



REVIEW

Extracellular Vesicles as Therapeutic Tools against Infectious Diseases

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ABSTRACT: Extracellular vesicles (EVs) have arisen as potential therapeutic tools in managing infectious diseases because EVs can regulate cell-to-cell signaling, function as drug transport mechanisms, and influence immune reactions. They are obtained from a myriad of sources, such as plants, humans, and animal cells. EVs like exosomes and ectosomes can be utilized in their native form as therapeutics or engineered to encompass antimicrobials, vaccines, and oligonucleotides of interest with a targeted delivery strategy. An in-depth understanding of host-pathogen dynamics provides a solid foundation for exploiting its full potential in therapeutics against infectious diseases. This review mainly offers an extensive summary of EVs, comprising their various origins, formations, and pathogen relationships. It further provides insights into the various techniques utilized in isolating and engineering these vesicles to target infectious diseases and how challenges involving large-scale production and cargo loading efficiency should be addressed for clinical application. Finally, preclinical and clinical implementations of EVs derived from animals, plants, and microorganisms are elucidated, stressing their promise for designing innovative antimicrobial approaches.

KEYWORDS: Extracellular vesicles; exosomes; ectosomes; infectious diseases; virus; bacteria; fungi; parasite; clinical applications; drug delivery system; vaccines

1 Introduction

Infectious diseases caused by viral, bacterial, and parasitic microorganisms remain a worldwide health challenge that has led to elevated morbidity and mortality rates globally [1]. Traditional therapeutic approaches against these diseases consist of antibacterial, antiviral, and antiparasitic agents. However, these agents have encountered constraints such as drug resistance, inability to travel across some biological barriers, and reduced effectiveness, especially in recently emerging diseases such as COVID-19 and Ebola [2]. Lately, extracellular vesicles (EVs) have appeared to serve as innovative therapeutic tools that can regulate immunological reactions, transport therapeutic contents, and also simulate pathogen-originated cues to regulate disease advancement [3].

EVs are small, membrane-bound structures released by cells into the external environment [4–7]. They are lipid-bound vesicles that encompass an assortment of cell-derived components consisting of lipids, proteins, genetic material such as DNA, messenger RNAs (mRNAs), microRNAs, and other biologically active molecules [7,8]. Depending on their cellular formation, dimension, and role, they are categorized into various primary subtypes: exosomes, microvesicles (MVs), and apoptotic bodies [7,9,10]. They have emerged as vital facilitators in cell-to-cell signaling and possess considerable potential as therapeutic tools in combating infectious diseases [8]. EVs are engaged in diverse physiological and pathological processes



comprising immune defense, inflammation, cellular stress, and the modulation of disease mechanisms in conditions like cancer and nervous system disorders [11,12]. Modern innovations have notably improved the purification and characterization of EVs, enabling a profound insight into their functions in health and illness [13].

EVs serve as modulators of both innate and adaptive immune reactions [14,15]. The therapeutic possibilities of EVs are extensive, with their use in vaccine formation, immune-regulating strategies, and transport of antimicrobial compounds [16,17]. The clinical capacity of EVs can be improved by modifying them to alter their exterior composition or packaging with the desired cargo [10,18]. These vesicles can also serve therapeutic purposes as it has been suggested to be used in sheathing adenovirus used in gene therapy and drug delivery strategies [19]. An in-depth understanding of the roles EVs play in disease progression and spread lays a foundation for their utilization as therapeutic interventions. Studies on viral host interaction have shown exosomes to be involved in the entry and spread of Coronavirus (CoV) via the exosomal protein transmembrane serine protease 2 (TMPRSS2) [20]. HIV, Ebola, Epstein-Barr Virus, and Hepatitis Virus were shown to exploit the host endosomal sorting complex required for transport (ESCRT) and miRNA machineries vital in exosomal biogenesis and formation. This exploitation has facilitated viral spread [21].

Current research has emphasized the complex functions of EVs in infectious illnesses, such as virus-bacteria concurrent infections, immune regulation, and immunization. For example, EVs enable cross-kingdom nutrient transport, increasing microorganism persistence during viral-bacteria co-infections [22]. Bacterial extracellular vesicles (BEVs) are currently acknowledged as crucial agents in host-microbe interaction and have prospects in detection and treatment approaches [23]. Protein-related examination of the outer membrane vesicle-based vaccine VA-MENGOC-BC[®] showed a complex composition that is effective against *Neisseria meningitidis* strains B and C [24]. Moreover, the immune-activating ability of meningococcal external membrane vesicles is strongly reliant on their biochemical structure, affecting their capacity to stimulate innate immune reactions [25].

This review mainly intends to offer an extensive overview of the diverse functions of EVs in infectious diseases. It will address the origins of EVs, their formation and content, innovations in the isolation process, and characterization. Additionally, this review covers the EVs' role in host-pathogen dynamics while also addressing their function as therapeutic tools and modification for improved effectiveness in infectious diseases. The investigation of EVs serving as therapeutic tools in contagious illnesses indicates a rapidly developing discipline with substantial effects on future treatment approaches [16].

2 Biogenesis and Composition of Extracellular Vesicles

In 1983, studies reported that vesicles from endosomes were used by mature reticulocytes to transport transferrin receptors [26,27], leading to the discovery of exosomes. Exosomes are formed from endosomal systems via endocytosis and successive multivesicular body (MVB) formation. It starts with the plasma membrane budding inwards and forming endosomes that contain cell surface proteins and extracellular macromolecules [28]. These early endosomes mature into late endosomes by changing their intracellular location, replacing some endosomal membrane proteins and lipids, such as sphingomyelin and RAB5, with ceramides and RAB11, respectively, and forming MVBs [29]. Within the MVBs are intraluminal vesicles (ILV) produced by the invagination of the endosomal membrane via an ESCRT-dependent or independent pathway [30]. These vesicles contain specific lipids, proteins, nucleic acids, and cytosolic molecules. MVBs have more than one fate after production; some fuse with lysosomes, and the cargo contained in the ILV is degraded, while the rest are transported along the microtubule network and fuse with the plasma membrane. The ILVs are exocytosed into the extracellular matrix as exosomes (Fig. 1) [28,29]. Small GTPases like

RAB 2B, 5, 7, 9A, 11, 27A, 27B, and 35 mediate ILV formation by promoting the transport of MVB along microtubules and membrane fusion, while RAB 31 hinders the fusion of MVB and lysosome fusion [31,32].

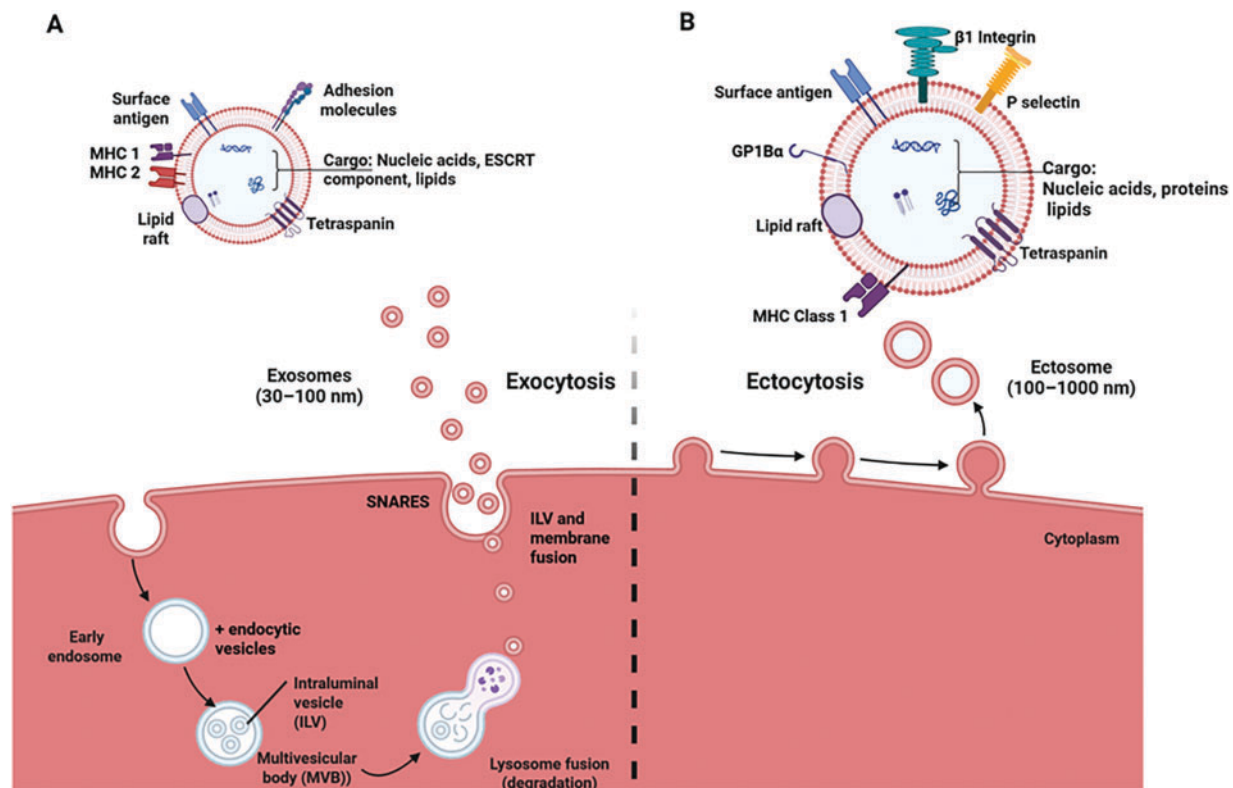


Figure 1: This depiction shows the biogenesis and composition of exosomes and ectosomes. (A) Exosome formation begins with the invagination of the plasma membrane. The endocytic vesicles fuse with the early endosomes to form the MVB, which contains intraluminal vesicles formed after the invagination of the early endosomes. MVBs fuse with lysosomes and are degraded, releasing the ILVs. These ILVs fuse with the plasma membrane and are exocytosed as exosomes comprising tetraspanins, ESCRT components (Alix, TSG101), and MHC 1 & 2, depending on the cell origin. (B) Ectosomes are formed via outward budding of the plasma membrane through a process called ectocytosis. These vesicles comprise tetraspanins, glycoprotein 1b α (GPIIb α), β 1 integrin, and MHC 1, depending on the cell origin. This image was created utilizing Bio Render

Ectosomes, also called microvesicles (MVs), are membrane-bound vesicles that evaginate from the plasma membrane of cells via exocytosis after a well-regulated pinching and scission event (Fig. 1). This formation origin/mechanism is the basis for differentiating these vesicles from exosomes. The series of events leading to the formation of ectosomes involves the reorganization of the cellular membrane's phospholipid mediated by scramblases and flippases due to an increase in cytosolic calcium level and pinching off vesicles regulated by the actomyosin contractile machinery [33–36]. According to Muralidharan-Chari et al., ADP-ribosylation factor 6 (ARF6) of the small GTP-binding proteins mediates the release of ectosomes into surrounding environments [37]. This study delved into its mechanism of action and reported that the phosphorylation of myosin II light chain (MLC) at Thr18/Ser19 generates the force needed for the scission of microvesicles via an actomyosin-based contraction at the vesicles' neck. Li et al. showed that a Rho A-dependent pathway is involved in the biogenesis of ectosomes [38]. The study discovered that

through a series of events ending with phosphorylation of cofilin on ser3, actomyosin contracts leading to ectosome biogenesis.

Molecular Make-Up of Extracellular Vesicles

Extracellular vesicles have been reported to carry a wide array of molecular cargoes, which can be influenced by the cellular origin and formation pathway of the vesicles [39]. They comprise genetic materials, lipids, and proteins, and these cargoes often play important roles in the progression of infectious diseases and their treatment (Table 1).

Table 1: Composition of extracellular vesicles and their roles in infectious diseases

Composition of extracellular vesicles	Relevance in infectious diseases
Cytoskeletal proteins: Actin, tubulin, cofilin, ezrin, profilin-1, moesin, tubulin beta chain, and vimentin [40,41].	Facilitate vesicle movement and cytoskeleton remodeling during pathogen uptake and spread.
Membrane trafficking and fusion proteins: Flotillin, annexins, dynamin, syntaxin, and RAB 2B, 5, 7, 9A, 11, 27A, 27B, 35 [36].	Regulate EV formation and cargo release, viruses such as Influenza A Virus, Hantavirus, and respiratory syncytial virus, exploit the Rab GTPase complexes for their replication and release via the plasma membrane [42]. Cytomegalovirus infection level was also shown to increase with the level of Rab 27A [43].
Heat shock proteins: HSPs 60, 70, and 90 [44].	The HSP70 surface protein receptor is essential for transporting exosomes to recipient cells.
Tetraspanins: Cluster of differentiation molecules CD9, CD37, CD53, 63, 81 and CD82 [36].	It promotes Human Immunodeficiency Virus (HIV-1) assembly and release through its association with the viral Gag (Group-specific antigen) protein [45]. CD9 was shown to assist Middle East respiratory syndrome coronavirus (MERS CoV) entry into cells [21].
ESCRT components: TSG 101 and Alix [39].	Hijacked by viruses (e.g., HIV) to facilitate viral budding; mediate sorting of viral components into EVs [21].
Transporters: ATP7A, ATP7B, MRP2, SLC1A4, SLC16A1, CLC1 and CLIC1 [46].	Alter cellular metabolism to favor pathogen survival; potential biomarkers in viral infections.
Integrins: β 1-integrin, integrin Mac-1. Matrix proteins: Membrane type-1 matrix metalloprotease MT1-MMP.	Promote adhesion to target cells; involved in inflammation and immune cell recruitment in infections. Aid in tissue remodeling and invasion during infections (e.g., tuberculosis, viral inflammation).

(Continued)

Table 1 (continued)

Composition of extracellular vesicles	Relevance in infectious diseases
Adhesion proteins: P Selectin [39,47].	Facilitate leukocyte migration; can enhance pathogen-induced inflammation.
Lipids: Ceramide, Phosphatidylinositol, Cholesterol, Phosphatidylserine, Glycosphingolipid, Phosphatidylethanolamine, Phosphatidylcholine, and Sphingomyelin [44,48].	The increased level of phosphatidylserine present in EVs can cause latent infections when usurped by microorganisms [49].
Nucleic acids: DNA; RNA (circular, long non-coding, non-coding, ribosomal, micro, messenger, small nucleolar, piwi-interacting).	Viruses such as Hepatitis have been shown to transfer their genetic material from infected cells to non-infected cells via exosomes [50]. Studies have shown that miRNAs such as miR-28, 29a, 149, 150, 198, 223, 324, 378, and 382 contained in exosomes from HIV infected cells have protective roles against HIV infection [21].

3 Extracellular Vesicles in Infectious Disease Progression

The interplay between hosts and pathogens is an intricate and active mechanism, and EVs serve as essential contributors [51]. Importantly, comprehension of the process of host and pathogen interplay via EVs plays a significant role in utilizing EVs as a therapeutic approach to contagious diseases [52]. EVs function as crucial intermediaries in the signaling between infectious agents and host organisms. Hence, they assist in the transfer of cellular messages, which can affect the result of diseases [53]. The host and pathogen interplay forms a complex relationship between infectious microbes, including viruses, fungi, bacteria, and parasites [54,55]. Such interactions happen within multiple stages, including cell-level, organismal, and biochemical processes. They are defined via the ongoing struggle between the host's immune responses and the microbe's approaches to avoid and utilize such safeguards [54,55].

Fig. 2 indicates that once an infectious agent invades a host, the pathogen needs to maneuver the immune barrier network [56]. On entry, pathogens produce unique molecular signals called pathogen-associated molecular patterns (PAMPs), which are detected using pattern recognition receptors (PRRs) at the outer layer of target cells [57]. Hence, initiating signal transduction cascades that stimulate the innate defense system [58]. Also, the interplay between the PAMPs and PRRs can initiate the intercellular signaling routes [58]. Such routes engage the gathering of numerous modulatory proteins and various activator signaling components. These eventually result in triggering immune responses, which are intended to remove the infectious agents [58–60]. However, pathogens possess diverse approaches to avoid the host defense responses [61,62].

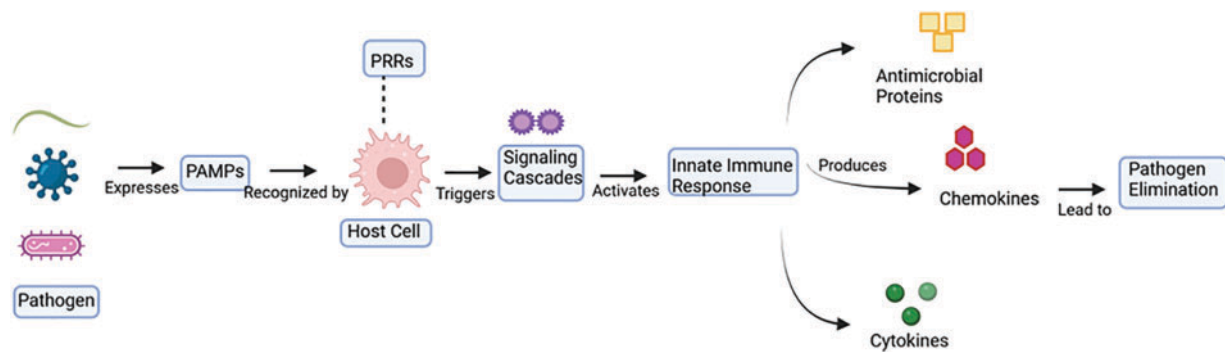


Figure 2: Schematic depiction of microorganism identification and innate defense mechanism initiation. This figure demonstrates the infectious agent recognition process and the innate immune defense initiation. Microorganisms produce Pathogen-Associated Molecular Patterns (PAMPs) that are detected via Pattern Recognition Receptors (PRRs) on the exterior of target cells. Then, this action detection stimulates inside the cell signaling cascades, initiating the innate defense response. This stimulated defensive reaction leads to the synthesis of antimicrobial proteins, chemokines, and cytokines. Together, these immune regulators assist in the removal of microorganisms. Image created utilizing Bio Render

Pathogens such as fungi, parasites, and bacteria emit EVs that encompass diverse biological molecules, such as DNA, RNA, and proteins [63]. These EVs can transfer pathogenic factors and regulatory genetic materials into the target cells, thereby regulating the immune defenses of hosts and assisting in microbe persistence and spread [64]. However, EVs originating from host cells such as macrophages serve a vital function in modulating immunological reactions and countering infections caused by viruses, bacteria, and parasites [65].

The external membrane particles derived from gram-negative bacteria are capable of delivering catalysts and harmful substances, which play a significant role in disease development as well as immune resistance [66]. Additionally, bacteria-derived EVs can trigger immune-inflammatory reactions via engaging with pathogen-sensing receptors like Toll-like receptors on host cells. This could result in the synthesis of pro-inflammatory cytokines. For instance, EVs originating from pathogens like *Staphylococcus aureus* that invaded vascular lining cells have the potential to trigger a pro-inflammatory reaction within the host cells via transferring pro-inflammatory cytokines, as well as supplementary immune stimulators [17,67]. In the *Mycobacterium tuberculosis* infection, EVs from infected cells can promote the disease progression by causing the recruitment of more macrophages and naïve T cells through stimulating chemokine release from non-infected macrophages. It was also shown in mouse models that administration of EVs from *M. tuberculosis* and *M. bovis* bacillus Calmette-Guerin (BCG) caused accelerated tumor necrosis factor alpha (TNF α) and Interleukin 12 (IL-12) production and migration of uninfected target macrophages, causing disease progression [68]. Bacterial EVs assist infectious agents in escaping the organism's immune reaction by transporting compounds that suppress complement-driven cell rupture and additional immune systems [66]. EVs derived from bacteria also transport infection-sourced elements and can act as durable indicators of disease presence, providing a promising detection tool [69]. Such EVs remain highly persistent in the bloodstream relative to dissolvable components and can be employed for observing the process of advancement of pathological conditions [68].

In viral infections, EVs transmit viral components such as proteins, genetic molecules, and receptors from infected cells to healthy cells, increasing susceptibility to infection. Research has found a crucial receptor in the fusion of SARS-CoV-2 virus particles with the host cell membrane, Angiotensin converting enzyme 2 (ACE2), in EVs generated by some cells; this suggests that these vesicles play a role in promoting

CoV infection [70]. As an alternative, coronaviruses (CoVs) can enter cells through the endocytic route that is dependent on caveolin-1, since caveolin-1 is a component of EVs and a crucial regulator of EV biogenesis. It has been proposed that EVs expressing caveolin-1 may aid in the propagation of SARS-CoV-2 [71]. Moreover, exosomal microRNAs like miR-145 and miR-885 are involved in the modulation of blood clot formation in CoV patients, corresponding with the D-dimer concentrations and causing endothelial impairment [72]. Research on lipid biochemical processes obtained from plasma small EVs in COVID-19 recovering patients has shown ongoing changes, suggesting prolonged metabolic effects due to the disease [73]. Endothelial EVs loaded with microRNA-34a are recognized as indicators of recently diagnosed diabetes in COVID-19 patients, providing knowledge of metabolic sequelae linked with the infection [74]. Additionally, the investigation of canine and feline CoVs and Crandell-Rees Feline Kidney cells and Canine fibrosarcoma cells (A-72) emphasizes the significance of comprehending the modification of EV production and its content after viral infection. Therefore, suggesting approaches to prevent future CoVs from companion animals [6,7,75–77].

EVs propagate HIV by transferring proteins to target cells, rendering them susceptible to infection. HIV protein Nef is sorted into EVs [78,79]. When these EVs are supplied to latent HIV-1 cells, they become activated and more susceptible to HIV infection. HIV-infected cells were also shown to produce EVs with trans activation response element (TAR) RNA that interacts with Tat protein and upregulates viral RNA production. The interaction results in resistance to apoptosis and sustenance of viral release [80]. Hepatitis C virus was initially assumed to collect in the cytoplasm and endoplasmic reticulum (ER); however, a study has shown that this virus is encapsulated into MVBs/exosomes and released via the exosomal secretory pathway, dependent on hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) [81]. Infection with cytomegalovirus has been shown to produce EVs containing lectin and DC-SIGN that are proviral while subsequently impairing the host's antiviral response [82]. Epstein-Barr virus utilizes EVs to enhance host defense evasion in throat cancer via loading programmed death-ligand 1 into small EVs through the LMP1-ALIX axis, as a result suppressing CD8⁺ T cell activity [83].

MVs from the fungus *C. neoformans* were the first studied before vesicles from other fungi were explored. This organism causes cryptococcosis, especially in immunosuppressed individuals. Within these EVs is the polysaccharide glucuronoxylomannan (a virulence factor) which confers protection against phagocytosis and prevents leukocyte migration [84]. These events promote fungal persistence and spread. Infections by *Candida albicans* cause the host to produce EVs rich in transforming growth factor beta 1 (TGF- β 1), which causes an increase in fungal immune tolerance and reduces host immune response, subsequently causing the organism to thrive in the host [85]. Also, EVs from *Saccharomyces cerevisiae* were shown to contain Sup35p prions in their infectious aggregated state [86]. These infectious prions were reported by Liu et al. to be taken up by recipient mammalian cells [87].

EVs are also produced from pathogens that can inhibit their host defense reaction by transporting substances capable of blocking complement-driven lysis and other defense pathways [15,88–90]. For example, EVs derived from *Trypanosoma cruzi* can suppress complement-driven parasitic lysis and facilitate microbial avoidance [91]. Furthermore, EVs enable inter-kingdom RNA transfer, in which microRNAs originating in infectious agents are transferred into host cells. This mechanism can control genetic activity and adjust the immunological mechanism [61]. This process is detected in diseases caused by parasites, whereby vesicles carry RNA variants homologous to the host microRNAs, hence resulting in immune regulation [64]. EVs derived from parasitic organisms such as *Trypanosoma cruzi* and *Echinococcus granulosus* carry microRNAs and additional non-coding RNAs, which can modulate the host defense mechanisms as well as enhance disease development [92]. An infection of erythrocytes by *Plasmodium falciparum* releases EVs with functioning 20S proteasome complexes that change the mechanical characteristics of healthy red blood

cells, subsequently increasing their vulnerability to infection. Also, high levels of the cytokine CXCL10 have caused these EVs to terminate the cytokine synthesis, thereby encouraging the thriving and survival of *P. falciparum* and malaria disease [92]. Additionally, cellular hosts secrete EVs due to pathogen invasion, which can influence immune defense and impact the infectious agents' capacity to invade and reproduce [62].

Finally, the function of EVs in host-pathogen relationships is complex and essential in facilitating the progress of EV-based treatment approaches in contagious diseases [68]. Through grasping and exploiting these relationships, investigators can develop novel treatments that regulate the immune reaction, convey treatment agents, as well as fight diseases with greater efficiency [93].

4 Technological Advances in Extracellular Vesicle Isolation and Characterization

The extraction of EVs is an essential phase in their research and application, especially within the scope of therapeutic tools for contagious illnesses [8,94]. EVs are mostly isolated using the differential ultracentrifugation method, commonly known as the gold standard for EV extraction [95]. It is lengthy, necessitates large sample quantities, and might not distinguish various categories of EVs, such as exosomes, MVs, apoptotic bodies, autophagic EVs, stressed EVs, matrix vesicles, and oncosomes effectively [96,97]. However, density gradient centrifugation enables enhanced purification [98–100]. Additionally, ultrafiltration employs membrane filters with distinct pore sizes to isolate EVs [101]. It is more efficient and does not necessitate costly equipment, but it can result in vesicle blockage and trapping within the filter [101,102].

Moreover, the immune affinity isolation method plays a significant role in the EV isolation process [94]. Size exclusion chromatography (SEC) also isolates EVs according to their dimension and molecular mass, thereby generating EVs with higher stability and unaltered vesicle characteristics [94]. It is especially effective for separating EVs from small specimen quantities and is also capable of being expanded and automated [103]. Importantly, the SEC can maintain the physical-biological and functional characteristics of purified EVs [104]. Furthermore, asymmetrical flow field-flow fractionation (AF4) is an advanced technique that isolates components according to their diffusion rate [105,106]. It needs a lesser volume of an initial substance than standard chromatography and isolates EV samples of elevated quality [107]. Another technique is precipitation, which involves utilizing polymers or salt to isolate EVs from the specimen [108]. The miRCURY, like commercial kits, employs sedimentation to separate EVs, which is rapid and simple [109]. However, this technique commonly leads to reduced purity and increased contamination with dissolvable peptides [103].

Characterizing EVs is crucial to comprehending EVs' dimensions, concentration, content, and functional attributes [94]. Various analytical techniques are utilized for this purpose. Dynamic light scattering (DLS) measures EV size distribution by analyzing photon scattering caused by Brownian motion [110–112] while nanoparticle tracking analysis (NTA) tracks individual particles in real time to assess their dimensions and density; however requires careful dilution to avoid interference [113,114]. Flow cytometry enables the identification and classification of functional EVs based on membrane markers and other features, subsequently offering a detailed profiling of EV subsets [115–117]. Electron microscopy (EM and Cryo-EM) is also employed in observing the morphology and dimensions of EVs at high resolution [118,119] as it offers significant details on EVs' dimensions and structure. Atomic force microscopy (AFM) scans EV surfaces with a fine probe, yielding detailed images of their surface topography and dimensional range [120–122]. Furthermore, tunable resistive pulse sensing (TRPS) quantifies EV dimension and concentration by detecting variations in electrical impedance while particles travel across a pore [94,123], offering high sensitivity [124]. Immunoblotting assays like ELISA, western, and dot blot are utilized to examine the protein composition of EVs [125]. Additionally, optical techniques such as super-resolution microscopy [126] help

evaluate the dimension, chemical makeup, morphology, and cellular source of individual EVs [127], offering comprehensive insight into the heterogeneity of EV preparations [128,129]. All these methods provide a comprehensive insight into EVs, which is crucial for advancing their application as therapeutic agents in infectious diseases.

5 Modes of Anti-Pathogen Action by Extracellular Vesicles

EVs are pivotal in modulating host-pathogen interactions via several means. Their mode of action against infectious organisms is highly dependent on their composition, cell of origin, and target specificity. These means are widely divided into two major groups, which are the natural cargo effectors that possess the fundamental biological action of the vesicles and the engineered cargo effectors that involve the alteration of EVs to deliver therapeutic payloads. These two groups of vesicles act on the pathogen or stimulate immunomodulatory effects that enhance pathogen clearance and reduce the pathological manifestation of the infection. The naturally released EVs contain PAMPs that stimulate PRRs and MHC class I and II molecules that lead to cytokine production and antigen-specific T-cell activation, respectively [130]. Furthermore, EVs are modified by loading their interior or surface with RNAs, CRISPR-Cas components, proteins, lipids, and antimicrobial compounds [131,132]. The surfaces of these EVs have targeted ligands, aptamers, and antibodies attached to them for targeted delivery [133].

Engineering Extracellular Vesicles for Therapeutic Purposes

EVs can be loaded endogenously by introducing functional oligonucleotides into parent cells to modulate gene expression. These oligonucleotides are packaged within the EVs and the EVs are released with the required therapeutic advantage during EV biogenesis [134]. In addition to endogenous loading, vesicles can also be exogenously loaded post-isolation by directly modifying the EVs. The incubation method is often considered a passive strategy that involves the direct incubation of drugs with EVs. It depends on the drug's hydrophobic nature, which allows it to interact with the lipid layer of the vesicle's membrane (Fig. 3B,C). It is a simple and affordable technique that does not involve high-throughput technology to transport hydrophobic cargoes only. Electroporation is the most common method. It was introduced for the loading of water-soluble drugs (Fig. 3B). It is also used to load small hydrophilic cytotoxin molecules like doxorubicin. This technique is referred to as advantageous because it produces an average loading efficiency compared to other substitutes, and it is done without additives. A higher field strength is applied to increase the membrane permeabilization when working with EVs [135], but this strong field causes damage to the vesicles and their cargo.

Sonication involves using sound waves to produce calm shearing forces that aid in disrupting the lipid bilayer and subsequently incorporating the desired molecule within the EVs (Fig. 3B). However, certain challenges, such as alteration of zeta potential [136], loss of exosomal protein, heat generation during the sonication cycle, and membrane disruption, are some drawbacks involved with this technique. During extrusion, the lipid bilayer membrane is disrupted through a small-sized polycarbonate porous membrane, and the molecules of interest are loaded within the vesicles (Fig. 3B). However, this strategy can increase EV toxicity by initiating mechanical stress that changes its membrane arrangement and zeta potential [136]. Freeze-thaw methods comprise an ephemeral induction of pores on the vesicles' membrane after freezing at -80°C and then thawing at 37°C multiple times to aid the entry of the desired molecules into these EVs (Fig. 3B). The membrane's integrity is preserved due to the reduced external force required [137]. However, incessant freeze-thaw cycles clump these vesicles, inactivate the protein, and increase their size. Therefore, it should be carried out with optimum care to ensure the integrity is intact [138].

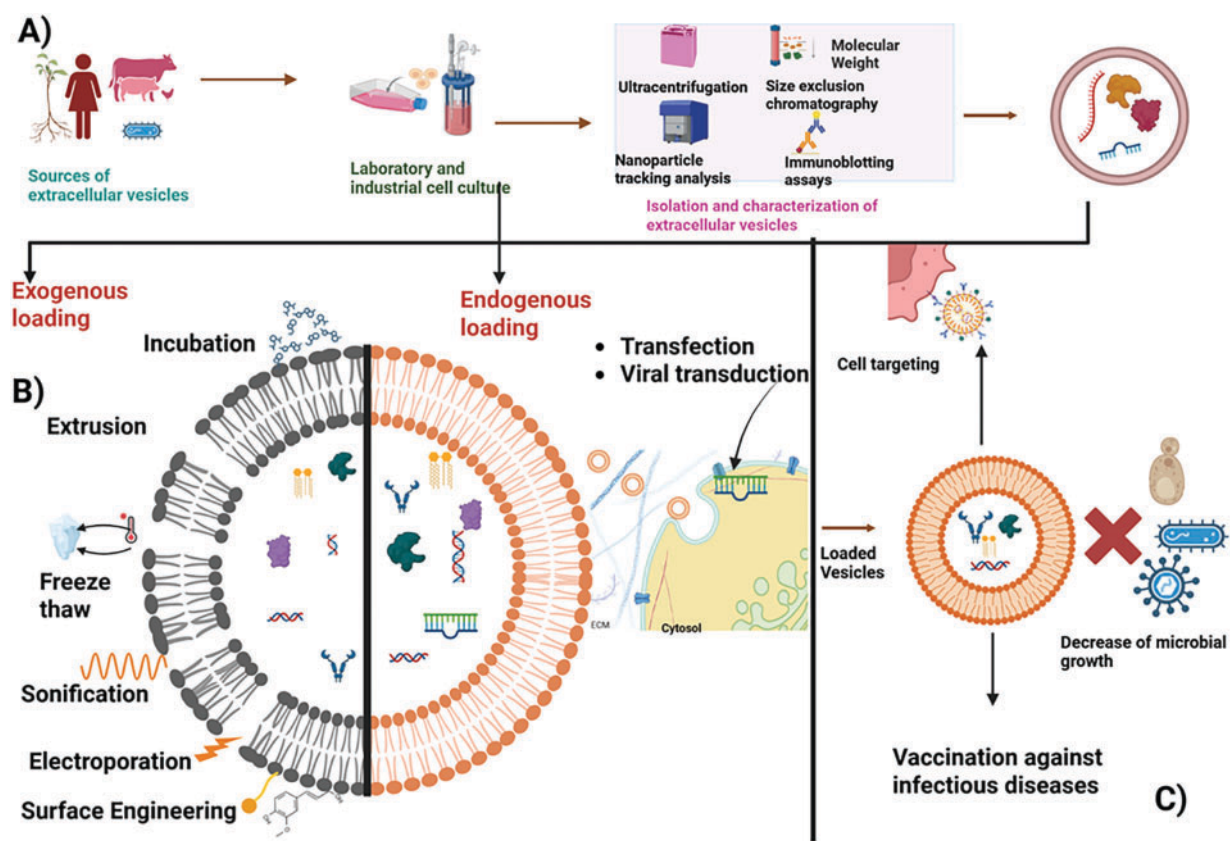


Figure 3: Presents a schematic illustration of the process of obtaining extracellular vesicles (EVs) and loading them with therapeutic payloads. (A) First, EVs are sourced from a variety of biological materials, including plants, cells, human and animal body fluids, and microorganisms. These cells can be cultured either in the laboratory or industrially. (B) The vesicles can be loaded with therapeutics through two methods: endogenous or exogenous loading. For endogenous loading, cells are transfected or virally transduced with the oligonucleotide of interest, after which the vesicles are released and isolated, carrying the therapeutic molecules. In exogenous loading, vesicles are first isolated and characterized using techniques such as ultracentrifugation, size exclusion chromatography, nanoparticle tracking analysis, and immunoblotting assays. Subsequently, the vesicles are loaded with the therapeutic payload via passive incubation or active methods that modify the phospholipid bilayer, such as electroporation, sonication, freeze-thawing, and extrusion. (C) These engineered vesicles enhance specific targeting of cells, microbial killing, and the delivery of vaccines for infectious diseases. Image created utilizing Bio Render

6 Pre-Clinical and Clinical Application of Extracellular Vesicles

6.1 EV-Mediated Antiviral Strategies

Viral diseases have taken countless lives throughout the ages, often eradicating significant segments of the global population, as demonstrated by the 1918 influenza epidemic and the devastating COVID-19 pandemic. Since 2019, the deadly SARS-CoV-2 virus has been responsible for over 6 million fatalities and 766 million illnesses [139]. These events have reechoed the importance of developing effective strategies for the treatment and prevention of viral infection.

Current studies have emphasized complex functions of EVs in viral disease development, such as COVID-19, and their application in the treatment. Animal-derived EVs, mainly from mesenchymal stem cells (MSCs), exhibit the possibility of treating severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) through regulating immune responses and decreasing inflammation [140]. The safety profile of

nebulized exosomes obtained from allogenic adipose tissue mesenchymal stromal cells in treating COVID-19-associated pneumonia was assessed in a phase 2 pilot clinical trial conducted in Wuhan, China. After 5 days of consecutively inhaling these vesicles, the patients' conditions were alleviated, which was characterized by decreased lung inflammation and lack of adverse events [141]. However, the study was challenged by the small number of patients enrolled. Additionally, treatment with nebulized EVs employing umbilical cord mesenchymal stem cell-derived exosomes demonstrated potential in managing COVID-19 lung infection, along with findings supporting improved lung damage absorption and a decrease in patient care duration [142]. Also, it was reported that exosomes from COVID-19 patients had ACE2 receptors on their surface. Through competition with the binding site of cellular ACE2, these vesicles can prevent SARS-CoV-2 infection in humanized ACE2 (hACE2) transgenic mice [143].

Recent studies have shown the therapeutic effect of oral administered extracellular vesicles from *Lactobacillus reuteri* (LrEVs) against influenza A virus (IAV) infections. The administration of the LrEVs *in vivo* was effective against IAV through its modulation of the immunological response of IL-17-producing cells in the intestine and gut. This involved inhibiting Th-17 cell development and transportation. It also contained miR-4239, which controlled the production of IL-17a, thereby suggesting its potential in IAV treatment [144].

Plant-derived extracellular vesicles (PDEV) have also been isolated [131,145–147]. PDEVs are utilized in therapeutics due to their inherent bioactive properties and their ability to withstand digestive enzymes without inducing inflammatory responses [148,149]. Exosomes from epithelial cells infected with SARS-CoV-2 (exosomes^{Nsp12Nsp13}) were shown by Teng et al. to have induced lung inflammation in mice, while Ginger exosome-like nanoparticles (GELN) were utilized in the inhibition of the SARS-CoV-2 gene via its inherent miRNA without causing side effects [150]. The GELN microRNA (miRNA aly-miR396a-5p) and rlc-miR-rL1-28-3p bound to multiple sites on the viral genome and inhibited spike gene and Nsp 12 expression. This miRNA and the host miRNA do not share homology in their sequence, therefore supporting the unlikelihood of side effects occurring. Plant-based exosomal microRNAs were discovered to suppress pulmonary inflammation caused by exosomes, including SARS-CoV-2 Nsp12, indicating a possible cross-kingdom medical approach [150].

Additionally, MSC-originated EVs have also shown potential as an anti-inflammatory agent and improved healing during severe pulmonary damage induced by the influenza virus [52,96]. Zou et al. utilized exosomes in delivering anti-HIV agents to infected cells and induced cell death [151]. This is important because of the adverse drug events faced after the treatment of HIV [152,153]. This was done by transfecting HEK293 cells with plasmids containing single chain variable fragment (scFv) of a high-affinity HIV-1-specific monoclonal antibody, 10E8, and subsequently loading the exosomes expressing scFv from these transfected cells (10E8_{scFv}-exos) with curcumin (a molecule that destroys HIV infected cells) or miR-143 (an apoptosis-inducing mRNA) [151]. MSC-derived EVs suppressed viral duplication within Hepatitis C virus-exposed cells via the function belonging to particular microRNAs such as miR-145, miR-199, miR-221, and let-7f [154]. In another study by Chen et al., exosomes from CRISPR/CAS9-expressing cells were endogenously loaded with Cas9 and human papillomavirus (HPV) or hepatitis B virus (HBV) specific single guide RNA (sgRNA). These vesicles were able to destroy transfected HPV and HBV genomes in the human cervical cancer cell line (HeLa) and the human liver cancer cell line (HUH7), respectively. However, the full-length Cas9 mRNA protein was not expressed in the exosome, which was suspected to have contributed to the weak gene editing activity. Also, this strategy could increase the off-target effect, thereby affecting the safe profile of the CRISPR/CAS 9 system via the intracellular communication of the vesicles with neighboring cells and tissues. Finally, no exosome distinctive isolation technique was utilized in this study, so microvesicles/ectosomes could have been involved in the delivery [132].

Anticoli et al. fused antigens from Influenza Virus NP, Hepatitis C Virus NS3, Ebola Virus VP24, NP and VP40, Crimean-Congo Hemorrhagic Fever NP, and West Nile Virus NS3 with a HIV Nef protein. For every studied viral protein, mouse intramuscular vaccinations produced a strong and targeted cytotoxic CD8⁽⁺⁾ T cell response. This vaccine platform maintains EVs' high biosafety profile while improving their low cytotoxic T lymphocyte immunogenicity [155]. To enhance the effectiveness of the therapeutic when loaded endogenously, the mRNA of interest is fused to an RNA binding domain (RBD) that encodes an EV sorting protein. Zickler et al. integrated the target mRNA expression cassette into the genome of the EV producer cell with CD63 fused to an optimized version of the designer Pumilio and Fem-3 mRNA-Binding Factor from *Caenorhabditis elegans* (FBF) homology domain. This prevented the carryover of the mRNA-encoding plasmid and subsequently increased assay robustness [156].

Additionally, exosomal miRNA from honeysuckle was shown to be highly stable and target a myriad number of influenza viruses, including H7N9, H5N1, and H1N1. It caused a significant reduction in the expression of H1N1-encoded NS1 and PB2 proteins [157]. Also, this microRNA was reported by Huang et al. to impair *varicella-zoster* virus replication through its target of the gene IE62 [158]. PDEV has not been extensively studied against viral diseases compared to its animal counterparts, so its studies are still at the laboratory phase. Pre-clinical investigations have indicated encouraging outcomes, and clinical studies remain essential to completely assess the safety and effectiveness of EVs in humans [159]. The application of EVs as treatment methods necessitates precise uniformity related to their extraction and identification [160]. Finally, guaranteeing the protection and effectiveness of these EVs in therapeutic applications is essential. Since they may transport toxic compounds [161].

6.2 EV-Mediated Antibacterial Strategies

EVs have shown promise as therapeutics against bacterial infections in their native or modified form by loading cargoes (Fig. 3A,B). In a study by Imparato et al., nontoxic, biocompatible EVs were obtained from mature biofilm formed by *Candida albicans*. When tested *in vitro*, it showed an antagonistic effect against *K. pneumoniae*, affecting its ability to form biofilms and inhibiting its attachment to mammalian cells [162]. A study by Gao et al. isolated cell membrane nanovesicles endogenously engineered to contain ceftazidime (CEF) and expressed Resolvin D1 (RvD1) on their surface via incubation. Co-delivery of ceftazidime and Resolvin D1 in a mouse model decreased bacterium-induced peritonitis [163]. However, this technique tends to offer lower encapsulation efficiencies [164]. Li et al. compared passive and active (electroporation and sonication) loading of LEV with Doxorubicin and assessed its effect against *Staphylococcus* species. *L. plantarum* WCFS1-derived extracellular vesicles (LDEVs) loaded passively showed lesser efficacy than actively loading, where the two approaches had similar efficacy. Also, doxorubicin loaded in LDEV showed increased antimicrobial activity compared to free Doxorubicin [165]. Yang et al. utilized exosomes for a targeted treatment of intracellular methicillin-resistant *Staphylococcus aureus* (MRSA) infection in macrophages. They loaded the mannosylated exosome with vancomycin or lysostaphin via mild sonication. This drug delivery system showed a better intracellular accumulation of the drug and inhibitory action against MRSA [166].

Gao et al. also coated PLGA NP loaded with vancomycin and rifampicin with BMV from *S. aureus* (NP@EV). This delivery platform showed improved targeting ability and alleviated *S. aureus* infection *in vitro* and *in vivo* [167]. Also, bacterial membrane vesicles (BMV) obtained from *E. coli* were used to modify the surface of a citrate-stabilized 30 nm gold (Au) nanoparticle (NP) [168]. The final BM-coated AuNP obtained was more stable in a biological environment than the BMV only. Furthermore, intravenous injection of this coated NP caused an increased and longer-lasting cellular and antibody response, showing its promise as an effective antibacterial vaccine. Animal-sourced EVs also possess demonstrated capability

in managing pulmonary infections such as *E. coli*-caused severe lung damage and bloodstream and gastrointestinal infections, mainly through regulating immune responses, boosting bacterial elimination, and facilitating cellular regeneration [169].

Ginger exosome-like nanoparticles (GELN) impacted the entry, adhesion, multiplication, and survival of *Porphyromonas gingivalis* in a phosphatidic acid-dependent manner when administered orally in a mouse model with periodontitis. This occurred due to the interaction of GELN with hemin-binding protein 35 (HBP35) on *P. gingivalis* [170]. However, more studies need to be carried out to assess the effect of GELN on polymicrobial biofilms. Ginger-derived EVs were further shown by Qiao et al. in a synergistic combination with a biomimetic nanoplatfrom (Pd-Pt nanosheets) to eradicate *Staphylococcus aureus* and its biofilm. The EVs in this nanoplatfrom ensured biocompatibility and sustained circulation in the blood and accumulation of the platform at the infection site, while the nanosheet provided electro-influenced catalytic and photothermal activity [171]. *Apis mellifera* honey-derived EVs were reported by Leiva-Sabadini et al. to possess antibacterial and anti-biofilm effects against *Streptococcus mutans* and *Streptococcus sanguinis*. The vesicle's cargo was assessed to understand the mechanism associated with this effect, and defensin-1, major royal jelly protein-1 (MRJP1), and jellein-3 were identified. These molecules have an antibacterial effect, which is expressed through the formation of pores, cell membrane disruption, and lysis [172].

Broadening the field related to EV-derived vaccines has uncovered their potential to function as both antigen transporters and immunological regulators in infectious illnesses. Outer membrane vesicle (OMV)-derived vaccines, such as VA-MENGOC-BC[®], have effectively proven defensive against *Neisseria meningitidis* [24]. Its immune-activating ability is based significantly on their structure [25]. Moreover, investigation and advancement in this field might improve our comprehension of EVs in the role of versatile tools intended for upcoming vaccine approaches [23].

Creating approaches that focus on the targeted transport of EVs to locations inside the body is important to optimize EVs' therapeutic capability (Fig. 3C) while at the same time reducing non-specific impacts [173]. Additionally, approaches aimed at suppressing secretion and activity involving bacterial-derived EVs have the potential to be investigated as a method for interrupting the pathogenicity of infectious agents [174].

6.3 EV-Mediated Antifungal Strategies

New approaches are desperately needed to prevent and treat fungal infections, which kill over 1.5 million people each year and impact over a billion people globally [175]. Furthermore, it is critical to stress that emerging fungal infections and their resistance to antifungals pose global health security risks, especially in immunocompromised individuals [176]. In contrast to other infectious illnesses, fungal infections have received much less research funding despite this public health burden [175]. In this regard, efforts to treat fungal infections, such as those utilizing EVs, can be advantageous.

EVs derived from *Aspergillus fumigatus* (AF), a prominent causative agent of fungal keratitis (FK), were assessed against *A. fumigatus* to understand their protective role in the disease *in vivo* and *in vitro* [177]. It was shown that *A. fumigatus* fungal keratitis was much less severe in mice who had EV injections beforehand, as evidenced by decreased fungus burden, inflammatory manifestations, and clinical ratings. The study highlights AF-derived EVs as a future in therapeutic development against FK.

The commensal microbe *Candida albicans* persists in the mucosal surfaces of healthy individuals. However, its overgrowth can be detrimental because it is the most common agent isolated from patients with extreme presentations of fungal infections [176]. Human oral mucosal epithelial cell line-derived EVs (Leuk-1 cells) were investigated against oral candidiasis in a mouse model. Results from this analysis showed enhanced responses of oral mucosal epithelial cells to *C. albicans* defense, detrimental changes

structurally and morphologically, and reduced hyphal invasion into mucosal epithelium, thereby impairing fungal growth.

Moreover, the mold *Aspergillus fumigatus* poses a great challenge due to the difficulty encountered during its diagnosis, its resistance to antifungals, and inadequate, nuanced therapeutic interventions. However, EVs released from polymorphonuclear cells after infection with wild-type and the melanin-deficient *pksP* mutant conidia from *A. fumigatus* showed an effect on hyphal growth after coincubation with conidia in a concentration-dependent manner. The authors attributed the antifungal ability to the cathepsin G and azurocidin present in the EVs and damage caused by the physical interaction of the EVs and the hyphae, leading to mitochondria fragmentation and changes and invaginations on the cellular surface [178].

An article published by Xiao et al. explained how *Cryptococcus neoformans*-derived EVs carry disease agents such as laccase, melanin, urease, as well as the *Ssa1* gene, enabling fungal disease-causing potential and immune system resistance. Therefore, these findings emphasize that EVs have the potential for therapeutic goals for novel antifungal treatments against cryptococcosis [179].

In addition, EVs from plants (PDEVs) have also shown prospects as therapeutics and delivery vehicles for treating fungal infections. EVs obtained from the sunflower seedlings *Helianthus annuus* contained a huge amount of defense proteins, so Regente et al. assessed the effect of these vesicles on phytopathogenic *Sclerotinia sclerotiorum* ascospores [180]. These EVs were internalized by the fungus, and they impaired spore growth and development, subsequently causing fungal death. PDEVs from *Arabidopsis* have also shown effects against the necrotrophic fungus *Botrytis cinerea* by transferring small RNAs (sRNA) into fungal cells to silence virulence-related genes [131]. Cumulatively, these findings pinpoint the need for extended and intense exploration of the therapeutic potential of EVs against fungal infections.

6.4 EV-Mediated Antiparasitic Strategies

The substantial burden of illness and mortality caused by protozoan and helminthic diseases globally, particularly in tropical countries, makes them important public health concerns. Approximately one in four people worldwide are infected with gastrointestinal parasites. Infections with soil-transmitted helminths (STH), such as *Trichuris trichiura*, usually cause stunted growth, anaemia, and delayed cognitive development [181,182]. However, existing drugs used in combating this parasite have reduced efficacy and are ineffective against recurrence, showing the need for better treatments and a vaccine [183]. According to an investigation by Shears et al., in a mouse model, administration of EVs garnered from the soluble material of *Trichuris muris* was protective against re-infection. The results showed an increase in IgG2a/c and IgG1 levels, thereby presenting these vesicles as strongly immunogenic. However, further studies should be carried out combining this strategy with adjuvants to better exploit its potential [183].

In Chagas disease, investigative studies showed that a parasite virulence factor (MASP) coated with *T. cruzi*-derived EVs coupled to keyhole limpet hemocyanin induced higher survival rates and lower parasite loads in the heart, liver, and spleen post-infection. It subsequently showed an increase in the levels of neutralizing antibodies and protective cytokine expression, indicating the suitability of this vaccine candidate [184].

The potentially fatal illness, malaria, is caused by parasites belonging to the genus *Plasmodium*. The parasite has been shown to utilize EVs to transfer antigenic materials to host cells, contributing to the parasite and infection spread. On the contrary, these EVs have been shown by research as an effective strategy against *Plasmodium falciparum*. In a study by Borgheti-Cardoso et al., tafenoquine and atovaquone, encompassed within EVs from plasmodium-infected red blood cells, impaired the growth of the parasite better than the drugs alone, thereby showing a stronger efficiency of hydrophobic drugs after sheathing with EVs [185].

Toxoplasma gondii is an apicomplexan parasite that causes toxoplasmosis [186]. This disease is a heavy challenge that can induce stillbirth and abortion in livestock [186]. The main treatments for toxoplasmosis have had failure rates, therefore stating the importance of alternative strategies and exploration of novel vaccine candidates [186,187]. Tawfeek et al. explored exosomes from human hepatoblastoma cell lines infected with *T. gondii* as potential immunizing agents. The strategy in this study involved conjugating exosomes to alum (an adjuvant) and comparing its efficacy to excretory secretory antigens (ESAs), the most superior vaccine candidates. The EV-alum candidate presented a more robust cellular and humoral immune response and higher protection against *T. gondii* challenge [187]. *Leishmania* species obtained EVs that decreased parasite levels and regulated immunological reactions, demonstrating therapeutic potential [52]. EVs extracted from schistosomes influence host defense mechanisms and have the potential to assist in creating medical interventions treating schistosomiasis [52]. These studies, in general, have provided a new direction for the development of therapeutics to combat *Toxoplasma* infections.

7 Conclusion

While ongoing research emphasizes the potential function of EVs from varied origins in fighting infectious illnesses, multiple crucial aspects require additional investigations to transition from basic discovery to clinical implementation. Collaborative efforts should be put into bridging the gap in diagnostic sensitivity, therapeutic specificity, and regulatory guidelines. Of high importance is the development of sensitive, high-throughput techniques that can analyze single EVs for applications in infectious disease control. Despite promising *in vitro* findings, challenges in standardized isolation methods, cargo loading, and large-scale production need to be surpassed to fully exploit the clinical application of this therapy in infectious diseases. Future studies should focus more on understanding EV biodistribution, improving the bioengineering of EVs, and ensuring their safety and application in human trials to combat infectious diseases. A synergistic utilization of EVs as delivery vehicles and nuance technology should be employed to achieve “Precision Medicine” and tailored medication therapy. Furthermore, as gene therapy and non-cellular treatment advance, the use of EVs will also play a vital role in these therapeutic approaches. Thus, EV-based drug delivery systems provide a future in the medical industry.

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