Journal of Drug Delivery Science and Technology 68 (2022)
103057 https://doi.org/10.1016/j.jddst.2021.103057

Exosomes and exosome-mimetics as targeted drug carriers: where we stand and what the future holds?

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Keywords: Exosomes, exosome-mimetics, drug delivery, nanocarrier, exosome-based drug delivery, drug cargo-loading

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Summary: Exosomes are a sub-group of extracellular vesicles, playing an important part in a cell-cell communication in many physiological and pathological conditions. Their size and competence for transferring material to recipient cells make them a promising nanocarrier for clinical use. Their non-immunogenic nature, similar to the body's own structure make them far superior transporters compared to liposomes and polymeric nanoparticles. This review, will provide an overview of exosome biogenesis, biological role, and purification methods. The focus of this manuscript will be to summarize specific applications of exosomes and exosome-mimetics as drug delivery systems in pharmaceutical drug development. We will describe drug-loading approaches, in vivo and in vitro exosome tracing methods, specific modifications and examples of the delivery of therapeutic and imaging molecules from a variety of biological origins. Challenges in the translation of exosome-based drug carriers to clinical use will also be discussed in this review.

1. INTRODUCTION

Past decades have seen a rise in the exploration of nanoscale drug delivery systems in order to improve the therapeutic efficacy of chemical and biomolecular drugs[1, 2]. There are two main problems in clinical translation of these systems: cytotoxicity of materials, and rapid clearance by the reticuloendothelial system (RES) or the mononuclear phagocyte system (MPS)[3]. Only a small number of nanoscale drug delivery systems have gained regulatory approval for human use[4].

Cell-Cell communication is essential for all multicellular organisms. To maintain homeostasis, cells continually interact with other cells and their surroundings through the secretion of molecules, which can be packed into different types of extracellular vesicles [5]. Classification of extracellular vesicles (EV) is typically based on a range of properties, including the process of biogenesis, density, or dimension[6]. Based on their mechanism of release and their size, EVs are categorized as exosomes (EXOs), microvesicles/shedding particles, and apoptotic bodies. Their content is different, depending on their cells of origin. Microvesicles and apoptotic bodies are released directly from the plasma membrane in living or dying cells, but EXOs are released after the fusion of late endosomes with the plasma membrane [7]. Apoptotic bodies are the largest of the EVs, followed by microvesicles, and finally, the smallest in this group are the EXOs, less than 150 nm in diameter. EXOs were first described in rat reticulocytes in 1983 and sheep reticulocytes in 1985 [8, 9]. Primarily, it was believed that EXOs are an alternative way to eliminate waste products, but later studies have shown that they play a role in important physiological and pathological processes, such as coagulation, intercellular signaling, immunosuppression and inflammation, tumor growth and metastasis [10, 11].

Pioneering reports describe the use of EXOs as nanocarriers. Their advantages include small size suitable for penetration into deep tissues, slightly negative zeta potential enabling long circulation [12], flexible cytoskeleton, and resemblance of EXOs membrane to cellular [13]. The biocompatibility of EXOs makes them an ideal natural nanocarrier for clinical application. In this review, the focus will be on strategies for

the translation of EXO into clinical practice from methods of their purification to their drug loading and modification strategies.

2. BIOGENESIS AND BIOLOGICAL FUNCTION

2.1 Biogenesis

Exosome formation is a tightly regulated and coordinated process phases driven by at least two different mechanisms: ESCRT (endosomal sorting complexes required for transport)-dependent, and ESCRT-independent (Fig. 1.).

Internalization of exosomal cargo begins from early endosomes then follows by formation of intraluminal vesicles (ILV) in the multivesicular bodies (MVBs) (Fig. 1). MVBs are important as an intermediary in the internalization of cargoes for membrane protein degradation and the regulation of cell-cell signaling. MVBs can either undergo lysosomal degradation or fuse with plasma membrane (PM) resulting in the release of ILVs into extracellular space. Which of these two processes will take place depends on the current state of the cell [14].

ESCRT machinery, consisting of four different protein complexes, ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and an additional associated protein such as the AAA ATP-ase-VPS4 complex, controls the formation of ILVs in MVBs (Fig. 1). They are all located on the cytoplasmic side of endosomal vesicles. ESCRT-0-I-II are soluble and stable multimeric complexes, containing ubiquitin-binding domains. ESCRT III is assembled from individual ESCRT III subunits [15].

Studies have demonstrated that the release of EXOs can occur without ESCRT (Fig 1.). Even with knockdown of all four subunits of ESCRT, ILVs are still formed in MVBs, thus indicating the possibility of the existence of another mechanism for releasing EXOs, specifically, an ESCRT-independent mechanism [16]. In this pathway, some additional proteins are important, such as tetraspanins: CD9, CD82, CD63, Tspan8 etc.

Lipid rafts, also known as the detergent-resistant domains (DRM) of the membrane, and lipid raft-associated proteins have been confirmed in EXO [17]. Together with proteins, they have been included in ESCRT-independent MVB formation. These lipid rafts contain, alongside cholesterol, a high amount of sphingolipids, which can then be converted to ceramides using the neutral sphingomyelinase family [18].

2.2 Biological function of EXO

The information that EXOs carry, as well as their function within an organism, depends on the state of the cells from which they originate. It was discovered that they have important roles in processes that are connected to both physiological and pathological states. EXOs have a role in apoptosis, coagulation, antigen-presentation, cell homeostasis, inflammation, intracellular signaling, angiogenesis, the growth and metastasis of tumors. It is well known that EXOs have a role in the communication between cells, however, the exact mechanism for the uptake of EXOs by other cells is still unclear. There are many known ways for endocytosis: phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, as well as caveolin, and clathrin-independent endocytosis [14]. EXOs may implement different mechanisms of internalization in the same cell depending on conditions and timing [15].

EXOs have an important role in promoting angiogenesis [19]. Tumor-derived EXOs are capable of promoting angiogenesis and remodeling of extracellular matrices. EXOs achieve their function via micro RNA molecules (miR132, miR210, miR126, miR21) that function as signal carriers, starting other processes in their target cells [20]. The role of EXOs in these processes indicates that they may be a future target for the development of novel therapeutic methods, for example in the treatment of ischemic diseases [21].

Apoptotic cells, release higher amounts of EVs in comparison to healthy cells [22]. These apoptotic EXO have unique protein markers such as Sphingosine 1-Phosphate Receptors 1 and 3, S1PR1/3. The expression of S1PR1/3 induces a proinflammatory response in macrophages, leading to the release of proinflammatory cytokines (IL1-β) resulting in the activation of NF-kB and p38 MAPK signaling pathways in macrophages [23].

Generally, EXOs that originate from cancer cells can inhibit natural killer cells, have cytotoxic effects, induce cell proliferation, and affect the apoptosis of T cells. The signaling pathway that induces T lymphocyte apoptosis is unknown, but research has shown that cancer-derived EXOs increase the apoptosis of T lymphocytes [24].

Increased levels of certain tetraspanins on the surface of EXOs can be directly correlated to specific illnesses. For example, EXOs that contain an increased amount of CD81 present a sign of active inflammatory response and are used as biomarkers for the detection of hepatitis C, while EXOs that contain α-synuclein are used as biomarkers for the detection of Parkinson's disease [25].

EXOs have an important role in cell proliferation and differentiation as well. Their role in cell proliferation can be seen with various tumors [26]. EXOs can carry certain molecules which can affect specific signaling pathways (PI3/AKT, MAPK/ERK), stimulating the proliferation of cancerous cells [27]. EXOs can also affect the differentiation of cells. Generally, the molecules present in EXOs can influence the direction of cell differentiation. Because of this, exosomes are important as potential tissue-specific grafts for the reparation of different types of tissues [28].

3. METHODS FOR PURIFICATION EXO-NANOCARRIERS

EXOs have been found in most bodily fluids such as pleural effusions, plasma, bile and urine, breast milk, ascites, amniotic fluid, semen (“prostasomes” and “epididymosomes”), saliva, nasal secretions, cerebrospinal fluid (CSF), bronchoalveolar lavage (BAL), synovial fluid [29].

EVs can be obtained and isolated from cell culture conditioned media. In this case, culturing and harvesting conditions such as passage number, seeding density, confluence, frequency, and interval of harvest, culture volume, oxygen or other gas tension, antibiotics, growth factors, etc. should be determined precisely and, depending on the conditions, the amount of released EXO will vary [30]. Blood is a good candidate for the isolation of EVs but has limitations in practical work due to complexity and the presence of cells, proteins, nucleic acid, etc. Also, serum and plasma are the other options for the isolation of EVs [31].

Various biophysical and biochemical properties can be used to isolate EXOs, including size, mass density, shape, charge, and antigen exposure (Table 1.) [32]. At the end of 2015, differential ultracentrifugation was the most commonly used method for EXO separation (81% of studies), according to a worldwide ISEV survey [33]. There are other techniques such as density gradients, precipitation, filtration, size exclusion chromatography, and immunoaffinity isolation used for that purpose. Most researchers combine one or more methods to achieve better results in the case of subtype separation or specificity of EVs. Each technique offers a set of advantages and disadvantages that need to be taken into account when formulating the protocol for EXO isolation [34], especially in cases where EV samples are isolated for clinical validation studies and liquid biopsy applications [35].

Gold standard method for EXO purification is ultracentrifugation (UC). It relies on the sequential separation of particles based on their size and density by successively increasing centrifugal force and duration [36] [37].

Despite the fact that UC has been the most commonly used method to isolate EXO, it is not suitable for clinical applications because it is time-consuming, laborious, and low throughput (Table 1.). UC cannot be used to isolate completely pure EVs due to the small difference in sedimentation rate, mass density, and size of different types of vesicles. There is also a good possibility that UC can lead to clumping of vesicles, co-isolation of non-EV components, including viruses and protein aggregates [38].

A variant of UC, density gradient UC, can be used to improve the previously mentioned disadvantages. Besides mass and size, this separation method includes separation based on density, using a pre-constructed density gradient increasing from the top to the bottom of the tube [39]. There is a number of gradient mediums that may be implemented; however, sucrose and iodixanol are most commonly used [40]. Gradient UC requires even more time than UC, but the obtained sample is of greater purity [34].

Ultrafiltration (UF) is used to separate particles based on their different size, using a membrane with specific pore diameters. Large particles are removed using a filter with pore diameters. The remaining, EXO-rich suspension is then separated through filters with pore diameters smaller than the EXOs (0.22 and 0,1 μm). In one run of ultrafiltration, EXOs may be concentrated up to 240-fold [41, 42]. The advantages of UF are that

it is comparatively less time and labor-intensive and does not require special equipment [43] but the main disadvantages of UF is that it is difficult to remove contaminating proteins.

Polymer precipitation is easy to use and does not require special equipment or skills. EXOs are most precipitated using Polyethylene glycol, a water-soluble, volume-excluding, non-toxic, and non-denaturating polymer. The precipitation method is based on an aqueous PEG solution that has the ability to aggregate EXOs, which can then be precipitated by low-speed centrifugation [44]. The purity of EXOs obtained in such a way is lower, compared to other methods due to the co-precipitation of non-EVs components such as immunoglobulins, viral particles, immune complexes, etc. (Table 1.)[39]. Overall, this method results in a high yield but a low purity of isolated exosome samples due to unspecific co-precipitation of contaminants. Users can process many samples at the same time, quickly, with ease, and at a relatively low cost without damage to the EXO. Besides the low purity of isolated EXO, the positive properties of this super hydrophilic polymer are notable and, make PEG a very interesting method for fast exosome extraction in clinical research [45].

Immunoaffinity-capture methods are based on the separation of EXOs, using certain antigens expressed on the surface of their membranes. These methods were developed to improve the purity of obtained samples, avoid contamination, and isolate specific sub-populations of EXOs. EVs express different surface protein markers based on their origin, but, CD9, CD63, CD81, EGFRVIII, CD163, and TAG72 are considered hallmarks EXO protein markers. There are many variations of the method as different antigens can be used for the capture, and different types of solid-phase for support (magnetic beads or gold ferric oxide nanocubes, microplate). CD63, CD9, CD81 are typically used as targets for the isolation of EXO from various sources, but are not specific enough to target different EXO sub-populations [39, 46]. Recently, an immunoaffinity approach for EXO isolation from different sources using single-domain antibodies has been described [47]. This approach does not increase specificity toward certain EXO subpopulation but significantly reduces the isolation costs [47].

Microfluidics-based isolation techniques have been rapidly developed over the past few decades and have the potential for application in the different fields: food, agriculture, and medicine [48].

In general, microfluidics is a high-throughput method that uses a small device to isolate EXOs based on their physical and chemical features such as size, density, and exposure of antigens. The development of these techniques is very promising and shows their potential as future tools in the field of exosome separation. In combination with other methods, this technique can result in better separation and purity.

All methods developed for microfluidic-based exosomal purification can be classified into three categories depending on the principle of the separation of EXOs: trapping EXOs based on an immune-affinity approach (capture assay), filtration approach, and magnetic approach [49].

The most commonly used method is the immune-affinity approach, which is similar to the conventional immunoaffinity capture assay. The device that has been first constructed using this principle is the "ExoChip", with CD63 antibodies [49].

The methods that implement microchips based on the filtration principle are similar to the standard ultrafiltration method. They contain nanomembranes with different cut-offs (of 30 to 200 nm) to separate EXO from other EVs and proteins. The membrane with larger pores (200 nm) was used for the removal of cells, cell debris, large EVs while the membrane with smaller pores (30 nm) was used to remove soluble proteins from EXO [50].

The magnetic separation technique has become the most commonly used method. In this method, antibodies are immobilized on the surface of magnetic beads. One such example combines the enrichment of captured EXO (1st stage- binding EXO on antibodies labeled magnetic beads), chemical lysis and immunoprecipitation of intra-vesicular proteins (2nd-stage), and chemifluorescence-assisted sandwich immunoassay (3rd stage- measuring). By measuring the intensity of fluorescence, it is possible to determine the presence of protein markers within isolated EXO [51].

Microfluidics-based isolation techniques have many advantages such as fast analysis of multiple samples at the same time, easy handling of devices, and small sample volumes (10μL). However, these methods are hard to standardize, have not been tested on large-scale clinical samples, and lack the methods for validation [49, 52].

Another size-based separation technique, used for the isolation of EXO is Size exclusion chromatography (SEC). Macromolecules and other components are separated through the macroporous stationary phase. The stationary phase can be packed with a number of polymers, including cross-linked dextran (Sephadex), polyacrylamide (BiogelP), agarose (Sepharose), or allyldextran (Sephacryl). The separation of particles using this method relies on the size of molecules, specifically their Stokes radii, which are in routine practice usually replaced with hydrodynamic radii [53]. EXOs with a bigger hydrodynamic radius cannot pass through many smaller pores and crevices and, as a result, are eluted from the column earlier [53]. The separation force in SEC is gravity, so EXO and other biologically active components can retain structural integrity and activity. However, it is a time-consuming method [54].

SEC cannot separate EXO from microvesicles, due to their similar size, so when the identification of a single EV population is required, SEC has limited applicability and the method needs to be combined with another technique, such as the immunoaffinity method (Table 1.).

Tangential flow filtration (TFF) is emerging a new and promising method for EV isolation [55]. The method is based on fluid that flows tangentially (horizontally) across a filter reducing the pore clogging that limits conventional dead-end filtration [56]. Controlled flow rates that are implemented during TFF result in low shear rates enabling separation of components of different size without any damage. TFF protocols usually use a system of two different size filters, one that separates EVs from cell debris and the second that separates EVs and smaller contaminants such as proteins, carbohydrates, lipids, and nucleic acids[55]. Since TFF includes both diafiltration and separation steps, larger volumes of starting material could be processed and reduced to smaller volumes in just a few hours [56]. TFF EV-yields can be as much as 5-times higher in

comparison to ultracentrifugation, when starting from the same material, making it particularly suitable for EV functional studies such as using cell culture assays and therapeutic animal studies[56]

Translation of EXO into clinical practice requires, first of all compliance with regulatory frameworks [57]. Considerable challenge in clinical translation of EXO is the standardization of production procedure and effective purification of large amounts of these nanovesicles. Crucial requirements are the manufacturing of homogeneous drug nanoformulations, and the production and quality control procedures. Additionally, biobanking of purified EXO, their downstream analysis in terms of storage stability control is still lacking. There is no recommended standard techniques established for the clinical grade production and quality control of EV-based therapeutics so far. Coupled with the fact that several manufacturing and safety considerations need to be addressed and appropriate quality controls have to be implemented and validated, it remains a challenge to set up platforms for the EXO clinical grade production that fulfils all necessary criteria for the successful approval of subsequent EXO-based clinical trials [57]

4. CELL-SOURCES FOR ENGINEERED EXOSOMES

Proper choice of cell-source of therapeutic exosomes plays a crucial role in their subsequent application. The composition of surface lipid and protein markers can determine the functionality of therapeutic EXO and maintenance of these characteristics is paramount. Careful consideration of the biological characteristics of intended exosomal drug carriers is crucial for successful therapeutic application.

EXO possess immunomodulatory properties. Dendritic cell (DC) derived EXO have the ability to stimulate an immune response by transferring peptide–MHC complexes from DCs that have been exposed to an antigen to another DC that has not had contact with the antigen [58]. EXOs derived from DCs stimulated tumor peptides can prime cytotoxic T cell response delaying tumor growth or completely eradicating established murine tumors [59]. Clinical trials conducted on patients with advanced-stage melanomas [60] or non-small cell lung carcinomas (NSCLC) [61] expressing melanoma-associated antigen, using the autologous DC EXOs in end-stage cancer, demonstrated effect on NK-cell effector functions in patients, but only minimal or no melanoma-associated antigen-specific T-cell responses [60, 61].

EXOs derived from antigen-presenting cells (APC) can exert an immunosuppressive role especially in the case the cells are expressing IL-10, IL-4, FasL, and indoleamine 2,3 dioxygenase (IDO) making them an effective tool in the treatment of autoimmune diseases such as rheumatoid arthritis [62, 63].

Mesenchymal stem cells (MSC) derived EVs exhibit unique properties in therapy of certain conditions such as diseases of cardiac injury, kidney injury, and brain injury[64]. MCS cell-derived EV therapies are quickly moving toward clinical applications particularly in the context of post-surgical wound healing, the treatment of colo-cutaneuous fistulas and ischemia–reperfusion injury [65, 66]. The MSC-derived EVs also have the potential to treat conditions such as cardiovascular ischemia, and kidney and liver diseases.[65, 67-69] It has been demonstrated that exosomes from cardiosphere-derived cells have limited injury and improved function in myocardial infarction[69]. Furthermore, evidence has been provided that exosomes purified from pericardial fluid of patients undergoing acute myocardial infarction are related to an improvement in myocardial performance, including arteriogenesis, and reduced cardiomyocyte apoptosis [70].

Tumor-derived EXOs have appealing properties as a vehicle for drug delivery or vaccine in oncological immunotherapy. Malignant effusions often contain an abundance of tumor-derived EXOs. Furthermore, tumor-derived EXOs carry on their surface specific antigens and MHC I molecules from parenting cells enabling the induction of T-cell specific immune response [71]. Tetraspanins and other surface molecules interact with different tissue ligands homing the EXOs specifically to the given tissue [72]. Despite having specific targeting abilities EXOs also tend to increase the metastatic potential of different cancer types [73]. Other molecules such as proteases (urokinase plasminogen activator, cathepsin D) and adhesion modulators (vimentin, galectin 3-binding protein, and annexin A1) prone to promoting cell movement and increasing metastatic potential have also been detected in cancer-derived EXO [74]. Furthermore, tumor-derived EXOs help tumor evade the immune system by enhancing the production of myeloid-derived suppressor cells [75], by inhibiting tumor-reactive effector T cells through the expression of apoptosis-inducing ligands, such as FasL and TRAIL or PDL-2 [76, 77]. For the above-mentioned reasons, the use of tumor-derived EXOs in the

therapy of malignancies carries significant risks of enhancing the malignant disease rather than improving it, making the right choice of EXO even more crucial.

EXO isolated from plants have been explored as alternative options for clinical use because they are derived for reliable sources and exhibit good safety profiles [78]. Food EXO are of particulate interest as they are commonly ingested and thus are generally considered safe [79]. Agricultural products are particularly interesting source of EXO since they are economically practical and scalable sources. There is an ongoing clinical trial aiming to assess the ability of plant derived EXO to deliver therapeutics to colon cancer [80]. EXO-like nanoparticles from grapes have been found that after oral administration, these vesicles induced growth and differentiation of intestinal stem cells and protected mice from intestinal damage initiated by dextran sulfate sodium[81]. Modified grapefruit-derived EXO have improved tumor targeting capability and after loading them with the anticancer agents, doxorubicin and curcumin, have been found to target inflammatory tumors and to have efficacy against inflammation in a mouse model [82].

Another agricultural source of reliable, scalable, and safe EXO for therapeutic delivery is bovine milk. EXO isolated from milk have been loaded with different therapeutic cargos, including both hydrophilic and hydrophobic small molecules and chemotherapeutic agents [79]. These drug-loaded EXO have enhanced biological efficacy on lung tumor cells in culture and in xenograft models when compared to free drug, However, despite of the high yield and superior safety profiles of EXO derived from food they are incapable of boosting host immune systems and lack immunotherapy benefits.

5. EXOSOME DRUG LOADING METHODS

The drug loading methodologies applied to exosomes, and their mimetics (Cellular Vesicles), are categorized into two main groups, the pre-isolation loading (pre-loading) methods, and the post-isolation loading (post-loading) methods. In pre-loading methods, the drug is initially produced or loaded in the parental cells, and the extracellular vesicles or cellular vesicles isolated/produced by them are already loaded with the desired drug. In the post-isolation strategies, the desired drug is loaded into the EXO after the purification procedure [83].

Understanding EXO structure has a pivotal role in successful drug loading to EXOs carriers. There are two major approaches for loading therapeutic molecules into EXOs carriers: passive (I) and active (II) encapsulation, resulting in different stabilities and loading efficiencies.

Characterization of exosomal features pre- and post-loading normally relies on use of different biophysical (Electron Microscopy, Nanoparticle Tracking Analysis Dynamic Light Scattering, Atomic Force Microscopy,) and molecular approaches (Western blot, Flow cytometry, Mass spectrometry)[84]. Drug loading does not affect some of the features of EXO, such as protein content. Presence of EXO specific markers was confirmed post loading indicating that the procedure itself did not affect the protein content of the vesicles [85, 86]. Morphological studies indicated that loading procedures of the did not change round and vesicular shape of EXO during drug encapsulation [85, 87]

5.1. Passive cargo loading methods

Passive cargo loading methods include incubation of the drug with donor cells (Fig 2a.) or with EXOs (Fig 2b.). Incubation with EXOs implies simple incubation of the drug with purified EXOs allowing for the drug to diffuse into the EXOs along the concentration gradient. After which, the cargo-loaded EXOs are obtained by removing unwanted cargo. The efficiency of loading is highly dependent on the hydrophobicity of the drug.

Many types of small-molecule cargo have been loaded to EXOs via incubation. Anti-inflammatory drug curcumin loaded to EXOs, as means for delivery to activated monocyte-derived myeloid cells in vivo have exhibited therapeutic and without apparent toxic effects [88]. Chemotherapeutic agents have also been loaded to carrier EXO using incubation. For instance, chemotherapeutic drug paclitaxel was loaded into prostate cancer cell-derived exosomes to generate drug-loaded exosomes with the potential for effective drug delivery to prostate cancer cells [89]. Peptides and proteins can be passively loaded into EXOs. For instance, brain-derived neurotrophic factors and catalase were loaded into EXO for a potent antioxidant for the treatment of Parkinson's disease [90] [91].

Incubation of cargo with EXOs has emerged as an easy-to-operate strategy with minimal destruction to the carrier. However, loading efficiency is limited and the loading quantity is difficult to control due to the physicochemical properties of cargos and EXOs. Efficacy is under the influence of the system setup and physicochemical characteristics of the drug, making a universal loading protocol hard to achieve. Hydrophobicity of the cargo can influence the distribution of the drug in different EXO compartments [92, 93].

Incubation of EXO donor cells with drugs is another way of loading cargo-to the EXO-carrier. Donor cells are treated with the drug, leading to the secretion of loaded EXOs. Different types of drugs could be loaded this way such as chemotherapeutics and nanomaterials. This method was used to load low-dose paclitaxel to mesenchymal stroma cells obtaining paclitaxel-loaded EXOs with inhibitory effects on tumor growth. A combination of paclitaxel and doxorubicin was loaded into EXOs from ovarian cancer cells and breast-to-lung metastatic cells enabling suppression of ovarian cancer cell growth [94].

Direct loading of CRISPR associated protein 9 (Cas9) to EV was achieved using passive incubation. Cas9 was bound to cationic lipids and further complexed with MDA-MB-231 cell-derived EVs through passive incubation. Size-exclusion chromatography was used to remove components that were not complexed with EVs. The ability of EVs obtained this way, to facilitate intracellular delivery of proteins was compared to conventional methods, such as electroporation and commercial protein transfection reagents. The results indicate that EVs retain native features following protein-loading with comparable ability of intracellular protein delivery but with decreased toxicity[95]

Different nanomaterials can also be loaded to EXOs this way. For instance, iron-oxide nanoparticles have been incubated with macrophages to create nanoparticle-loaded EXOs[96]. Citrate-coated gold-nanoparticles, modified with polyethylene glycol and folic acid, were incubated melanoma cells for targeting of melanoma cells in vivo [97].

Similarly, direct incubation of cargo with EXO donor cells is simple but has low loading capacity and is hard to control loading effic iency. The amount of drug that diffuses into the cell and is packed in EXOs is

hard to predict and experimentally manipulate. Furthermore, drugs themselves can damage the cells and disrupt the production and integrity of secreted EXOs.

5.2. Active cargo-loading methods

EXOs from donor cells are sonicated (Fig 3a.) with cargo drug using a homogenizer probe. Physical force disrupts the EXO membrane integrity and allows the drug to diffuse into the EXO interior. Membrane microviscosity is significantly reduced during sonication without significantly affecting the membrane-bound proteins or the lipid contents of the exosomes [98]. The integrity of the membrane is restored after one hour at 37°C. In some cases, drugs are not only incorporated but also attached to the surface of EXOs, so the release of drugs can occur in two phases: the first burst release phase, when the surface attached drug is released, and the second slow release phase when the incorporated drug is released [98].

Sonication method has been used for incorporation of gemcitabine with pancreatic cancer cell-derived exosomes leading to greater than 4-fold in loading capacity compared to the incubation method [99]. Similarly, paclitaxel can be loaded to macrophage-derived EXOs using sonication with higher capacity in comparison with simple loading methods [100]. Furthermore, catalase [91] and gold-nanoparticles [101] can be loaded to EXO using sonication making sonication an effective method of loading different types of cargo to the EXO.

Extrusion is another method for loading drugs to EXOs during which EXOs are mixed with a drug, and the mixture is loaded into a syringe-based lipid extruder with 100–400 nm porous membranes under a controlled temperature, leading to disruption of EXO membrane during vigorous mixing (Fig. 3b.). EXO membrane collapses and a homogenous mixture with the cargo is obtained after several repetitions under certain parameters [102]. This strategy has been utilized to produce catalase-loaded EXOs that are rapidly uptaken by neural cells in vitro and in vivo, protecting them from degeneration in Parkinson's disease models [91]. Breast-cancer EXOs loaded with porphyrin with changed zeta-potential exhibited cytotoxicity whereas porphyrin-EXOs obtained using different methods did not show significant cytotoxicity [92].

Extrusion has high loading capacities but a rearrangement of EXO surface structure possibly changes their immune-privileged status.

Electroporation is based on using an electric field in a conductive solution for the creation of temporary membrane pores facilitating the diffusion of the drug into EXOs (Fig. 3c.). This method was employed for loading nucleic acids such as siRNA and miRNA into EXOs because these relatively large molecules cannot spontaneously diffuse into EXOs. Electroporation is far superior for loading siRNA into EXOs than chemical transfection [103]. Besides RNA, hydrophilic small molecules and nanomaterials can also be loaded to EXOs using electroporation. For example, 5,10,15,20-tetrakis (1-methyl-4-pyridinio) porphyrin tetra (p-toluene sulfonate) or TMP [92]. Chemotherapeutics have been loaded with greater efficiency into EXOs using electroporation when compared to incubation. This way drugs such as doxorubicin [104] and paclitaxel [98] were loaded to dendritic cell-derived and macrophage-derived EXOs respectively. Nanomaterials, such as gold nanoparticles coated with polymethacrylates and coupled with chlorin-6 contained bovine serum albumin, were electroporated into EXO for cancer imaging and photodynamic therapy [105].

Surfactants such as saponin and triton can generate membrane pores increasing membrane permeabilization (Fig.3d.) [106]. Surfactants significantly promote the loading capacity of different types of drugs, compared to simple incubation methods [107]. Saponin-assisted loading of hydrophilic porphyrins is 11-fold more effective when compared to simple incubation [92]. Saponin has also been successfully used for loading catalase to EXOs in order to induce neuroprotective effects in Parkinson's disease model post intranasal administration [91]. Surfactants can degrade the EXO cargo potentially limiting the effectiveness of therapy. Furthermore, saponin is haemolytically active in vivo, thus saponin concentration needs to be limited, and loaded EXOs need to be purified before use [106].

Freeze and thaw cycles have been used in the reconstitution of liposomes [108] and due to the fast expansion of EXO research this method has been employed for EXO reconstitution (Fig. 3e.) [91]. In this method, EXOs are incubated with drugs for a fixed amount of time after which they are rapidly frozen at -80 °C or in liquid nitrogen. This is repeated at least 3 times so that the drug gets encapsulated into EXO [109].

However, this method can induce EXO aggregation leading to broadening of EXO diameter. This method can be used for fusion between EXO and liposomes developing rationally designed exosomes as hybrid nanocarriers for use in advanced drug delivery systems [109]. The freeze-thaw approach is simple and effective for different types of drugs. However, the method has low loading capacity and could lead to the inactivation of membrane proteins [91] inducing EXO aggregation

5.3. Parental cell transfection

Transfection is the most widely-used and efficient method for the loading of protein- or oligonucleotide-drugs into EXO. Using transfection reagents, specific plasmids are transduced into cells to ectopically express desired nucleic acids, proteins, or peptides that are packaged into exosomes afterward. Cell transfection can be achieved by using the calcium phosphate method or by a commercially available lipid transfection reagent such as Lipofectamine. This way exosomes could be loaded with gene promoters and/or silencers regulating transcription levels in recipient cells. Also, certain proteins could be overexpressed on the membrane surface or in the EXO lumen (Fig. 4a.) [83].

miRNAs have been successfully introduced into EXOs by using miRNA expression vectors. Transfecting modified miR-143 in THP-1 macrophage cells led to the successful loading of the modified miRNA into the exosomes. This demonstrated that overexpression of miRNA in the parental cells leads to their passive loading into EXOs (Fig 4b.) [81]. siRNA can similarly be loaded into the EXOs. For instance, BCR-ABL siRNA has been transduced into human embryonic kidney 293 (HEK293) cells and exosomes collected from the culture medium afterward were used to target chronic myeloid leukemia cells overcoming pharmacological resistance [110].

Transduction of syndecan-1 into mouse pulmonary microvascular endothelial cells by lentiviral-based vector generated two types of exosomes with either high or low levels of syndecan-1 that could be used for ameliorating pulmonary edema [111]. HEK293 cells were transfected with vascular stomatitis virus glycoprotein to enhance the fusion of EXOs with the plasma membrane of the recipient cells in a process

known as “membrane-editing” [112]. Similarly, HEK293 cells were transfected with CD9 fused to human antigen R, an RNA binding protein, to facilitate the loading of miR-155 into exosomes [113].

6. EXOSOME MIMETICS IN DRUG DELIVERY

There are two types of exosome mimetics: (I) Artificial exosome-mimetics, and (II) semi-synthetic mimetics, corresponding to artificial, or structures generated from cultured cells, respectively. Artificial EXO-mimetics are lipids assembled into a bilayer structure (which resembles the membrane of the exosome), functionalized with proteins, or created from larger substrates (cells) that are reduced to units for the creation of small size vesicles [114]. Only small unilamellar vesicles are ideal precursors for the preparation of vesicles that mimic EXOs due to their similar size range and membrane disposition. Classical methods used for the preparation of small unilamellar liposomes such as thin-film hydration method, reverse-phase evaporation method, ethanol injection method, ether injection method, microfluidic-based methods, extrusion techniques, etc., give rise to the vesicles in the EXO size range.

Recently, a promising strategy for the creation of exosome-mimicking liposomes was formulated using DOPC/SM/Chol/DOPS/DOPE and utilized for delivery of VEGF siRNA to A549 and HUVEC cells. These liposomes exhibited decreased cytotoxicity and higher storage stability and anti-serum aggregation effect compared with Lipo 2000 and DOTAP liposomes. Cellular uptake of such formulation was dependent on cell types and included several pathways from membrane fusion, caveolae-mediated endocytosis, to micropinocytosis. Although this formulation exhibited significantly higher cellular uptake and silencing efficiency than PC-Chol liposomes it remained far from satisfactory and warranted further improvement in the future research [115].

Artificial lipid vesicles containing bioactive APO2L/TRAIL were used as a treatment in a rabbit model of antigen-induced arthritis. These artificial-exosomes increased APO2L/TRAIL bioactivity and resulted in a more effective treatment of antigen-induced arthritis compared to unconjugated APO2L/TRAIL [116]. Also, they have been shown to reduce synovial inflammation and hyperplasia in rabbit knee joints Furthermore,

liposomes with bioactive APO2L/TRAIL have been shown to greatly increase APO2L/TRAIL activity showing apoptosis-inducing ability on cell lines from hematological tumors [117].

EXO similar nanoplatforms that simulates tumor derived EXO in terms of structure and functionality, but having a controlled composition have been used for delivery of oligonucleotide therapeutic to lung adenocarcinoma cells [118]. Using established liposome technology engineering EXO mimetics that can be loaded with therapeutic compounds, and tailored with specific proteins (integrin α6β4) providing them organotropic properties. These carrier-systems have natural similarities with respect to their physicochemical properties, drug loading capacity, and ability to interact with the cancer target cells in vitro and in vivo, but are easier to manufacture, can be produced at high yields, and are safer by definition. This multifunctional and highly versatile nanoplatform provides an important advantages in terms of production methodology and regulations and is suitable for a broader range of applications [118].

Specific targeting of synthetic vesicles can be achieved by either specific targeting molecules such as antibodies antibody fragments or by the formation of "protein corona" (PrC) [119]. Protein corona is self-assembled biomolecular coating, made up of mainly proteins representing a sort of bio-nanointerface, between synthetic vesicles and biological systems mediating cytotoxicity, cellular internalization and specific targeting to cancer cells [120]. It is formed during contact of artificial vesicles and biological fluids such as plasma [121]. PrC on the surface of EVs is a major determinant of their pharmacokinetics and bio distribution within body fluids and is mainly affected by their surrounding environment and intrinsic features. It has been shown that generating EV-like structures with certain PrC properties could potentially circumvent the limitations associated with the systemic delivery of EVs to the therapeutic targets [122] Similarly, artificial PrC derived from human plasma can drastically prolong circulation if coated lyposome by drastically reducing capture by circulating leukocytes in whole blood [123].However, type of plasma proteins that make the PrC and their relative abundance can affect the intracellular uptake through modulating the relative internalization of modified synthetic vesicles, with different formulations, in healthy and cancer human cells [124].

Semi-synthetic cellular vesicles are inspired by extracellular vesicles but represent a different type of endogenous mimetic. Cellular vesicles are produced from the cultured cell as membrane fragments that retain surface characteristics of their parental cells making them highly biocompatible with efficient intrinsic targeting ability. Moreover, they do not require further surface functionalization. Their natural origin yields higher retention time in the circulation and a reduced clearance rate. These stable and long-circulating endogenous vesicles protect the drug cargo from degradation, increasing drug delivery to targeted tissues [125]. Cell-derived EXO mimetics can be created using various methods including manual and centrifugal-induced extrusion over polycarbonate filters and microfluidic-based pressurization over hydrophilic microchannels and slicing with silicon nitride blades [126].

Cellular vesicles have been employed as drug carriers in the treatment of various conditions. For cancer treatment, cell-derived vesicles show enhanced permeability and retention effect on leaky vasculature of tumors due to their nanomolecular size and biocompatibility. Cellular vesicles have been employed for the successful delivery of different chemotherapeutic substances, such as doxorubicin, at high amounts to tumor tissues significantly reducing tumor growth without the adverse effects observed with equipotent free drug [127]. Successful application of cellular vesicles describes the engineering of anti-PSMA peptide-decorated EXO mimetics for targeting advanced prostate cancer (PC). EXO mimetics were produced from anti-PSMA peptide, (WQPDTAHHWATL) in U937 monoblastic cells, followed by successive extrusion cycles. The engineered EXOs were nanosized and produced at a high yield. They displayed the anti-PSMA peptide, exosomal markers and monocytes proteins on their surface. Prostate cancer cell lines (LNCaP and C4-2B) exhibited higher cellular uptake of the modified EXO nanocarriers compared to unmodified EXO mimetics. Furthermore, higher tumour targeting was observed in solid C4-2B tumors, following intravenous administration, confirming their targeting ability in vivo [128].

The anti-tumour effect of the EXO mimetics derived from NK cells was confirmed in vitro and in vivo. These mimetics have morphological features and composition similar to EXO, however their production is far simpler. These EXO mimetics have exhibited in vitro the cytotoxicity against different cancer cells

(glioblastoma, breast carcinoma, anaplastic thyroid cancer and hepatic carcinoma. Xenograft glioblastoma mouse model confirmed in vivo activity of NK derived EXO mimetics by the significantly decreasing size and weight of the tumor compared with the control group. Moreover, NK derived EXO mimetics affected the expression level of the cell survival markers p-ERK and p-AKT, and increased levels of apoptosis protein markers cleaved-caspase 3, cytochrome-c and cleaved-PARP in glioblastoma cells [129].

There is also a well-established therapeutic praxis to use EXO mimetics in regenerative studies. Recent studies have demonstrated the utility of cellular vesicles as carriers in liver regeneration studies. Cellular vesicles produced by serial extrusions of primary hepatocytes were injected intravenously into mice. It was clearly demonstrated that these vesicles promote hepatocyte proliferation and liver regeneration by significantly enhancing the content of sphingosine kinase 2 in the recipient cells [130].

Extrusion technique has also been employed to amass EXO mimetics from human mesenchymal stem cells (hMSCs). The collected mimetics had a significantly increased proportion of vesicles positive for the EXO-specific CD-63 marker compared with MSC-derived EXO. Using genetically modified hMSCs in which expression of noggin, a natural bone morphogenetic protein antagonist, was down-regulated, enhanced the osteogenic properties of EXO mimetics. Moreover, the administration of hMSC-EXO mimetics in conjunction with an injectable chitosan hydrogel into mouse nonhealing calvarial defects demonstrated robust bone regeneration via inhibition of miR-29a [131].

7. ENHANCING EXO AND EXO MIMETICS THERAPEUTIC POTEINTIAL VIA SURFACE MODIFICATION

EXOs and EXO-mimetic nanovesicles have been found to be effective therapeutics or drug delivery vehicles in a wide range of human diseases including cancer and neurodegenerative diseases. However, therapeutic efficiency of EXO and EXO-mimetics is limited by rapid clearance by the reticuloendothelial system. One way to circumvent this problem is to modify EXO surface. This can be achieved either by before isolation (pre-isolation strategies) or after isolation (post-isolation) strategies.

Pre-isolation strategy usually involves transfection of parental cells with an expression vector either to express protein inexistent in the cell or to modify naturally occurring protein. Expression of these modified or inexistent proteins in the cells modifies the EXO proteome as well creating EXO that can be traced in vivo [132], reach specific tissues or functionalize EXO for nanomedicine purposes [133].

For example, Lamp2b encoding plasmids have been constructed and transfected into dendritic cells. The EXOs harvested from transfected donor cells have been found to fuse strongly to the neuron-specific rabies viral glycoprotein (RVG) peptide through Lamp2b. These targeted EXOs can effectively deliver siRNA to the brain in a mouse model [134]. Similarly, using engineered EXO from immature dendritic cells in which Lamp2b has been fused with αv integrin-specific iRGD peptide (CRGDKGPDC) facilitated their targeting to tumor tissue [135]. Another example is the delivery of let-7a miRNA in a targeted manner to EGFR-overexpressing breast cancer mouse xenograft model allowing for the successful therapy of EGFR-expressing cancerous tissues with nucleic acid drugs [136].

Exosomal surface protein and lipid content, especially adhesion molecules and ligands, naturally target them to certain types of recipient cells. EXO natural targeting ability is based on the donor cell. For example, neuroblastoma-derived EXOs express on their surface glycosphingolipid glycan groups that bind amyloid-β aggregates, a driver of Alzheimer's disease pathology ameliorating their build-up [137]. Another direct use of pre-existing exosome surface components is to transmit membrane receptors and ligands to attenuate the function of autoreactive CD4 lymphocytes isolated from experimental autoimmune encephalitis (EAE) mice using mesenchymal stem cell (MSC) derived EXOs [138].

Post-isolation strategy involves modifying EXO or EXO mimetics after the isolation procedure. In that regard several methodologies have been employed such as click-chemistry, direct membrane fusion and pegylation. Click-chemistry, a popular tool for protein labeling, has found its way to directly attach molecules to the surface of exosomes via covalent bonds. Crosslinking of an alkyne chemical group and an azide chemical group is catalyzed with or without copper resulting in a triazole linkage formation [139]. This

novel technique can be used to functionalize the surfaces of EXO with small molecules, large bio-macromolecules, and polymers, and to monitor the exosome bio-distribution in vivo [139].

Glioma-targeting EXO were generated using the click chemistry technology [140]. In this study, EXO derived from Raw274.6 murine macrophage-like cells loaded with superparamagnetic iron oxide nanoparticles and curcumin, acting as anti-tumor agents, were conjugated using click-chemistry with neuropilin-1-targeted peptide (RGE-peptide). These modifications allowed EXO to cross the BBB and efficiently reach the tumor site exerting significant antitumoral effects. Importantly, the authors of this study also reported that the click chemistry method did not affect EXO properties and the modification itself was stable up to 4 weeks at −80 °C storage [140]. Using the copper-free approach EXO derived from MSC were modified with a peptide with high affinity to integrin αvβ3, a protein expressed in reactive endothelial cells after ischemia. Modified EXO were injected systemically in a transient middle cerebral artery occlusion (MCAO) mice model successfully crossing the BBB, targeting the lesion region of the ischemic brain and accumulating in it in a greater proportion than unmodified exosomes [141]. Additionally, when these modified EXO were loaded with curcumin or miR-201 they exert a strong suppression of the inflammatory response and cellular apoptosis in the lesion region, increased the angiogenesis in the ischemic zone and consequently animal survival [141, 142]

The direct membrane fusion between exosomes and synthetic liposomes is a useful methodology to modify exosomes membrane surface: using either freeze–thaw method or polyethylene glycol [143]. Direct membrane fusion allows the modification in EXO through the artificial functionalization modifying their type and proportions of phospholipids and protein content. However, despite the efficiency and chemical-free nature of these modifications, change in the EXO lipid composition affect the cellular uptake efficiency [109]. This technique has been successfully employed as means of introducing large plasmids such as CRISPR–Cas9 expression vectors in exosomes [144]. Noncovalent strategies have also been employed for stable EXO surface modification. Cationic lipids have been bound to the EXO surface using electrostatic modifications enhancing EXOs cellular uptake [145].

PEGylation is the coating of EXO with PEG. In this way the coated surface is protected from aggregation, opsonization and phagocytosis, prolonging the half-life in vivo [146]. Additionally, PEG can be used as a linker for to conjugation different molecules to the surface of EXO. Using this approach, nanobodies specific for the epidermal growth factor receptor (EGFR) were conjugated to phospholipid (DMPE)-PEG derivatives creating the nanobody-PEG-micelles. These micelles were mixed with EXO creating EXO with surface incorporated nanobodies without changes in their morphology, size distribution or protein composition. [147]. These modified EXO Exosomes successfully targeted EGFR + tumor cells in vitro, without affecting their bio-distribution pattern. In addition, this modification enhanced EXO circulation time in the bloodstream in comparison with unmodified EXO. In a similar approach, Si et al. developed an efficient surface tagging technique to generate monoclonal antibody (mAb)-EXO that targeted SSTR2-overexpressing neuroendocrine cancer cells [148]. mAb against somatostatin receptor 2 and EXO were both modified to display an 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-PEG (DSPE- PEG) 'arm', successfully linking them together without altering their physical and biochemical characteristics. These modified EXO were able to deliver histone deacetylase inhibitor romidepsin efficiently, inducing the cytotoxicity in cancer cells in vitro and in vivo [148].

9. TRACING EXO IN VITRO AND IN VIVO

In order to fully comprehend and track EXO movement and pharmacokinetic properties efficient labeling strategy must be introduced. The most challenging aspects of EXO labeling are derived from their relative small size and heterogeneity, making universal strategy for labeling hard to formulate [149]. Most commonly different types of lipophilic dyes, such as the PKH family, DiL-family, and FM-family, have been used by researchers in order to stain the lipid membrane of EXO [150-152]. This strategy was successful in labeling human bladder cancer cell derived EXO PKH-26, and tracing their uptake by bladder cancer cells [150].

Using nanoparticles to label EXO for theranostic approach is another promising and widely used method [153, 154]. For instance, hollow gold nanoparticles were loaded within EXO secreted by human placental mesenchymal stem cells by targeting EXO biogenesis pathway allowed for the release loaded EXO. This

labelling allowed to demonstrate the selective transfer of the secreted EXO only to the cell type of origin when studying different cell types including cancer, metastatic, stem or immunological cells [155].

Exosomes membrane anchoring is also a suitable surface modification technique that assists cellular uptake. It is dependent on surface lipids or peptide components of EXOs [156]. A recent report on development of a new surface modification system for EXOs that consists of an anchor, spacer, ligand, and doxorubicin has been published [157]. The author used a fluorescent lipophilic boron-dipyrromethene (BODIPY), as a cell anchor. PEG was used as a spacer to conjugate the BODIPY-anchors, increasing EXO half-life and preventing their aggregation at higher concentrations. Arg-Gly-Asp (RGD) peptide was used as a specific targeting ligand for integrin, which is overexpressed in melanoma, increasing cellular uptake, and specific target accumulation [157]

10. TRANSLATING EXO INTO CLINICAL PRACTICE

There is an extensive clinical effort to translate EXO into clinical application from many condition from cancer to inflammatory conditions and COVID-19. A large-scale production EXO-based formulation is critical for success of clinical translation. Recent publications have reported good manufacturing procedures for EXO from different sources [158-160]. Several companies and scientific institutions are involved in dealing with short-term safety of EXO formulations [161]. Cellular sources of the EXO used in these trials range from dendritic cells, reticulocytes, erythrocytes, monocytes, macrophages to mesenchymal and human induced pluripotent stem cells [162].

However, despite the great potential of EXO based therapy, there are still many obstacles to be overcome before they can be used in clinical practice. The technology of exosome production and quality control is flawed. There are still many pitfalls in large scale GMP EXO production and the consensus on the technical standards for exosome production and isolation are lacking [163]. Moreover, the storage and stability of EXO are also not clearly studied: freezing can lead to aggregation and increase in size, proteomic content can be affected and consequently biological function [164]. In addition, the long term safety of EXO based therapy is hard to predict as biological functions of EXO have not been fully understood yet [165].

11. FUTURE DIRECTIONS AND CONCLUDING REMARKS

Many features make EXOs very good drug carriers: low-immunogenicity, long half-life, good biocompatibility and stability in vivo. The practical application of EXOs is limited by the low purification yield, cargo loading inefficiency, and difficulty of quality control. Furthermore, there are risks associated with EXO use, such as immunosuppression and reversion to tumorigenesis [166, 167]. There is a constant search to find a cell source of clinically safe EXO therapeutics and using non-tumor cells in order to circumvent the risk associated with using tumor-derived EXOs. Artificial optimization can enhance the therapeutic potential of these vesicles [168, 169].

Many challenges lie in the clinical application of EXOs. First of all, a large-scale purification method of EXOs for clinical use is still missing. Cargo loading strategies, such as incubation, transfection, electroporation, and sonication further restrict utilization of exosome-based carriers in clinical practices. These physical treatments enhance loading efficiency but bring potential damage and contamination to exosomes and also require more exploration of experimental conditions to precisely control the formation of micropores or the process of membrane recombination. Incubation strategy is not efficient enough and is hard to control, hampering its potential application in clinical settings.

EXO-mimetics have not yet reached clinical translation. Current obstacles in the use of EXO mimetic include among other things, the establishment of regulatory protocols and mode of actions as well as clarification of safety aspects. Commercialization also requires scaling-up procedures and automatization of purification steps. Research into the improvement of the manufacturing methods of both approaches: semi- and fully synthetic artificial exosomes would require a multidisciplinary approach including the contribution of molecular biology, chemistry, and engineering.

Nevertheless, the concept of utilizing exosomes as delivery vehicles is enticing and promising. However, more systematic in vivo studies regarding the potency and toxicology of EXOs are needed for bringing them a step closer to clinical practice.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

This work was supported by the Ministry of Education, Science and Technological Development of Republic of Serbia Contract number: 451-03-9/2021-14/ 200168.

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Mechanism of separation	UC	UF	PEG	IA	MF	SEC
Specificity	intermediate	low	low	high	high	intermediate
Recovery	intermediate	high	high	intermediate	low	high
Purity	high	low	low	high	high	high
Sample Volume	intermediate	intermediate	low	intermediate	low	low
Time	high	high	intermediate	high	intermediate	low
Cost	low	intermediate	low	high	high	low
Efficiency	intermediate	intermediate	intermediate	intermediate	high	high
Functionality of EVs	yes	yes	yes	no	no	yes
Specialized equipment	yes	no	no	no	yes	no

Table 1. Comparison of different methods for EXO purification: Ultracentrifugation (UC), Ultrafiltration (UF), Poly-Ethylene Glycol -Based Precipitation (PEG), Immunoaffinity Capture (IA), Microfluidics-Based Isolation Techniques (MF), Size-exclusion chromatography (SEC). Relative scaling to: (low); (intermediate); (high); in the case when criteria was not applicable yes or no was used as an equivalence.

Method	Advantages	Drawbacks	Examples
Incubation with drug	East to operate Does not require special equipment Minimal destruction to carrier EXO	Low loading capacity Difficult to control Efficacy is under the influence of the system setup and physicochemical characteristic of the drug	Curcumin [88] Doxorubicin [170, 171] Brain-derived neurotrophic factors [90] Catalase [91]
Incubation of parental cells with drug	East to operate Does not require special equipment	Low loading capacity Difficult to control The amount of drug that diffuses into the cells and loads into EXO is unpredictable	Paclitaxel[91] Paclitaxel and doxorubicin[92] Curcumin [172] iron-oxide nanoparticles[96] Citrate-coated gold-nanoparticles modified with polyethylene glycol and folic acid[97]
Sonication	Increased loading capacity Effective method of loading different types of cargo	Disruption the EXO membrane integrity Attachment of drugs to the surface of EXO	Gemcitabine[99] Paclitaxel[100] Catalase[91] Gold-nanoparticles [101]
Extrusion	High loading efficacy	Rearrangement of EXO surface structure possibly changing their immune privileged status	Catalase[91] Porphyrin[92]
Electroporation	Loading large biomolecules	Disruption ox membrane integrity	siRNA[103] Small molecules [92] Doxorubicin [104] Paclitaxel [98] Nanomaterials [105]
Surfactants	High loading capacity	EXO degradation Hemolytically active *in vivo* need for further purification	Porphyrins[92] Catalase[91]
Freeze-thaw method	Simple method Effective for different types of drugs	EXO aggregation Low loading capacity Can lead to inactivation of membrane proteins	Fusion between EXO and liposomes [109]
Transfection	Efficient method for the loading of proteins or oligonucleotides	Need for commercial transfection reagents	miRNA[81] siRNA[110] syndecan-1[142] CD9 fused to human antigen R[144]

Table 2. Comparison of different EXO drug-loading methods

Fig. 1. ESCRT dependent and ESCRT independent EXO biogenesis pathways: Exosome formation begins with the formation of intraluminal vesicles (green) in multivesicular bodies (MVBs), the transport of MVBs to the plasma membrane (PM), their fusion with the PM, and the subsequent release of EXO into extracellular space. ESCRT (endosomal sorting complex recquired for transport)

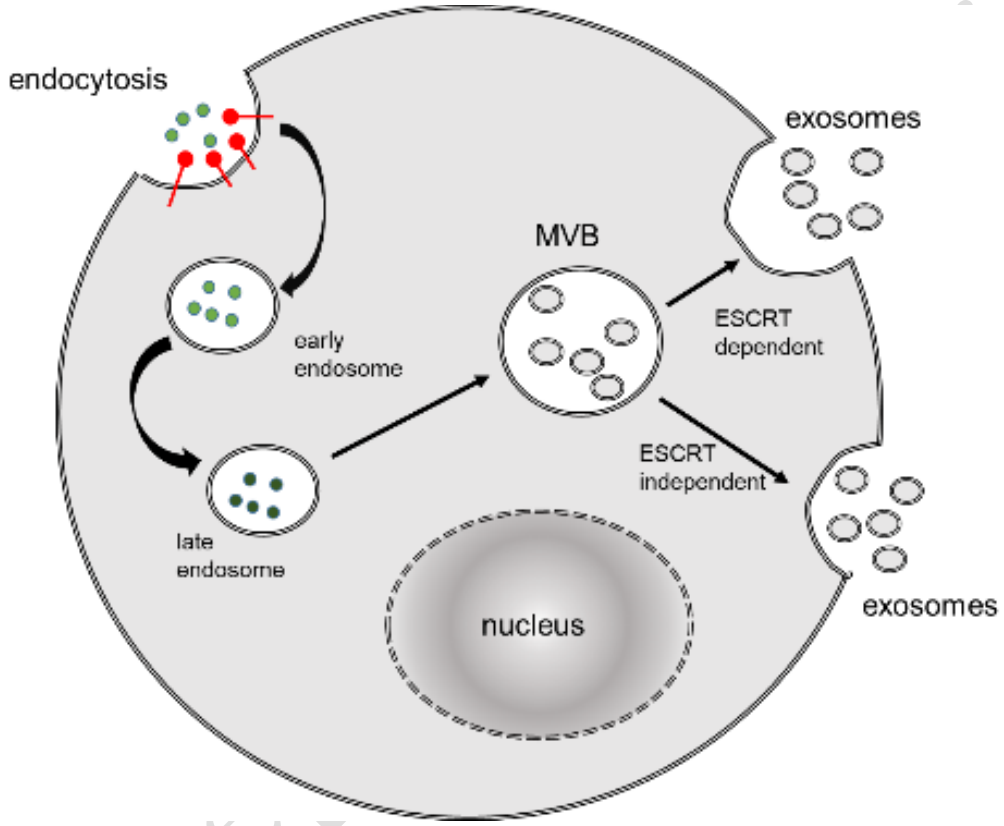

Fig. 2. Schematic representation of methodology for passive drug-cargo loading into EXO using: a) Incubation with donor cells; b) Direct incubation with EXO

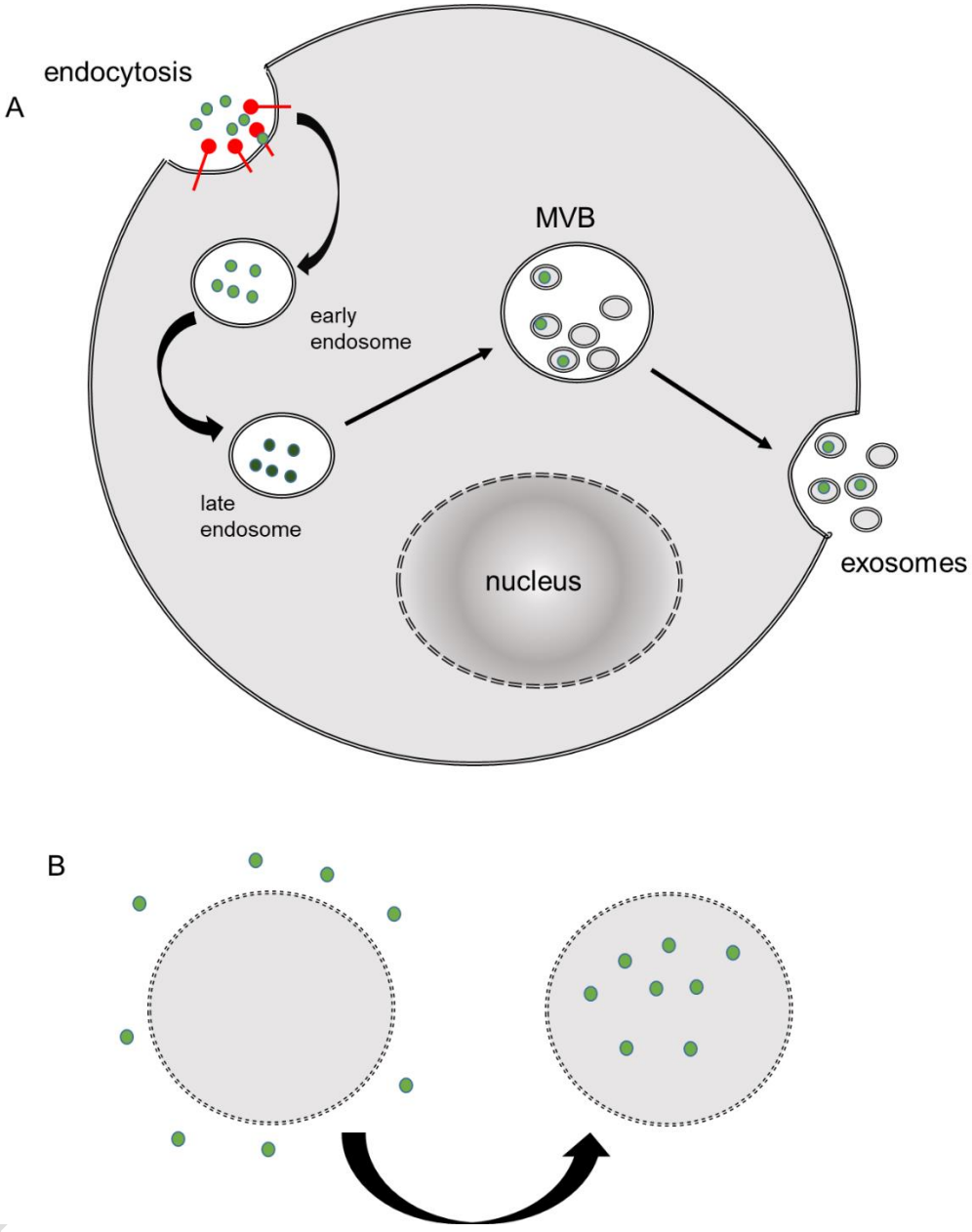

Fig. 3. Schematic representation of methodologies for active drug-cargo loading into EXOs: a) Sonication; b) Extrusion; c) Electroporation d) Surfactant method d) Freeze and thaw method

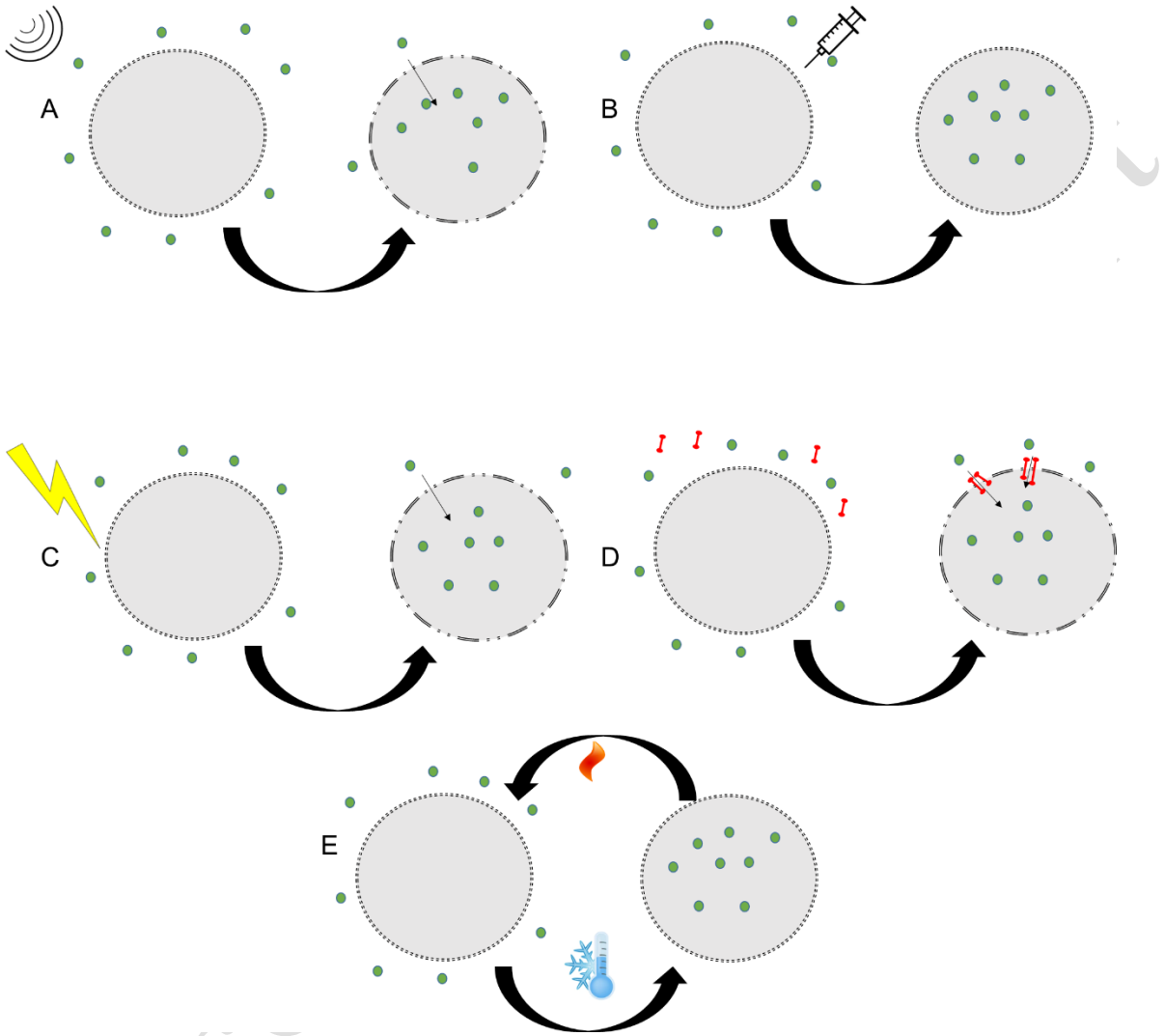

Fig. 4. Loading large biomolecules such as protein and RNA into EXO via a) Parental cell transfection and overexpession of desired molecules as part of EXO cargo or surface b) Direct transfection of target molecule into EXO