REVIEW

School of Pharmaceutical Sciences[1], Shandong University, Jinan; Marine College[2], Shandong University, Weihai, Shandong, China

Drug loading techniques for exosome-based drug delivery systems

Xiao-Ming Xi[2], Chen-Meng[2], Shu-Jun Xia[2], Rong Lu[1,2,*]

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**Corresponding author: Rong Lu, School of Pharmaceutical Sciences, Shandong University, Jinan, Shandong, 250012; Marine College, Shandong University, Weihai, Shandong, 264209, China*
lurong@sdu.edu.cn

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Exosomes are bilayer membrane-coated extracellular vesicles measuring between 40 and 100 nm in diameter. As a natural carrier, exosomes have the advantages of low immunogenicity, high stability in blood, and direct delivery of drugs to cells. Exosomes can be transported between cells and thus are conducive to the exchange of substances and information between cells. They change the functional state of recipient cells by loading exogenous drugs (e.g., small-molecule drugs, transmembrane proteins, and nucleic acid drugs). The key to using exosomes as drug carriers is the effective loading of exogenous drugs into exosomes; however, this task poses a challenge in studying the functionalization of exosomes as drug carriers. Currently, sonication, electroporation, transfection, incubation, extrusion, saponin-assisted loading, transgenesis, freeze-thaw cycles, thermal shock, pH gradient method, and hypotonic dialysis have been applied to load these drugs into exosomes. This review aims to provide an overview of the advantages and disadvantages of various drug loading technologies for exosomes.

1. Introduction

In the 1960s, Bonucci observed the physiological changes in articular cartilage and discovered secretory vesicles (Bonucci 1967). Trams et al. (1981) studied vesicle-related ATPases and nucleotide enzymes and defined exosomes as secretory vesicles with physiological functions. Mature early endosomes develop multiple indentations and shoots to form late endosomes with intraluminal vesicles (ILVs). Multivesicular bodies (MVBs) are endosomes rich in ILVs. Some MVBs integrate with the cell membrane and release ILVs known as exosomes into the extracellular environment (Fig. 1) (Hessvik and Llorente 2018). Exosomes are bilayer membrane-coated vesicles with a diameter of 40–100 nm (Huang et al. 2019); these vesicles are formed from the plasma membrane and a few buds on the membrane of late endosomes (Bai et al. 2019). In addition, they are loaded with cytoskeletal proteins, membrane receptors, mRNAs, miRNAs, and other biological carriers (Chen et al. 2020). Once released, exosomes participate in various physiological activities, such as development, immunity (Fan et al. 2020; Zheng et al. 2020), tissue homeostasis (Wu et al. 2020; Kalluri and LeBleu 2020), cancer, and neurodegenerative diseases (Candelario et al. 2020), in the extracellular space and play an important role in intercellular communication under physiological and pathological conditions (Zhou et al. 2020). Exosomes are heterogeneous (Brown et al. 2020). Each exosome generally carries specific proteins, lipids, RNA, and DNA (Ivica et al. 2020). These encapsulated molecules have different intracellular sources, but exosomes from the same cell may also contain different products (Yunusova et al. 2016). Exosomes fuse with the cell membrane of the receptor to transport drugs (Nicholson et al. 2020) in acidic environments (Logozzi et al. 2019). For example, exosomes are rich in the tetraspanin protein CD9 (Riquelme et al. 2020), which fuses with the cell membrane expressing the receptor to transport the target drug for therapy. Therefore, studies have started to explore whether exosomes represent an effective carrier for *in vivo* nucleic acid drug delivery.

Abbreviations
EVs=extracellular vesicles; PTX=paclitaxel; AMO-21=antisense miRNA oligonucleotides; mir-21=microRNA-21; T7-exo=T7 peptide-modified exosome; 5-fu=5-fuorouracil; miR-21i=microRNA-21 inhibitor; EGFR=epidermal growth factor receptor; EDTA= ethylene diamine tetraacetic acid; MPS=mononuclear phagocytic system; Cltc=clathrin heavy chain; AQP5=aquaporin-5; STAT3=signal transducer and activator of transcription-3; HGNs Hollow gold nanoparticles; hsiRNAs=hydrophobically modified small motor RNAs; AuNPs=-gold nanoparticles; PBS =phosphate buffered saline; MSCs=mesenchymal stem cells; %=per cent.

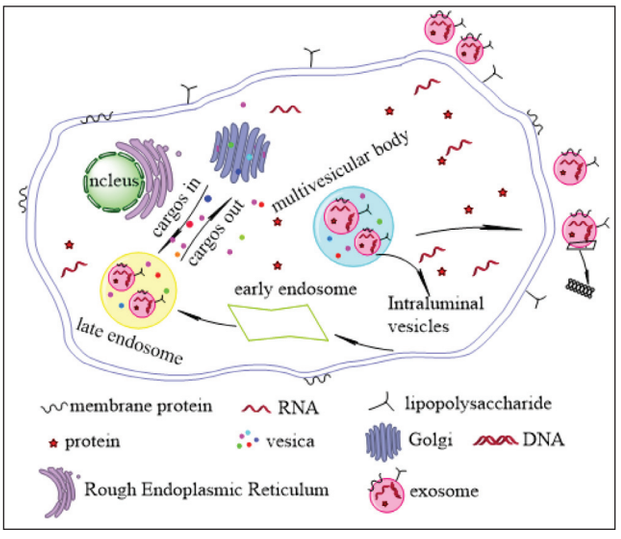

Fig. 1: Model of exosome biogenesis. After endocytosis, cells form early endosomes, and early endosomes form late endosomes in many pits of the membrane of early endosomes. Material exchange occurs between late endosomes and Golgi bodies. Multivesicular bodies (MVBs) are rich in intraluminal vesicles. Some MVBs bind to cell membranes and release ILVs into the extracellular environment. ILVs released into the extracellular environment are called exosomes.

As a natural carrier, exosomes target and protect cargo drugs and prolong the drug circulation time. Exosomes also mediate long-distance cell-cell communication. Given their high biocompatibility, small size, and low immunogenicity, exosomes are potentially useful as natural carriers of small-molecule drugs, transmembrane proteins, and nucleic acid drugs. Currently, the clinical application of exosomes as drug carriers still faces several challenges, such as methods to import drugs into exosomes efficiently. Exogenous drugs have been loaded into exosomes using various methods, including sonication, electroporation, transfection, incubation, extrusion, saponin-assisted loading, freeze-thaw cycles, thermal shock, pH gradient method, and hypotonic dialysis. However, these methods have their own drawbacks and advantages. This review aims to provide an overview of the advantages and disadvantages of various drug loading technologies for exosomes (Table).

blastoma multiforme cells was greater than the free PTX solution. The exosome delivery system also effectively bypassed the effect of P-glycoprotein exosome proteins and solved the multidrug resistance of tumor cells. In other experimental groups (Kim et al. 2016), PTX was loaded into exosomes *via* incubation, electroporation, and ultrasound. The macrophage-derived exosome delivery system significantly improved the cytotoxicity and multidrug resistance of PTX.

The blood-brain barrier limits the effects of most drugs on neurological diseases (Page et al. 2018; Uddin et al. 2020); thus, nanoscale drugs have been developed over the years to target this structure (Khan et al. 2020). However, the efficacy of nanoscale drugs is not satisfactory because of their toxicity and ingestion by the mononuclear phagocytic system (MPS) *in vivo* (Jo et al. 2015). Haney et al. (2015) developed a novel exosome-based peroxidase

Table: Advantages and disadvantages of drug loading techniques for exosome-based drug delivery systems

Methods	Pros	Cons
Sonication	Drug loading and continuous drug release are highly efficient.	It causes exosomes to aggregate and affects the surface protein structure.
Electroporation	The method is simple to operate and has been widely applied to encapsulate siRNAs or miRNAs.	It may lead to RNA precipitation or exosome aggregation, thus reducing drug loading efficiency.
Transfection	It results in higher efficiency and molecular stability than other methods.	Transfection agents have certain toxicity and safety problems, and they may lead to changes in gene expression in donor cells producing exosomes; thus, the nucleic acid drugs and biological activities delivered by exosomes are affected.
Direct incubation	The packaging efficiency depends on the polarity of the drug.	The encapsulation rate is relatively low.
Indirect incubation	It is mainly used for small-molecule chemical drugs with low cytotoxicity.	The encapsulation rate is relatively low.
Extrusion	The drug loading efficiency of this method is high, and the size of exosomes obtained is uniform.	Researchers have not clearly determined whether the law of mechanical force will change the properties of the secreted exosome membrane (e.g., zeta potential and membrane protein structure, etc.).
Saponin-assisted loading	It is highly efficient.	Saponins are difficult to remove completely and may cause a continuous increase in exosome membrane permeability, cytotoxicity, and hemolysis.
Freeze–thaw cycle	It is simple to operate, uses mild conditions, and rarely destroys bioactive substances.	It can induce exosome aggregation, and the encapsulation rate is generally lower than ultrasound or extrusion.
Thermal shock	It does not affect the exosome morphology and may improve the immunogenicity of exosomes.	It affects the fluidity of exosome membranes and the stability of the cargo.
pH gradient method	The efficiency of this method is comparable to ultrasound or electroporation, and the stability of nucleic acid drugs is not affected.	It reduces or aggregates the total protein content in EVs.
Hypotonic dialysis	The loading efficiency may be remarkably improved.	It induces peak broadening and a shift in the size distribution.

2. Sonication

As shown in Fig. 2A, a homogenizer probe was used for ultrasonic treatment after the exosomes were mixed with drugs (Liu et al. 2019). In the initial stage of ultrasound treatment, the mechanical shear force damaged the integrity of the exosome membrane, and the drug entered the exosome during the membrane deformation stage. After ultrasound, the exosomes were incubated to restore the integrity of the membrane (Wang et al. 2019).

Salarpour et al. (2019) used ultrasound and an incubation to inject paclitaxel (PTX) into exosomes from the U-87 cell line for the treatment of glioblastoma multiforme. During sonication, the mixture was treated with ultrasound at a 20% amplitude for six cycles. The load rate of ultrasound was greater than the incubation (loading efficiency rates were 0.92% and 0.74% for sonication and incubation at 37 °C, respectively), and the exosomes were larger when the drug was loaded using ultrasound than when it was loaded using incubation. Based on the experimental data, the zeta potential of the PTX exosomes exerted a minimal effect on their stability, and the toxicity of the PTX-loaded exosomes toward glio-

delivery system for the treatment of Parkinson's disease. They loaded the drugs into exosomes using incubation, saponin-assisted loading, freeze-thaw cycling at room temperature, ultrasound, or extrusion. Ultrasound produced the highest efficiency, and exosomes were absorbed efficiently by target cells, while their sizes and shapes inevitably changed. The exosome delivery system not only protects and slowly releases peroxidase but also increases the rate of drug deposition in nerve cells. Ultrasound has the advantages of a high drug loading efficiency and continuous drug release. In contrast to electroporation, ultrasound does not cause nucleic acid aggregation and thus may lead to exosome aggregation and affect the surface protein structure; in addition, long-term ultrasound treatment may lead to nucleic acid degradation (Haney et al. 2019).

3. Electroporation

Electroporation forms small holes on the exosome membrane under the action of an electric field (Fig. 2B), thereby increasing membrane permeability (Pan et al. 2020). Drugs or nucleotides then

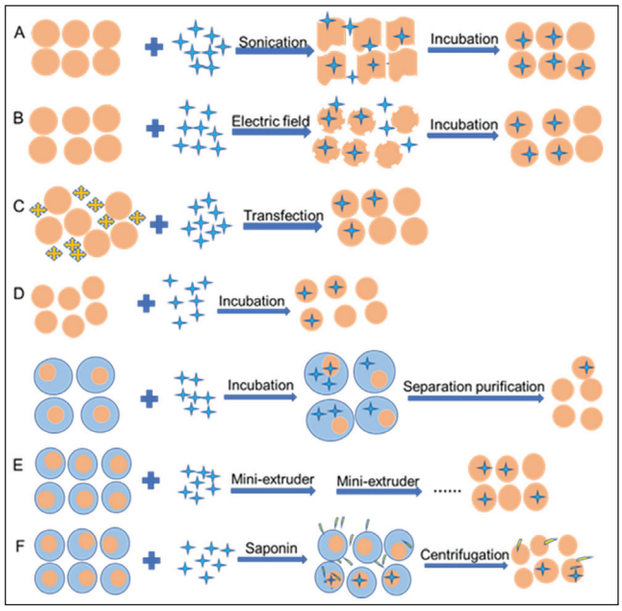

Fig. 2: Exosome loading methods. (A) During ultrasound, the exosome membrane deforms, the membrane permeability increases, and drugs enter the exosome. After incubation, the recovery of the exosome membrane is improved. (B) Electroporation technique: under the action of an electric field, a small hole is formed in the exosome membrane, which is conducive to the drug entering exosomes, and then the integrity of the exosome membrane is restored through an incubation. (C) The permeability of the membrane is improved by the transfection reagent and thus allows the drug to enter the vesicle. (D) Incubation methods are divided into direct and indirect types. Direct incubation is the direct incubation of the drug with exosomes. Indirect incubation is the process by which the drug is incubated with donor cells, and then the exosomes carrying the drug are isolated and purified. (E) After the drug is mixed with exosomes, extrusion is performed using a small extruder. After repeated extrusion steps, exosome membrane deformation is conducive to the entry of the drug, and exosomes enveloping the drug are obtained through automatic recovery of the membrane. (F) Saponin-mediated osmosis, an auxiliary method, can be used alone or in combination with ultrasonic electroporation. Saponins form complexes with vesicle membranes, leading to pore formation and drug entrance into exosomes.

enter the exosome through diffusion, and the exosome membrane quickly recovers its integrity after drug loading. In this method, large nucleotides, such as siRNAs or miRNAs, are preferred for loading into exosomes (Pomatto et al. 2019).

Kim et al. (2020) electroporated antisense miRNA oligonucleotides against miR-21 (AMO-21) into exosomes for the treatment of glioblastoma. The mixture of exosomes and AMO-21 was electroporated at 400 V in a 4 mm cuvette. Then, the unloaded AMO-21 was removed by supercentrifugation at 100,000 x g using the Beckman NVT 90 rotary cup for 1 h. Ultracentrifugation results showed a loading efficiency of 1.68±0.23 %. The fusion protein of Lamp2b on T7 and the exosome membrane was mixed into the exosome membrane to obtain T7 peptide-modified exosomes (T7-exos) and improve the targeting of the target carrier. T7 is the overexpression of the transferrin receptor on the surface of glioblastoma cells. T7-exos display a high brain targeting levels, cross the blood–brain barrier, bind to transferrin on tumor cells and reduce the tumor size. A major challenge in the use of 5-fluorouracil (5-FU) as a treatment for colorectal carcinoma is drug resistance; nevertheless, multicomponent administration effectively improves the treatment rate (Xu et al. 2020). Liang et al. (2020) loaded a microRNA-21 (miR-21) inhibitor and chemotherapy drugs into exosomes *via* electroporation. They measured the optimal parameters for electroporation, and a time constant of 10 ms and a voltage of 1000 V result in the highest loading efficiency. Under these conditions, high-performance liquid chromatography and quantitative real-time PCR results showed that the respective loading capacities of 5-FU and the microRNA-21 inhibitor in exosomes were approximately 3.1% and 0.5%, respectively. After fusion with LAMP2, Her2 was expressed on the exosome surface to achieve cancer cell targeting. Her2 is a widely expressed membrane protein involved in tumor progression and inhibition. The HHERer2-lamp2 fusion protein promotes targeted uptake in colorectal cancer cells through epidermal growth factor receptor-mediated endocytosis. Compared with single drug therapy, this multicomponent targeted delivery system not only improves the sensitivity of colorectal cancer cells to the drug but also significantly improves the antitumor effect of the component drug. Importantly, the component drug significantly enhances the cytotoxicity of 5-FU-resistant colon cancer cells. Lamichhane et al (2015) studied the loading of DNA into exosomes *via* electroporation at two pulses of 400 V and 125 µF. They observed similar levels of nucleic acid accumulation caused by electroporation before and after the addition of ethylenediaminetetraacetic acid (EDTA). The efficiency of DNA loading *via* electroporation is related to the DNA size. The loading efficiency of linear DNA into extracellular vesicles (EVs) is higher than plasmids; however, the overall efficiency is lower. The parameters for transferring DNA into exosomes *via* electroporation have been explored. Exosomes are preferentially absorbed by the MPS in the liver and spleen through endocytosis. The function of exosomes in target organs, such as the myocardium, is limited (Koh et al. 2017). Clathrin heavy chain (CLTC)-dependent endocytosis shows high activity in the MPS, but the activity of caveolin-1 in the myocardium is much higher than CLTC; thus, exosome uptake mechanisms vary in different cell types (Guo et al. 2015). Wan et al. (2020) loaded an siRNA targeting CLTC into exosomes originating from HEK293T cells *via* electroporation to block the endocytosis mediated by the MPS in the spleen and liver; consequently, the distribution of miR-21-based exosomes in other target organs was significantly improved. The exosomes were electroporated at 70 V/150 mF in an electroporation pool with a diameter of 0.4 cm. The team also found that the inhibition of CLTC significantly blocks the endocytosis of exosomes by the MPS upon the injection of miR-21-loaded exosomes. Researchers have suggested further modification of exosomes with CD47 to maximize the likelihood of MPS escape and prevent the MPS from ingesting exosomes through other mechanisms.

Kooijmans et al. (2013) performed electroporation in 0.4 cm cuvettes at 400 V/125 µF; according to the results, 3.7% of the siRNA was loaded into the EV pellet. For the insoluble siRNA formed after electroporation, the researchers adopted the addition of EDTA or the use of citrate electrode buffer to reduce the formation of insoluble siRNA. The use of citrate was more effective than the addition of EDTA at reducing the precipitation of siRNA. Electroporation theoretically induces strong siRNA aggregation and a high level of siRNA encapsulation. Therefore, the main cause of precipitation is derived from the metal ions derived from the electrode and the formation of hydroxyl compounds in the buffer. However, the removal of one or all of the influencing factors may decrease the encapsulation rate. A high electric field intensity may increase the accumulation of exosomes, but the appropriate concentration of trehalose can alleviate this situation (Hood et al. 2014). In addition, the accumulation of nucleic acids has been reduced by lipid complexation and increases the drug loading rate (Sen et al. 2002).

In summary, electroporation is easy to perform and has been widely used to encapsulate siRNA, microRNA or miRNA, but it may also lead to RNA precipitation or exosome aggregation, thereby reducing drug loading efficiency.

4. Transfection

Transfection is a method of loading drugs into exosomes with transfection agents (Lu et al. 2019) (Fig. 2C). Efficiency and molecular stability of this method are higher than with other methods, but the efficiency is not fixed (Zhang et al. 2017). However, the application of transfection methods has been limited because of the toxicity and safety concerns of transfection agents (Momen-Heravi et al. 2014). Transfection agents may alter gene expression in exosomes produced from donor cells, thereby affecting the nucleic acid drugs and biological activities carried by exosomes (Ramanathan et al. 2019).

According to Kanada et al. (2015), plasmid DNA that is integrated into EVs *via* transfection using Lipofectamine 2000 targets recipient cells *via* exosomes and microvesicles. However, only DNA associated with microvesicles results in the expression of functional proteins. Furthermore, the parameters and therapeutic potential of EVs for DNA delivery are almost completely uncertain. Choi et al. (2019) found that the exosomes derived from preosteoblasts under growth conditions are enriched in let-7 miRNAs, which regulate osteogenic differentiation upon the transfection of exosomes with miRNA inhibitors. Genetically modified exosomes have been used as developmental regulatory biomaterials, which regulate bone formation and improve diseases, such as neurological and infectious diseases and cancer, by regulating the expression of endogenous miRNAs. Aquaporin-5 (AQP5) plays an important role in the migration of breast cancer cells (Zhu et al. 2018). Park et al. (2020) isolated the exosomes containing the interleukin-4 receptor binding peptide from HEK293T cells through the transfection and modification of exosomes. The effects of exosomes loaded with miR-1226-3p, miR-19a-3p, and miR-19b-3p on AQP5 protein abundance and breast cancer cell migration were determined. AQP5-targeting miRNAs effectively reduce the migration of low-density lipoprotein-induced cancer cells and the abundance of AQP5 proteins. Exosomes that express tumor-targeting peptides efficiently deliver miRNAs into cancer cells. However, the mechanism of miRNA-mediated AQP5 targeting remains to be clarified. High expression of signal transducer and activator of transcription-3 (STAT3) causes downregulation of miR-320d expression in myocardial cells in atrial fibrillation. Liu et al. (2019) obtained exosomes loaded with miR-320d mimics after the transfection of adipose mesenchymal stem cells (MSCs). The target gene of miR-320d was STAT3, and the constructed exosomes were cocultured with cardiomyocytes in atrial fibrillation.

5. Incubation

Two types of incubation methods have been developed. The first (Fig. 2D) is the direct incubation of drugs and exosomes. The efficiency of this method depends on drug hydrophobicity, which allows the drug to interact with the lipid layer of the exosome membrane (Sun et al. 2010; Li et al. 2016; Yang et al. 2018). The second type is the incubation of drugs with donor cells of exosomes and the subsequent isolation of drug-carrying exosomes, which are mainly used for small-molecule chemical drugs with low cytotoxicity (Lv et al. 2012; Torreggiani et al. 2016). The use of either method results in a relatively low loading efficiency (Pomatto et al. 2019).

Hollow gold nanoparticles (HGNs) play an important role in the labeling of living cells, imaging, and diagnosis (Choi et al. 2020). Sancho-Albero et al. (2019) used electroporation, passive loading by diffusion, sonication, and saponin-assisted loading to encapsulate HGNs in exosomes, but did not obtain a satisfactory result; the loading rate was approximately 15%. These methods considerably affected the morphology and integrity of exosomes. Moreover, drug-carrying exosomes were obtained *via* exosome generation, and murine melanoma cells were co-incubated with PEGylated HGNs. Heterozygous exosomes were isolated and purified with a loading rate of approximately 50%. Therefore, the unique characteristics of exosome delivery carriers and theranostic nanoparticles as a signaling or therapeutic agent have been utilized well. HGNs in exosomes are the key to the realization of near-infrared "surface plasmon resonance," which may be used for hyperthermia *in vivo* (Qiu et al. 2019). Linezolid has been used to treat intractable staphylococcal infections (Cabrera et al. 2020). However, *Staphylococcus aureus* can escape from endosomes/phagocytes (de Vor et al. 2020) and multiply in the cytoplasm (Trafny 2012), which complicates the treatment of staphylococcal infections. Based on these problems, Yang et al. (2018) developed a new countermeasure by directly mixing exosomes from RAW264.7 cells with a linezolid solution (dimethyl sulfoxide) and incubating the mixture for 1 h at 37 °C while maintaining a final solvent concentration <10% (v/v). The selected dimethyl sulfoxide solvent was also used on the premise that exosome quality was not affected after the experiment. The drug-loading rate was 5.06±0.45%. Exosomes that carry drugs were used to treat infections of macrophages by methicillin-resistant *S. aureus*. The results showed that exosomes are effective carriers of therapeutic antibiotics and effectively kill the bacteria hidden in macrophages. Because the exosome yield is low, Jang et al. (2014) continuously extruded mononuclear cells or macrophages in the presence of adriamycin and then gradually reduced the filtration membrane aperture (10, 5, and 1 μm). Drug-carrying vesicles with similar properties to exosomes were obtained, the yield of which was 100 times that of exosomes. Compared with heterozygous exosomes incubated with adriamycin and exosomes, these vesicles are useful as vehicles to effectively deliver chemotherapy drugs for the treatment of cancer. Nucleic acid drugs are degraded through the nuclear lysosomal pathway (Yi et al. 2020). Exosome-loaded nucleic acid drugs bypass this pathway and the efficacy of nucleic acid drugs is improved. However, no stable and extensible method is able to load RNA into exosomes. Nucleic acid electroporation, which has been developed in recent years, easily destroys the integrity of exosomes, is substantially affected by the environment and is difficult to scale up. In a study on the treatment of Huntington's disease, Didiot et al. (2016) developed a U87 glioblastoma cell line source coincubation method to obtain an exosome-based hydrophobically modified small motor RNA (hsiRNA) delivery system. The 3' end of the passenger strand binds to cholesterol on the cell membrane, mediating the binding of hsiRNAs to the exosome membrane or into the exosome body cavity. Notably, 10–50% of the hsiRNAs in the solution bind to the exosome. The authors estimated that exosomes carry 1000–3000 hsiRNAs. The coincubation of hsiRNAs with exosomes provides a reliable, efficient, and highly reproducible method for loading exosomes with chemically synthesized oligonucleotides. This method also provides new ideas for the delivery of other therapeutic oligonucleotides, such as microRNAs, antisense oligonucleotides, or antagomirs.

6. Extrusion

In this method, a mixture of exosomes and drugs is loaded into a lipid extruder with a porous membrane (aperture: 100–400 nm), and the drug is loaded. The drug loading efficiency of this method is high, and the size of exosomes obtained by this method is uniform; however, researchers have not clearly determined whether the mechanical force of this method changes the properties of the exosome membrane (e.g., zeta potential and membrane protein structure).

In recent years, gold nanoparticles (AuNPs) have played a vital role in medicine, particularly in therapy, imaging, drug delivery (Laksee et al. 2020; Shah et al. 2020), and the so-called "theranostic (combined therapy and diagnosis) system" (Demir et al. 2018); however, their application is limited because of the lack of specificity in distinguishing the fragments of target cells and other cells (Burygin et al. 2020; Zhu et al. 2020). Khongkow et al. (2019) obtained AuNP-wrapped exosome particles *via* continuous extrusion (Fig. 2E). Before extrusion, AuNPs and exosomes were co-incubated in phosphate-buffered saline at pH 7.4 for 30 min. Then, the particles were extruded through 400, 200, and 100 nm polyporous membranes using mini-extruders. The particles were passed through each porous membrane repeatedly 10 times to ensure a high loading rate. The team also concluded from the experimental results that the surface charge of the composite is determined by AuNP/exosome ratios, with an optimal ratio of 1:2. This delivery system has the advantage of low toxicity in brain therapy and provides prospects for the development of brain imaging methods. Exosome delivery systems may reduce the effects of drugs on normal cells. After continuous extrusion through filters with different apertures, Kalimuthu et al. (2018) mixed PTX with MSCs and obtained drug-loaded, synthetically personalized exosome mimetics. The concentration of PTX substantially alters the drug loading rate. When PTX was applied at a concentration of 50 μg/mL, the drug loading capacity reached a maximum of 76.1 ng/μg. This system not only effectively targets cancer cells but also reduces the cardiotoxicity and neurotoxicity associated with chemotherapy drugs. In a study of exogenous drug coatings, Fuhrmann et al. (2015) summarized the parameters of

the drug-loading method, which laid a foundation for future drug loading research. The hydrophobic porphyrin drug was studied using the extrusion method. The EVs and the drug were extruded at 42 °C using a syringe-based hand-held mini-extruder with a heated block. Drug-carrying vesicles were obtained using polyporous membranes with a 400 nm pore size (track etch membrane, Whatman) after each sample was extruded 31 times.

7. Saponin-assisted loading

Saponins are efficient membrane-penetrating agents that form complexes with membrane cholesterol (Orczyk and Wojciechowski 2015) (Fig. 2F) and produce holes/pores, which increase cell membrane permeability. This method is efficient (Cao et al. 2020). Sancho-Albero et al. (2019) loaded HGNs into exosomes *via* incubation using saponins as auxiliary agents. Saponins facilitate the opening of pores in exosome membranes and thus are advantageous to HGN encapsulation. The addition of saponins does not affect the morphology of exosomes and generally increases the encapsulation rate. However, the application of saponins *in vivo* is limited due to the hemolysis effect. Thus, exosomes should be purified after the incubation with saponins (Lin and Wang 2010).

As drug carriers, drug activity in exosomes is important; however, the production of EVs must be evaluated further. Richter et al. (2019) studied the stability of EVs (size, particle concentration, and form) and their activity during drug preservation. They aimed to develop a simple procedure to evaluate the effects of different storage conditions on EVs. The preservation of cargo activity was evaluated by encapsulating the cargo into EVs with the enzyme betaglucuronidase through a process mediated by saponin. First, saponin and betaglucuronidase phosphate-buffered saline (PBS) solutions were mixed by vortexing for 3 s. The exosomes were then incubated with the cargo for 10 min at room temperature. The mixture was terminated by gently knocking the wall of the tube and finally purified using size-exclusion chromatography. The experiments laid a foundation for researchers to identify the optimal storage conditions for EVs and thus promote the development of EVs for use in clinical applications. Oshchepkova et al. (2019) used natural EVs and two types of artificial mimics derived from primary human endometrial MSCs to load synthesized single-stranded oligonucleotides into exosomes *via* saponin infiltration, freeze-thaw cycling, and ultrasound. The loading capacity of oligonucleotides and the ability to transport the cargo to target cells under different conditions were compared to evaluate the yield of natural EVs and artificial imitations. The loading levels of the three methods were similar. In the saponin permeation step, nanovesicles were treated with a concentration of 0.02–0.2% saponins extracted from Quillaja bark for 30 min; the process was repeated 2–7 times, with samples shaken at 700 rpm at room temperature. Among the simulators and natural exosomes, cytochalasin-B-inducible nanovesicles were the most efficient at releasing oligonucleotides, showing high potential for clinical application.

8. Alternative exosome loading techniques

The treatment of acute pancreatitis mainly focuses on inhibiting inflammation and apoptosis. Wang et al. (2019) conducted an experiment in which Klotho was overexpressed in MSCs to explore the pathogenesis of acute pancreatitis and the molecular mechanism of apoptosis. Klotho is an antiaging gene that plays an important role in acute pancreatitis. The authors used pLVX-Puro lentiviral vector-produced constructs. Klotho was inserted into the plvx-puro vector at the EcoR I and BamH I sites and used to infect MSCs. MSCs were infected for 1 week. Then, the exosomes of MSCs were isolated to obtain a gene delivery system. Klotho-overexpressing MSC exosomes altered the expression of apoptosis-related genes, NF-κB translation, and NF-κB translocation.

The particle size of exosomes increases after drug loading via freeze-thaw cycles because of the accumulation of exosomes during cycling (Bosch et al. 2016), and the repeated freeze-thaw cycles may lead to the degradation of RNA in exosomes (Bosch et al. 2016). The freeze-thaw cycling method is simple to operate, uses mild conditions, does not easily destroy bioactive substances, and has potential for mass production. However, it may induce exosome accumulation, and the sealing rate is generally lower than ultrasound or extrusion methods. Exosome-based drug delivery systems not only maintain the integrity of drugs but also promote cell proliferation and differentiation (Lee et al. 2020). Recent studies have shown that exosomes have great potential in promoting cartilage proliferation (Tan et al. 2020). Cartilage is a poorly vascularized tissue composed of extracellular matrix and chondrocytes with a low proliferation capacity (Li et al. 2020). Cartilage damage is caused by lesions or damage and is treated primarily through surgery. Surgery only alleviates symptoms, but does not heal cartilage (Fehske et al. 2020). Lee et al. (2020) prepared exosomes-containing miR-140 using the freeze–thaw cycle method. The exosomes isolated from plasma were used as a drug delivery system to deliver the miR-140 gene, which was effectively applied to repair cartilage *in vivo*.

Sancho-Albero et al. (2019) used thermal shock and various methods to load HGNs into the body but did not obtain satisfactory results, probably because thermal shock affects the stability of the cargo and the mobility of the exosome membrane.

In an attempt to address this issue, Jeyaram et al. (2020) developed the pH gradient method to promote nucleic acid loading. The HEK293T-derived EVs were internally acidified by a pH gradient method to promote the loading of negatively charged cargo. The EVs were dehydrated using 70% ethanol, rehydrated in acidic citrate buffer (pH 2.5), and then dialyzed against 1X HEPES-buffered saline (pH 7) to replace the surrounding acidic environment, and a pH gradient was formed inside and outside the exosome membrane. This method has the same efficiency as ultrasound or electroporation, without affecting the stability of nucleic acid drugs. However, the total protein content in EVs is either reduced or aggregated. Experimental results showed that the optimal load parameter of the cargo is incubation at room temperature (22 °C) for 2 h at pH 2.5.

In addition, a hypotonic dialysis method has been developed for drug delivery systems based on exosomes. Fuhrmann et al. (2015) performed the hypotonic dialysis of drugs and exosomes in PBS using a dialysis membrane at room temperature. Then, the solution was stirred at room temperature for 4 h. The loading efficiency was dramatically increased by coincubation with 0.01% (w/v) saponin or by hypotonic dialysis, but not by extrusion. However, hypotonic dialysis induces peak broadening and a shift in the size distribution.

9. Conclusions and future perspectives

Exosomes in acidic environments are likely ingested by hypoxic cancer cells, and exosomes can be targeted to recipient cells by secreting molecules (Giusti et al. 2016; Li et al. 2020). The exosomal expression of the tetraspanin CD9 promotes rapid membrane fusion with target cells, thus facilitating drug delivery. CD47 interacts with regulatory proteins on the exosome surface to generate a signal (Ha et al. 2016; Sancho-Albero et al. 2019; Lu and Huang 2020) that prevents phagocytosis by the MPS and substantially reduces immunogenicity.

Exosomes are easily modified during biosynthesis because they are derived from cells. In addition, exosomes of human origin have low immunogenicity. Exosomes target receptors on cells, including fusion proteins, adhesion molecules, and integrins, through one or more cell-specific antigen modifications at the surface. In addition, the immunogenicity of exosomes can be artificially enhanced by genetic modification or fusion with specific antigens. In clinical applications, exosomes can be lyophilized and recombined to retain their morphology and other characteristics, thus laying a foundation for preparation and storage in clinical applications. Compared with all drug loading methods, electroporation and ultrasound are efficient but suitable for small-scale use in the laboratory. Their safety in industrial production is low and unsuitable for large-scale applications. Electroporation easily causes the degradation and loss of exosome nucleic acid content, whereas ultrasound readily affects the structure of surface proteins on the exosome membrane.

Methods such as thermal shock, hypotonic dialysis penetration, and extrusion have high equipment requirements and high costs. Some drug delivery methods require the addition of other substances, such as saponins and transfection reagents, which are highly toxic and affect the safety of drug use. Although the incubation method results in a relatively low drug load (7.40±0.37 ng/μg) (Salarpour et al. 2019), it is the simplest of all methods. Incubation does not destroy the stability of the exosome membrane and has the advantages of a low cost and high safety. In view of the low drug loading rate of the incubation approach, we can adopt appropriate methods, such as increasing the drug content and stirring during incubation. The incubation environment can also be optimized. For example, exosomes are stable in acidic environments (Ban et al. 2015). Therefore, the incubation method has broad prospects in clinical applications.

However, in the laboratory phase, visualizing and tracking exosome uptake by target cells are not technically challenging. Exosomes can be labeled with fluorescent dyes or antibodies and visualized or tracked under a confocal microscope or using flow cytometry. In terms of the purification and separation of exosomes, many techniques, including sequential filtration, antibody-assisted isolation, magnetic bead-based isolation, and membrane-based isolation, have been used. However, none of the methods meet the requirements of the large-scale separation and effective purification of exosomes. Exosomes were extracted from a wide range of raw plant materials, such as ginger, grapefruit, and tomato, and the cost of raw materials was low (Xiao et al. 2018). The yield of EVs from plant sources obtained using multistep differential centrifugation is relatively high. Statistical analyses show that 320–450 mg of EVs can be extracted from 100 g plant raw materials (Jingyao et al. 2014). This result is highly conducive to the large-scale production and application of plant-derived EVs, thus effectively addressing the clinical demand for exosomes. In addition, large quantities of exosomes are required for clinical use, and the visualization and tracking of uptake by target cells are technically challenging. Furthermore, the mechanism of exosome drug metabolism in vivo must be further studied, for which numerous samples are usually required for purification and labeling. However, purifying and separating large quantities of exosomes is difficult, and specificity is lacking. A method for preparing synthetic exosomes has been developed to solve this problem (Rayamajhi et al. 2019; Lu and Huang 2020). In the future, the purification, synthesis, stability, and loading of exosomes should be considered to achieve high-efficiency loading of exosomes. The use of antibiotic-loaded exosome platforms to treat intracellular infections has attracted extensive attention. Thus, the mechanism by which exosomes reach the target requires further study.

Exosomes, which are efficient, economical, and safe drug carriers, have been widely used for drug and gene delivery because of their advantages over traditional nanocarriers. An in-depth study of drug loading methods will substantially improve the therapeutic effect on diseases, but many problems remain to be solved. The current drug loading methods suffer from problems in terms of mass production and the drug loading rate. In the future, an industrial exosome drug loading industry may emerge. Therefore, researchers should explore other efficient drug loading schemes in the future.

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