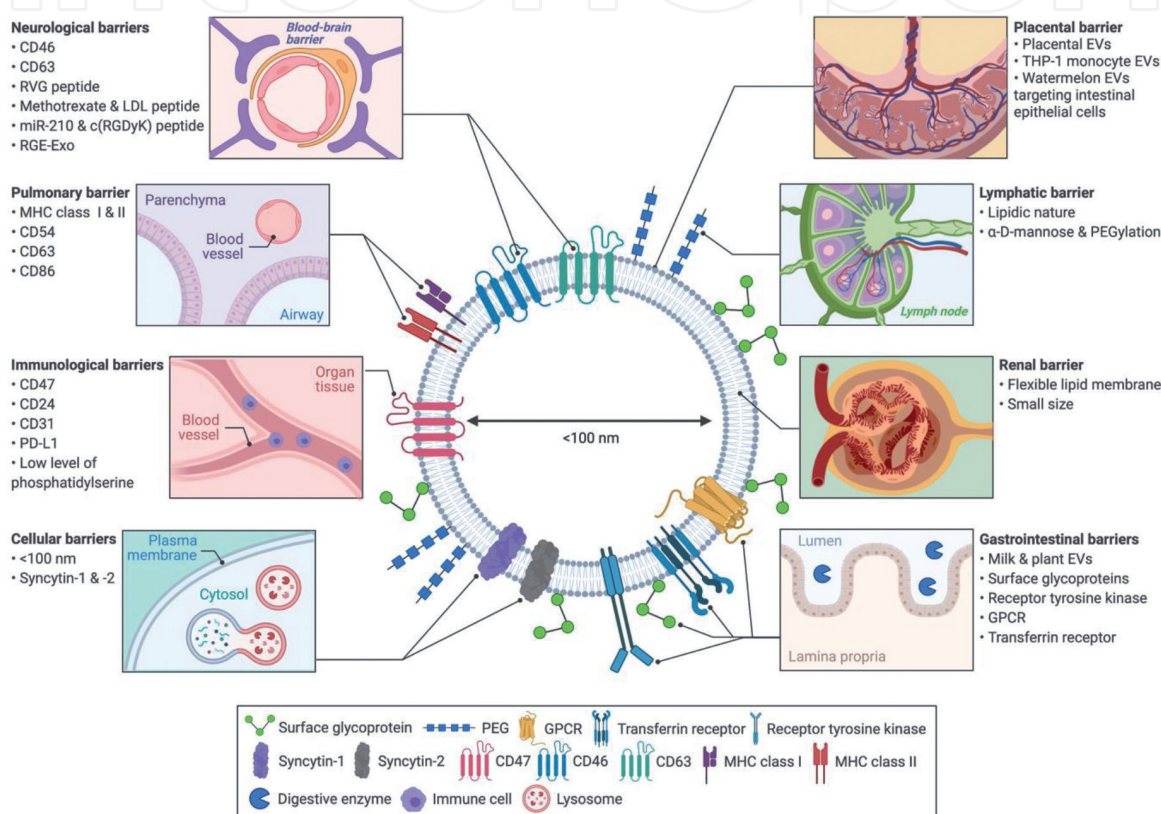
Figure 4.
*Recommendation on how to engineer extracellular vesicles (EVs) to overcome various biological barriers for drug delivery applications. (1) **Gastrointestinal barriers** can be overcome by deploying milk and plant-derived EVs to withstand the harsh acidic and enzymatic conditions, while also ensuring that EVs possess surface glycoproteins to enable trans-endocytosis across the small intestinal barrier. (2) **The placental barrier** can be overcome directly via the use of placental and THP-1 monocyte EVs, or indirectly via engineering watermelon EVs to target intestinal epithelial cells (IECs) which can communicate with the placenta. (3) **Immunological barriers** can be overcome by having a high proportion of CD47, CD24, CD31 and PD-L1 to produce the "don't eat me" signal, and probably a low level of phosphatidylserine (PS) on the surface of EVs to minimize the chances of being engulfed by macrophages. (4) **Neurological barriers** can be overcome minimally by incorporating high amounts of CD46 and CD63 into EVs as the quantity of these tetraspanins are positively correlated with the ability of EVs to cross the blood-brain barrier (BBB). (5) **The lymphatic barrier** can be overcome by EVs naturally as their lipidic nature enables them to cross the reticular network of the blood-lymph barrier (BLyB). Adding α-D-mannose and PEGylating EVs may also enhance their passage across the barrier. (6) **The pulmonary barrier** may be targeted by EVs derived from bronchoalveolar lavage fluid (BALF), which possess MHC classes I and II, CD54, CD63 and CD86. (7) **The renal barrier** can be overcome by EVs naturally, probably due to their small size and fluid lipid membranes which might allow them to squeeze through the tiny pores of the glomerular filtration barrier. (8) **Cellular barriers** can be overcome by EVs naturally via retrograde trafficking, endocytic recycling, direct fusion with the plasma membrane or other mechanisms. Engineering EVs with a size of <100 nm might help to reduce the chances of EVs being internalised via macropinocytosis which tends to lead to lysosomal degradation more than other mechanisms accessible to smaller EVs. Incorporating syncytin-1 and -2 into EVs might also enable them to fuse with the plasma membrane directly, allowing them to evade the endosomal and lysosomal membranes completely (created with BioRender.com).*

5. Conclusion—the future of EVs

Through critically analysing the relationship between the key components of EVs and the biological barriers EVs overcome, this review is the first to put together a recommendation in such a manner (**Figure 4**) to engineer EVs as suitable DDSs based on various studies. The implementation of EV drug carriers would revolutionise the global worldview of therapeutic treatments, as EVs unlock a whole new realm of endless possibilities in achieving the ideal therapeutic for patients, one of maximum efficacy and biocompatibility with negligible side effects. Even now, efforts have been made to transform the notion of personalised medicine into a reality, and having EVs as fully-approved personalised DDSs is worth pursuing. As past findings are limited due to the complex nature of EVs and various biological membranes, it is hoped that the mechanisms of EVs and their interactions with various biological membranes can continue to be more fully delved into, and that EV engineering can be carried out *via* efficacious yet sustainable methods, bearing in mind the availability and accessibility of natural EV sources, and hence the cost-effectiveness of the engineering processes.

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