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Review

Engineering exosomes as refined biological nanoplatforms for drug delivery

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Abstract

Exosomes, a subgroup of extracellular vesicles (EVs), have been recognized as important mediators of long distance intercellular communication and are involved in a diverse range of biological processes. Because of their ideal native structure and characteristics, exosomes are promising nanocarriers for clinical use. Exosomes are engineered at the cellular level under natural conditions, but successful exosome modification requires further exploration. The focus of this paper is to summarize passive and active loading approaches, as well as specific exosome modifications and examples of the delivery of therapeutic and imaging molecules. Examples of exosomes derived from a variety of biological origins are also provided. The biocompatible characteristics of exosomes, with suitable modifications, can increase the stability and efficacy of imaging probes and therapeutics while enhancing cellular uptake. Challenges in clinical translation of exosome-based platforms from different cell sources and the advantages of each are also reviewed and discussed.

Keywords: exosome; nanoparticles; drug delivery systems; imaging probes; therapeutics

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Introduction

In the past decade, nanoscale drug delivery systems (DDS) have attained considerable prominence[1]. Various nanobased drug formulations have been used to improve the therapeutic efficacy of chemical and biomolecular drugs[2–4]. However, clinical translation of these systems faces two distinct issues: cytotoxicity of materials, and rapid clearance by the reticuloendothelial system (RES) or the mononuclear phagocyte system (MPS)[5]. Consequently, only a small number of nanobased DDS have been approved by the FDA for use in humans[1, 6]. Although PEGylation can extend the circulation time of these nanoparticles, it can also hinder the interaction between the nanobased DDS and target cells, thus decreasing the drug biodistribution in diseased tissues[7–9]. Endogenous DDS, as compared with synthetic nanoformulations, have shown promising results in enhancing drug delivery and therapeutic efficacy because of their native biocompatibility in vivo[10–13].

Exosomes are nanosized (30–120 nm) membrane vesicles secreted by various types of cells[13]. Since the discovery that exosomes function as intercellular communication tools that transfer their cargo to recipient cells, they have garnered considerable research attention[14]. Exosomes originate in multivesicular bodies (MVBs) and are released into the extracellular environment after fusion with the plasma membrane[15]. They relay information by transferring their contents from donor cells to recipient cells (Figure 1), thus resulting in functional changes and/or differentiation of target cells[16]. Exosomes contain and protect a specific mRNAs, regulatory microRNAs, lipids and proteins[11, 12]. Thus, exosomes are important mediators that facilitate intercellular communication without direct cell-to-cell contact[17]. Several pioneering reports have shown the advantages of using exosomes as nanocarriers[12]. These advantages include their small size for penetration into deep tissues[12], slightly negative zeta potential for long circulation[18, 19], and deformable cytoskeleton, as well as their similarity to cell membranes[20]. In addition, some exosomes also exhibit an increased capacity to escape degradation or clearance by the immune system[11]. Overall, exosomes are ideal natural nanocarriers for clinical application because of their naturally biocompatible characteristics.

Exosomes are engineered at the cellular level under natural conditions, but the successful modification of exosomes requires further exploration[21]. As compared with the cellular level endogenous method, which relies on biological approaches, exogenous methods, after production in cell cul-

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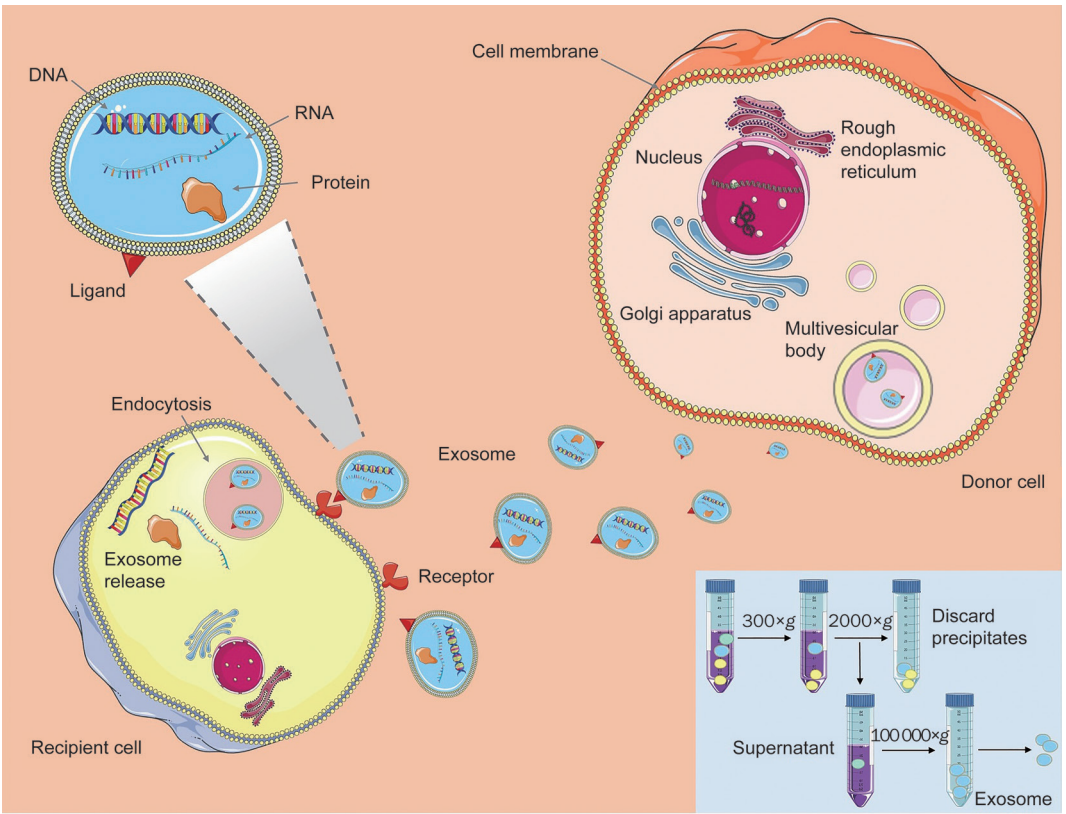

Figure 1. Exosome generation, secretion and cargo transfer from the donor cells to the recipient cells. Exosome are small membrane vesicles secreted by most cell types. Internal vesicles form by the inward budding of the cellular compartments known as multivesicular body (MVB). When MVB fuse with the plasma membrane, these internal vesicles are released as exosomes, which can transfer the DNA, RNA and proteins to the distant recipient cells, and influent various aspects of cell behavior and physiology. The inset shows a typical ultracentrifugation protocol. In consecutive rounds of centrifugation and pouring off, the RCF (g) and the centrifugation time are increased to pellet smaller particles. After first 200×*g* and 2000×*g* centrifugations, pellets that contain dead cells and cell debris are discarded, and the supernatant is kept for the next step. In contrast, after the 100 000×*g* centrifugations, pellets (containing EVs) are kept, and supernatants are discarded. The pellets are resuspended in phosphate buffered saline (PBS) for further analysis.

ture, make exosome application more reliable when exosome source cells are not amenable to customized modification[12]. In addition, exogenous methods expand the possible sources for exosomes, thereby greatly facilitating mass production. Successful post-isolation modification of exosomes relies on a complete understanding of their structural characteristics and underlying cellular biology and is necessary to facilitate future diagnostic and therapeutic interventions. The yield of exosomes and the physicochemical properties that affect their pharmacokinetics may vary with the type of donor cell[22]. Because these factors are expected to greatly influence the therapeutic efficacy of exosomes, it is necessary to select the appropriate type of donor cells for the development of exosome-based DDS[11, 12]. Here, we will mainly focus on different exogenous modification strategies for exosome-based therapeutic and diagnostic applications.

Appropriate source cells for engineered exosomes

Exosomes from a vast variety of cells have been investigated for use in clinical therapeutic applications[12, 22, 23]. Exploring different cell sources for therapeutic exosomes is of interest, because the lipid and surface protein composition of exosomes may be crucial to their function, and preservation of these characteristics is very important[24]. Additionally, surface markers, depending on the cell of origin, may have functions that jeopardize the intended effect of the exosomal therapy and even endanger the recipient. It is therefore crucial to carefully study and consider the biological characteristics of exosomes derived from different cell types and to weigh their benefits and drawbacks for therapeutic purposes.

A number of groups have investigated the use of tumor cell-derived exosomes for delivery of chemotherapeutic agents or other anticancer agents[12, 25]. There are appealing aspects to the use of tumor-derived exosomes for delivery of therapeutic agents and vaccines for immunotherapy. For example, tumor cells and tumor exosomes can be found in high numbers in malignant effusions, and it has been shown that tumor exosomes carry tumor-associated antigens specific to the tumors from which they are derived, as well as MHC class I molecules. Tumor exosomes can deliver antigens to

dendritic cells and consequently induce a T-cell-mediated immune response against tumor cells[26]. In fact, a phase I clinical trial has been completed on the release of tumor exosomes, which were presumed to bear tumor-specific antigens ready for presentation to immune cells and to stimulate the immune systems of glioma patients to clear remaining tumor cells after resection[27]. In addition, tumor targeting and selective drug delivery using tumor-derived exosomes has been proposed as a possibility because of their specific expression of tetraspanins, which preferentially interact with ligands in different tissues[28]. On the one hand, tumor-derived exosomes demonstrate specific targeting capabilities[25]; on the other hand, they have been shown to signal an increase in metastatic behavior in less metastatic cancer cell types[29] and to contain tetraspanins, which mediate tumor growth. Proteases, such as urokinase plasminogen activator, which promotes tumor cell invasion, and cathepsin D, and adhesion modulators, such as vimentin, galectin 3-binding protein, and annexin A1, have also been found in tumor-derived exosomes[30]. miRNAs and other nucleic acids, which can lead to malignant changes in target cells, have been found in tumor cell exosomes[31]. In addition, exosomes from melanoma patients have been shown to enhance the production of myeloid-derived suppressor cells (MDSCs), in an important mechanism by which tumors avoid immune recognition[32, 33]. Most importantly, tumor exosomes can inhibit tumor-reactive effector T cells through the expression of apoptosis-inducing ligands, such as FasL and TRAIL or PDL-2[34, 35]. Tumor exosomes exhibit potential drawbacks, including induction of apoptosis in activated cytotoxic T cells, impairment of monocyte differentiation, induction of myeloid-suppressive cells and T regulatory cells, suppression of lymphoid activation signaling molecules, and induction of a pro-inflammatory microenvironment[33]. The potential risk of tumor exosomes aggravating a patient's malignancy, instead of improving it, makes choosing the right exosomes crucial for therapy.

For the reasons mentioned above, exosomes isolated from fruits and plants have been explored as alternative options for clinical use because they come from reliable sources and have better safety profiles[36]. Exosomes derived from food have garnered attention, owing to the obvious conclusion that these exosomes are known to be commonly ingested and thus are generally considered safe[37]. In addition, agricultural products such as fruits and milk are relatively economically practical and scalable sources from which to isolate exosomes. A phase I clinical trial has been undertaken for the use of exosomes derived from fruit to deliver curcumin to the colon for the treatment of colon cancer[38]. Other groups are also isolating exosomes from plants for use in therapeutic applications. For example, Ju *et al* have isolated exosome-like nanoparticles from grapes and have found that oral administration of these vesicles induces growth and differentiation of intestinal stem cells and protects mice from intestinal damage initiated by dextran sulfate sodium[36]. Another group, led by Wang *et al*, has modified exosomes from grapefruit to improve their tumor targeting capability and loaded them with the anticancer agents doxorubicin and curcumin. These modified exosomes have been found to target inflammatory tumors and to have efficacy against inflammation in a mouse model[39]. Another agricultural source of reliable, scalable, and safe exosomes for therapeutic delivery is bovine milk. Munagala *et al* have isolated exosomes naturally present in milk and loaded them with different therapeutic cargos, including both hydrophilic and hydrophobic small molecules and chemotherapeutic agents. They then tested these drug-loaded exosomes on lung tumor cells in culture and in xenograft models and have found enhanced biological efficacy of exosome-encapsulated formulations over free drug, especially when the tumor targeting ligand folic acid is added to the exosomes[37]. Regardless of the high yield and superior safety profiles of exosomes isolated from food, these exosomes are incapable of boosting host immune systems and lack immunotherapy benefits.

Immune cell-derived exosomes, as a consequence, are receiving a great deal of attention regarding drug delivery and therapeutic vaccine applications[40–42]. Monocytes and macrophages in particular have been investigated as sources of exosomes for immunotherapy[43]. These exosomes have been shown to be especially good at evading immune phagocytosis, a clearing mechanism that is a drawback associated with other exosome types, thus allowing these exosomes to circulate longer and therefore extend their efficacy[44]. However, the future looks brightest for dendritic cell (DC)-derived exosomes for vaccine delivery, because they have been proven safe in multiple phase I trials in different types of cancers[40]. Phase II clinical efficacy studies have recently been completed using these exosomes loaded with tumor antigen as a vaccine against non-small cell lung cancer in combination with cyclophosphamide[45]. It has been shown that DC-derived exosomes can facilitate tumor rejection *in vivo* by transferring peptide-MHC complexes from DCs that have been exposed to an antigen to other DCs that have not been in contact with the same antigen[41–43, 46].

Engineering methods for encapsulation of therapeutic agents and imaging probes

Exosomes are composed of a lipid membrane bilayer structure that expresses the surface ligands and receptors from source cells[24]. The exosome lipid membrane surrounds and contains a hydrophilic core[24]. A thorough understanding of exosome structure is useful for manipulating the function and cargo packaging of modified exosomes. Therapeutic agents are incorporated into exosomes or exosome mimetics using two major approaches: (I) active or (II) passive encapsulation. These approaches result in different loading efficiencies and stabilities of the drugs in the exosome vesicles.

Passive cargo-loading methods

These methods are relatively simple and do not require the addition of active substances into the system.

Incubation with exosomes

Exosomes are simply incubated with drugs, and the drugs dif-

fuse into the exosomes along the concentration gradient. The loading efficiency depends on the hydrophobicity of the drug molecules. Hydrophobic drugs can interact with the lipid layers of the vesicle membrane[47]. For instance, in one study, mouse lymphoma-derived exosomes have been incubated with curcumin in phosphate-buffered saline (PBS) at 22 °C for 5 min before purification by gradient centrifugation[47]. In another study, Haney *et al* have successfully loaded the enzyme catalase, a tetramer protein of 250 kDa, into exosomes extracted from RAW264.7 cells in PBS buffer at room temperature for 18 h[44]. The main drawback to this method may be its low loading capacity.

Incubation with donor cells

The donor cells are treated with a drug, and these cells then secrete exosomes loaded with the drug. Pascucci *et al* have treated SR4987 mesenchymal stroma cells with a low dose of paclitaxel for 24 h, then washed the cells and reseeded them in a new flask with fresh medium[48]. After 48 h of culture, the cell conditioned medium was collected, and exosomes were isolated. The paclitaxel-loaded exosomes from the treated cells had significant, strong anti-proliferative activities against CFPAC-1 human pancreatic cells *in vitro*, as compared with the exosomes from untreated cells. Interestingly, the exosomes from the untreated SR4987 cells also showed some anti-proliferative effect, possibly as a result of the presence of certain proteins or nucleic acids within the exosomes that can change the tumor microenvironment[48].

Active cargo-loading methods

Sonication

Exosomes from donor cells are mixed with drugs or proteins and subsequently sonicated by using a homogenizer probe. The mechanical shear force from the sonicator probe compromises the membrane integrity of the exosomes and allows the drug to diffuse into the exosomes during this membrane deformation. Kim *et al* have demonstrated that exosome membrane microviscosity significantly decreases after sonication[49]. Nevertheless, this membrane deformation process does not significantly affect the membrane-bound proteins or the lipid contents of the exosomes. The membrane integrity of the exosomes has been found to be restored within an hour when the exosomes are incubated at 37 °C[49]. However, in some cases, drugs are not only encapsulated inside the exosomes but also attached to the outer layer of the membrane; as a result, two phases of drug release are observed. The first burst release phase results from the release of the drug attached to the outer layer of the exosomes, and this is followed by the slow release of the drug encapsulated inside the exosome vesicles[49].

Extrusion

Exosomes from donor cells 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. During the extrusion, the exosome membrane is disrupted and vigorously mixed with the drug. Whether the harsh mechanical force used in this method changes the membrane properties, such as zeta potential, and membrane protein structures is still unclear. Fuhrmann *et al* have reported that loading exosomes extracted from MDA-MB231 breast cancer cells with porphyrin using the extrusion method alters the zeta potential of the original exosomes and causes cytotoxicity, whereas porphyrin-loaded exosomes prepared using other methods do not show significant cytotoxicity[50]. These observations might have resulted from the intensive extrusion process (the exosomes were extruded 31 times) transformed the vesicle constitution[50]. However, catalase loaded into RAW264.7 macrophage-derived exosomes by using 10 extrusions has been found to have no cytotoxicity and to demonstrate greater neuroprotective activity than that of the exosomes prepared by freeze/thaw or simple incubation methods[44]. Therefore, further detailed studies into this method are required.

Freeze and thaw cycles

In this procedure, drugs are incubated with exosomes at room temperature for a fixed amount of time, and then, the mixture is rapidly frozen at -80 °C or in liquid nitrogen and thawed at room temperature. This process is repeated for at least 3 cycles to ensure drug encapsulation[51]. However, the method can induce aggregation of the exosomes, thus resulting in a broad size distribution of the drug-loaded exosomes. The drug loading capacity of the freeze/thaw method is generally lower than that of the sonication or extrusion methods. Interestingly, this method can be used for membrane fusion between exosomes and liposomes and has been used to create exosome-mimetic particles, as reported by Sato *et al*[51], who have isolated exosomes from RAW264.7 macrophages and fused the exosomes with several types of phospholipid-based liposomes. The fused exosome-liposomes were characterized using a fluorescence resonance energy transfer (FRET) assay[51], and the number of freeze/thaw cycles has been found to affect the lipid dilution ratios, thus resulting in changes in the fluorescence intensity.

Electroporation

This technique creates small pores in the exosome membrane through application of an electrical field to exosomes suspended in a conductive solution. The electrical current disturbs the phospholipid bilayer of the exosomes, thus resulting in the formation of temporary pores. Drugs or nucleotides can subsequently diffuse into the interior of the exosomes via the pores. The integrity of the exosome membrane is then recovered after the drug loading process. This method is widely used for loading siRNA or miRNA into exosomes, because these nucleotides are relatively large and cannot diffuse into the exosome spontaneously, as do small hydrophobic molecules. Electroporation leads to superior loading of siRNA over chemical transfection[52]. However, electroporation may cause RNA aggregation and exosome instability, thereby resulting in a low loading capacity. Johnsen *et al* have reported that electroporation in an optimized buffer such as trehalose disaccharide can aid in maintaining the structural integrity and can

inhibit the aggregation of exosomes extracted from adipose-derived stem cells[53]. Electroporation not only enhances RNA loading into exosomes but also increases hydrophilic small molecule loading into exosomes, for example, 5,10,15,20-tetrakis (1-methyl-4-pyridinio) porphyrin tetra (*p*-toluenesulfonate) or TMP, which is used for photodynamic effects[50].

Incubation with membrane permeabilizers

Saponin is a surfactant molecule that can form complexes with cholesterol in cell membranes and generate pores, thus leading to an increase in membrane permeabilization[54]. Saponin enhances the loading capacity of catalase into exosomes, as compared with the simple incubation method. Although saponin is a surface-active agent, it does not degrade the catalase, whose activity is preserved[44]. Saponin can also assist in loading hydrophilic molecules into exosomes. A study has shown that incubation of a small hydrophilic molecule with saponin increases drug loading into exosomes 11-fold compared with passive loading without saponin[50]. However, there are concerns regarding the *in vivo* hemolytic activity of saponin when this compound is used[54]. Therefore, the concentration of saponin used for drug loading should be limited, and the exosomes should be purified after incubation with saponin.

Click chemistry method for direct conjugation

Chemistry methods can also be used to directly attach molecules to the surfaces of exosomes via covalent bonds. Copper-catalyzed azide alkyne cycloaddition, known as click chemistry, is ideal for bioconjugation of small molecules and macromolecules to the surfaces of exosomes[11]. An alkyne chemical group and an azide chemical group react to form a triazole linkage. The reaction is rapid and efficient, as compared with traditional cross-linking reactions, such as maleimide-thiol coupling, and provides better control over the conjugation site. It is also suitable for modification of biological macromolecules, because it can occur in aqueous media[55]. Smyth *et al* have reported that an exosome cross-linked with alkyne groups using carbodiimide chemistry can be conjugated to a model azide, azide-fluor 545[56]. This conjugation has no effect on the size and internalization of the exosomes, thus suggesting that the reaction is mild and does not affect exosome structure or function.

Antibody against exosomal proteins

Recent studies have indicated that exosomes carry the genetic and proteomic contents of their parent cells. Fluorophores and microbeads conjugated to highly specific antibodies can bind a particular antigen on the cell surface. Higginbotham *et al* have demonstrated the feasibility of using fluorescence-activated vesicle sorting to analyze and sort individual exosomes isolated from DiFi cells[57]. EGFR and the exosomal marker CD9 has been used to conjugate Alexa-647 to the surfaces of exosomes for detection. Because exosomes carry the antigens of their parent cells, it is logical to assume that specific antigen-conjugated microbeads can be used for exosome isolation and tracking *in vivo*.

The various approaches used to load cargos into exosomes each have different loading capacities, which depend upon the properties of the cargo such as hydrophilicity, hydrophobicity, and molecular weight. Haney *et al* have reported that sonication and extrusion provide the highest catalase loading into exosomes, as compared with freeze/thaw cycles and passive incubation, which yield the lowest drug loading efficiency[44]. However, loading efficiency is not the only factor that must be considered. Exosome membrane integrity and stability are also important in drug delivery. As mentioned earlier, although catalase loading into exosomes using sonication and extrusion results in good enzymatic activity, other studies have found that membrane integrity is compromised, and the membrane-bound protein structure may change, thus affecting the therapeutic effect of the drug-loaded exosomes. Therefore, further investigation is necessary.

Engineering exosomes for delivery of therapeutic and diagnostic molecules

The previously described strategies for engineering exosomes further highlight the unique advantages of exosome-based nanoplatforms for cargo delivery. A series of good examples of these strategies that have been successfully applied for exosome-mediated delivery of functional molecules are presented in Table 1.

Delivery of small therapeutic molecules

Several small molecules, both hydrophobic and hydrophilic, have been incorporated into exosomes by using the different methods mentioned in the above section (Figure 2A, 2B). In most cases, exosomal delivery leads to higher drug accumulation in target cells and improved small molecule stability and blood circulation time, thus improving the potency of small molecule drugs and lowering the IC_{50}. Munagala *et al* have used exosomes isolated from bovine milk to encapsulate chemotherapeutic and chemopreventive agents, such as paclitaxel, doxorubicin, and withaferin, by passive loading[58]. These drugs are slowly released from the exosomes in a time-dependent manner. The researchers have demonstrated that withaferin-loaded exosomes and paclitaxel-loaded exosomes have a lower IC_{50} than those of the free drugs for anti-proliferative activities against A549 lung cancer cells. They have also confirmed the anti-tumor efficacy *in vivo* by using a tumor-bearing mouse model. Withaferin-loaded exosomes exhibit a significantly greater inhibitory effect on tumors compared with that of controls after intraperitoneal injection at a sub-optimal dose[58]. Sun *et al* have demonstrated that curcumin-loaded exosomes isolated from the EL-4 mouse lymphoma cell line show better curcumin stability *in vitro* and higher blood concentration *in vivo*[47]. As a result, the level of curcumin increases in the CD11b+Gr-1+ target cells, and significant suppression of inflammatory cytokines such as IL-6 and TNF-α has been achieved in a model of lipopolysaccharide-induced septic shock in immunocompetent mice[47]. Kim has reported that paclitaxel-loaded macrophage-derived exosomes, com-

Table 1. Examples of engineering exosomes for cargo delivery.

		Advantages	Disadvantages	Model drugs
I) Passive loading	a) Incubation of exosomes and free drugs	Simple Do not compromise membrane integrity	Low drug loading efficiency	Doxorubicin[60] Paclitaxel[48] Catalase[44] Paclitaxel[58]
	b) Incubation of the donor cells with free drugs	Simple Do not compromise membrane integrity	Low drug loading efficiency Drugs may cause cytotoxicity to the donor cells	
II) Active loading	a) Sonication	High drug loading efficiency	Compromise membrane integrity	Catalase[44]
	b) Extrusion	High drug loading efficiency	Compromise membrane integrity	Porphyrin[50]
	c) Freeze/thaw	Medium drug loading efficiency Liposome-exosome fusion	Aggregations	Porphyrin
	d) Electroporation	Loading with large molecules such as siRNA, miRNA	Aggregations	let-7a miRNA[66] MAPK1 siRNA[52]
	e) Incubation with saponin	Enhanced drug loading	Toxicity	Catalase[44] Porphyrin[50]
	f) Click chemistry	Quick and efficient Better control over the conjugation site		Azide-fluor 545 for *in vitro* tracking[56]
	g) Antibody binding	Specific and easy to operate		CD9 antibody with Alexa-647[57]

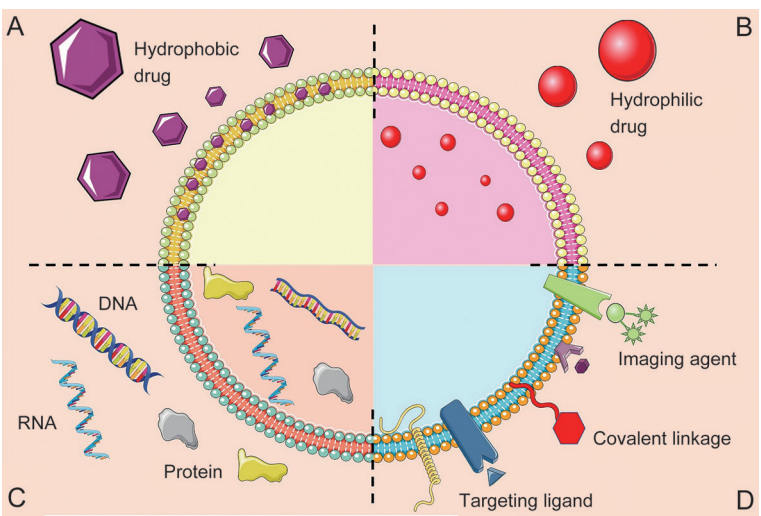

Figure 2. Schematic representation of the different types of exosomes drug delivery systems. Exosomes consist of a lipid bilayer that can be composed of lipids, which encloses an aqueous core. Both the lipid bilayer and the aqueous space can incorporate hydrophobic (A) or hydrophilic compounds (B), respectively. (C) Exosomes can be used for DNA, RNA and protein delivery. (D) Theranostic or imaging probes, specific targeting ligands and covalent linkage can be attached to exosome surface.

pared with paclitaxel-loaded liposomes, significantly increases cellular uptake in the 3LL-M227 murine Lewis lung carcinoma cell line[59]. Interestingly, paclitaxel-loaded exosomes have been found to overcome the drug resistance problem in MDCK MDR1 cells. The IC_{50}s of paclitaxel-loaded exosomes are significantly lower than those of free paclitaxel and Taxol[59]. Thus, exosome delivery can bypass the P-glycoprotein (P-gp) drug efflux system. However, the exosomes themselves do not inhibit P-gp, and the mechanism by which they overcome the drug resistance remains unclear.

Delivery of therapeutic RNA

RNA is a large molecule and is difficult to deliver *in vivo*. Several approaches have been explored, such as use of cationic liposomes[60, 61], dendrimers[62], and cationic polymer-based particles[63]. However, these carriers are not suitable for use in clinical practice, owing to safety, stability and off-target issues[64]. The most commonly used technique to incorporate

siRNA or miRNA into exosomes is electroporation, because this method destabilizes the vesicle membrane and allows siRNA or miRNA to penetrate the vesicles. However, aggregation of exosomes and RNA during electroporation has previously been reported, as discussed above. Alvarez-Erviti *et al* have utilized murine self-derived dendritic exosomes targeted with Lamp2b protein to deliver GADPH siRNA and BACE1 siRNA across the blood-brain barrier in mice. The data show that the housekeeping gene, GADPH, and BACE1-loaded exosomes strongly suppress mRNA expression and β-amyloid in the brains of wild-type mice after intravenous injection, as compared with Lipofectamine and siRNA-RVG-9R[65]. Wahlgren *et al* have demonstrated that siRNA loaded in exosomes isolated from human plasma successfully target monocytes and lymphocytes and subsequently silence the MAPK1 gene. The data also have shown that siRNA-loaded exosomes co-localize in the cytoplasm of recipient cells. However, only *in vitro* studies have been undertaken, in which the exosomes were co-cultured with the recipient cells; as a consequence, these recipient cells take up the exosomes more easily than they would in an *in vivo* situation[52]. Ohno *et al* have reported the delivery of GE11-targeted exosomes containing let-7a miRNA to epidermal growth factor (EGFR)-overexpressing breast cancer cells in a mouse xenograft model. GE11-targeted exosomes show higher tumor accumulation than do controls. Moreover, the exosomes isolated from donor cells previously transfected with let-7a miRNA inhibit tumor growth in mice[66]. These examples suggest that exosomes are a promising method for gene delivery with better safety profiles than those of viral vectors, cationic lipids, or polymer based particles.

Delivery of therapeutic proteins

Utilizing exosomes is one of the most promising methods for delivering macromolecular proteins (Figure 2C). Proteins can be loaded into exosomes through genetic engineering of the donor cells or through direct loaded into the exosomes. In the first method, donor cells are transfected with a plasmid carrying the gene of interest. Consequently, the cells synthesize the protein encoded by the inserted gene, and these proteins are subsequently secreted into the extracellular vesicles. At this stage, the extracellular vesicles can be isolated by collecting the cell culture supernatant and then purified. In the second method, proteins are directly loaded into exosomes as mentioned in the previous section. Catalase, a redox enzyme, loaded in exosomes extracted from macrophages has neuroprotective effects against oxidative stress in mice with acute brain inflammation. Catalase-loaded exosomes significantly decreases microgliosis and astrocytosis in the mouse brain and decreases rotation after apomorphine induction. These data suggest that exosomes are able to preserve catalase function and deliver catalase to the mouse brain after intranasal administration[44].

Delivery of imaging molecules

Currently, a number of reports demonstrating post-isolation strategies to modify exosome surface structures have described methods to more effectively track exosomes *in vitro* or *in vivo* (Figure 2D). Lai *et al* have engineered human embryonic kidney 293T exosomes expressing a membrane-bound Gaussian luciferase fused to a biotin receptor domain and then complexed the biotin expressed on the exosomes with fluorescent Alexa Fluor 680-streptavidin[67], thus allowing the exosomes to be tracked *in vivo* either by bioluminescence or fluorescence. In addition, Wilna *et al* have used fluorophore-conjugated antibodies against the exosomal proteins CD24 and aquaporin 2 (AQP2) to identify a subpopulation of CD24- and AQP2-positive exosomes by using nanoparticle tracking analysis (NTA) *in vitro*[68]. Wang *et al* have used anti-CD63 antibody-conjugated microbeads and secondary antibody-conjugated Q-dots to track endothelial cell exosomes by using NTA[69]. Exosomes are initially formed during the inward budding of late endosomes and are subsequently stored inside of multivesicular bodies (MVBs) before being released into the extracellular space. Thus, it is not surprising that some endosomal forming and sorting proteins (Rab5, Rab27 and Rab35), heat-shock proteins, and tetraspanins (CD9, CD63 and CD81) are enriched in exosomes. Because of this enrichment, and the presence of other donor cell-derived factors within exosomes, the selection of source cells has a great impact on the function and distribution of exosomes and must be considered carefully.

Engineering targeted molecules on exosomes

Because exosomes are extracellular vesicles secreted from cells, they intrinsically express some lipids and cell adhesion molecules and ligands that naturally target certain types of recipient cells. Several studies have shown that exosomes have natural targeting ability based on donor cells. For instance, exosomes isolated from neuroblastoma intrinsically express glycosphingolipid glycan groups that can bind to the aggregates of amyloid-β in the brain, and therefore may provide an effective treatment for Alzheimer's disease[11]. Targeting ligands on the surfaces of exosomes can also be engineered. The most commonly used technique is to insert the gene encoding the targeting proteins into the donor cells. The donor cells then secrete this protein in the exosomes. For example, plasmids encoding Lamp2b have been constructed and transfected into dendritic cells. The exosomes harvested after the donor cells have been transfected and found to fuse strongly to the neuron-specific rabies viral glycoprotein (RVG) peptide through Lamp2b on the exosomal membrane. The expression of Lamp2b on the exosomes has been confirmed by western blotting. These targeted exosomes can effectively deliver siRNA to the brain in a mouse model[65]. Another study has used exosomes to deliver let-7a miRNA in a targeted manner to EGFR-overexpressing breast cancer cells in mice. GE11 or EGF was cloned into a pDisplay vector and transfected into HEK299 cells. The data suggest that intravenous injection of the let-7a-loaded GE11-targeting exosomes can deliver the gene to the EGFR-expressing tumor in a mouse xenograft model[66].

Click chemistry is rapidly becoming a popular tool for mod-

ification of biomacromolecules. Smyth *et al* have reported a novel technique that can be used to functionalize the surfaces of exosomes with small molecules, large biomacromolecules and polymers and to monitor the exosome biodistribution *in vivo*[56]. It is expected that use of click chemistry may affect exosome biodistribution through conjugation of targeting moieties, such as RGD derivatives, with azide-terminated groups, such as those that have been used in other nanocarriers[56, 70]. In addition, some noncovalent strategies have been used to provide stable modification of the exosome surface. Amstrong *et al* and Nakase *et al* have used electrostatic interactions to bind cationic lipids on the surfaces of exosomes, which enhance the exosome uptake[71, 72]. In another study, Qi *et al* have successfully bound the surfaces of blood exosomes with transferrin-conjugated superparamagnetic nanoparticles targeting the native transferrin receptors present on the exosome membrane[73]. Despite these successes, achieving efficient exosome targeting via surface modification is nontrivial. The reaction conditions must be strictly limited to avoid exosome disruption and aggregation due to inappropriate temperature, pressure and osmotic stress[71].

Future directions and concluding remarks

The advent of nanotechnology for use in medicine has heralded a new chapter in drug delivery. Exosomes are promising for use as vectors for clinical application, owing to their strong biocompatibility. However, there are risks associated with use of exosomes as well, such as immunosuppression and reversion to tumorigenesis[74–76]. Therefore, the relentless search for safe and effective exosome-based nanoformulations has led to exogenous modification of exosomes as a viable path. Because exosomes can be derived from various types of cells, the issues associated with the use of tumor exosomes can be circumvented. It is highly probable that modified exosomes will be engineered for clinical use, the exosome source will be immune cells[43], and the exosomes will be artificially optimized by incorporation of specific payloads.

Despite the advances outlined in this article, there are still many challenges ahead. One challenge is achieving large-scale production of exosomes for clinical use[77]. It has previously been reported that large-scale manufacturing of therapeutic exosomes can be achieved via rapid purification[78]. However, this technique still requires further testing with different types of cells. Furthermore, the question of which cell type to use for exosome derivation still remains to be answered. Nevertheless, the concept of utilizing exosomes as delivery vehicles is attractive and promising. Hence, exosomes may be the answer to the ongoing search for a clinically suitable nanoplatform. More systematic *in vivo* studies regarding the potency and toxicology of exosomes are imperative for bringing this exciting development a step closer to clinical reality.

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