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REVIEW

Exosomes: Biogenesis, targeting, characterization and their potential as "Plug & Play" vaccine platforms

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Abstract

Exosomes are typically characterized as spherical extracellular vesicles less than 150 nm in diameter that have been released into the extracellular environment via fusion of multivesicular bodies (MVBs) to the plasma membrane. Exosomes play a key role in cell-cell communication, vary widely in their composition and potential cargo, and are reportedly involved in processes as diverse as angiogenesis, apoptosis, antigen presentation, inflammation, receptor-mediated endocytosis, cell proliferation, and differentiation, and cell-signaling. Exosomes can also act as biomarkers of health and disease and have enormous potential use as therapeutic agents. Despite this, the understanding of how exosome biogenesis can be utilized to generate exosomes carrying specific targets for particular therapeutic uses, their manufacture, detailed analytical characterization, and methods of application are yet to be fully harnessed. In this review, the author describes the current understanding of these areas of exosome biology from a biotechnology and bioprocessing aspect, but also highlight the challenges that remain to be overcome to fully harness the power of exosomes as therapeutic agents, with a particular focus on their use and application as vaccine platforms.

KEYWORDS
biogenesis, bioprocessing, exosomes, extracellular vesicles, surface display, vaccine platform

Abbreviations: Ac4ManNAz, tetraacetylated N-azidoacetyl-D mannosamine; Ag85B, *Mycobacterium tuberculosis* antigen 85B; ALIX, ALG-2 interacting protein X; APC, antigen presenting cell; BM-DC, bone marrow-derived dendritic cell; BCG, Bacillus Calmette–Guérin; CEA, carcinoembryonic antigen; cGMP, current good manufacturing practice; CFP, culture filtrate protein; CHMP4, charged multivesicular body protein 4; CMV, cytomegalovirus; DBCO-Cy5, dibenzyl cyclooctyne-conjugated Cy5; DC, dendritic cell; DNA, deoxyribonucleic acid; eGFP, enhanced green fluorescent protein; EBV, Epstein Barr Virus; ELISA, enzyme-linked immunosorbent assay; ESAT6, 6 kDa early secretory antigenic target; ESCRT, endosomal sorting complex required for transport; EV, extracellular vesicle; FACS, fluorescence activated cell sorting; GTP, guanosine-5'-triphosphate; GTPase, guanosine-5'-triphosphate hydrolase; HER2, human epidermal growth factor receptor 2; HEK293T, human embryonic kidney 293T cell line; HIV, human immunodeficiency virus; HPV, human papillomavirus; HS, heparin sulphate; HSP, heat shock protein; HSPG, heparan sulphate proteoglycans; HUVEC, human vascular endothelial cells; IgG, immunoglobulin G; ILV, intraluminal vesicles; INF-γ, interferon gamma; lncRNAs, long non-coding ribonucleic acid; MAGE 3, melanoma-associated antigen 3; MHC, multi-histocompatibility complex; miRNAs, micro-ribonucleic acid; MoDC, monocyte-derived dendritic cells; MRC-5, medical council research strain 5 (fetal lung fibroblast cell line); MVB, multivesicular bodies; NK, natural killer; nSMase2, type II sphingomyelinase; OMV, outer membrane vesicle; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PDCD6IP, programmed cell death 6-interacting protein; PLP, proteolipid protein; PRRSV, porcine reproductive & respiratory syndrome virus; qRT-PCR, quantitative real-time polymerase chain reaction; RABV, rabies virus; RFP, red fluorescent protein; RNA, ribonucleic acid; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-Cov-2, severe acute respiratory syndrome coronavirus (2019 strain); SEA, soluble egg antigen; siRNA's, small interfering ribonucleic acid; SNAP, synaptosomal-associated protein; SNARE, synaptosomal-associated protein receptor; TAA, tumor-associated antigen; TEM, transmission electron microscopy; TEMs, tetraspanin-enriched microdomains; TLR, toll-like receptor; TNF- α, tumor necrosis factor α; tRNA, transfer ribonucleic acid; TSG101, tumor susceptibility gene 101; t-SNARE, target-associated synaptosomal-associated protein receptor; VLP, virus-like particle; v-SNARE, vesicle-associated synaptosomal-associated protein receptor; VSV, vesicular stomatitis virus.

1 | INTRODUCTION

1.1 | Definition of exosomes

Of the three main subcategories of extracellular vesicles (EVs) derived from mammalian cells (Table 1), exosomes are the smallest in size. Exosomes are typically defined as spherical EVs of <150 nm in diameter that are released into the extracellular environment via fusion of multivesicular bodies (MVBs) to the plasma membrane.[1] Other subcategories of EVs include microvesicles, formed by outward budding of the plasma membrane, and apoptotic bodies, a by-product of programmed cell death, both being generally >100 nm in diameter.[1] There is also another class of EV recently discovered, termed exomeres, although there are only two published studies detailing methods for their isolation and characterization,[2,3] and one investigating their potential as carriers of functional cargo.[4] They are distinct from exosomes in their size (<50 nm),[2] differ in protein composition and lack encapsulation in a lipid bilayer.[3]

1.2 | The composition and functions of exosomes

It has been more than 50 years since the first description of EVs as "plasma dust"[5] and nearly 40 years since the visualization of their release from cells via endocytic pathways in rat[6] and sheep reticulocytes.[7] These observations led to the pioneer of the field, Rose Johnstone, naming them "exosomes." Initially, the function and impact of exosomes were widely debated and largely underappreciated. However, their potential as diagnostic biomarkers, vehicles for drug delivery, potential in regenerative medicine and possible use as viral and cancer vaccine candidates has increased interest in exosomes alongside an improving knowledge of their biosynthesis and role(s) in cell-cell communication. Here we focus on exosomes, although we note they are not the only technology for vesicle-based delivery and presentation, with virus-like particles (VLPs), liposomes and bacterial outer membrane vesicles (OMVs) as alternative options.

Exosomes show a large variation in their molecular composition, often dependent on the cell type they are released from and function/target requirements. There are, however, a number of exosome protein markers including transmembrane tetraspanins (CD81, CD63, CD9), flotillin, cytosolic heat shock proteins (HSP70 & HSP90), Rab proteins (Rab5 & Rab7), annexins, TSG101 and MHCs.[8–13] A comprehensive evaluation of exosome compositions is available in the databases ExoCarta (www.exocarta.org),[14] Vesiclepedia (www.microvesicles.org/)[15] and Exosome-RNA (https://exosome-rna.com)[16] that collectively catalogue the various protein, lipid and nucleic acid compositions reported in exosomes and other EVs from a wide range of cell types. Multiple biological functions of exosomes have been reported and include roles in angiogenesis, apoptosis, antigen presentation, inflammation, receptor-mediated endocytosis, cell proliferation and differentiation, and cell-signaling, and it is now apparent that exosomes can also be biomarkers of health and disease[17] (Table 2).

1.3 | The biogenesis of exosomes

1.3.1 | The ESCRT pathway

The biosynthesis of exosomes involves two major events; an endocytic event at the plasma membrane, which, following the maturation of early endosomes to late endosomes, progresses to the formation of intraluminal vesicles (ILVs) through inward budding of the endosomal membrane to form MVBs[18] (Figure 1). A prominent component in MVB formation is the ESCRT (endosomal sorting complexes for transport) machinery, comprised of four different protein complexes (ESCRT-0, -I, -II, and -III).[1] Whilst the ESCRT-0 complex works to cluster ubiquitinylated cargo, the ESCRT-I and -II complex are

TABLE 1 Summary of the three main defined subcategories of extracellular vesicle, including size, origin and mechanism of biosynthesis, and the properties unique to each subcategory

EV subcategory	Size range (diameter, nm)	Origin and mechanism of biosynthesis	Unique properties
Exosomes	50-150	Fusion of MVBs with the plasma membrane to release ILVs, termed exosomes on release to the extracellular space	Smallest EVs. Heavy involvement in cell-cell communication. Have distinct universal molecular compositions alongside cell specific attributes. Carry unique cell origin-specific surface proteins. Isolation methods: mainly ultra-centrifugation and immunoaffinity capture.
Microvesicles	100-1000	Outward budding (exocytosis) of the plasma membrane	Also known as ectosomes, shedding vesicles and oncosomes. Molecular composition is more dependent on cell-type origin. No unique surface marker proteins. Isolation methods: mainly ultracentrifugation.
Apoptotic bodies	100-5000	Released from cells undergoing apoptosis	Carry cellular debris away from dying cells and trigger phagocytes to clear the area of waste produced in apoptosis. Isolation methods: centrifugation.

TABLE 2 Biomarkers of exosomes found in various cancer patient bodily fluids and patient-derived cell line culture media, reported to accurately predict cancer status. The methods for exosomal biomarker detection and measurement are also outlined

Exosome biomarker associated disease	Biomarker molecule(s)	Localization of biomarker	Method of detection
Lung cancer[101]	miR-378a, miR-379, miR-139-5p, miR-200b-5p	Blood plasma	qRT-PCR
Esophageal squamous cell cancer[102]	miR-21	Blood serum	qRT-PCR
Colorectal cancer[103]	let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, miR-23a	Blood serum	qRT-PCR
Pancreatic cancer[104]	Glypican-1	Frozen blood serum	FACS analysis, immunogold TEM
Breast cancer[105]	miR-1246, miR-21	Blood plasma	qRT-PCR
Bladder cancer[106]	miR-4454, miR-21, miR-15b-5p, miR-126-3p, miR-93-5p, and miR-150-5p, miR-720/3007a, miR-205, miR-200c-3p and miR-29b-3p	Tumor tissue, urine and white blood cells	Nanostring miRNA assay, Droplet digital PCR
Acute myeloid leukemia[107]	let-7a, miR-99b, -146a, -155, -191, and -1246	Blood serum	qRT-PCR
Glioma[108]	miR-21	Cerebrospinal fluid	qRT-PCR
Gastric cancer[109]	lncUEGC1	Blood plasma, cell culture media	qRT-PCR
Head and neck cancer[110–112]	Human Papillomavirus 16 (HPV16) DNA for HPV driven oropharyngeal cancer, GAPDH, ALDOA, LDH, PKM	Saliva	qPCR, Mass spectrometry, transmission electron microscopy
Liver cancer[113]	tRNA-ValTAC-3, tRNAGlyTCC-5, tRNA-ValAAC-5 and tRNA-GluCTC-5	Blood plasma, cell culture media	qRT-PCR

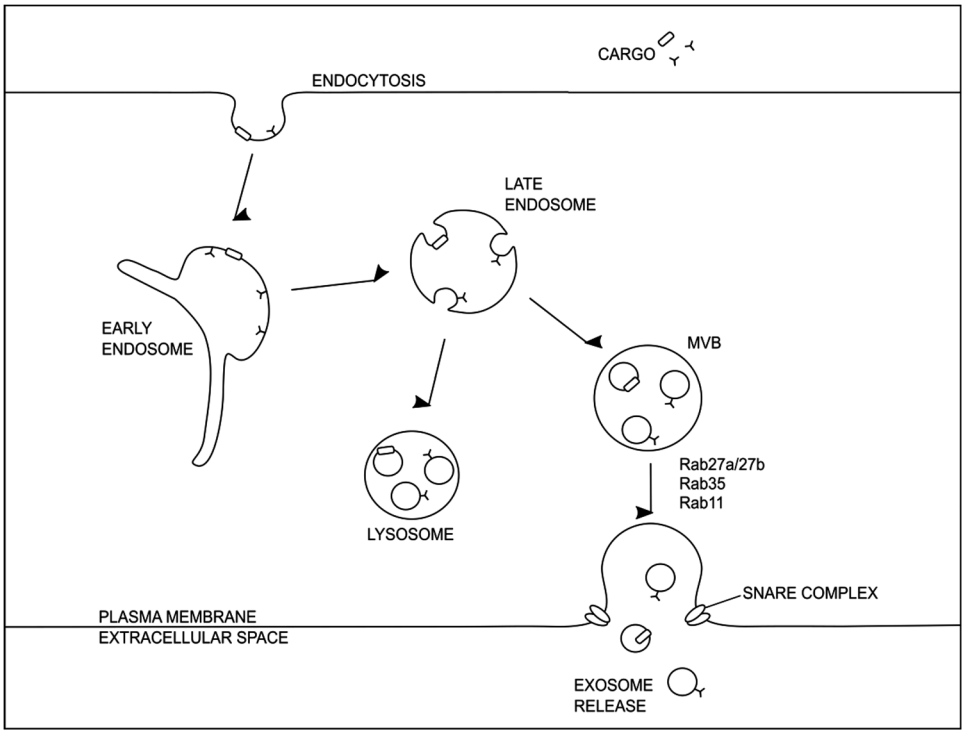

FIGURE 1 Schematic depicting exosome formation and release from cells. Cargo taken in by endocytosis is sorted at early endosomes which is then packaged into either lysosomes for degradation, or multivesicular bodies (MVB) containing intraluminal vesicles (ILV) formed by budding of late endosomal membranes. ILVs are then released as exosomes into the extracellular space when MVBs fuse with the plasma membrane

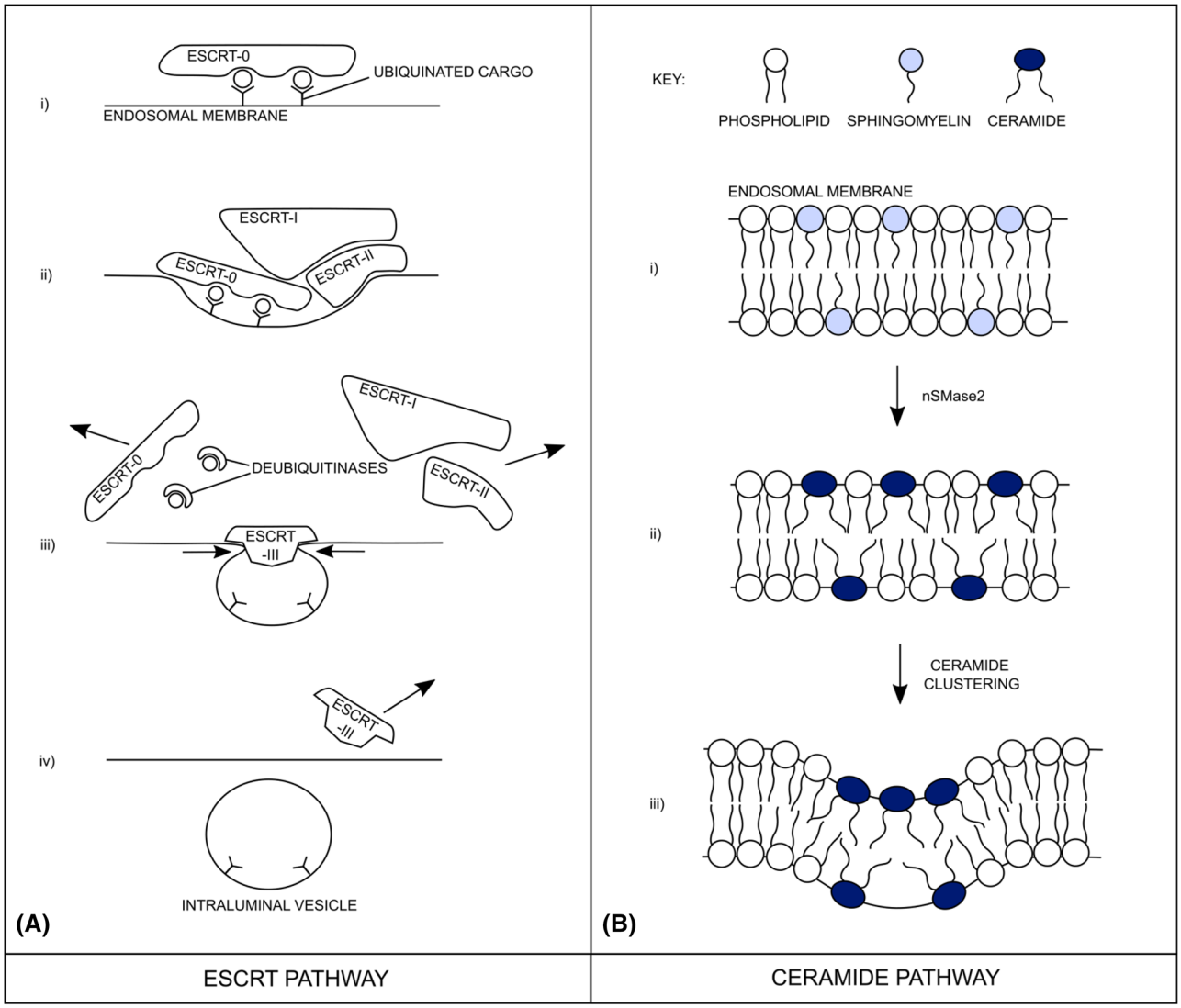

FIGURE 2 (A) Vesicle formation via the ESCRT Pathway in which ubiquitinated proteins at the endosomal membrane are recognized by ESCRT-0 (i), which then binds with ESCRTI and -II to begin invagination of the endosomal membrane (ii). This process results in the budding of the endosomal membrane, encapsulating the ubiquitinated cargo which is then deubiquitinated (iii), and the vesicle is formed when the membrane opening of the developing bud is fused by ESCRT-III (iv). (B) Endosomal membrane budding for vesicle formation via the Ceramide Pathway in which conversion of sphingomyelin (i) to ceramide within the membrane by nSMase2 (ii) results in clustering of ceramide to create the curvature of the membrane (iii) due to its more branched, unsaturated fatty acid tail structures

involved in membrane budding followed by ESCRT-III driving membrane scission[19] (Figure 2). Completion of exosome production is achieved after MVBs are trafficked to the plasma membrane along microtubules by Rab GTPases and released to the extracellular space via fusion with the plasma membrane mediated by SNARE (soluble N-ethylmaleimide-sensitive fusion attachment protein receptor) proteins.[20]

The small GTPases of the Rab family, within the Ras superfamily, play a part in exosome biogenesis and guide vesicles in trafficking, motility and docking before their fusion with the plasma membrane via SNARE protein complex formations and release into extracellular space.[21] The Rab GTPases, once recruited to MVB membranes, act as molecular switches cycling between a GTP-bound active form and a GDP-bound inactive form to facilitate vesicle trafficking.[22] The GTPases Rab27a and Rab27b have been linked to docking of MVBs at the plasma membrane with their silencing leading to changes in the distribution and targeting of exosomes.[23] Rab35 and its GTPase activating proteins have been proposed as regulators of the docking and tethering of endocytic vesicles to the plasma membrane.[24] The final release step is when v-SNAREs (vesicle-associated) on MVB membranes come together with t-SNAREs (target-associated) on the plasma membrane to form a stable ternary SNARE complex.[25]

1.3.2 | The ceramide pathway

Independent of the ESCRT machinery, there is an MVB formation pathway involving a type II sphingomyelinase, nSMase2, a lipid-modifying

enzyme that converts sphingomyelin to ceramide creating membrane curvature required for vesicle formation and whose knockdown results in a reduced exosomal release of proteolipid protein (PLP) in Olineu cells.[26] This conversion to ceramide at sphingolipid-rich membrane lipid microdomains results in the clustering of domains to form lipid rafts and triggers membrane invagination and budding[27] (Figure 2). Knockdown of the key ESCRT complex components Hrs, Alix, and Tsg101 does not affect exosome secretion via this pathway,[26] confirming its independence from ESCRT machinery. It has been suggested that the fate of MVBs may be determined by which pathway they are produced, with ESCRT-dependent mechanisms producing MVBs likely to be fused to lysozymes for degradation and ceramide-dependent mechanisms leading to exosome release.[28]

1.3.3 | The syndecan-syntenin-ALIX pathway

Syndecans are a family of transmembrane proteins prominent in cell surface signaling that regulate and bind molecules through their heparan sulphate (HS) chains.[29] The binding of syndecan cargo via HS chains leads to a clustering of syndecans and creates assemblies capable of recruiting syntenin-1 and ALIX to support membrane budding.[30] Syntenin-1 (SDCBP), is a cytosolic factor containing two PDZ domains with a high affinity for interaction with syndecans, which facilitates the recruitment of syntenin to membranes. The N-terminal region interacts with ALIX, or PDCD6IP, capable of interaction with the ESCRT proteins TSG101 and CHMP4 via three LYPX(n)L motifs.[31] The three proteins can form tripartite complexes, syndecan-syntenin-ALIX, in vitro[31] and through these interactions are thought to ensure adaptation for ESCRT budding machinery at the endosomal membrane.[32] Alongside syntenin and ALIX, the tetraspanin CD63 co-accumulates in exosomes produced by this pathway, possibly due to its ability to bind with the PDZ domain of syntenin, producing a $CD63^+$ exosome subpopulation that bypasses lysozyme degradation.[31] Tetraspanins, including CD9, CD63 and CD81, are considered biomarkers of exosomes and are found in tetraspanin-enriched microdomains (TEMs) which may act as "specialised cargo platforms" capable of directing components to exosomes.[33]

1.4 | Exosomes and elicitation of immune system mechanisms

Exosomes can present antigens to the immune system and trigger an immune response via a number of mechanisms as outlined in Figure 3. Indeed, the benefits of using exosomes as antigen-carrying agents include their general low or non-immunogenicity[34]; however, changes to the constituent molecules within an exosome can inhibit or activate the immune response.[35] For example, the presence of the programmed and cell death ligand-1 (PD-L1) transmembrane protein in exosomes can result in tumor immune escape which leads to immunotherapy resistance arising.[36] The role that exosomes may play in activating immune responses, and how these could present antigens to the immune system such as would be required in the case of vaccines, is discussed in more detail in Section 2.2 below.

2 | EXOSOMES AS MESSENGERS AND MEDIATORS OF IMMUNE RESPONSES

2.1 | Shuttling properties of exosomes

As "messengers" of cell-cell communication, exosomes carry cargo between cells such as antigens to raise a direct response in immune cells,[37–42] or nucleic acid molecules including DNA,[43] mRNA,[44] microRNAs,[44–49] and lncRNAs.[50,51] Messenger molecules are delivered when the exosomes are internalized by the recipient cells via endocytosis. Manipulation of this "shuttling" function using exosomes either harvested or engineered for carrying specific cargos has been successful for delivery of specific miRNA's,[52,53] siRNA's,[54,55] and antigens[56–59] to targets, with work being undertaken to use exosomes as nanoshuttles for drug delivery.[60]

2.2 | Roles of exosomes in immunity

Exosomes play a major role in immunity, acting as both mediators and modulators during infections. Exosomes released from infected cells can carry pathogen-associated molecular patterns (PAMPs), unprocessed antigens and even antigens displayed on the exosomal membrane by major histocompatibility complexes (MHCs) (see Figure 3).[40,61,62] These EVs are then received by their recipient cells either via cell adhesion at the cell surface, via specific receptors, or can be internalized by the cell into endocytic compartments.[61]

Activation of the innate immune system can be triggered by the recognition of PAMPs by toll-like receptors (TLRs), meaning that exosomes released from infected cells bearing PAMPs can promote immune responses (Figure 3). Antigen presentation is also propagated by exosomes released from infected cells, capable of inducing CD4+ and CD8+ T cell responses. This can be through direct or cross-presentation (Figure 3), in which antigens are presented as part of MHC-peptide complexes to antigen-specific T cells or transferred alone to antigen-presenting cells (APCs) which then process and load them onto their own MHCs for display.[40] It is also possible for exosomes to transfer antigenic MHC-peptide complexes onto dendritic cells (DCs) which then engage in T cell presentation (Figure 3), known as cross-dressing.[63] This method of presentation has been exploited in the development of "Dexosomes" (Dex), DC-derived exosomes capable of transferring antigenic MHC-peptide complexes onto DCs that have not encountered the antigen, which have been trialed as an immunotherapy to treat cancers[64–66] (Table 3).

Investigations into the use of exosomes released from cells of an infected host as a possible immunization agent have also been explored for vaccination against viral,[57,59,67–71] bacterial,[72,73] and parasitic[52] pathogens (see Table 3). These studies have shown that serum-derived exosomes from infected hosts can elicit T cell responses

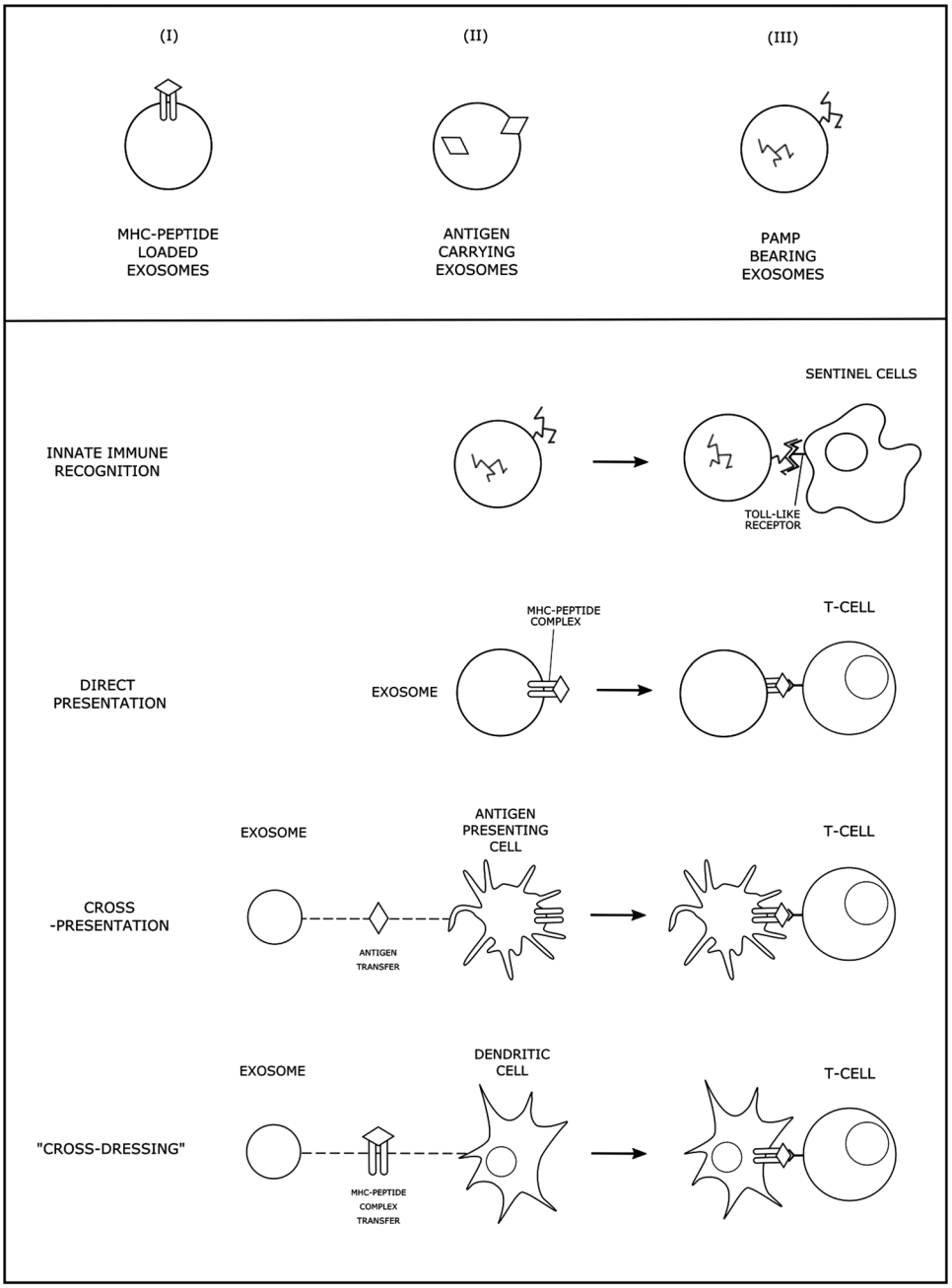

FIGURE 3 Variations on the types of exosome-cargo loading (I-III) and their possible presentation strategies to the respective immune cells, dependent on cargo type, to illicit immune responses

in naïve hosts, an immunization method that has the potential to be safer than live-attenuated vaccines but more effective than subunit vaccines which often require adjuvants and boosters.

Naturally, derived exosomes may not be viable as a treatment for some infections such as those caused by enveloped viruses. These pathogens can hijack the exosome biogenesis pathways of host cells which can promote viral reproduction.[74,75] This utilization of the exosome biogenesis pathways also produces exosomes with both PAMPs and host factors, which are capable of hiding from the immune system, as well as exosomes containing docking receptors which increase the infectivity of the invading pathogen.[62] In this case, genetically engineered exosomes designed to only bear specific antigens rather than serum-derived exosomes can be used, although this approach may be preferable in any potential exosome-based vaccine development endeavors.

2.3 | Exosomes and cancer

Exosomes are also reportedly important in the development of some cancers, shuttling proteins and nucleic acids.[76] They are also present in most bodily fluids,[77] presenting an opportunity to extract vesicles,

TABLE 3 Published experimental exosome-based vaccinations against various bacterial, parasitic and viral targets as well as immunotherapies against autoimmune disease and cancers, including the details of exosome therapeutics production and the outcome of each study/trial

Therapeutic target	Target type	Exosome vaccine production method	Experimental model	Outcome
M. tuberculosis[72]	Bacterial	Exosomes isolated from murine macrophage cell line (Raw 264.7) treated with *M. tuberculosis* culture filtrate protein (CFP).	Mouse	CFP exosome vaccination in mice stimulated a T_H1 immune response, but a limited T_H2 response compared with responses seen BCG-vaccinated mice. However, prime-boost models showed no difference.
Mycobacterium bovis[73]	Bacterial	Exosomes isolated from a BCG-infected murine macrophage cell line (J774)	Mouse	BCG^+-macrophage exosomes activated naïve T cells, which also respond with IFN-γ production upon restimulation with BCG antigens ex vivo, as well as effective memory $CD4^+$ and $CD8^+$ T cells.
Toxoplasma gondii[52]	Parasite	Isolated from *T. gondii* culture media	Mouse	A significantly increase of $CD8^+$ T cells was observed in the exosome-immunized mice as well as a humoral immune response with increased IgG levels, both important factors in fighting a *T. gondii* infection. However, for this study, complete protection was not achieved as all immunized mice died when challenged with intraperitoneal injection of the parasite.
Porcine reproductive respiratory virus (PRRSV)[67]	Viral	Exosomes isolated from sera of PRRSV-infected pigs	In vitro	This study found that PRRSV viral antigens were present in both viremic and non-viremic pigs serum-derived exosomes, with non in the naïve animals sera. It also showed in an ELISA assay, that sera of viremic pigs reacted specifically to exosomes purified from non-viremic pigs but not naïve sera, suggesting these exosomes have the immunogenicity that make for a potential vaccine candidate.
EBV, CMV & Influenza[68]	Viral	Exosomes isolated from human monocyte-derived dendritic cells loaded with a mix of 23 immunogenic peptides from EBV, CMV and influenza	In vitro	MoDC-exosomes were able to induce IFN-γ production in $CD8^+$ T cells, without the addition of DCs, in a dose-dependent manner. The MoDC exosomes were found to have greater T cell stimulatory capacity in their lipopolysaccharide-matured form and that exosomes could also induce TNF-α production.
CMV[69]	Viral	Exosomes isolated from CMV-infected human vascular endothelial cells (HUVECs).	In vitro	It was observed that $CD4^+$ T cells were not directly activated by CMV-infected HUVECs, but by the antigens carried on exosomes released from them. Purified exosomes from HUVECs were able to activate $CD4^+$ T cells in the absence of cocultured endothelial cells.
Rabies virus[70]	Viral	Exosomes isolated from a rabies-infected human embryonic lung fibroblast cell-line (MRC-5)	In vitro	Exosomes isolated from cells infected with the rabies virus (RABV) carry a microRNA (miR)-423-5p, which as part of a feedback inhibition loop suppress RABV replication. Isolated exosomes carrying miR-423-5p, when delivered intracellularly, inhibited RABV replication in MRC-5 cells.
HPV[71]	Viral	Engineered exosomes were produced endogenously in hosts after delivery of a DNA vector expressing fusion protein (HIV Nef^{mut}/HPV E7) via intramuscular injection	Mouse	Mice inoculated with the Nef^{mut}/E7 expressing DNA vector developed $CD8^+$ T cell response to both Nef and E7, but not to vectors expressing them alone – likely due to non-incorporation into exosomes. Successful immunogenic exosome production was further proven by E7-specific $CD8^+$ T cell responses in mice injected with the plasma of DNA-treated mice. The $CD8^+$ T cell response induced by the DNA injection was also effective in producing an anti-tumor effect in mice implanted with TC-1 tumor cells.

(Continues)

TABLE 3 (Continued)

Therapeutic target	Target type	Exosome vaccine production method	Experimental model	Outcome
Ebola Virus VP24, VP40 and NP, Influenza Virus NP, Crimean–Congo Hemorrhagic Fever NP, West Nile Virus NS3, and Hepatitis C Virus NS3[57]	Viral	Engineered exosomes were produced endogenously in hosts after delivery of a DNA vector expressing fusion protein (HIV Nef^{mut}/target viral antigen) via intramuscular injection	Mouse	Mice injected with the DNA vectors for expression of various fusion antigen proteins, all developed a well-detectable $CD8^+$ T cell response. The response raised by the exosomes produced from these injections was also able to neutralize peptide-loaded and/or antigen-expressing syngeneic cells.
SARS-CoV[59]	Viral	Engineered exosomes produced via cell line HEK293T transfected with a vector expressing a fusion protein (SARS-S protein/VSV-G protein).	Mouse	S-containing exosomes were able to induce neutralizing antibody titers when tested in mouse models. When used to prime alongside an adenoviral vector vaccine booster, neutralizing antibody titers exceeded those observed in convalescent serum of s SARS patient.
Metastatic melanoma[64]	Cancer	Exosomes isolated from cultured human monocyte-derived dendritic cells (MoDCs), which were then loaded with the target (MAGE 3) peptides/MHC-peptides.	Human	Of the 15 patients who took part in this trial, one exhibited a partial response whilst one minor, two stable and one mixed response was also observed at skin and lymph node sites. Whilst no MAGE3-specific $CD4^+$ and $CD8^+$ T cells responses were detectable in the blood; the trial did highlight the safety of exosome administration to humans.
Non-small cell lung cancer[65]	Cancer	Exosomes isolated from cultured human MoDCs, which were then loaded with the target (MAGE) peptides/MHC-peptides.	Human	Increases in systemic immune responses against MAGE in three of the nine patients who had no reactivity prior to immunization. NK cell activation was increased, but minimal increases were seen of antigen-specific T cell activity in in vitro assays of circulating PBMCs.
Mastocytoma/ adenocarcinoma[66]	Cancer	Exosomes were isolated from either human MoDCs or murine bone marrow-derived dendritic cells (BM-DCs) which were then loaded with the target peptides.	Human	Administration of BM-DC-derived exosomes to mice with established mastocytoma (P815) tumors resulted halted tumor growth within a week and 40%–60% of mice were tumor free by day 60. This proved to be a long-lasting immune response as they were able to reject a tumor challenge with P815 (though not with syngeneic leukemia L1210).
Inflammatory bowel disease[114]	Autoimmune Disease	Exosomes were isolated from *Schistosoma japonicum* soluble egg antigen (SEA)-treated BM-DC cultures	Mouse	Mice with induced acute colitis were given intraperitoneal injections of exosomes over 7 days, after which there was a significant reduction in disease activity indexes, weight loss as well as improved colon health. The exosomes also exhibited prophylactic properties, preventing colon damage in acute colitis-induced mice.

non-invasively, and examine for any known indicators of malignancy as a form of diagnosis. Using exosomes as diagnostic biomarkers would allow for less invasive sampling procedures and the capability of detecting cancers very early,[78] which is known to heavily impact patient outcomes.[79] There have been several exosome-related cancer biomarkers identified in various body fluids across multiple cancers that can accurately predict the cancer status of patients (Tables 2 and 3).

Exosomes secreted by tumor cells are also often immunologically active, capable of carrying native tumor antigens for cross-presentation, contributing to immune surveillance and enhancing opsonization.[63] Cases of antigen presentation and T cell activation have been observed in human melanoma where DCs which have taken up exosomes can activate tumor-specific $CD8^+$ T cells and promote tumor rejection.[38] Furthermore, these melanoma-derived exosomes were able to induce $CD8^+$ T cell cross-priming in vivo[38] and this effect has been re-produced ex vivo by pulsing monocyte-derived dendritic cells (MoDCs) with the melanoma-derived exosomes to induce restricted $CD8^+$ T cells responses.[80] The authors reported that MoDCs pulsed with tumor-exosomes could be more efficient than those loaded with soluble antigens, providing a potential exosome-based method for cancer therapy. Alternatively, exosomes that carry MHCII-peptide complexes can directly activate $CD4^+$ T cells[81] and exosomes engineered to carry specific antigen-MHC-II complexes could be used as vaccines. Unfortunately, tumor-derived exosomes have the potential to both promote and inhibit tumor growth.[63]

Inhibitory behaviors can include exosome-mediated suppression of T cell and natural killer (NK) cell activity whilst activating myeloid-derived suppressor cells. Tumor progression can also result from the production of exosomes which facilitate the expulsion of anti-cancer drugs and block their delivery by enhancing fibroblast growth, leading to decreased efficacy of cancer treatment regimens.[82]

The contradictory promoting and inhibiting effects of tumor-exosomes can be attributed to different local nano-environments containing heterogeneous populations of exosomes.[83] The inconsistent impact of exosomes in tumor immune responses likely arises from the fact that exosomes can both promote and suppress immunity but do so at different intensities and times.[84] Thus, by identifying the peak time for generation of immunity-promoting exosomes within a disease timeline, effective anti-tumor therapy could be developed.[84] This carries risk in that such a timepoint must be identified and appropriate exosomes delivered to promote immunity rather than suppress it. Nevertheless, the development of vesicle-based therapeutics remains a highly desirable goal despite these complications due to their ability to move unrestricted across tissue barriers such as the blood-brain barrier.[85]

3 | DEVELOPING AN EXOSOME-BASED PLUG AND PLAY ANTIGEN DISPLAY SYSTEMS FOR VACCINE DEPLOYMENT

3.1 | Host-derived exosome targeting components

Creating an effective "Plug & Play" system, to display proteins of interest on the surface of exosomes, is the first step toward developing exosome-based therapies. There have been several methodologies developed for the manipulation of exosome structural and biogenesis pathway components for antigen incorporation (see Figure 4 for key examples). An early exosome display technology exploited the C1C2 domain of Lactadherin, which mediates binding to lipid surfaces and secretion of Lactadherin in association with exosomes, by replacing the EGF-like domain with a protein of interest to produce exosomes displaying the target protein (Figure 4).[86] This method has been utilized to target the tumor-associated antigens (TAAs) CEA and HER2 to exosomes, which showed improved antigen-specific immune responses and enhanced therapeutic anti-tumor responses in mouse models.[87]

Ubiquitin is another molecule shown to direct proteins to exosomes (Figure 4), thought to be an artifact of its involvement in trafficking molecules to vesicles for lysosomal degradation.[88] Utilizing this, vectors expressing fusion proteins of ubiquitin with eGFP, HER2 or *Mycobacterium tuberculosis* antigens Ag85B and ESAT6 have been expressed in human embryonic kidney 293 (HEK293) cell lines, each of which resulted in exosomes containing eGFP, HER2, or Ag85B-ESAT6, respectively.[88] The authors found that concentrations of the Ag85B-ESAT6 protein targeted to exosomes increased approximately 10-fold compared to controls and that a direct correlation was observed between exosome fusion protein concentrations and the number of Ag85B and ESAT6 specific INF-γ-secreting T lymphocytes in mice which were inoculated with Ag85B-ESAT6^{+} exosomes.

3.2 | Viral-derived exosome targeting components

Components of some enveloped viruses can be modified and re-appropriated as signal sequences to target proteins of interest to exosomes due to their natural ability to hijack host cell exosome biogenesis pathways for their own propagation. Modification of the HIV protein, Nef, which mediates exosome biogenesis hijacking and directs proteins for incorporation into exosomes, and fusion to an antigen is an efficient way of incorporating proteins of interest into exosomes (Figure 4).[89] These Nef^{mut}-engineered exosomes are capable of cross-presentation levels similar to lentiviral Nef^{mut} VLP carrying the same proteins.

Another exosome targeting method that relies on the exploitation of enveloped viral proteins is to use the G protein from vesicular stomatitis virus (VSV), of which the transmembrane and cytosolic domains can be replaced with a protein of interest for targeting exosomes. The application of this was tested with the spike protein (S) of the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) to produce a vaccine to respond to potential future SARS outbreaks.[59] The approach successfully generated S-containing exosomes which, when administered to mice, induced neutralizing antibody titers, but only exceeded titers in convalescent serum of SARS patients when boosted with an adenoviral vector vaccine also expressing SARS-S. The authors suggested that due to the tumor challenge model used to avoid direct work with infectious SARS-CoV, the model may not be a completely accurate representation of the vaccine in human subjects.

3.3 | Exosome proteins as anchors for surface display

Other strategies for targeting exosomes focus on using molecules known to be common throughout subpopulations, exosomal marker proteins, as an anchor for attaching and displaying molecules on the surface of exosomes. This can be done biologically with engineered chimeric marker-display proteins for natural expression of exosomes that display the protein of interest. The transmembrane tetraspanin exosome marker proteins, CD9, CD63, and CD81, are effective anchors for such display technology and can display fusion proteins on the surface of exosomes in an internal and/or external orientation (Figure 4).[90] Using trimeric fusion proteins of GFP and RFP, with the reporters inserted at the internal or external transmembrane loop regions of the tetraspanins, Stickney et al. demonstrated a novel and simple to replicate method for producing a potential vaccine manufacturing platform although no reports have as of yet shown the size limitations of this display technology, or it's efficacy for antigen presentation.

Chemical-based methods are also available for labelling exosomes.[91] The approach described here involved treating cells with

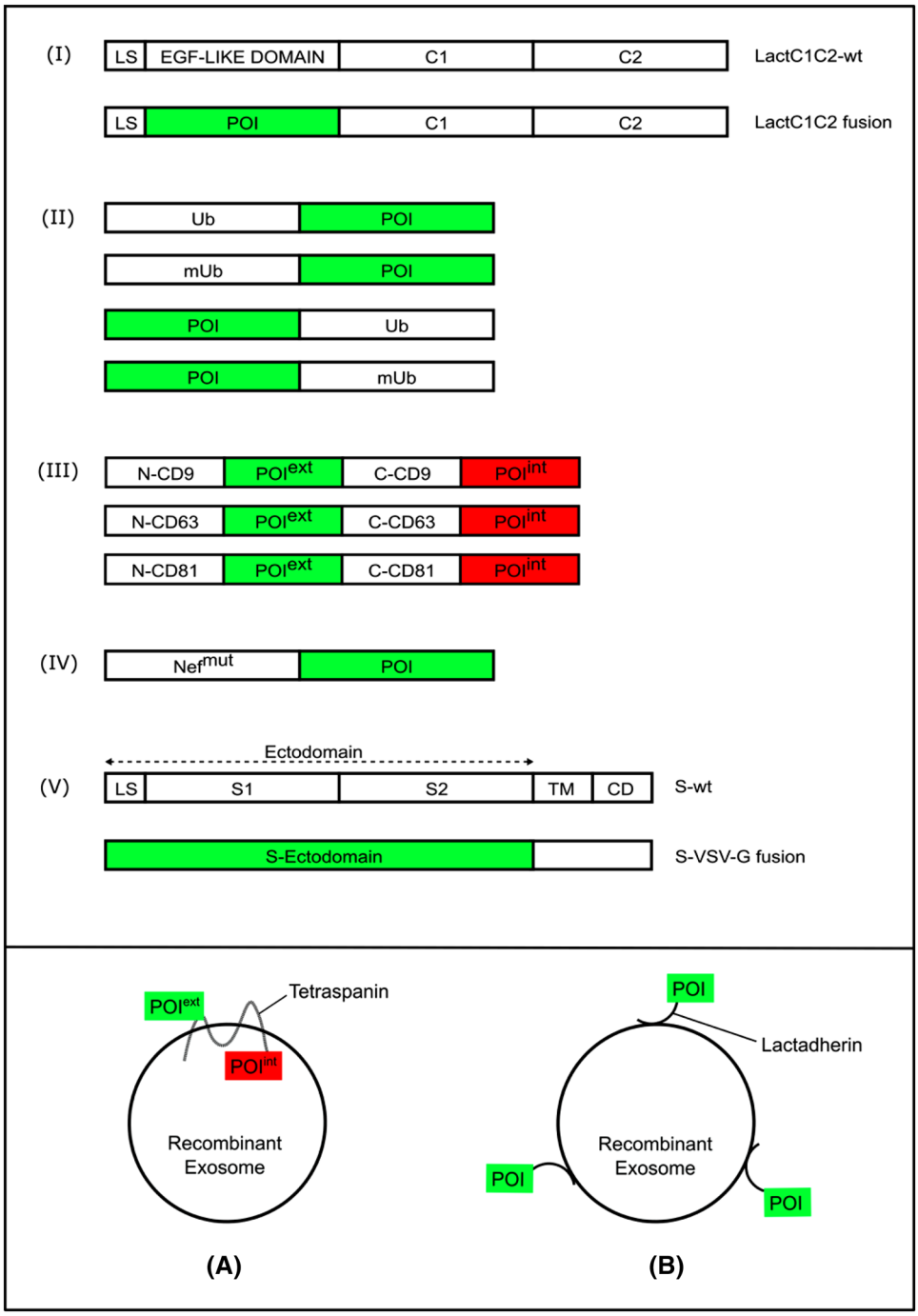

FIGURE 4 Engineered genetic constructs for insertion via plasmid transfection into mammalian cell cultures for generation of recombinant exosomes which display a protein(s)of interest (POI). (I) Lactadherin modification: Replacement of the EGF-like domain of lactadherin with a POI.[86] (II) C-terminal and N-terminal tagging of POIs with ubiquitin or a mutated ubiquitin.[72] (III) Tri-fusion proteins that insert POIs at the external (POIext) and internal (POIint) transmembrane loop domains of the tetraspanins CD9, CD63 and CD81.[90] (IV) HIV Nef protein modification: Tagging of a mutated HIV-Nef protein (Nefmut) with a POI.[89] (V) VSV-G tagging: SARS-Cov S protein has been truncated and tagged with the G protein of the vesicular stomatitis virus.[59] Proposed display conformations (A & B): (A) Resulting recombinant exosomes displaying POIs internally and externally via tri-fusion tetraspanin expression.[90] (B) Resulting recombinant exosomes displaying POIs fused to the lactadherin C1C2 domains present at the vesicle membrane via modified lactadherin expression[86]

tetraacetylated N-azidoacetyl-D Mannosamine (Ac4ManNAz) to generate unnatural azide groups on the cell surface, followed by labelling with the fluorescent dye dibenzyl cyclooctyne Cy5 (DBCO-Cy5) via biorthogonal click chemistry. The cell surfaces bearing labelled azides are taken into the cell to form endosomal compartments and from this, exosomes within MVBs bearing the labelled azides, which the cell then secretes as a result of MVBs fusing with the plasma membrane. This "in situ one-step" labeling strategy and the possibility for labelling with more complex molecules such as antigens opens up the possibility of novel vaccine manufacturing exosome-based platforms.

3.4 | Challenges to overcome in the development of bioprocesses for the production of exosome-based vaccines

Although exosomes offer an exciting new potential approach for vaccine delivery and presentation of antigens, their application for this purpose requires a number of challenges in their manufacture and characterization to be overcome. These include; (1) a need for improved methods for the isolation of more homogeneous exosome preparations that can be used under GMP conditions at scale and reduce the current heterogeneity found in many exosome preparations, (2) new and/or improved analytical technologies and markers that provide certainty over the identification and composition of exosomes that allows robust characterization of exosome preparations and their heterogeneity/homogeneity, (3) precise and controllable methodology for loading of targets into/displayed on exosomes that provide a more uniform distribution of target(s) into exosome preparations, (4) an improved understanding and control of how cellular lipids, proteins and nucleic acids are assembled into exosomes and the impact and safety of these when administered to patients, (5) further development and understanding of sources of exosomes and controlled bioprocesses associated with these. With regard to (5), there are potentially multiple sources of exosomes although cell culture-based systems such as HEK293, epithelial, dendritic and mesenchymal stem cells have been used to produce exosomes used in preclinical development.[92] Such cell culture-based systems can benefit from the knowledge developed around the use of such systems for the large-scale manufacture of recombinant biotherapeutic proteins,[93,94] subunit vaccines[95] and gene therapy viral vectors[96] although scale-up, reproducibility and cell culture bioprocesses all need further development for exosome production. Improved purification methods that provide more homogenous exosome populations may include affinity-based methods whilst engineering of cell systems to enhance cargo loading into, and displayed from, exosomes will need to be developed for exosome systems to become more widely used as vaccines. The chemical conjugation of antigens to specific proteins/sites on exosomes is also an area that warrants further development, which would allow better control over the stoichiometry and homogeneity of exosome labelling with cargo. Thus, before a "plug and play" exosome vaccine technology can emerge, bioprocesses based around well-controlled cell culture fermentation systems associated with improved antigen cargo targeting, advances in downstream large-scale purification that reduces heterogeneity alongside improved analytical characterization that differentiates between subpopulations of exosomes need to be developed to deliver a robust platform for the manufacture of exosomes as vaccines and other applications.

4 | CONCLUSIONS AND FUTURE DIRECTIONS

Exosomes are an exciting potential delivery mechanism for a variety of cargoes and as candidates for vaccine platforms. Their intrinsic low immunogenic profile without risking the introduction of genetic material pathogenic in origin gives this platform an advantage over VLP-based products, while still harboring multiple possible approaches for antigen display engineering. Natural exosomes make excellent vaccine candidates due to their natural shuttling capabilities for communicating and producing immune responses. Engineering exosomes with a "plug and play" system to generate antigen-presenting exosomes could provide a platform technology for the development of rapid, safe vaccines that can be manufactured at scale and produce strong immune and protective responses to disease-causing agents with no viral culturing or adjuvant production required. The potential for exosome-based vaccines as a response to seasonal outbreaks or situations such as the current global pandemic (SARS-CoV-2) is attractive due to the prospective speed of production, as well as in the area of non-communicative diseases such as cancer. Large-scale production of exosome-based products could be achieved using established cGMP production and recovery methods,[97] as well as the potential for products to be lyophilized[98] for cheaper and simpler distribution. With the development of DNA vaccines able to induce endogenous production of engineered exosomes,[57] future production of exosome-based vaccines may eliminate the need for complex production and distribution procedures whilst offering fast adaptability for antigen shift of current infections or sudden emerging outbreaks and pandemics.[99] Prospects for exosome-based therapeutics could even expand to gene therapy, with the delivery of nucleic acids via EVs.[100] However, to achieve these further developments in exosome isolation, analytics for their characterization, more defined exosome targeting strategies and more well-defined cell culture processes and the impact of these on exosome production, will need to be realized.

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CONFLICT OF INTEREST

The authors declare no commercial or financial interests.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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