Therapeutic Application of Small Extracellular Vesicles (sEVs): Pharmaceutical and Pharmacokinetic Challenges

Yoshinobu Takakura,* Akihiro Matsumoto, and Yuki Takahashi

Graduate School of Pharmaceutical Sciences, Kyoto University; 46–29 Yoshida-Shimo-Adachi, Sakyo-ku, Kyoto 606–8501, Japan.
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Small extracellular vesicles (sEVs), including exosomes as typical example, are cell-derived vesicles comprising lipid bilayer with a diameter approximately 100 nm. sEVs are endogenous delivery vehicles that deliver their contents such as nucleic acids and proteins to recipient cells. Because of their potential nature as endogenous delivery vehicles, therapeutic applications of sEVs as delivery systems of various drugs are expected. To develop sEV-based therapeutics, a variety of challenges should be overcome. In this review, we summarize the current status and future perspectives of therapeutic applications of sEVs. Several pharmaceutical and pharmacokinetic challenges will be discussed.

Key words  exosome; small extracellular vesicle (sEV); therapeutic application; pharmaceutical challenge; pharmacokinetic challenge

1. INTRODUCTION

Recent advances in cell biology have revealed that extracellular vesicles (EVs) secreted from various types of cells can be found in our body fluids and play very important physiological roles. One of the EVs are classified as small extracellular vesicles (sEVs); these are small membrane vesicles with a diameter of approximately 100 nm.1,2) Historically, the discovery of sEVs occurred more than 30 years ago; sEVs, then known as exosomes, were found during maturation of reticulocytes to erythrocytes.3) However, sEVs did not attract a great deal of attention from researchers for many years. The history dramatically changed when a Swedish group reported that sEV might be involved in genetic exchange between cells by transfer of mRNAs and microRNAs into other cells, in 2007.4) After this epoch-making discovery, extensive studies have been carried out in interdisciplinary research fields.

Our present understanding is that sEVs are very important intercellular communication systems in physiological and pathological states and involved in intercellular transport of important biological molecules such as endogenous RNAs and proteins. Based on their interesting and unique characteristics, sEVs are expected to play roles as novel and powerful delivery systems of various drugs. Extensive studies on the therapeutic application of sEVs are ongoing.5–9)

Recently, scientific societies such as International Society for Extracellular Vesicles (ISEV) and Japanese Society for Extracellular Vesicles (JSEV) have been established so as to accelerate research activities on EVs including sEVs. Very recently, ISEV published guidelines for studies of EVs (MISEV2018).10) containing recommendations on nomenclature, isolation and characterization methods, and so on. They recommend that the term “small EVs (sEVs)” should be used instead of “exosomes” because it remains difficult to support the definition of exosomes experimentally due to our still limited knowledge of their specific molecular machineries of biogenesis and release, as compared with other biophysically similar EVs. Therefore the term “sEV” is used in this review irrespective of use of the term “exosomes” in published literature cited in this review.

sEVs have potential advantages as drug delivery systems for therapeutic application. However, from the viewpoint of pharmaceutical sciences, there are several challenges to be overcome, including pharmaceutical challenges such as production and drug loading and pharmacokinetic (PK) challenges (Fig. 1). In this review, we summarize the current status and future perspectives of sEV-based therapeutic applications. Pharmaceutical and pharmacokinetic challenges will be discussed.

2. PHARMACEUTICAL CHALLENGES

sEVs can be classified into a cell-derived formulation. Therefore, for therapeutic application of sEVs, careful considerations are required as the formulation from the pharmaceutical viewpoint.

2.1. Production of sEVs  Production of sEVs is one of the major challenges for therapeutic application because of their heterogeneity and low productivity. For example, the yield of sEVs is typically less than 1 µg sEV protein from 1 mL of culture medium, while approximately 10–100 µg sEV protein/mouse was used in most animal experiments. Thus large amounts of sEVs are required for in vitro and in vivo experiments to evaluate their feasibility for therapeutic application. Several efforts have been attempted to improve large-scale production; however, there remain concerns to be overcome. Details on production issues have been discussed in

* To whom correspondence should be addressed.  e-mail: takakura@pharm.kyoto-u.ac.jp

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Selection of cell type is also an important factor. We evaluated some characteristics including the yield of sEVs derived from five different types of mouse cell lines: B16-BL6 murine melanoma cells, C2C12 murine myoblast cells, NIH3T3 murine fibroblasts cells, MAEC murine aortic endothelial cells, and RAW264.7 murine macrophage-like cells. sEVs were collected by differential ultracentrifugation method. C2C12 and RAW264.7 cells produced more sEVs than the other types of cells, although the physicochemical and PK properties were similar.

Selection of isolation method is another key factor for sEV production. There are many options for isolation method such as ultracentrifugation-based methods (simple pelleting, density cushion method, density gradient method), size-based separation (ultrafiltration, size exclusion chromatography), polymer-based methods (polymer-based precipitation, aqueous two-phase system), and affinity-based separation (surface protein affinity method, phosphatidylserine affinity method). Appropriate methods should be selected depending on the purpose because each method has advantages and disadvantages. Among them the ultracentrifugation-based methods are the typical and most widely used methods for sEV isolation from cell culture medium. As far as our comparative study between the ultracentrifugation-based methods is concerned, simple ultracentrifugation caused more contamination of proteins and aggregation of sEVs compared with density cushion and density gradient methods. Therefore simple ultracentrifugation method, the most commonly used method for sEV isolation, might not be suitable for therapeutic application.

Very recently, Zhang et al. reported a unique isolation method, so-called asymmetric flow field-flow fractionation (AF4). These authors fractionated cultured cell-derived sEVs collected by classical ultracentrifugation into three fractions according to size by this technique. They identified two sEV subpopulations (large vesicles, 90–120 nm; small vesicles, 60–80 nm) and discovered an abundant population of non-membranous nanoparticles termed “exomeres” (approximately 35 nm). Interestingly, each population had distinct characteristics in terms of lipid composition, sugar chain modification pattern, protein composition, DNA and RNA profiles, and biodistribution in mice. The AF4 method could be a powerful tool to overcome challenges in sEV isolation.

Storage of sEVs is also an important issue from the pharmaceutical point of view. Generally, sEVs are stored frozen at −80°C; however, this condition is not suitable for their handling or transportation. Our recent study demonstrated that sEVs can be preserved at room temperature using lyophilization in the presence of trehalose, a cryoprotectant, as can liposome formulations. Lyophilization without cryoprotectant resulted in aggregation of B16-BL6 melanoma-derived sEVs, whereas addition of trehalose prevented aggregation during lyophilization. Proteins and RNA of sEVs were protected following lyophilization in the presence of trehalose, and lyophilization had little effect on PK of the sEVs after intravenous injection into mice. Moreover, lyophilized sEVs retained the activity of loaded reporter protein, Gaussia luciferase (gLuc), and immunostimulatory CpG DNA for approximately 4 weeks even when stored at 25°C.

### 2.2. Drug Loading into sEVs

A variety of drugs or therapeutic agents have been studied for therapeutic application of sEVs to various diseases. Representative drugs include lipophilic drugs with low molecular weight and RNA-based drugs such as small interfering RNA (siRNA).
Efficient drug loading into sEVs is key to successful therapies. Drug loading of small lipophilic drugs is relatively easy because they interact with the lipid bilayer of sEV membrane through hydrophobic interaction just by mixing and incubation. Successful drug loading has been reported for curcumin, doxorubicin, among others. On the other hand, RNA-based drugs require special loading strategies, i.e., exogenous or endogenous loading as sEV cargos.

2.2.1. Exogenous Loading
RNA-based drugs are naturally hydrophilic and have little interaction with the hydrophobic lipid bilayer membrane of sEVs. One approach to drug loading is chemical modification of RNA with hydrophobic moieties. Several studies have reported successful loading into sEVs using hydrophobically modified siRNA conjugated with cholesterol and fatty acids. On the other hand, loading of unmodified RNA-based drugs into sEVs requires membrane permeabilization strategies, including electroporation and sonication. The most commonly used method is electroporation, which has been widely used for cell transfection. Small pores are transiently generated in the lipid bilayer membrane by electrical stimuli. Successful loading of siRNA (approximately 20% of the input) was reported for bioengineered sEVs targeted to the brain and plasma sEVs for delivery to human blood cells. Where- as, Kooijmans et al. reported that true loading efficacy by electroporation was extremely low (below 0.05% of the input), and apparently high loading efficiency was an artifact caused by formation of insoluble siRNA aggregates. Careful attention should be paid to the equipment and protocol conditions for electroporation. Cellular damage or denaturation of sEVs caused by electroporation procedure is another concern.

2.2.2. Endogenous Loading
RNA-based drugs can be endogenously loaded into sEVs by transfection of sEV-producing cells with RNA or RNA-encoding vectors. sEVs containing the RNA are collected after secretion from the transfected cells. In contrast to exogenous loading with electroporation, there are no denaturation concerns of sEVs because the RNA is sorted into sEVs based on the natural mechanism of endogenous RNAs. However, the loading efficiency of RNA into sEVs is not so efficient.

It was reported that RNAs enriched in sEVs frequently contain specific sequences such as zipcode-like sequences and some sequence motifs. Transfection of plasmid DNA encoding a reporter protein RNA ligated with a zipcode-like sequence resulted in an increase in the amount of the reporter mRNA in sEVs, suggesting that such sequences might be helpful to increase the loading efficiency of RNAs in sEVs. We found a typical RNA sequence by systematic evolution of ligands by exponential enrichment (SELEX) method to screen sEV-tropic RNAs. The sequence found by SELEX was enriched in sEVs after transfection to B16-BL6 cells, suggesting that the sequence can be used to load functional RNAs into sEVs by conjugation. Further studies are necessary to enhance the efficacy of endogenous loading of RNA-based drugs.

3. PHARMACOKINETIC CHALLENGES
PK of sEVs is one of the most important issues to develop sEV-based therapeutic systems. Availability at the target tissues/cells after administration of sEVs is the key determinant for the desired effect of an sEV-based therapeutic system. Quantitative information regarding the elimination profile from the blood circulation and biodistribution of sEVs is also required for rational design of sEV-based therapeutic systems.

3.1. Labeling Methods of sEVs
The key technology for PK studies is to develop sEV labeling methods (Fig. 2). There are several methods applicable to qualitative and quantitative
cells after transfection with plasmid DNA encoding gLuc-LA. Using gLuc-LA-labeled B16-BL6 sEVs, the in vivo distribution of the B16-BL6 sEVs was visualized by detecting chemiluminescence emitted from gLuc-LA after intravenous injection of the gLuc-LA-labeled B16-BL6 sEVs. We have already provided more than 10 labs in the world with the plasmid for this labeling by exchanging material transfer agreement.

3.1.3. Luciferase Labeling at Inner Leaflet

Luciferase labeling is also possible at the inner side of sEV membranes by genetic engineering of sEV-producing cells. In our recent study, we developed a novel method to label the inner space of sEVs using Gag protein, which is derived from Moloney murine leukemia virus (MLV).39 Gag localizes to the inside of plasma membrane through interaction between the polybasic region of the Gag protein and phosphatidylinositol 4,5-bisphosphate in the plasma membrane.40,41 We labeled sEVs derived from B16-BL6 melanoma cells through transfection of a plasmid encoding a fusion protein consisting of Gag protein and gLuc (Gag-gLuc). Luciferase labeling at inner leaflet can be a useful method to study the role of surface proteins on sEV membranes in PK.39

3.1.4. Radiolabeling

Radiolabeling of sEVs is a useful method to study the PK characteristics including biodistribution in more quantitative manner.42,43 For this purpose, we have established a radiolabeling method by genetic engineering. We labeled B16-BL6-derived sEVs with iodine-125 (125I) based on streptavidin (SAV)-biotin system. A plasmid vector encoding fusion protein, SAV-LA, was constructed, and B16-BL6 cells were transfected with the plasmid to obtain SAV-LA-labeled sEVs. SAV-LA-labeled sEVs were incubated with (3,125I-lodobenzoyl)norbiotinamide (125I-IBB) to obtain 125I-labeled B16-BL6 sEVs. Quantitative biodistribution and single photon emission computed tomography (SPECT)/CT imaging studies of the B16-BL6 sEVs were carried out in mice after intravenous injection.43
rapidly.

We also demonstrated that macrophages are the main contributor of sEV uptake in the liver and spleen whereas vascular endothelial cells are responsible for uptake in the lung, using fluorescent-labeled sEVs in combination with immunohistochemical techniques. Moreover, it was also directly shown that macrophages play a significant role in the clearance of sEVs from the blood after systemic injection in macrophage-depletion experiments using clodronate-containing liposomes in mice. Similar PK characteristics were also observed for gLuc-LA-labeled sEVs produced from five different types of cultured cells, suggesting that these PK properties are common characteristics of sEVs regardless of the origin of the producing cells. In addition, B16-BL6 sEVs radiolabeled with [125I]labeled sEVs after systemic administration with no significant effect on the liver and spleen accumulation of sEVs.

Cellular uptake mechanisms of sEVs after systemic administration were also examined in our studies. Macrophages are well-known to take up negatively charged nanoparticles such as chemically modified lipoproteins and anionic liposomes by recognition with scavenger receptors. sEVs are extracellular vesicles with strong negative charges on the surface, that is, the zeta potential is determined approximately −30 to −40mV. One major reason for the negative charges is due to a negatively charged membrane phospholipid, phosphatidylserine (PS), because PS is abundantly present on the sEV surface regardless of the production cell type. Sulfated polysaccharide derived from proteoglycans, glycolipids, and other cell membrane components may also be involved in the strong negative charges of the sEV surface. We have demonstrated that preinjection of PS-containing liposomes significantly delayed elimination of gLuc-labeled B16-BL6 sEVs from blood circulation and decreased accumulation of [125I]labeled sEVs in the liver after intravenous injection in mice, suggesting that negative charges play an important role in recognition of sEV by liver macrophages (Kupffer cells) probably via scavenger receptors. Watson et al. also reported that macrophage scavenger receptors are involved in sEV recognition using dextran sulfate, a scavenger receptor inhibitor. In addition, CD169-positive macrophages in the spleen take up sEVs through recognition of negatively charged α2-3 sialic acid on sEV membrane. Taken together, the physicochemical properties of sEVs as nanoparticles, in particular, negative surface charges, are important in cellular recognition in vivo after systemic administration.

Regarding the mechanism of sEVs distribution in the lung, we have shown evidence that sEV surface proteins are involved using B16-BL6 sEVs labeled with luciferase at inner leaflet (Gag-luc) as described above. Since Gag-luc-labeled sEVs have a luminescent probe, gLuc, inside the sEV, it is possible to detect luminescence derived from the sEVs even after surface protein degradation by protease treatment. We found that protease treatment significantly decreased the distribution of sEVs to the lungs after intravenous injection, although their distribution to the liver was not affected, suggesting that surface proteins of sEVs play an important role in the uptake by the lung, probably by endothelial cells of this organ. Our findings are in agreement with a report that pulmonary epithelial cells recognize tetraspanin proteins and integrins present on the sEV surface.

3.3. Pharmacokinetics of sEVs after Local Administration

For therapeutic applications, sEVs are often administered from local routes such as subcutaneous, intradural, and intratumoral injection. After local injection, nanoparticles including sEVs are absorbed predominantly into lymphatic capillaries rather than into blood capillaries from the interstitial space of the injected tissues because of their large size in relation to the physiological structures of those capillaries. Srinivasan et al. reported that sEVs labeled with fluorescent dyes are rapidly transported within minutes from the periphery to the lymph node by lymphatics after subcutaneous injection in mice by in vivo NIR imaging. The authors also identified macrophages and B cells as key players in sEV uptake in lymph nodes.

Another study compared the PK characteristics of cell- and serum-derived sEVs after subcutaneous injection in mice. Serum-derived sEVs labeled with Rhodamine (less than 50nm in size) were delivered to lymph nodes more efficiently than cell-derived sEV (over 100nm in size), suggesting that smaller serum-derived sEVs might serve as efficient carrier systems of immune stimulators to lymph nodes for desired immune responses.

Shimoda et al. also reported that PKH26-labeled sEVs were transported into lymph nodes after subcutaneous injection in mice. The authors also observed internalization of the sEVs by antigen-presenting cells (APCs), particularly those expressing CD11b positive for cell-surface-bound sialic acid-binding immunoglobulin-like lectins (siglecs), but not by APCs negative for siglecs, suggesting that cellular uptake of sEVs involved recognition of sialic acids of sEVs by siglecs. As reported by this paper, interaction between glycans on sEVs and lectins on the recipient cells might play an important role in PK of sEVs after local administration.

We investigated PK properties of B16-BL6 sEVs in mice bearing B16-BL6 melanoma cells after intratumoral injection. After intratumoral injection of [125I]labeled sEVs, most radioactivity was detected within the tumor tissues of mice and part of the radioactivity detected in the liver and lungs. Fractionation of cells present in the tumor tissue showed that PKH26-labeled sEVs were mainly taken up by B16-BL6 cells. Interestingly, tumor growth was significantly promoted after intratumoral injection of the sEVs, suggesting that B16-BL6 cells secrete and take up their own sEVs to induce their proliferation and inhibit their apoptosis, thereby promoting tumor progression.

4. THERAPEUTIC APPLICATIONS OF SEVS

A variety of diseases could be targets of sEV-based therapeutics. Some examples of therapeutic applications of sEVs are discussed herein.

4.1. Cancers

Extensive studies have been carried out for the treatment of cancers based on various strategies. Successful studies on sEVs loaded with small molecule anti-
cancer drugs and RNA-based drugs were reported. It has been reported that drug delivery of anticancer drugs doxorubicin and miRNA to tumor by sEVs may be achieved by modifying sEVs with RGD peptide or GE11 peptide targeting epidermal growth factor receptor (EGFR).\textsuperscript{[54–57]} Recently, Kamker et al.\textsuperscript{[58]} reported that CD47-expressing sEV with long retention in the circulation loaded with siRNA targeting KRAS, an oncogene, can be successfully delivered to pancreatic cancer model mice after intravenous injection. CD47 is a protein known as “do not eat me signal” that inhibits phagocytosis by macrophages through binding to receptors expressed on their cellular surface. Gong et al.\textsuperscript{[57]} demonstrated that sEVs loaded with doxorubicin and cholesterol-modified miRNA 159 can be delivered to breast cancer cells inoculated in nude mice after intravenous injection. The sEVs derived from THP-1 cells were functionalized by stimulation with phorbol 12-myristate 13-acetate to have a higher cellular uptake via integrin-mediated interaction.

Another representative example of therapeutic application of sEVs is cancer immunotherapy, especially tumor vaccine. Validity of this approach is supported by the finding that sEVs are rapidly taken up by macrophages, a typical APC, one of the important target cells in cancer immunotherapy as described in the previous section. Dendritic cells (DC) are another potent APC. DC-derived sEVs collected from DCs pulsed with tumor antigens have been used as a tumor vaccine because the sEVs contain not only tumor antigen but also MHC molecules presenting tumor antigen epitopes. Autologous DC-derived sEVs have been tested in clinical trials; however, sufficient therapeutic effects have not been observed.\textsuperscript{[59]} For the development of effective sEV-based tumor vaccine, not only tumor antigens but also adjuvants should be delivered to APC by sEVs.

Tumor cell-derived sEVs (TEV) are another typical tumor vaccine.\textsuperscript{[60]} One particular advantage of TEV is that identification of tumor antigen is not required since TEV contains endogenous tumor antigens. Therefore the TEV vaccine approach can be applicable, in principle, to any types of tumors. We have developed a tumor vaccine, an efficient TEV-based tumor antigens-adjuvant co-delivery system using genetically engineered TEV containing endogenous tumor antigens and immunostimulatory CpG DNA.\textsuperscript{[61]} We employed B16-BL6-derived sEVs expressing SAV-LA used in PK studies,\textsuperscript{[39]} and were conjugated with biotinylated CpG DNA. Immunization with CpG DNA conjugated TEX exhibited stronger in vivo antitumor effects in B16-BL6 tumor-bearing mice than simple co-administration of sEVs and CpG DNA, indicating that the genetically engineered TEV conjugated with CpG DNA is an effective sEV-based tumor antigens-adjuvant co-delivery system that may be useful for cancer immunotherapy.

Very recently, we developed a novel TEV-based tumor vaccine, a DNA-anchored sEVs superstructure in which B16-BL6 derived sEVs were assembled with each other to achieve prolonged tissue residence and APC targeting.\textsuperscript{[62]} We prepared TEV modified with immunostimulatory CpG-DNA containing an additional “sticky end,” which was mixed with an oligonucleotide duplex containing the sequence complementary to the “sticky end” of the CpG-DNA, resulting in self-assembly of CpG-TEV into a micrometer-sized superstructure. The assembly was selectively taken up by APCs, and efficiently activated DCs in vitro. Moreover, the assembly formation significantly prolonged tissue residence and increased immune responses of CpG-DNA intradermally injected into mice. These results indicate that CpG-TEX assembly is an effective system that may be useful for tumor immunotherapy.

In our recent study, the effects of localization of antigen proteins in antigen-loaded sEVs on efficiency of antigen presentation was investigated.\textsuperscript{[63]} Ovalbumin (OVA) was used as a model antigen protein. OVA-inner-loaded (Gag-OVA) sEVs and OVA-outer-loaded (OVA-LA) sEVs were prepared by the genetic engineering technique used for gLuc-labeled sEVs in PK studies.\textsuperscript{[23,34]} Gag-OVA sEVs showed enhanced class I antigen presentation capacity as compared with OVA-LA sEVs, indicating that loading of antigen proteins inside sEVs helps in efficient antigen presentation. This information would be useful when tumor antigens are exogenously loaded by the genetic engineering technique in designing tumor vaccines.

4.2. Inflammatory Diseases Mesenchymal stem cells (MSC) have attracted much attention in cell-based therapy because of their potent and various biological activities such as tissue regenerative capacity, immunoregulatory function, and anti-inflammatory ability. It is well known that MSC-derived sEVs significantly contribute to many of these biological activities of MSCs. Multiple experimental studies have reported that MSC-derived sEVs can mimic the biological activities of MSCs. Based on these findings, multiple efforts are ongoing in developing MSC sEV-based therapeutics.\textsuperscript{[5]}

Brain drug delivery can be possible after intranasal administration. This route has been applied for delivery of anti-inflammatory drugs to the brain by sEVs.\textsuperscript{[64]} Long et al.\textsuperscript{[65]} reported that MSC-derived sEVs intranasally administered to mice can be delivered to microglia, resident macrophages in the brain, and to induce anti-inflammatory effects.

4.3. CNS Diseases Alvarez-Erviti et al.\textsuperscript{[20]} designed a fusion protein containing rabies viral glycoprotein (RVG), a brain targeting peptide, and Lamp2b, a protein present on the sEV membrane surface, for the purpose of delivering siRNA to the brain. It has been reported that siRNA can be delivered to the brain by systemic administration of RVG-modified sEVs encapsulating siRNA targeting Alzheimer’s disease-related gene to mice, which resulted in knockdown of the target gene in the brain. Haney et al.\textsuperscript{[66]} reported that catalase-loaded sEVs administered nasally provide neuroprotective effects in Parkinson’s disease model mice.

5. CONCLUSION

sEVs have attracted much attention for their therapeutic applications to the treatment of a variety of diseases. Extensive studies on the basic and applied aspects of sEVs are ongoing in interdisciplinary research fields. From the viewpoint of pharmaceutical sciences, continuous and multiple efforts are required to overcome pharmaceutical and PK challenges.

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