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Review

Drug delivery application of extracellular vesicles; insight into production, drug loading, targeting, and pharmacokinetics

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Abstract: Extracellular vesicles (EVs) are secreted from any types of cells and shuttle between donor cells and recipient cells. Since EVs deliver their cargos such as proteins, nucleic acids, and other molecules for intercellular communication, they are considered as novel mode of drug delivery vesicles. EVs possess advantages such as inherent targeting ability and non-toxicity over conventional nanocarriers. Much efforts have so far been made for the application of EVs as a drug delivery carrier, however, basic techniques, such as mass-scale production, drug loading, and engineering of EVs are still limited. In this review, we summarize following four points. First, recent progress on the production method for EVs is described. Second, current techniques of drug loading methods are summarized. Third, targeting approach to specifically deliver cargo molecules for diseased sites by engineered EVs is discussed. Lastly, strategies to control pharmacokinetics and improve biodistribution are discussed.

Keywords: drug delivery system; drug loading; exosome; extracellular vesicle; gene therapy; microvesicle; nucleic acid therapeutics; pharmacokinetics; targeting

Abbreviations:

BBB	blood brain barrier;	DDS	drug delivery system;
EE	encapsulation efficiency;	EPR	enhanced permeability and retention;
EV	extracellular vesicle;	LC	loading capacity;
miRNA	microRNA;	MPS	mononuclear phagocyte system;

MSC	mesenchymal stem cell;	NP	nanoparticle;
SEC	size exclusion chromatography;	siRNA	short interfering RNA;
PEG	polyethylene glycol		

1. Introduction

Drug delivery system (DDS) is one of the key technologies to achieve safe medication, since without DDS, drug molecules can easily diffuse throughout body and affect non-disease sites [1]. In the case of cytocidal drugs including anti-cancer drugs, side effects are very severe and often reduce the quality of life in patients. According to the need for avoiding the side effect of conventional small molecule drugs, nanostructured materials, so-called nanocarriers, have been used for delivering drugs to diseased site [2]. When the drugs are encapsulated in the nanocarriers and administered into body, they can remain in the body for longer period compared to those of drug molecule without nanocarriers. This is because nanocarriers over 5 nm can circumvent renal excretion [3], and encapsulated drugs can be protected from metabolism process. In addition, encapsulated drug can be released over time in a controlled manner when the nanocarriers are rationally designed. Most importantly, drugs can be targeted to specific sites in body by engineering nanoparticles (NPs) with targeting ligand that binds to targeting cells. Furthermore, enhanced permeability and retention (EPR) effect contribute to the accumulation of nanocarriers to tumor tissues and inflammation site [4,5]. In spite of these advantages of NPs, efficient drug delivery has never been fully achieved [6,7]. This is because of lack of truly specific molecular target for targeted drug delivery, inability for intracellular delivery, and low bioavailability of synthetic NPs. Thus, entirely new approach or materials is needed urgently to overcome the difficulties of conventional NPs for efficient drug delivery.

Extracellular vesicles (EVs) are released by any type of cells and have several tens of nanometer to micrometer in diameter. The term of “EV” includes exosome, microvesicle, and other membranous vesicles [8,9]. Their discrimination is still problematic since the characteristics of these membranous vesicles overlaps each other. International Society for Extracellular Vesicles (ISEV) recommends to use “EV” that means any kinds of extracellular vesicles to eliminate confusion. Throughout the all types of EVs they have common features; EVs are composed of closed lipid bilayer as shell with integral membrane proteins; in the inner space there are soluble proteins, nucleic acids, and other molecules (Figure 1). EVs are secreted by cells, however the origin of the EVs is varied; exosomes are from multivesicular bodies and secreted by exocytosis, while other type of EVs including microvesicles are considered to be released from plasma membrane [10,11]. In 2007, Valadi et al. found that EVs carry nucleic acids, mainly RNAs and it could be functionally delivered to recipient cells [12]. Since then, there is growing evidence that EVs deliver proteins and nucleic acids for intercellular communication in physiological conditions [13,14,15]. According to these facts, researchers had realized that EVs could deliver exogenous cargo molecules to cells of interest for therapeutic applications.

Since EVs have been expected to have tropism for specific organs or cells, targeted drug

delivery would be achieved by utilizing intrinsic mechanism of EVs. Furthermore, EVs are used in physiological condition for intercellular communication. Thus, EVs are potentially non-toxic as therapeutic use. These properties of EVs are advantageous over conventional synthetic nanocarriers. In this review, we review current progress on the application of EVs for DDS. Especially, we discuss about the key features of EVs for DDS application, such as production, drug loading method, targeting by engineering EVs, and improving pharmacokinetics of EVs (Figure 2).

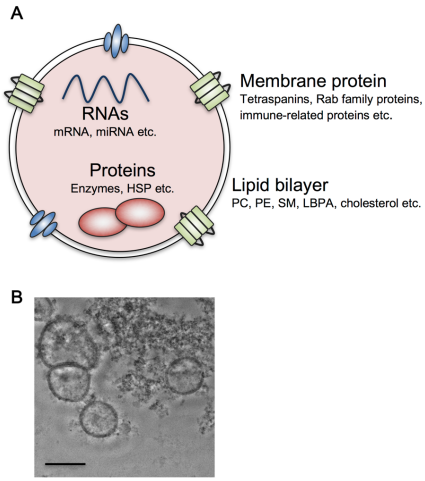

Figure 1. (A) Schematic representation of EV. EVs are composed of closed lipid bilayer, integrated membrane proteins, and encapsulated proteins and nucleic acids, mainly RNA. Abbreviations indicate; HSP, heat shock protein; LBPA, lysobisphosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin. (B) serum-derived EV from healthy donor observed by phase contrast-transmission electron microscope. Bar represents 100 nm.

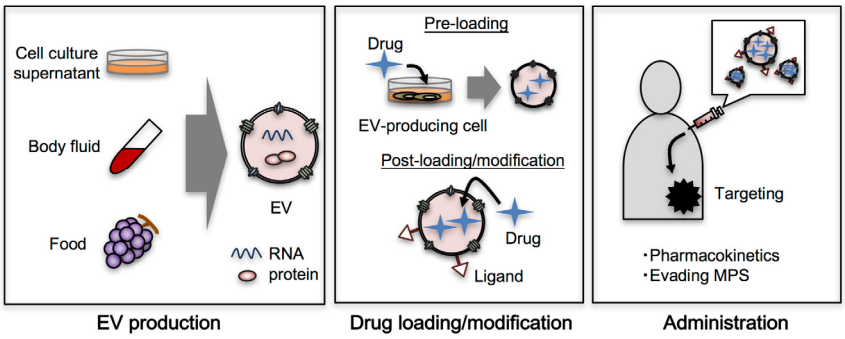

Figure 2. Workflow for the EV as DDS application. Left, EVs can be isolated from various raw materials such as cell culture supernatant, body fluid, and food. Middle, drug molecules can be encapsulated into EVs by introducing drug molecules into EV-producing cells (pre-loading method, upper) or directly introducing drug molecules into EVs (post-loading method, lower). Furthermore, targeting ligand can be conjugated on the surface of EVs. Right, efficient drug delivery to diseased site can be achieved by improving pharmacokinetics by evading mononuclear phagocyte system (MPS) and targeting specific organs or cells.

2. Production

Compared to conventional synthetic nanocarriers, EVs are composed of complex biomolecules including proteins, lipids, and nucleic acid, and totally manufactured by living cells. Reconstitution of EVs from chemically defined materials has never been accomplished. Therefore, large-scale production of EVs is exceedingly difficult compared with conventional synthetic nanocarriers. For the application of EVs as a DDS nanocarrier, EVs should be produced in mass-scale at reasonable cost. We summarized flowchart of mass-scale production of EVs in Figure 3. In this chapter, we discuss the ideal purification method and source to isolate large amounts of EVs.

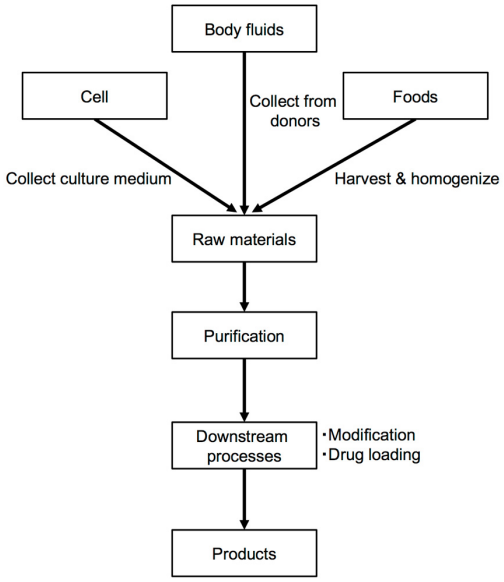

Figure 3. Flowcharts of the production of EVs. The sources of EVs are processed and collected as raw materials. After that, EVs are purified and concentrated, followed by the downstream processes such as modification or drug loading of EVs. As the pharmaceutical products, all the processes must be performed under good manufacturing practice.

2.1. Purification method for EVs

As well as the source of EVs, purification method is important to obtain substantial amounts of EVs. Various types of purification method have been reported, however, each method has pros and cons. Importantly, EVs should be manufactured in mass-scale, and highly purified and concentrated as pharmaceutical products. In this section, we summarize current progress on the purification method for EVs.

2.1.1. Ultracentrifugation

For the isolation and purification of EVs, ultracentrifugation is performed in the most of studies.

Under the high gravity of ultracentrifugation (over 100,000×*g*), EVs can be pelleted due to its sedimentation properties, while other components in raw materials are not. Based on this principle, EVs are purified and concentrated from raw material. For the laboratory-scale experiments, ultracentrifugation method is sufficient to obtain fairly pure EVs. However, in the clinical setting, ultracentrifugation is not suitable for mass-scalable production of EVs. Additionally, some report argued that high gravity during ultracentrifugation may affect the integrity of EVs. Yamashita et al. showed that EVs isolated by ultracentrifugal pelleting is likely to be aggregated [16]. According to these flaws, it is necessary to develop other purification method for mass-scale production of EVs.

2.1.2. Size exclusion chromatography

Size exclusion chromatography (SEC) is simple and feasible method to isolate EVs from raw materials. Due to its large size of EVs compared to non-EV components, even in the protein-rich and crude material such as plasma, SEC was shown to clearly separate EVs from non-EV proteins [17,18]. SEC can be adopted for mass-scale purification of EVs. Nevertheless, physicochemical properties of lipoproteins, which are nano-sized structure and composed of lipids and proteins, in the raw materials are similar to EVs, and can be contaminated in the EV fraction. Therefore, for the isolation of pure EVs, SEC should be combined with more specific purification method, such as affinity purification as described in the next section.

2.1.3. Affinity purification

Affinity purification is supposed to be the most promising approach to obtain EVs with high purity. Since EVs have typical proteins on their surface, such as tetraspanins, these EV marker proteins can be the target of affinity purification. Antibody-mediated purification is most reliable and selective for EV isolation. Microbeads conjugated with specific antibodies to EV markers can be used for the purification of EV. Several companies are providing antibody-immobilized microbeads for EV isolation. In addition, phosphatidylserine (PS)-binding protein Tim4 can be used for EV isolation. Since EVs contain PS abundantly in the lipid bilayer, Tim4-immobilized microbeads can capture EVs specifically [19]. However, these approach using antibodies and recombinant proteins, is difficult to use in mass-scale production due to the high cost of ligands. Furthermore, EVs must be mildly handled during purification processes, while affinity between proteins is sometimes excessively intense for dissociation in mild condition. Thus, these approach is not the best option for mass-scale isolation of EVs.

EVs have been reported to bind to heparin and this interaction is necessary for the cellular uptake of EVs [20]. By utilizing the affinity between heparin and EVs, heparin affinity chromatography was developed [21]. Heparin-immobilized sepharose beads was used and the EVs purified by this method contain less protein contaminants than those of ultracentrifugation method. Although heparin affinity chromatography is useful for EV isolation, in this report, the purification steps need up to three days. Taken together, there is still no perfect method for the purification of pure EVs, which is suitable for mass-scale production. Simple and scalable purification method must be developed for EV isolation to realize practical use of EVs in clinical setting.

2.2. Source of EVs

For the therapeutic application, EVs should be produced in mass-scale. Although cell culture-derived EVs have been mainly used, other types of EVs is investigated (summarized in Table 1). We focus on EVs from culture supernatant, body fluid, and food, and describe characteristics of these EVs.

Table 1. Source of EVs for DDS application.

Source		Yield (mg/kg-raw material)	Mass-scalability*	Notes	Reference
Culture supernatant	Various cells	0.5–2.0	-	Yield largely depends on cell type	[22]
	HEK293	~ 60	+	Bioreactor culture system	[23]
Body fluid	Plasma	N.D.	-	Possible to use autologous EVs	[24]
Food	Grape	1760	++	Clinical trials are now ongoing	[25]
	Grapefruits	2210	++		[25]
	Tomato	440	++		[25]
	Bovine milk	200–300	++		[22]
	Ginger	~ 50	++		[26]

*-, difficult; +, possible; ++, suitable for mass-scale production

2.2.1. Cell culture-derived EVs

In the field of EV research, most of the studies used cell culture supernatant. Since any type of cells secrete EVs, culture supernatant can be readily used for EV isolation. Usually, fetal bovine serum-free medium is used for culturing EV-producing cell since serum contains abundant serum-derived EVs and non-EV components, such as proteins, lipids, and other molecules, which are difficult to separate from EVs of interest. Among the variety of cell lines, HEK293 cells are frequently used for the production of engineered EVs due to the competency to overexpress exogenous gene. Watson et al. developed EV-producing bioreactor utilizing hollow-fiber system. Using the bioreactor, the yield of EVs from HEK293 was increased up to 10-folds compared to conventional two dimensional culture condition [23]. This kind of high-density culture system is essential to obtain cell culture-derived EVs for the therapeutic application of EVs. However, the yield of EVs from culture supernatant is still very low compared to that of conventional recombinant protein therapeutics (> 5 g/L of culture medium [27]).

2.2.2. Body fluid-derived EVs

Body fluid is alternative source for EV isolation, since many kinds of body fluid are enriched with EVs. EVs from body fluid are of interesting to perform autologous administration of EVs. If EVs were isolated from patient's body fluid, the EVs are recognized as self and might be completely safe for patient. Previously, plasma-derived EVs were shown to be used for siRNA delivery [24]. Plasma-derived EVs show similar surface protein markers and size to cell culture-derived EVs. However, limited amounts of raw material and the complexity of raw materials are problematic in this strategy.

In addition to plasma, other body fluids can be a source of EVs. For instance, urine was shown to be enriched with EVs [28]. Since approximately one litter of urine can be noninvasively obtained from individuals every day, urine might be more feasible source than blood for EV isolation.

2.2.3. EVs from foods

EVs are reported to be present in various foods including milk, vegetables, and fruits, and contain biomolecule cargos [26,29–33]. The physiological role of these EVs is largely unknown, however, it is speculated that food-derived EVs can deliver biological cargo molecules and contribute to interspecies communication between human and the origin of food-derived EVs. In fact, for instance, bovine milk-derived EVs was revealed to functionally deliver their cargo to human cells [30,34,35]. Since these food-derived EVs might be safe for human body and resistant to digestive juice, these vesicles can deliver drugs *via* oral administration. Based on these reports, food-derived EVs are attractive DDS.

Bovine milk-derived EVs might be a most promising source to obtain large amounts of EVs. Bovine milk is generally consumed around the world, thus bovine milk-derived EVs are considered as safe material. Munagala et al. reported that EVs from bovine milk can be applied as DDS to deliver anti-cancer drugs [22]. According to the report, yield of bovine milk-derived EVs is at least 100-folds higher than those of cell culture-derived EVs. Upon the oral administration of bovine-milk EVs, they showed no toxic effect in rats and mice.

Besides bovine milk, fruits are promising source of EVs. Grape-derived EVs was shown to be specifically taken up by intestinal stem cells upon oral administration and have therapeutic effect in colitis mice [36]. In addition, grapefruits-derived EVs have been used for various drugs [25,37–39].

According to these literatures, food-derived EVs might be capable of mass-scale production and safe DDS. However, it is carefully considered that these food-derived EVs are tolerable in human body upon systemic administration. It is estimated that oral administration of food-derived EVs is tolerable, while administration from other route, such as intravenous injection, can unexpectedly induce severe immunoreaction against the components of EVs. Especially, individuals who has food allergy cannot accept food-derived EVs. In addition to the tolerability in human, quality control is essential for manufacturing food-derived EVs with constant characteristics as pharmaceutical products. If the tolerability and quality control of food-derived EVs are guaranteed, foods might be an ideal source of EVs.

3. Drug Loading

For the delivery of therapeutic molecules by EVs, the cargos should be properly encapsulated in the EVs. When the endogenous cargos in EVs are utilized as the therapeutic molecules, loading method is somewhat easy; purify the cargo-containing EVs from EV-producing cells and apply to medication. This methodology, namely pre-loading method [9] is discussed in section 3.1. On the other hand, loading exogenous cargos into EVs after purification is more attractive approach, since the amounts of drugs in EVs can be controlled in this method, namely, post-loading method. This methodology is discussed in section 3.2.

As for the efficiency of drug loading method, we used two criteria in this review, such as encapsulation efficiency (EE) and loading capacity (LC), which are defined as equation (1) and (2), respectively.

$$\text{EE}\ (\%) = \frac{\text{Encapsulated molecules (mol)}}{\text{Input molecules (mol)}} \times 100 \quad (1)$$

$$\text{LC}\ (\%) = \frac{\text{Encapsulated molecules (wt)}}{\text{EVs (wt)}} \times 100 \quad (2)$$

EE represents the yields of drug molecules during encapsulation process, where LC represents the actual amounts of drug molecules in the EVs. For example, clinically used liposomal anti-cancer drug, such as DOXIL, showed ~ 95% of EE and around 10% of LC [40]. Ideally, drug molecules are encapsulated into EVs as well as conventional liposome.

3.1. Pre-loading methods

Pre-loading method is that drug molecules are encapsulated into EVs by natural sorting process, and when they are secreted by EV-producing cells, drugs are already inside the EVs. Once establishing the drug containing-EV-producing cells, it is unnecessary to encapsulate drugs into EVs after EV isolation. In this section, pre-loading technique to encapsulate therapeutic molecules into EVs are summarized.

3.1.1. Endogenous therapeutic molecules

Endogenous cargos, including proteins and miRNAs are functionally delivered by EVs to recipient cell. By utilizing this phenomenon, pre-loaded endogenous molecules in EVs could be a therapeutic molecule. Previously, we found that normal epithelial prostate PNT-2 cells suppress the growth of prostate cancer cell line PC-3M by transferring miRNA-143 *via* EV-mediated mechanism [41]. According to this result, endogenous anti-cancer miRNA could be a promising therapeutic molecule for cancer treatment.

Our group also reported that therapeutic effect of mesenchymal stem cell (MSC)-derived EVs. Enzymatically active neprilysin, β-amyloid peptide-degrading enzyme, is included in the EVs released from adipose tissue-derived MSCs. Furthermore, once the neprilysin-containing EVs are

taken up by neuroblastoma cells, the intracellular β-amyloid level was decreased [42]. This result suggested that endogenous proteins in EVs could functionally work and induce therapeutic effect.

Endogenous molecules in EVs are therapeutically valuable, however, the major problem of utilizing endogenous molecules is that the amount of cargo is uncontrollable. As for the nucleic acid, especially microRNA (miRNA), natural EVs contain less than one copy of miRNA within one EV [43]. As for the proteins, in our experiment, the amounts of active neprilysin in EVs was very few (equivalent to approximately 0.3 ng of recombinant neprilysin in 1 μg EVs as protein; *i.e.*, LC = 0.03%) [42]. Until now, sorting mechanism of cargo molecules into EVs has remained unknown. By hijacking or manipulating sorting mechanism of cells, endogenous therapeutic molecules can be efficiently loaded into EV.

3.1.2. Exogenous expression of therapeutic molecules

EV-producing cells should be engineered to obtain EVs containing substantial amounts of therapeutic molecules. One solution is to overexpress therapeutic molecules, for instance, miRNA, shRNA, and proteins, in EV-producing cells. In our previous experiment, shRNA-overexpressing cell secretes EVs containing substantial amounts of functional siRNA [44]. In a similar method, miRNA could be overexpressed in EV-producing cells and sorted into EVs.

To efficiently load specific miRNA into EVs, understanding the sorting mechanism is important. Currently, we and another research group proposed that certain types of proteins are responsible for miRNA sorting into EVs. Annexin A2 can bind to miRNAs in sequence-independent manner and sort broad range of miRNAs into EVs [45]. Similarly, Y-box protein 1, RNA-binding protein, was found to be necessary to sort miRNA-223 into EVs in cell-free system and cultured HEK293 cells [46]. These findings are important to load miRNA of interest into EVs. Additionally, there may be other RNA binding proteins that is responsible for miRNA sorting into EVs. It is anticipated that overexpression of these proteins in EV-producing cells could facilitate miRNA sorting into EVs.

As for the loading of therapeutic proteins, several group succeeded to load proteins of interest into EVs. One approach is to simply overexpress protein of interest in EV-producing cells. However, the sorting of therapeutic protein is difficult since specific proteins are usually sorted into EVs. In fact, it was shown that exogenously expressed proteins are rarely sorted into EVs [14,47].

Another approach is to fuse therapeutic protein with EV-marker or EV-binding proteins. Lactadherin (also called MFG-E8), the EV-binding protein [48], can be used as anchor for therapeutic proteins. Several reports showed that exogenous proteins could be sorted into EVs by fusing with lactadherin [49,50]. Similarly, tetraspanins such as CD9, CD63, and CD81 can be used for protein sorting into EVs [51].

Currently, novel protein-sorting system that utilizes light-responsive protein was reported [47]. In this method, namely, EXPLORs (exosomes for protein loading *via* optically reversible protein-protein interactions), cargo proteins can be efficiently loaded by blue light-induced interaction between CRY2 (cryptochrome 2) and CIBN (CRY-interacting basic-helix-loop-helix 1 with mutation). Using EXPLORs, it was estimated that 1.4 molecules of cargo proteins was encapsulated in one EV. This efficiency is so much higher than those of conventional protein loading method.

3.1.3. Direct transfection of exogenous molecules into EV-producing cells

Many reports support that nucleic acids-transfected cells secret EVs containing transfected nucleic acids [52]. Due to this feature, EVs can encapsulate therapeutic nucleic acids by directly transfecting EV-producing cells. Ohno et al. succeeded to encapsulate let-7a into EVs by transfecting HEK293 cells. After the transfection, HEK293 cells secreted let-7a-containing EVs [53]. Another research group unveiled that MSCs-derived EVs can include anti-miRNA-9 by transfection. Anti-miRNA-9 in EVs can be functionally delivered to cancer cells [54]. However, the amount of encapsulated materials in EVs is not controllable and sorting efficiency might be low. In addition, large part of transfected nucleic acids might be used in the EV-producing cells and rarely sorted into EVs.

3.2. Post-loading methods

Drug molecules can be encapsulated into EVs after EV isolation. This approach, namely, post-loading method seems to be more feasible than pre-loading method. This is because that EE and LC could be controllable in post-loading method. As mentioned above, EVs have closed lipid bilayer as shell (Figure 1). Due to this feature, after the EV isolation, it is unlikely that drug molecules can be spontaneously packed into EVs. Especially, hydrophilic molecules, including therapeutic nucleic acids and proteins, could not penetrate into the inner space of EVs. Therefore, EVs has to be processed to load drug molecules. In the following sections, we describe three different modalities for encapsulation of therapeutic molecules into EVs.

3.2.1. Electroporation

Gold standard for the encapsulation of exogenous materials into EVs is electroporation. The principal of encapsulation by electroporation is that the electric pulse induces pores of lipid bilayer temporally, and exogenous drugs can be transferred into the inner space of EVs. The first report on this method was described in 2011 [55]. siRNA can be encapsulated into EVs with optimized condition and EE was estimated to ~ 35%. Following this report, there have been several research that various drug molecules including siRNA [24,56], miRNA [57,58], anti-cancer drug paclitaxel [59] and doxorubicin [60,61], and therapeutic protein saporin [62] can be encapsulated in EVs.

Although electroporation has so far been used for encapsulation of exogenous drug molecules, the EE of electroporation is sometimes overestimated. For example, Ohno et al. reported that miRNA could not be encapsulated by electroporation [53]. Furthermore, another report revealed that electric pulse induce aggregation of siRNA [63]. Even after the optimization of electroporation conditions, the EE was estimated to less than 0.05%. From this observation, without any support, exogenous molecules might not be actively loaded into EVs. Therefore, development of other encapsulation method for EVs is an urgent need.

3.2.2. Chemical methods

Some companies supply chemical transfection reagent to load exogenous materials into EVs. In this method, conventional transfection reagents, such as cationic lipids are used. Nucleic acids are mixed with cationic lipids and form complex first, and then mixed with EVs. As a result, nucleic acids can be encapsulated into EVs. Several reports argued that chemical transfection works well and EVs loaded with siRNA or miRNA can deliver into cells *in vitro* [24,64,65]. However, it is nearly impossible to avoid the possibility that chemical transfection reagent is solely responsible for delivery of nucleic acids into cells, as evidenced by the fact that complex of nucleic acids and chemical transfection (without EVs) can functionally deliver them into cells [65]. From this point of view, chemical transfection method is not suitable for loading drug molecules into EVs.

3.2.3. Physical methods

Another option for the encapsulation is physical methods. Lipid bilayer prevents encapsulation of exogenous drug molecules into the inner space of EVs. Especially, hydrophilic molecules, such as nucleic acids and proteins, cannot diffuse through lipid bilayer of EVs. In other words, if lipid bilayer is destabilized, drug molecules can be loaded into EVs.

When EVs are treated with physical stress, such as sonication, freeze-thawing cycle, or making pore in lipid bilayer by detergent, drug molecules can be encapsulated into EVs. Fuhrmann et al. succeeded to encapsulate porphyrins into EVs by saponin treatment, which transiently make lipid bilayer of EV permeable [66]. Haney et al. compared these physical method to encapsulate catalase, a candidate therapeutic protein for Parkinson's disease, into EVs [67]. In this report, sonication was most efficient to encapsulate catalase into EVs (EE and LC were 26.1% and ~ 20%, respectively). Another report also succeeded to encapsulate anti-cancer drug paclitaxel into EVs by sonication (LC = 28.3%) [59]. According to these results, sonication is one of the most feasible methods to encapsulate therapeutic molecules into EVs. It is noted the EE and LC could be varied depends on the physicochemical properties of drug molecules (e.g. molecular size, hydrophobicity, stability against physical treatment, etc.).

As for the hydrophobic molecules, they could be inserted into hydrophobic lipid bilayer of EV by simply mixing with EVs. By the simple incubation at room temperature, rhodamine 123, paclitaxel, and doxorubicin were encapsulated into EVs with 0.79%, 0.73% and 13.2% of LC, respectively [68]. Similarly, curcumin, a hydrophobic anti-inflammatory drug, can be encapsulated into EVs by simple mixing [69,70]. Actinomycin D can be loaded into EVs by simple mixing [71].

Organic solvents, such as ethanol, promote the solubilization of hydrophobic drugs and encapsulation of drugs into EV. This method was applied to various drugs, including withaferin A, anthocyanidins, curcumin, paclitaxel, and docetaxel (EE ranging from 10 to 40%, depends on drugs) [22,72].

Taken together, various methods have so far been reported to encapsulate drugs into EVs. Since each drug has inherent properties, the encapsulation method should be carefully chosen in each drug.

4. Targeting

In addition to the drug loading, targeting is another crucial point for the efficient drug delivery. Efficient targeting contributes to reduce side effect of the drug and the dose of drug can be reduced to obtain therapeutic effect. In this chapter, we discuss the intrinsic specificity of EVs and engineering approach to endow EVs with targeting ability.

4.1. Inherent targeting ability of EVs

EVs have been considered to have tropism to specific cells. In 2015, Hoshino et al. reported that integrins on the cancer-derived EVs determine the tropism of cancer metastasis [73]. Similarly, various kinds of "shipping tag" are displayed on the surface of EVs and recognize specific molecules of recipient cells. Various types of these interaction is well-summarized in the previous review [74]. According to these facts, EVs may have intrinsic tropism. This ability is now anticipated to utilize for targeted drug delivery. By understanding the targeting mechanism of EVs at molecular level, it could be translated into conventional nanocarrier DDS.

Interestingly, several articles reported that EVs can be accumulated in brain across blood brain barrier (BBB) after systemic injection [67,68], although conventional NPs have been unable to pass through BBB. Previously, we reported that brain metastatic breast cancer cells secret miRNA-181c-containing EVs that can disrupt BBB [75]. According to these results, EVs may be a powerful DDS for delivering drugs into brain by systemic injection.

4.2. EV engineering for active targeting

In the context of targeting, the shipping tags on EV ideally bind to specific target molecules of target cells. For this purpose, various modification methods to display specific ligand on EV have been reported. Exosome display technology was reported in 2005 [50]. In this report, C1C2 domain of lactadherin was used for the conjugation of antibodies. Lamp2b is also used as an EV-targeting molecule and fused with RVG peptide and iRGD peptide for targeting neuronal cells [55] and tumor cells [61], respectively. Transmembrane domain of platelet-derived growth factor receptor can be used for peptide display on EVs for tumor targeting [53].

Post-conjugation method is of interesting for targeting specific cells. Using click chemistry, various ligand molecules can be easily modified on the surface of EVs [76]. Additionally, Nakase et al. succeeded to conjugate octaarginine (R8) peptide, typical cell-penetrating peptide, on the surface of EVs *via* stearyl group as anchor. R8-conjugated EVs are highly competent for delivering cargo molecules into cells [62].

Koppers-Lalic proposed that hybrid DDS of EVs and viruses is promising for future drug delivery application [77]. Other options for targeting specific cells include membrane modification. Sato et al. developed hybrid type of EVs containing synthetic cationic lipids by the fusion between EVs and cationic liposome [78]. By combining inflammation-homing property of lymphocyte-derived membrane, EVs can be endowed with targeting ability to inflammation sites [38]. Taken together, by utilizing conventional technique such as ligand conjugation or other

method for the targeting, EVs may be a useful platform for targeted delivery of therapeutic molecules.

5. Pharmacokinetics

For the therapeutic purpose, pharmacokinetics of EVs is crucial to deliver drug molecules to specific site in the body. Basically, upon the systemic administration, NPs accumulate rapidly in liver, spleen, and lung, so-called mononuclear phagocyte system (MPS) or reticuloendothelial system (RES) [79]. Avoiding the capture by MPS is the solution to improve pharmacokinetics of EVs. In this chapter, *in vivo* biodistribution of EVs from various sources is summarized. Furthermore, the engineering approaches to improve pharmacokinetics of EVs are discussed.

5.1. Biodistribution of EVs upon administration

When the EVs are administered systemically, almost of EVs are suddenly captured by MPS [49,57,80,81]. Macrophages in MPS are responsible for this rapid clearance of EVs from blood stream [82]. This is not the particular phenomenon for EVs, because generally, NPs are likely to be taken up by MPS. Furthermore, the negative charge on the surface of EVs, descended from negatively charged phospholipid phosphatidylserine (PS), is the main cause of uptake by macrophage since PS can be recognized by PS-binding receptor molecules on the macrophages [83]. The source of EVs is also crucial on the pharmacokinetics of EVs. This is because each EV has different composition. EVs from different cell types showed slightly different biodistribution [84].

Administration route severely affect the pharmacokinetics of EVs. As mentioned above, intravenous injection of EVs results rapid clearance from blood and accumulation in MPS-related organs. Intranasal administration is the unique route to deliver drugs into brain [70]. By using food-derived EVs, oral administration is of promising approach to deliver drugs due to its minimum invasiveness [22,39], although it is still unclear whether EVs can be transferred into blood circulation *via* gastrointestinal absorption, maintaining the integrity of EVs.

5.2. Improvement of pharmacokinetics

In general, NPs are taken up by MPS upon systemic administration. For evading the capture by MPS, PEGylation might be most promising approach. Polyethylene glycol (PEG) is hydrophilic polymer and when attached on the NPs, PEG chains cover the surface of NP. Thanks to the steric hindrance effect by PEG chains, the interaction of NPs and proteins/cells can be reduced and half-life in blood circulation is prolonged [79]. Such strategies can be applied to EVs for improving pharmacokinetics.

One paper described the PEGylation of EVs [85]. PEG chains were conjugated on the surface of EV *via* lipid anchor. PEGylated EVs showed longer half-life in blood circulation. However, unexpectedly, significant tumor accumulation of EVs was not observed. This is probably due to the detachment of PEG chains during circulation. Rational and robust conjugation for PEG chain rather than lipid anchoring is necessary for stable PEGylation and improving pharmacokinetics of EVs.

Watson et al. reported that scavenger receptor class A family (SR-A) is responsible for uptake of EVs by macrophages *in vivo*. By utilizing this finding, they blocked SR-A by injecting dextran sulfate and reduced liver clearance of EVs [23]. Similarly, Matsumoto et al. reported that pre-injection of liposome composed of PS or phosphatidylglycerol prolonged the circulation time of EVs upon systemic administration [83]. These strategies are valuable to improve pharmacokinetics of EVs and facilitate targeted delivery to specific cells or organs.

6. Conclusion

In this review, we summarize current progress on research field of EVs for DDS application. Conventional NPs were believed as "magic bullet" to deliver drugs to diseased site with minimized side-effects, but unfortunately, efficient drug delivery has not yet been entirely achieved [6,7]. Many researchers and we believe that applying EVs as DDS can be a breakthrough of drug delivery.

Table 2. Therapeutic applications of EVs as DDS.

Stages	Source of EVs	Drugs	Therapeutic outcomes	Reference
In vitro	MDA-MB231 cells	porphyrin	phototoxic effect	[66]
	HUVECs			
	hESCs and hMSCs			
	LNCaP and PC-3 cells	paclitaxel	anti-cancer effect	[72]
	hMSCs	anti-miR-9	reduce chemoresistance of cancer cells	[54]
	HeLa cells	saporin	anti-cancer effect	[62]
In vivo	milk	withaferin A	anti-cancer effect	[22]
	grapefruit	JSI-124/paclitaxel	anti-cancer effect	[25]
	mouse dendritic cells	doxorubicin	anti-cancer effect	[61]
	Raw 264.7 cells	paclitaxel	anti-cancer effect	[59]
	Raw 264.7 cells	catalase	neuroprotective effect in Parkinson's disease model	[67]
	HEK293 cells	let-7a	anti-cancer effect	[53]
Clinical trials	grape	curcumin	ongoing phase I study	NCT01294072*
	dendritic cells	tumor antigen	ongoing phase II study	NCT01159288*

*Cf. ClinicalTrials.govh ESC, human embryonic stem cell; hMSC, human mesenchymal stem cell; HUVEC, human umbilical vein endothelial cell.

Therefore, much efforts so far been made to realize DDS utilizing EVs. In the table 2, we summarized the therapeutic application of EVs as DDS. Some EV-mediated drugs are used in clinical trials, however, we are still facing problems discussed in this review. Particularly, mass-scale production and drug loading are the bottleneck for the application of EVs as DDS. As mentioned above, large amounts of source for EVs should be obtained at reasonable cost. Furthermore, as

achieved in liposomes or other DDS nanocarriers, drug loading method should be rationally designed to efficiently encapsulate therapeutic molecules into EVs. Other two factors, including the targeting and pharmacokinetics are also important for the drug delivery by EVs. Technologies used in conventional nanocarriers, such as ligand conjugation or PEGylation are well-established in NP-mediated drug delivery. It is expected that these technologies contribute to the improvement of targeting efficiency and pharmacokinetics of EVs. Furthermore, understanding the biology of EVs will prompt the EV-mediated delivery as promising platform for forthcoming DDS.

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Conflict of Interest

The authors have no conflict of interest to declare.

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