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Engineering of Extracellular Vesicles as Nano Therapy for Breast Cancer

Sapna Jain and Manjari Singh

Abstract

Extracellular vesicles are membrane-derived nanoparticles that represent a novel mechanism of cell-to-cell communication. It is well reported that EVs play a central role in the tumor microenvironment by mediating intercellular signaling among cancer cells. This has resulted in the development of therapeutic strategies targeting various EV signaling pathways in cancer. However, because of their small size and endogenous origin, they have been extensively explored for cancer drug delivery. Hence, owing to their natural ability to mediate intercellular communication, high stability, and low immunogenicity, they have emerged as an attractive platform for cancer treatment. However, limited production and insufficient loading with therapeutic moieties are some of the issues constraining their clinical translation. In this chapter, recent research studies performed in an attempt to develop EVs as cancer biomarkers or drug delivery systems will be discussed. Further, it will also discuss various strategies such as direct and indirect cell surface modification, which can be employed to make EVs successful as cancer therapeutics. Furthermore, it will highlight the current and completed clinical trials using naturally derived EVs as cancer therapeutics.

Keywords: breast cancer, extracellular vesicles, engineering, drug delivery, biomarker

1. Introduction

In women, breast cancer is a prevalent cause of cancer worldwide [1]. It affected 2.3 million women globally in 2020, with 685,000 deaths. It has been diagnosed in 7.8 million women in the past 5 years, making it the most common type of cancer in the world [1]. Although breast cancer diagnostic methods and therapeutic procedures have improved in the past decade, the long-term survival of these patients remains low due to a high rate of postsurgical relapse. The efficacy of breast cancer treatment is limited by drug toxicity, multidrug resistance, and a lack of definitive prognostic biomarkers [2]. Thus, there is an urgent need to develop novel biomarkers and therapeutics to cure the disease.

In recent years, many studies have suggested that intercellular communication plays a key role in driving various cellular functions and homeostasis in physiological as well as pathological conditions such as cancer, cardiovascular diseases, and neurological disorders. Cancer development is mainly dependent on interactions between cancerous cells and their microenvironment components. Some of these interactions are mediated by extracellular vesicles, which alter the phenotype of recipient cells [3–5].

Extracellular vesicles (EVs) are spherical nanoparticles shed by all types of cells, including archaea, prokaryotes, eukaryotes, and fungi in the extracellular milieu [6]. These typically range from 30 nm to 5 μ m in diameter based on their type and vary widely in composition [7]. In addition to being released during disease pathology, EVs allow various cells to send and receive messages to crosstalk with other cells, thus carrying out various biological functions [7]. These are mainly composed of different proteins, lipids, nucleic acids, and enzymes [8]. EVs circulate through many body fluids, such as blood, serum, and urine. Owing to their structural similarity to the parental source, they are considered potential biomarkers for diseases such as cancer [9]. To study the characteristics and functions of EVs, they are isolated using different techniques such as differential ultracentrifugation, size-exclusion, and ultrafiltration [10].

EVs are generally categorized into exosomes, microvesicles, and apoptotic bodies according to their release mechanism, size, and composition [3]. Exosomes are 30–150 nm in diameter and are formed by inward budding of the plasma membrane of the cell [9]. Microvesicles are formed by direct outward budding of the cell's plasma membrane and range in size from 100 to 1000 nm in diameter. Consequently, they are reported to contain mainly cytosolic and plasma membrane proteins, such as tetraspanins. Apoptotic bodies are shed during cell death into the extracellular space, ranging from 50 to 5000 nm in diameter. These generally contain intact organelles, glycosylated proteins, and chromatin, unlike the other two types of EVs. Among these, exosomes have been widely studied since their role in intercellular communication has been reported. This chapter will focus on exosomes and their potential applications as therapeutics for breast cancer.

2. Biogenesis, contents and functions of exosomes

Exosomes are generated by the endocytic pathway from late endosomes (LE) [11, 12]. LEs are formed by inward budding of the multivesicular body (MVB) membrane. LE membranes invaginate to form intraluminal vesicles (ILVs) within MVBs. During this process, some proteins are engulfed and packaged within the ILVs. ILVs then fuse with the cell's plasma membrane and release the vesicles into the extracellular space.

As reported previously, the formation of ILVs can occur either dependent or independent of the ESCRT complex. The ESCRT complex is a set of proteins that function together to facilitate the formation of MVBs, vesicle release, and protein cargo sorting [13–15]. ESCRT 0 has two subunits, HRS and STAM 1/2, which bind together and recognize specific ubiquitinated proteins in early endosomes. This leads to the recruitment of ESCRT 1 containing Tsg 101, Vps28, Vps37, and Mvb 12, which further recruits ESCRT II. ESCRT II is composed of four subunits, Vps22-EAP30, Vps36—EAP45 and Vps25—EAP20 which starts the invagination of endosomal membranes encapsulating different molecules/cargo such as proteins and nucleic acids.

The ESCRT II subunit Vsp25 then binds with Vsp20 to activate and recruit ESCRT III. It deubiquitinates proteins and allows complete membrane invagination, generating ILVs. Other adaptor proteins such as Vps4 interact with ESCRT III to finally start budding of the membrane, ECSRT subunit removal, and cargo delivery. Hence, the ESCRT complex regulates the whole process of vesicle budding and cargo sorting into exosomes [16, 17]. In cancer, an increased amount of exosomes is often observed in the bodily fluids of cancer patients as a result of deregulation of exosomal formation and secretion [18]. Specifically, in breast cancer, the amount of exosomes released by the human tumor cell line B42 clone 16 was much larger than that released by the parental normal mammary epithelial cells (HMEC B42), as shown by Azmi et al. [19].

Exosomes are composed of a heterogeneous set of cytosolic, nuclear, mitochondrial, ribosomal, and membrane-bound proteins derived from donor cells [20]. Some of these proteins are conserved irrespective of their origin; therefore, they are considered exosomal markers such as tetraspanins, ESCRT proteins, and major histocompatibility complex (MHC) molecules [21]. In addition, some proteins are related to the phenotype of producing cells, such as cancer-derived exosomes, which in turn determines their biological mechanisms. The lipid bilayer membrane of exosomes contains transmembrane proteins, transporter proteins, adhesion molecules, and lipid raft-associated proteins. Exosomes contain nucleic acids such as DNA (ssDNA, mtDNA, dsDNA, and RNA (mRNA, miRNA, and lncRNA) [22]. Exosomal miRNAs and mRNAs are transferred from donor cells to recipient cells, thus modulating the latter's phenotype. Although there are numerous reports indicating the presence of DNA within exosomes, the mechanisms leading to this phenomenon remain unclear. Exosomes also exhibit an exclusive set of lipids distributed in their bilayers, such as sphingolipids, arachidonic acid, cholesterol, phosphatidylserine, and

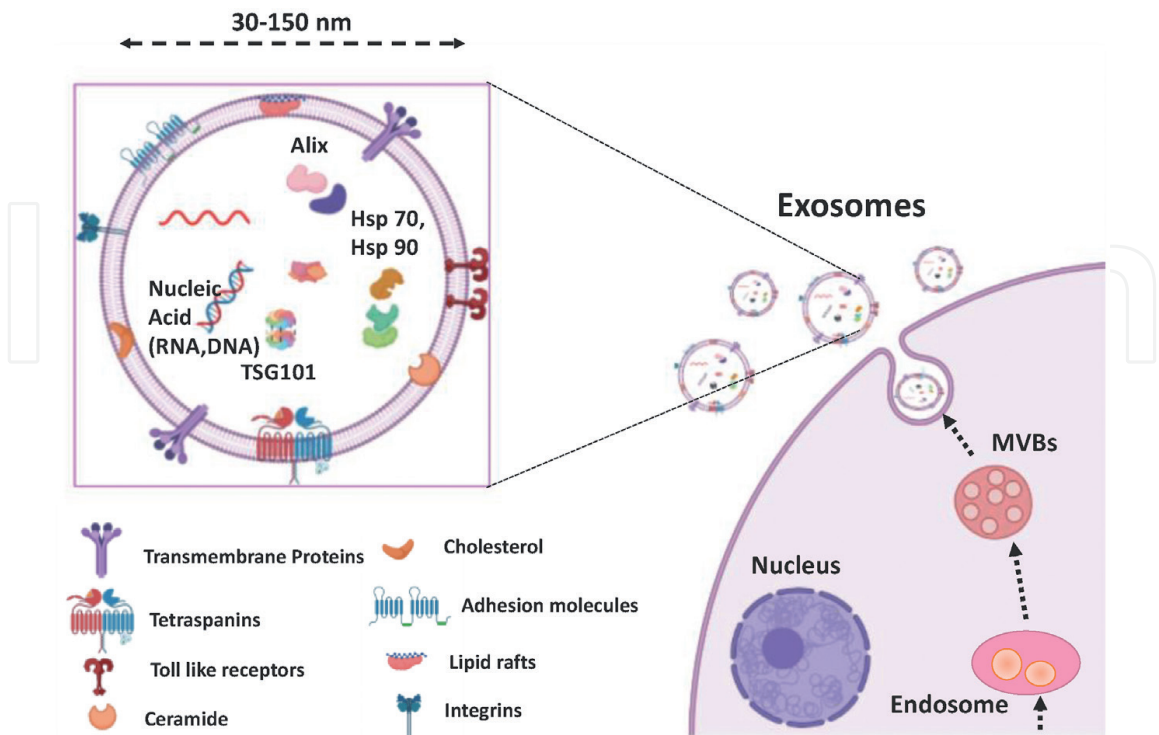


Figure 1.
Schematic representation of exosome biogenesis and composition. Exosomes originate from multivesicular bodies and shed into extracellular space packaging motley of proteins such as ESCRT associated protein, chaperones, along with ssDNA, RNA and dsDNA, miRNA and lncRNA.

ganglioside [23, 24]. Lipids such as lysobisphosphatidic acid are abundant in the inner membranes of multivesicular bodies and play a crucial role in exosome formation [25, 26]. ExoCarta is a database containing all the data on exosomal content, with over 47,000 protein, mRNA, and lipid entries. Furthermore, ExoCarta is an excellent source of information for exosome characterization (**Figure 1**) [27].

3. Functional role of exosomes in breast cancer development

Metastasis is the process by which primary tumor cells/tumor cells/cancer cells invade the surrounding tissues and colonize the blood vessels to proliferate and give rise to the tumor [28]. Controlling metastasis, which is mainly responsible for high patient mortality, is the main challenge in breast cancer therapy. Hence, several investigations are ongoing to understand the molecular mechanisms underlying metastasis in breast cancer. Recently, exosomes have attracted great attention as key players in regulating complex intracellular pathways from initiation to progression to metastasis in the development of breast cancer [29–31]. These mainly interact with the recipient cells in three ways: direct fusion with the cell membrane, interaction with the surface receptors, or internalization via endocytosis. Upon cellular uptake, exosomes deliver their cargo and initiate a cascade of events leading to various biological functions. Many breast cancer cell lines have been shown to release exosomes containing several proteins with signaling molecules, miRNAs, and long non-coding RNAs involved in migration, invasion, angiogenesis, and metastasis [32–34]. Proteomic profiling of exosomes secreted from breast cancer cell lines was shown to contain matrix metalloproteinases, which might be linked to the enhanced metastatic properties of breast cancer cells [32]. These findings suggest that exosomes act as key mediators in the tumor microenvironment by communicating various signaling molecules essential for breast cancer development [31].

Exosome-mediated transfer of genetic material from breast cancer cells has been shown to mediate resistance to chemotherapy and enhance tumor growth [35, 36]. Accumulating evidence suggests that exosomes may also play a role in the resistance of breast cancer radiotherapy and cancer immunotherapy [37, 38]. In breast cancer, drug-resistant cancer cells transmit resistance in drug-sensitive cells via the intercellular horizontal transfer of exosomal miRNAs [38]. Exosomes also transfer the drug efflux pump from docetaxel-resistant to sensitive ones in MCF-7 breast cancer cells [39]. Lv MM et al. showed that exosomes from drug-resistant cancer cells contain miRNAs that alter the phenotype of recipient breast cancer cells by altering their transcriptome [40]. Exosomes from stromal fibroblasts transmit non-coding RNA to breast cancer cells, thus contributing to treatment resistance by expanding therapy-resistant cells [41]. Thus, exosomes contribute to drug resistance in breast cancer.

4. Therapeutic implication of EVs in breast cancer

The role of exosomes in carcinogenesis has been extensively investigated in recent years. Cancer cells have been shown to use exosomes as a novel mechanism to transfer the malignant phenotype to normal healthy cells and establish a niche for tumor growth. Cancer cell-derived exosomes are reported to contain miRNAs, proteins, or long non-coding RNAs that mediate cancer development, growth, and progression [42, 43].

Exosomes derived from breast cancer cells contain a variety of proteins and RNAs that are transmitted among these cells as well as normal cells, thus altering the phenotype of healthy mammary epithelial cells. Wang J et al. showed that cancer exosomes were able to transform normal mammary epithelial cells into cancerous cells via transfer of microRNAs packaged within exosomes [44]. Similarly, Melo et al. showed enhanced expression of exosomal miR-10b in metastatic breast cells compared to non-metastatic or non-malignant breast cells [45]. Thus, it can be used as a therapeutic target for breast cancer therapy.

These characteristics make exosomes ideal biomarkers, and exosomal profiling in the absence of tissue holds great promise for early diagnosis. Owing to their crucial functional role in breast cancer, exosomes have been investigated for their potential development as breast cancer biomarkers and therapeutic targets. Singh R et al. have shown that psoralen reduces the formation and secretion of exosomes, thus reversing multidrug resistance in breast cancer cells [46]. The presence of diverse content within and on the surface of exosomes has led to their application as biomarkers, diagnostics, and drug delivery. A large number of exosomes circulate within bodily fluids of not only healthy individuals but also cancer patients, according to some studies. Since exosomes play various significant roles in breast cancer, exosomes can be developed as potential therapeutic agents in biomarkers, diagnostics, and drug delivery. Kumar et al. investigated the release of exosomes from breast cancer stem cells to characterize their constituent exosomal markers. They detected tetraspanin proteins, Alix, and tumor susceptibility gene-101 (TSG101) in breast cancer stem cell-derived exosomes. This study indicates that secreted exosomes can be utilized as biomarkers for breast cancer to understand their development, progression, and metastasis [47]. Kumar et al. showed that miRNAs 155 and 205 are expressed in serum exosomes derived from breast cancer cells and modulate the epithelial-to-mesenchymal transition (EMT), growth, and metastasis of cancer, suggesting their employability as breast cancer biomarkers [48]. Zhang et al. studied the role of long non-coding RNA MALAT 1 which is highly expressed in exosomes derived from breast cancer cells in tumor progression, representing a potential treatment strategy for breast cancer [49]. Dong et al. investigated the role of exosomal long non-coding RNA in the chemoresistance of HER2+ breast cancer cells. They found that exosomal lncRNA-SNHG14 was not only upregulated in trastuzumab-resistant cells but also transmitted the lncRNA into drug-sensitive cells, thus disseminating trastuzumab resistance. Furthermore, when compared to patients who responded to trastuzumab, the expression level of serum exosomal lncRNA-SNHG14 was higher in patients who were resistant. This suggests that lncRNA-SNHG14 is a promising therapeutic target for HER2+ breast cancer patients [50].

5. Engineering exosomes as therapeutics for breast cancer

Exosomes are emerging as promising therapeutic agents because of their role in tumor-related processes and their ability to deliver their cargo, such as proteins, lipids, and nucleic acids, into the tumor sites. However, their full clinical applicability has not yet been realized. This is because of many factors, including low yield and relatively low percentage loading to the therapeutic moiety. As such, new approaches for mass production and enhancement of the percent loading need to be explored. In general, these approaches are divided into two categories: passive and active loading, which are discussed in detail in the following sections.

Direct modification, also known as non-cell-based loading or exogenous loading, refers to the direct loading of therapeutic moieties such as siRNA, miRNA, drugs, and proteins after the isolation and purification of exosomes from the cells. This may encompass a series of procedures such as incubation, freeze-thaw cycles, sonication, and electroporation, and thus can further be categorized into passive and active loading. Passive loading includes loading of therapeutic moiety into exosomes by diffusion; on the other hand, active loading includes disrupting the exosomal membrane by electroporation, sonication, or freeze thawing, thus allowing the therapeutic moiety to enter into these vesicles. In passive drug loading, exosomes are incubated with drugs and allowed to diffuse into vesicles along a concentration gradient. Because exosomes consist of a lipid bilayer, the drug loading efficiency depends largely on the hydrophobicity of the drugs. Dong et al. loaded curcumin into milk exosomes by incubating at 4°C overnight and reported 70.46% drug loading using an incubation method [51]. Similarly, Sun et al. incorporated curcumin into exosomes derived from a mouse lymphoma cell line by incubating in PBS at room temperature (22°C) for 5 min and showed a binding capacity of 2.9 g curcumin to 1 g of exosomes [52]. Sun et al. packaged Cho-miR159 (cholesterol-modified miRNA 159) along with doxorubicin into exosomes derived from the human monocytic cell line THP-1 by incubating in PBS at 37°C to deliver to triple-negative breast cancer cells [53]. Linezolid was incorporated into exosomes derived from the mouse macrophage cell line RAW 264.7, by mixing both and incubating at 37°C for 1 h, resulting in ~5% drug loading. The exosomal formulation of linezolid was more effective against MRSA infections than the free drug [54]. Although several studies have reported the use of incubation with exosomes for drug or any therapeutic agent loading, it often suffers from issues of low percent drug loading, urging a requirement for improved methods for higher drug loading percent. Another method (less common) of passive loading includes incubating the exosome donor cells with the drugs/therapeutic agents. First, the donor cells are exposed to drugs or therapeutic agents, followed by isolation of released exosomes (supposedly) containing the loaded drugs or therapeutic agents. This method was used in a study by Pascucci et al., wherein they exposed bone marrow-derived mesenchymal stromal cells (MSCs) with a very high concentration of paclitaxel followed by incubation at 37°C for 24 h. After incubation, the cells were washed twice with PBS, trypsinized, and seeded in a fresh flask for 48 h. After 48 h, cell-conditioned medium was collected to isolate exosomes containing paclitaxel. They found that MSC-PTX-derived exosomes had a greater inhibitory effect on tumor cell proliferation (**Figure 2**) [55].

For active cargo loading, the exosomal membrane is temporarily disrupted using different methods and then restored once the drug/therapeutic agent was loaded. These methods may include sonication, extrusion, freeze-thawing, electroporation, use of membrane permeabilizers, conjugation using click chemistry, and antibodies against exosomal surface proteins. Electroporation uses an electric field to generate small pores in the exosomal membrane to disturb the phospholipid bilayer of exosomes. Drug/therapeutic agents can enter these vesicles via the generated pores. Once they entered, the pores were closed to recover the exosomal membrane integrity. This method has mostly been used to encapsulate siRNA or miRNA into exosomes and has been reported to enhance the percent loading compared to the simple diffusion method. Jia et al. loaded exosomes derived from RAW 264.7 cells with curcumin and superparamagnetic iron oxide nanoparticles (SPIONs) synchronously using optimal electroporation conditions of 400 V, 150 μ F, and 1 ms discharge time. They observed that electroporation had no effect on the membrane integrity of exosomes and

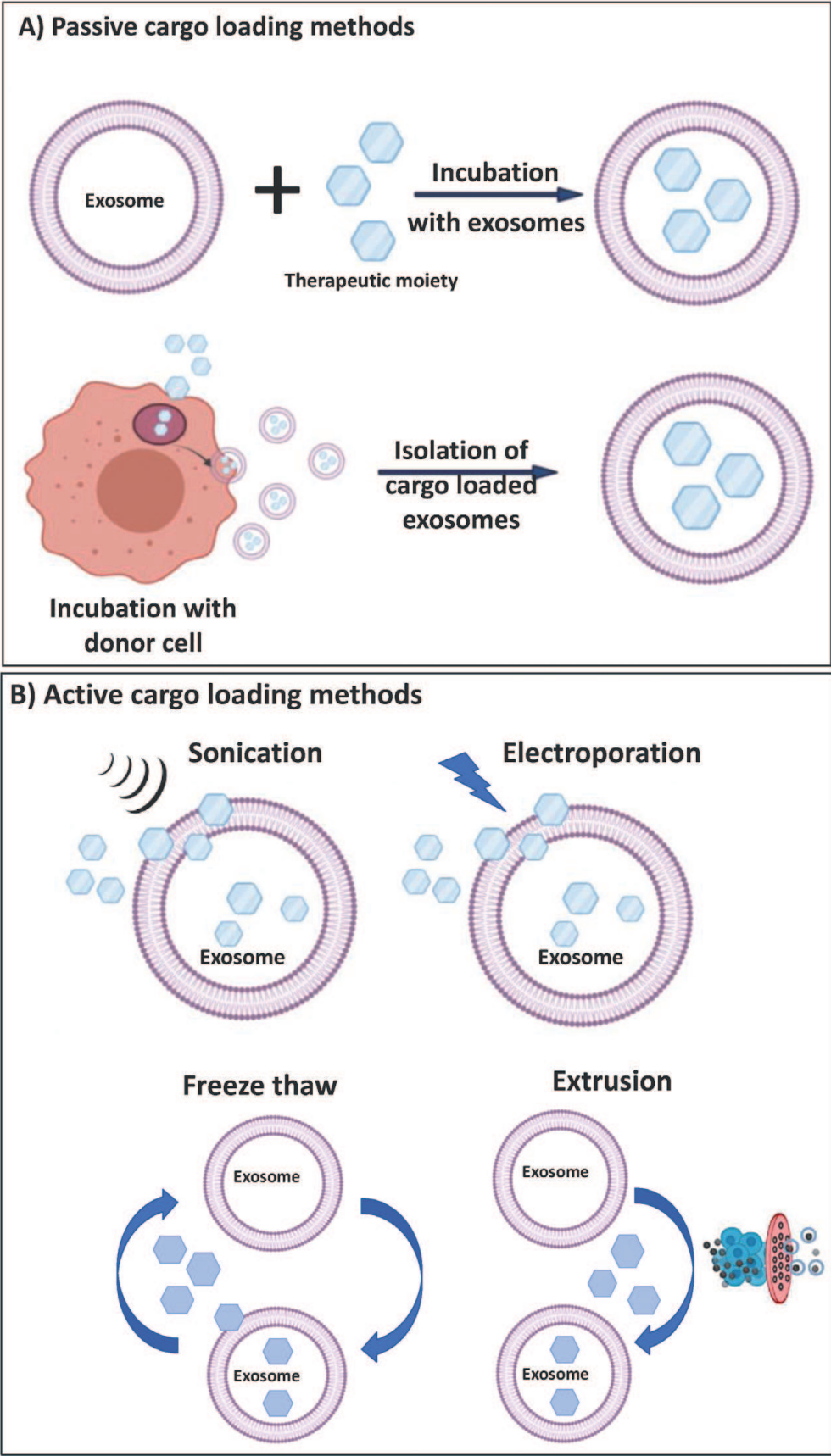


Figure 2.
Illustration representing different methods of cargo loading in exosomes. (A) Passive cargo loading is achieved by incubating the therapeutic moiety directly with isolated exosomes or by exposing to the exosome secreting donor cells followed by isolation of loaded exosomes. (B) Active cargo loading methods include use of physical treatments to disrupt the membrane integrity thus allowing entry of cargo in the interiors of exosomes. These treatments include sonication, electroporation, freeze thawing cycles and extrusion method.

efficiently encapsulated curcumin and SPIONs [56]. Similarly, Jia et al. incorporated doxorubicin into exosomes isolated from MDA-MB-231 and HCT-116 cell lines using an electroporation method, which resulted in ~1.5% drug loading [57]. According to published studies, although electroporation enhanced the percentage of drug loading in exosomes compared to the incubation method, it was still low. Therefore, scientists have employed sonication methods to load cargo more efficiently. The mechanical shear force of a sonicator/homogenizer probe is applied to disrupt the membrane integrity of exosomes, thus allowing the mixed drug/therapeutic agent to enter into the exosomes. In 2017, Kim et al. compared the incubation, electroporation, and sonication method of cargo loading in RAW264.7 cell derived exosomes to develop an exosomal formulation of paclitaxel (PTX). For the incubation method, the authors mixed and incubated PTX with exosomes at 37°C for 1 h. Using electroporation, exosomes and PTX were added to a pre-chilled electroporation cuvette and applied at 1000 kV for 5 ms followed by incubation at 37°C for half an hour to fully recover the exosome membrane. For sonication, the PTX-exosome mixture was sonicated at 20% amplitude, given 6 cycles of 30 s on/off for 3 min and a 2 min cooling period between each cycle. After sonication, the solution was incubated at 37°C for 1 h to fully recover the membrane of the exosomes. They showed the highest percent drug loading of ~28% using sonication followed by ~5% using electroporation and the lowest at ~1.4% with the incubation method [58].

In the extrusion method, exosomes mixed with the drug are passed through a syringe-based lipid extruder with a membrane ranging from 10 to 400 nm pore size. In this process, the membrane of exosomes is disrupted by the extensive mechanical force of the extruder. In a study by Kim et al. when breast cancer cell-derived exosomes loaded with porphyrin were extruded, it altered the surface charge of blank exosomes, leading to cytotoxic effects [59]. On the other hand, in another study by Fuhrmann et al., loading cargo in exosomes using the extrusion method did not render them cytotoxic [60]. In the freeze-thaw method, the drug was first incubated with exosomes at ambient temperature and then frozen at –80°C. The mixture was then repeatedly thawed at room temperature to ensure drug loading into these vesicles. The main disadvantage is that this method often leads to particle aggregation, resulting in a wide size distribution. This method has also been reported to result in a lower percent drug loading than other methods, such as sonication.

6. Exosomes for drug delivery in breast cancer: progress and future promise

Although exosomes have been shown to mediate cancer development, they are an emerging platform for drug delivery to cancerous sites because of their excellent biocompatibility, low immunogenicity (since they are derived from the patient's own cells), good tolerance, and remarkable biodistribution. Owing to their small size, they can readily pass through different bodily barriers such as the blood-brain barrier [61]. Compared to synthetic nanoparticles, exosomes are relatively easy to manipulate through surface modification in order to enhance their targeting efficiency to cancer cells. Recently, the use of exosomes for drug delivery in breast cancer cells has been proven to be efficient. Alvarez-Erviti et al. delivered the chemotherapeutic drug doxorubicin to breast cancer tissues in a mouse model [62]. First, they engineered these cells by expressing Lamp2b, a lysosome-associated membrane glycoprotein 2b, on their surface and fused with a targeting peptide for integrins. They then isolated

exosomes from immature dendritic cells (with low immunogenicity because of the absence of immunostimulatory markers on their surface) and used an electroporation technique to load doxorubicin within. They have shown that the exosomal formulation of doxorubicin has greater efficiency in targeting mouse tumors and hence, exhibits a novel propitious approach in breast cancer treatment in the clinical context. Li et al. loaded milk exosomes with doxorubicin to target CD44 overexpressed human breast cancer cell lines and found an exosomal formulation capable of delivering the drug into cancerous sites in a target-specific manner [63]. Vakshiteh et al. used dental pulp-derived mesenchymal stem cells to isolate exosomes and loaded them with miRNA, which was then targeted to breast cancer cells. They found that exosomes significantly decreased the proliferation of cancer cells and reduced the migratory and invasive properties of breast cancer cells *in vitro* [64, 65]. These studies indicate that exosomes are promising candidates for drug delivery in breast cancer therapy.

Currently, there are some hurdles in realizing the clinical potential of exosomes as drug delivery nanovehicles. These include low yield, long-term stability, and lack of understanding of their therapeutic effects. Hence, more research is required to develop techniques that can be used universally to enhance the yield in a time-efficient manner and increase the stability of exosomes.

Conflict of interest

The authors declare that they have no competing interests.

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