Extracellular Vesicles as Natural Nanosized Delivery Systems for Small-Molecule Drugs and Genetic Material: Steps towards the Future Nanomedicines

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ABSTRACT - A new platform for drug, gene and peptide-protein delivery is emerging, under the common name of "extracellular vesicles". Extracellular vesicles (EVs) are 30-1000 nm-sized cell-derived, liposome-like vesicles. Current research on EVs as nano-delivery systems for small-molecule drugs and genetic material, reveal that these tiny, biologically-derived vesicles carry a great potential to boost the efficacy of many therapeutic protocols. Several features of EVs; from efficacy to safety, from passive to active targeting ability, the opportunity to be biologically or chemically labelled, and most importantly, their eobiotic origin make them promising candidate for development of the next generation personalized nanomedicines. The aim of this article is to provide a view on the current research in which EVs are used as drug/genetic material delivery systems. Their application areas, drug loading and targeting strategies, and biodistribution properties are discussed.

INTRODUCTION

Each active pharmaceutical ingredient undergoes a formulation step before administration to the patients. Formulation of drug delivery systems using proper excipients helps to improve their therapeutic activity and reduces side effects (1). The ultimate goal of the formulation and drug delivery studies is to help patients by designing clinically relevant, effective and safe formulations. Advancements in drug delivery technologies have led to development of various clinically acceptable formulations with improved patient compliance and ease of administration (2).

Among them, nanoparticle-based systems represent one of the most promising innovations in the field of drug delivery. These systems include polymeric nanoparticles, dendrimers, nanocapsules, liposomes, solid lipid-based nanoparticles, protein conjugates, micro- and nanoemulsions, inorganic nanoparticles, carbon nanotubes etc (3). They have been studied for the development of formulations with increased drug solubility (4–7), improved bioavailability (7–12), modified biodistribution parameters (10,11,13), improved drug activity (14–16), reduced drug toxicity (12,15,17), reduced dosing regimen (18), and for drug targeting (15,16,19). Furthermore, these systems are essential tools for enabling systemic delivery of otherwise unstable nucleic acid therapeutics (20–24), peptides and protein drugs (19,25–27).

Difficulties with currently available nanoparticle delivery systems

Although appreciable improvement has been achieved in designing advanced drug delivery systems, there are still issues in which significant effort should be made to cover the ground of a clinically adequate therapeutic delivery system. Despite their versatility and enormous potential in therapeutics delivery applications, conventional nanoparticles have a considerable drawback; their xenobiotic origin, which means that they are composed of constituents that are alien to the organism to which they will be administered. Materials of xenobiotic origin often lead to unwanted immune reactions, and unexpected toxicity (28). So their safety is the subject of substantial criticism (29–34). From regulatory point of view the exposure safety of nanoparticles has long being considered limited as there are still insufficient data related to the acute and chronic toxic effects of nanoparticles upon administration to humans (35–38).

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Predominant part of studies dealing with development and application of nanoparticles for therapeutics delivery are of *in vitro* testing, or *in vivo* animal experiment character. Wherever *in vitro* data can be contradictory with the results obtained *in vivo* (39), it is the same when *in vivo* animal data are to be extrapolated to clinical trials (40). Thus, the toxicity of drug nanocarriers and the difficulty to reliably prove their safety are the main impediments for the development of an efficacious and clinically safe nanosized delivery system (41).

Extracellular vesicles: short description

Because of the obstacles with the currently available nanoparticle drug delivery systems, researchers continuously seek for innovative nanosized materials with biological origin. A group of biological nanosized vesicles, produced during natural processes of cell cycle are getting increasingly attractive for this purpose. These cell-derived endogenous vesicles are known as extracellular vesicles (EVs). EVs are small, lipid bilayer-enclosed vesicles which are released from the cells. They are logistic systems of the cells that carry proteins, lipids, mRNA, microRNAs, and DNA. The term ''extracellular vesicle'', indeed, is a general term for all secreted vesicles, and based on their biogenesis they are classified into; exosomes, microvesicles (MVs) and apoptotic blebs (42).

Exosomes are first reported by Johnstone and co-workers as vesicles secreted from reticulocytes (43). Exosomes are small membrane-bound vesicles that are formed inside endosomes during endosomal maturation and recycling (44). Their size varies between 40–100 nm. Microvesicles or ectosomes are directly formed by budding of the plasma membrane, and their size varies in a much wider range as compared to exosomes (50–1000 nm) (45,46). Apoptotic blebs are formed during late stages of the programmed cell death (47,48). Table 1 shows the main characteristics of different EV types (49). EVs have crucial roles in both normal physiological and pathological processes such as angiogenesis (50), inflammation (51), immune response (52), cell survival (53), autophagy (54), cardiovascular diseases (55), drug resistance (56), and cancer (57).

Biogenesis of exosomes

In cells, during the endosomal maturation three distinct types of cellular vesicles are formed; early endosomes, late endosomes and recycling endosomes. Early endosomes accept incoming cargo internalized from the plasma membrane and sort it to different intracellular destinations (58). Early endosomes mature into late endosomes. During this maturation, contents fated to be degraded or exported out of the cell are enriched in vesicles that bud inward to the lumen of late endosomes. Late endosomes contain Intraluminal Vesicles (ILVs). This form of the late endosomes is known as Multivesicular Bodies (MVBs). MVBs are either targeted to fuse with lysosomes for destruction of the cargo, or fuse with plasma membrane to secrete the ILVs to the extracellular space in form of exosomes. A superfamily of membrane proteins known as Tetraspanins and endosomal sorting complexes are required for the formation of ILVs. Tetraspanins CD9, CD63, CD37, CD81, or CD82 are specially enriched in the membrane of exosomes and they are often used as exosome biomarkers (59).

Several complex proteins are involved in the formation of ILVs. These are named endosomal sorting complex required for transport (ESCRT). These complexes work together with associated proteins like VPS4, VTA1, and ALIX (60). Interactions of ESCRT complexes and associated proteins with phosphatidylinositol 3-phosphate and ubiquitin leads to inward budding of the endosomal membrane and cargo sorting into the ILVs (61–63).

Biogenesis of Microvesicles

MVs, also known as ectosomes or microparticles, are formed directly by budding of the plasma membrane. Compared to exosomes, MVs are larger (50 – 1000 nm) and more heterogeneous in morphology. Activation of MVs is different from one cell to another. For instance; endothelial and circulating blood cells release MVs when exposed to complement attack, monocyte budding is induced by bacterial cell wall components, platelets release MVs by activation through thrombin, fibroblasts release MVs in response to stress relaxation (64).

Table 1. Properties of different extracellular vesicle types (49)

EV type	Shape	Reported size range (nm)	Origin
Exosomes	cup shaped-to-spherical	~ 30 - 100	Multi vesicular bodies
Microvesicles	spherical-to-irregular	~ 200 - 500	Plasma membrane
Apoptotic blebs	irregular	~ 1250-2500	Entire membranous cell components

Specific lipid composition of the vesicle membrane and cell cytoskeleton provides the formation of microvesicles (65). Aminophospholipids; phosphatidylserine and phosphatidylethanolamine are segregated in the inner leaflet, whereas phosphatidylcholine and sphingomyelin are enriched in the external leaflet. Flippase and floppase enzymes transfer phospholipids between the inner and outer leaflets (48). Reverse sided translocation of the phosphatidylserine induces the membrane budding and microvesicle formation. The formation of exosomes, MVs and apoptotic blebs is schematized in Figure 1.

EVs AS EMERGING DRUG DELIVERY NANOVESICLES

The structural feature that makes EVs especially attractive for drug delivery purpose is their analogy to liposomes. As liposomes are composed of phospholipids, they mimic the properties of cell membranes. As such, they are currently widely used in studies for delivering active pharmaceutical ingredients and several liposome-based products have taken their place in the market (67). Know-how established in the field of liposome research provides some fundamental knowledge about the properties of EVs like physicochemical characteristics, drug loading, drug release, targeting and stability (44,68–75). In addition to their structural similarity to liposomes, the mode of production of EVs is another feature which makes them more advantageous. They are produced by the cells themselves, which means that they are composed of the cell membrane lipids and proteins. This allows them to mimic the cell membrane in an extent even greater than liposomes. This means when EVs originating from a living organism‘s own cells are administered to the same organism *in vivo*, possess the ability to deliver molecules even through hard-to-cross barriers like blood-brain barrier (76).

EVs in small molecule drug delivery

EVs should be effectively loaded with drug molecules in order to be used as drug delivery systems. Several strategies are described for small molecule and genetic material loading to their synthetic counterparts; liposomes (75,77–82). However, most of these strategies are not feasible for drug loading into exosomes, as they require modification of the vesicle inner content, inclusion of cationic lipids, or pre-dissolving lipids in an organic solvent (75).

Two major strategies have been suggested for loading EVs with small molecule drugs. These strategies are: 1) loading after EV isolation and 2) loading during EV biogenesis (83). Table 2 summarizes the studies collected for small molecule drug delivery with EVs and the methods employed for drug loading.

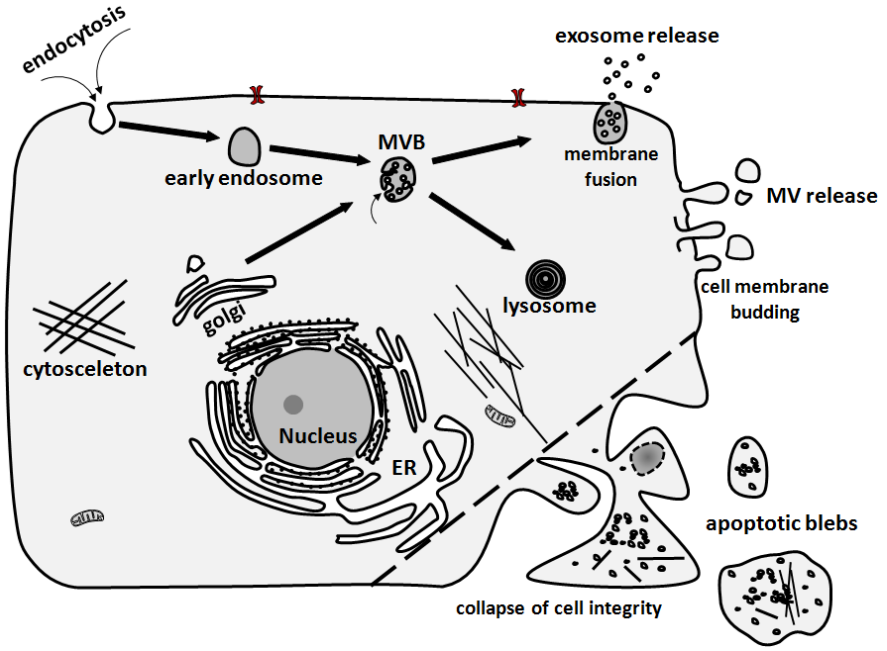

Figure 1. Illustration of extracellular vesicle release from cells. Exosomes are formed inside multivesicular bodies by inward budding of the endosomal membrane. MVs are directly shed from the cell membrane by outward budding. Apoptotic blebs are formed during the collapse of the cell integrity as a result of the late processes of apoptotic cell death. Abbreviations: MVB – multivesicular body; MV- microvesicles; ER- endoplasmic reticulum. Adapted with permission from J Cell Biol 2013; 200(4): 373–83 ©2013 The Rockefeller University Press (66)

Table 2. Studies on the small molecule drug delivery using EVs

Vesicle type	Drug	Loading method	Key findings
Exosomes	curcumin	Incubation at RT for 5 min	Increased solubility and stability *in vitro.* Increased bioavailability and enhanced anti-inflammatory effect *in vivo* (84).
Exosomes	cucurbitacin-I and curcumin	Incubation at RT for 5 min	Effective uptake by microglia cells after intranasal administration, and enhanced anti-inflammatory and anticancer effect *in vivo* (85).
Exosomes	doxorubicin	Incubation at 37°C for 2 h	Enhanced *in vitro* and *in vivo* antitumor efficiency is reported as compared to free doxorubicin. No toxicity assessment for exosomes is reported (86).
Exosomes	doxorubicin	Electroporation	Exosomes with targeting moiety delivered doxorubicin directly to tumor tissue and inhibited tumor growth *in vitro* (87).
Exosomes	doxorubicin	Incubation at 37°C for 2 h	Intra tumoral injection revealed greater impact on tumor size reduction as compared to liposomal doxorubicin. Intravenous injection caused asphyxiation, a condition with heavy breathing, and clearance by innate immune system is reported (88).
Exosomes	doxorubicin, paclitaxel	Incubation at 37°C for 2 h	Enhanced *in vitro* cytotoxicity of both drugs is observed. Exosomal doxorubicin and paclitaxel crossed the blood-brain barrier (BBB) *in vivo* in *Danio Rerio* embryo model, while drugs alone did not (89).
Exosome-mimetic nanovesicles	doxorubicin, gemcitabine, 5-fluorouracil, carboplatin	Serial extrusion of drug-treated cells through filters or membranes	Researchers found higher encapsulation and production efficiency for drug loaded exosome-mimetic nanovesicles (86).

Small molecule drug loading after EV isolation

Small molecule drug loading after EV isolation may be achieved by simple incubation of the drug of interest with isolated exosomes. This allows loading of lipophilic molecules in the lipid bilayer of the EVs. Examples for drugs loaded by incubation are curcumin (84,85), cucurbitacin-I (85), doxorubicin (88,89), paclitaxel (89). After intranasal administration exosomal JSI124 and exosomal curcumin have efficiently taken up by microglia cells, while the unencapsulated compounds have failed to cross the blood brain barrier and did not show any therapeutic effect (85). Doxorubicin has been loaded into unmodified exosomes isolated from 4T1, MCF-7 and PC3 cell lines through incubation at 37°C for 2 h. Exosomal doxorubicin was reported to inhibit tumor growth with a significantly higher extent than liposomal doxorubicin in this study (88). Yang et al. loaded doxorubicin and paclitaxel to exosomes from U-87MG cells and bEND.3 cells (89). Enhanced *in vitro* cytotoxicity of both drugs is observed in the exosomal form. The enhancement seems to be dependent on the exosome donor cells. Brain uptake of exosomal doxorubicin and paclitaxel is observed, while drugs alone did not cross the blood-brain barrier (BBB) *in vivo* in a zebra fish embryo model. *In vivo* experiments revealed that crossing through BBB also depends on the exosome donor cells (89).

Another more invasive strategy to load active compounds to isolated exosomes is applying electroporation (87). This process is somehow harmful for the exosomal membrane and requires additional incubation period at 37°C after the electroporation process in order to ensure that the membrane is recovered. Researchers explore the possibilities to minimize the risk of vesicle disruption and particle size enlargement. The method of trehalose pulse media to produce exosomes loaded with super paramagnetic iron oxide seems promising in that respect (90). For the future it seems beneficial for improvement of the efficiency of electroporation method for exosome drug loading. Unsuccessful attempts to load small molecule drugs to exosomes by electroporation are also reported (88). Apart from these methods recently freeze-thaw cycles (91), ultrasonication, saponin mediated loading, extrusion (91,92), and hypotonic dialysis (92) were used for loading biological molecules into EVs. Application of these methods to small molecule drugs can be considered promising strategies as well.

Small molecule drug loading during EV biogenesis

Small molecule drug loading during EV biogenesis has not been directly addressed to date. It is most likely that this occurs based on the natural mechanisms of cells to excrete xenobiotic or cytotoxic molecules out of the cell via EVs. In an early research, not concerned with the drug delivery potential of EVs, it was reported that increased lysosomal accumulation of cisplatin occurs when the drug is administered to resistant cancer cells (93). In addition to the increased lysosomal accumulation, researchers also reported that lysosomal compartment is markedly reduced, and that cisplatin resistant cells release exosomes with 2.6 fold higher cisplatin content than sensitive cells. Another cytotoxic drug; daunorubicin has also been shown to accumulate in the lysosomes in anthracyclin-resistant cells, but the potential of excretion through exosomes was not investigated (94,95). A study by Yamagishi and co-workers suggests that p-glycoprotein, an important member of the cell membrane proteins which is responsible of the efflux pump-type drug resistance, may be involved in this phenomenon (96). They concluded that chemoresistance in multidrug resistant cells involves accumulation of doxorubicin in the lysosomes. It implies that multivesicular bodies carry cell membrane proteins of efflux pump family associated to their membrane. Given the fact that ILVs are already formed in the endosomes, they have enough incubation time with the "excreted" drug in the lysosomal lumen, and become drug-loaded until being released out of the cell (Figure 2). Recently this was demonstrated with paclitaxel, which is another p-glycoprotein substrate. Researchers incubated bone marrow mesenchymal stromal cells (SR4987), which express high levels of p-glycoprotein, in a medium containing paclitaxel and subsequently isolated exosomes from the culture supernatant. It was shown that this chemotherapeutic agent was incorporated into exosomes during their biogenesis (97). However, it was not clarified whether exosomes have become drug-loaded in the cells, or later in the growth medium, after being released.

Another strategy for drug loading before exosome isolation is incubation of selected cells with chemotherapeutic drugs and then performing serial extrusion of these cells through membranes with consecutively narrowing pore sizes. By this means exosome-mimetic nanovesicles are produced. Examples of drugs loaded to exosome-mimetic nanovesicles include doxorubicin, gemcitabine, 5-fluorouracil or carboplatin (86). These exosome-mimetic nanovesicles showed concentration depended drug loading efficiency, good *in vitro* cell inhibitory activity and good *in vitro* and *in vivo* targeting ability.

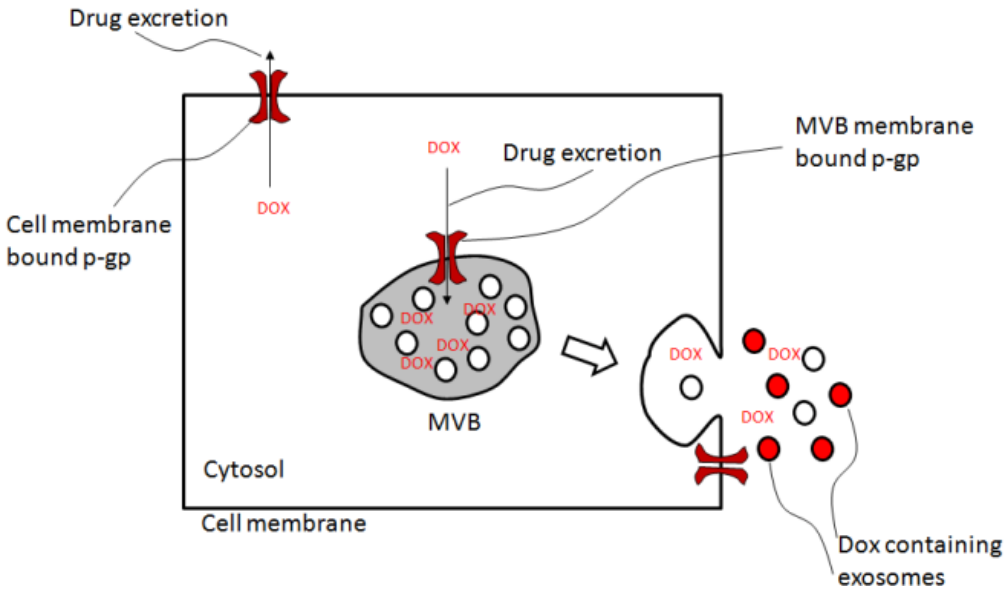

Figure 2. Illustration of the p-glycoprotein mediated lysosomal drug efflux and its possible contribution to the production of drug-loaded exosomes during their biogenesis. P-glycoprotein associated to the endosomal membrane excretes the internalized drug into the endosomal lumen. Here, newly formed exosomes are literally incubated with the drug and become "drug-loaded" before being released from the cell. Abbreviations are: MVB; multivesicular body, DOX; doxorubicin, p-gp; p-glycoprotein. Adapted with permission from J Biol Chem 2013; 288(44): 31761–31771. ©2013 The American Society for Biochemistry and Molecular Biology (96)

EVs in genetic material delivery

It is widely recognized that free genetic material (DNA, RNA) is rapidly cleared from the circulation via degradation by nucleases or filtration by the kidneys and cannot accumulate in the tissues or cells of interest. Additionally, NAs are unable to pass cellular membranes because of their electrical charge which is similar to outer cell membrane charge that prevents them from crossing the membrane (98–100). For this reason NAs intended for therapeutic application should be suitably formulated.

Recent evidence has shown that different kinds of RNA are being transported by exosomes or microvesicles as a normal route of cell-cell communication. These RNA molecules are called exosomal shuttle RNA (esRNA) which have important roles in cell-cell signaling between a variety of cell types and contribute to cancer progression and metastasis (101–105). RNA sequencing has shown that MicroRNAs (miRNAs), that are post-transcriptional modulators of gene expression are enriched in the multivesicular bodies in form of miRNA-RISC complexes and released in exosomes (106). When taken up by recipient cells, these complexes serve as ready-to-use components of posttranslational gene silencing machinery and represent an attractive alternative approach for therapeutic miRNA delivery. Therefore, the majority of the research on delivery of therapeutics by EVs is based on employing their natural feature of genetic material transfer during cell-cell communication.

During natural communication or cell functioning the genetic material to be delivered is synthesized and loaded to EVs naturally in the cells (107). Apart from this, there are several experimental methods for loading genetic material to EVs for therapeutic purpose (74). These include electroporation (108,109), chemical transfection (110), transfection/transduction of EV producer cells (111,112), and activation of cells with outer signals (113). Among these methods, transfection of EV producing cells seems the most effective way of introducing desired genes to EVs. In this method, once a desired gene is introduced to the cells they can provide a continuous overexpression of the transgene and constantly produce EVs containing the gene of interest.

As is the case with small molecule drug loading, the specific mechanism of NA enrichment into EVs during their biogenesis is not fully understood. Yet, some recent research papers give clues about how specific RNA molecules are overly sorted into EVs. In some studies it has been shown that cells transfected with a vector for overexpression of a specific miRNA, release EVs with increased level of this miRNA (104,111). Enrichment of miRNAs in EVs and their transfer to acceptor cells has been shown to be modulated by endogenous mRNA levels. When the expression of endogenous mRNAs that are target for miRNAs in the cells is downregulated, these miRNAs are enriched in exosomes, and vice versa (111). Fusing zipcode-like sequences to the desired genes can enhance the loading specificity of specific NAs (114).

Attempts to investigate the EVs for therapeutic genetic material delivery considerably increased after the pioneering works of groups interested in development of EV systems for siRNA/miRNA delivery (76,115). Alvarez-Erviti and co-workers tested the potential of exosomes from self-derived dendritic cells to deliver siRNA to the mouse brain after systemic injection (76). Authors isolated dendritic cells from mice and transfected them with a plasmid encoding an exosomal membrane protein genetically fused to a targeting ligand. In this way authors aimed to produce exosomes with specific brain-targeting property. Isolated exosomes have been loaded with GAPDH siRNA by electroporation and were administered to mice. As the result, specific GAPDH mRNA suppression in different brain parts, such as striatum, midbrain and the cortex has been observed (76).

Bolukbasi and co-workers have demonstrated that specific sequences can help to enrich mRNA's into vesicles (114). They concluded that such sequences may help to develop cell lines that produce vesicles, loaded with specific mRNAs, shRNAs or non-coding regulatory RNAs of therapeutic value.

A group of researchers attempted to use bacterial outer membrane vesicles (OMV) for siRNA delivery (119). For this purpose they employed a mutant *E. coli* strain that exhibits reduced endotoxicity towards human cells. They loaded the OMVs by electroporation and observed that this process did not affect the properties of OMVs. These OMVs internalized to SCOV3 cells and the carried siRNA escaped from lysosomes, a process which is very important in order to transfection to take place. High gene silencing and anticancer effect are observed *in vitro*. Also, high antitumor efficacy is observed *in vivo* in comparison to free siRNA as demonstrated by tumor xenograft growth regression.

Table 3. Recent examples of EVs used for genetic material delivery

Type of EVs [a]	Control carrier [b]	NA	Loading method [c]	Key findings
MVs	ND	miRNA	During biogenesis in transfected cells	Demonstration of secretion of transfected miRNAs *in vitro* and *in vivo*. *In vivo* delivery of desired miRNA is achieved by injection of *ex vivo* transfected self-macrophages to tumor- bearing mice (115).
Exosomes	Lipofectamine 2000	siRNA	Electroporation	Cell type-specific *in vitro* gene knockdown comparable to control carrier. Specific knockdown of selected genes has been achieved in targeted brain tissue *in vivo*. Implication for *in vivo* toxicological and immunological tolerability of exosomes through *in vitro* tests (76).
MVs/exosomes	Conventionally isolated AAVs	AAV [d]	During biogenesis in transfected cells	Demonstrated for the first time that AAV is incorporated in or associated to EVs. AAV containing EVs exhibit enhanced gene transfer as compared to conventionally isolated AAVs (112).
Exosomes	ND	anti-miRNA	During biogenesis in transfected cells	Contact independent transfer of anti-miR-9 from transfected mesenchymal stem cells to glyoblastoma multiforme (GBM) cells *in vitro*. Decreased level of P-gb and enhanced sensitivity of GBM to temozolomide is observed (116).
Exosomes	"self-delivering siRNA"(sdRNA) and Lipofectamine 2000	siRNA	Lipofectamine, electroporation	Chemical loading provides higher silencing efficiency. Higher silencing effect with exosomes as compared to Lipofectamine control group (110).
Exosomes	ND	siRNA miRNA	During biogenesis in transfected cells	The encapsulated siRNA loaded to targeted EVs significantly inhibited expression of the target gene *in vitro*. Significantly high targeting and inhibition of tumor growth are achieved *in vivo* in tumor xenografted mice with miRNA loaded EVs (117).
Exosomes	Oligofectamine	siRNA	Electroporation	Higher gene silencing efficiency as compared to free and scrambled siRNA, comparable results with oligofectamine (109).
MVs	ND	siRNA	during biogenesis in transfected cells	Demonstrated the antitumor effect of microvesicle delivered anti-TGF-β1 siRNA *in vitro* and *in vivo* (118).
Exosomes	HiPerFect, FuGENE® HD	miRNA mimic or miRNA inhibitor	Electroporation	Exosomes provided higher miRNA inhibition and subsequently higher TNF-α level reduction as compared to commercially available transfection reagents. Significantly lower cytotoxicity *in vitro* as compared to commercial reagents. Efficient miR-155 mimic delivery to the liver *in vivo* (108).
OMVs[e]	ND	siRNA	Electroporation	Electroporation did not affect the properties of OMVs. High gene silencing and anticancer effect *in vitro* and *in vivo*. Even higher effect with targeted OMVs *in vivo*. No evidence for non-specific *in vivo* side effects were reported (119).

[a] as indicated by the authors, [b] commercially available or conventionally used carrier system, [c] the method used for nucleic acid loading into extracellular vesicles, [d] AAV stands for adeno associated virus which contains single stranded 4.7 kb long DNA (120), [e] OMV stands for bacterial outer membrane vesicles. **ND** – not defined.

The fact that researchers observed no evidence for non-specific *in vivo* side effects underlines the huge potential of using special bacterial strains as producers of biological membrane vesicles for drug delivery (119). Table 3 summarizes some recent studies conducted for NA delivery via EVs.

When considering a nanoparticulate system for therapeutics delivery, its ability to protect the cargo molecules against a variety of environmental factors is a pivotal feature. These include both biochemical factors (enzymatic degradation by nucleases) and physicochemical factors (oxidation, pH or temperature changes). Almost any pharmaceutical formulation study is accompanied by stability experiments to prove if the developed formulation will maintain loaded drug molecules or NAs in their native form for a suitably long period of time. Although this is very important from pharmaceutical viewpoint there are still only limited stability studies performed

with EVs. Example for long-term storage stability of EVs is the work of Ge and co-workers. They showed that plasma-derived EVs stored at -20°C are able to retain their miRNA content unchanged for 5 years (121). Limited number of studies showed that EVs were able to protect internalized RNA molecules from degradation by RNase (108,122). For the future, in addition to comprehensive storage stability experiments, *in vitro* nuclease treatment and serum stability studies would reveal valuable information about the shelf life, and possible *in vivo* stability concerns of experimentally designed EV systems for NA delivery.

BIODISTRIBUTION AND TARGETING STUDIES ON EVs

During development of new delivery systems, the biodistribution after administration to the body is a very important issue that should be investigated, as the site of distribution determines the efficacy and toxicity of drugs. So, in order to avoid side effects many drugs, especially those for cancer treatment, are desired to accumulate only at the diseased site of the body. Providing targeted delivery of drugs to the site of interest not only reduces the side effect but also increases the potency and efficacy of the treatment. Nanoparticulate drug delivery systems provide versatile ways of achieving targeted drug delivery. Owing to their nano-size they offer passive targeting to sites with enhanced capillary permeability. Also, they provide active drug targeting by attaching to their surface, targeting ligands which recognize specific molecules existing on the diseased cells. EVs share these common features of synthetic nanoparticles. Currently the biodistribution of EVs is being investigated with employment of four different strategies. These are 1) using lipophilic fluorescent dyes (see EV-1 in Figure 3) (84,88,91,123,124), 2) using radio conjugate molecules (see EV-2 in Figure 3) (88,125), 3) applying genetic engineering to label EVs with proteins (see EV-3 and EV-4 in Figure 3) (124,126,127), and 4) modifying the surface of EVs and chemically labeling them by click chemistry (see EV-5 in Figure 3) (128).

Gaussia luciferase fused to an exosomal membrane protein has been used for *in vivo* visualization and dynamic tracking of exosome biodistribution (126,127). Takahashi *et al*. suggested that labeling exosomes with a chemiluminescent protein probe could provide a means of *in vivo* tracking and quantification of exosomal biodistribution (126). For this purpose they constructed a plasmid encoding the fusion protein; gLuc-lactadherin. This fusion protein consists of two domains; one domain is the exosomal outer membrane protein (lactadherin), and the other domain is *Gaussia* luciferase domain (gLuc). B16-BL6 cells transfected with this plasmid produced exosomes that contain *Gaussia* luciferase on their surface which provided the *in vivo* imaging after intravenous administration and *Gaussia* luciferase substrate (coelenterazine) application. Their results showed that intravenously administered exosomes mainly distributed to liver and lungs of white BALB/c mice. 4 hours after the injection the fluorescence remained mainly in lungs and the spleen. They compared chemically labeled exosomes (with PKH26) to gLuc-lactadherin exosomes and observed comparable distribution patterns (126). Lai *et al*. also developed *in vivo* tracking method by coupling *Gaussia* luciferase to the EVs' surface (127). For this purpose they transduced HEK293T cells with viral vectors and used these cells to produce the luciferase conjugated EVs. They administered athymic nude mice intravenously with these exosomes and observed a predominant localization of these EVs in the spleen and liver. Authors noted that EVs coupled to *Gaussia* luciferase undergo faster elimination as compared to earlier reports with dye-labeled exosomes (see Refs. 8, 13, 14, 17, 22 in Lai et al., 2014). They also applied *Gaussia* luciferase-labeled exosomes to tumor-xenografted athymic nude mice, and observed that they were rapidly accumulated in subcutaneous tumor xenografts in the first 60 min after injection (127). This finding implied that EVs could be passively targeted to the tumor site by the Enhanced Permeability and Retention effect.

Morishita and coworkers showed once again that the main site for localization of the labeled exogenous exosomes is the mononuclear phagocyte system (liver, lungs and spleen). They provided pharmacokinetic parameters of the radiolabeled exosomes using a two-compartmental model (125). As an alternative strategy to *Gaussia* luciferase, they engineered cells with streptavidin-lactadherin fusion protein. These exosomes, carrying streptavidin on their surface, were able to bind a radiolabeled biotin complex [(3-^{125}I-iodobenzoyl)norbiotinamide] by simple incubation. This enabled quantitative measurement of the total amount of exosomes accumulated in each organ and improved the *in vivo* tracking of EVs (125). The radioactivity in the blood of BALB/c mice, intravenously administered with these radiolabeled exosomes

decreased rapidly and distributed to the mononuclear phagocyte system.

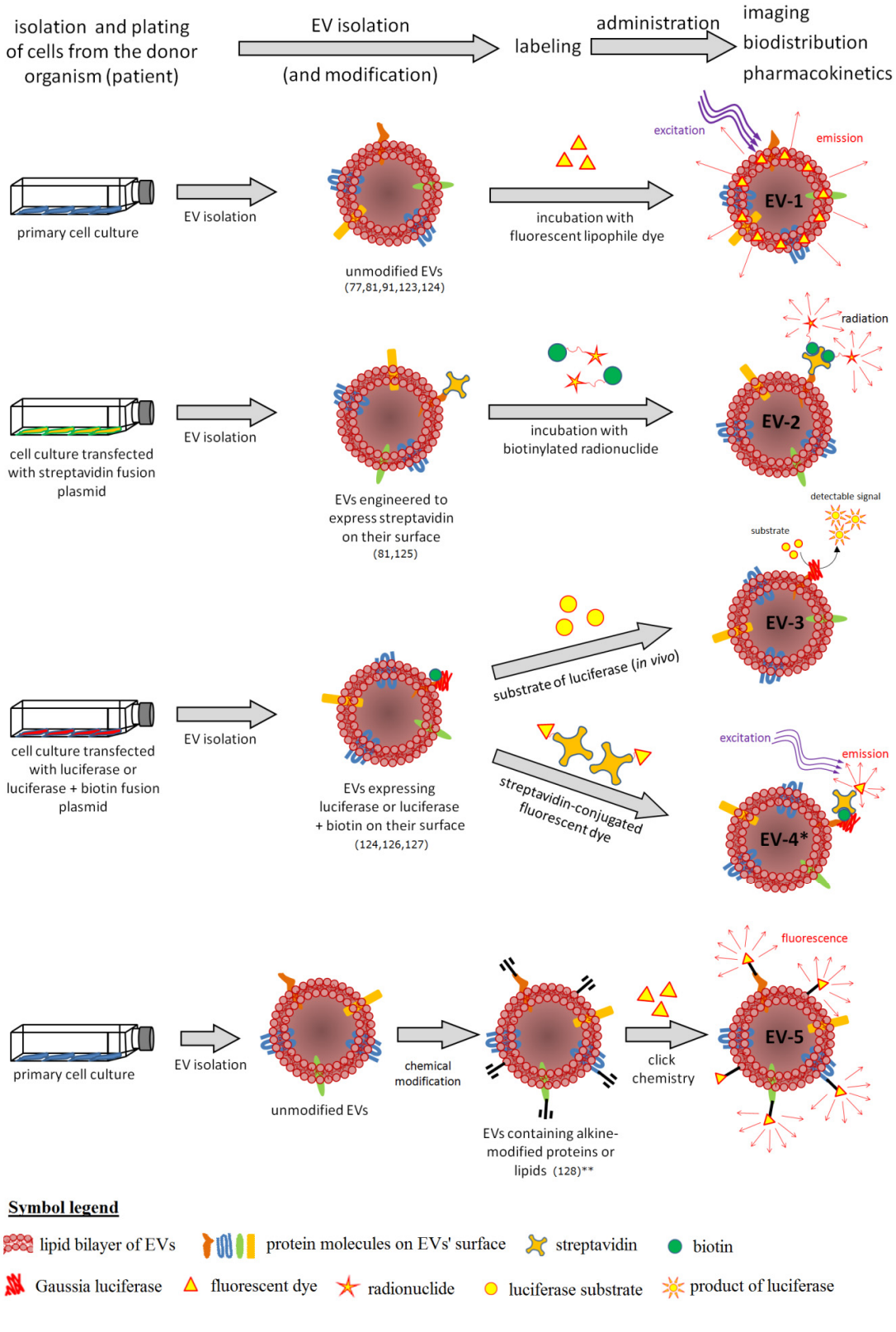

Figure 3. Representation of different strategies used for extracellular vesicle labeling. * Adapted with permission from ACS Nano 2014; 8(1): 483–494 American Chemical Society (127). ** Adapted with permission from Bioconjug Chem 2014; 25(10): 1777–1784. ©2014 American Chemical Society (128).

Grange *et al*. investigated the biodistribution of mesenchymal stem cell-derived, infrared dye-labeled EVs in nude mice with acute kidney injury (123). After intravenous administration of labeled EVs to the mice their distribution was visualized at different time points and researchers reported that EVs accumulated in the region of the injured kidneys, spleen and liver, and the signal retains in this region even after 24 hour. In healthy mice they observed signal only at the left dorsal and central abdominal region and concluded that this corresponded to spleen accumulation. Distribution in liver and spleen has also been shown in *ex vivo* images (123). They also compared two staining methods for EVs in order to be used for *in vivo* biodistribution studies; i) preincubated mesenchymal stem cells with the near infrared dyes DiD or DiI and after that isolated exosomes, or ii) initially isolated exsosomes from the cells and then incubated them with dyes. The second method provided better imaging and produced highest signal in injured kidneys.

Smyth et al. investigated the ability to functionalize the surface of exosomes by "click chemistry" (128). They firstly modified exosomal outer membrane proteins or lipids with alkine groups. After stepwise addition of a copper salt solution, *L*-ascorbic acid solution, bathophenanthrolinedisulfonic acid disodium salt trihydrate, and azide-fluor 545 and stirring for 3h at RT they labeled the exosomes' surface by the alkine-azide click chemistry (128). These experiments showed that the physicochemical properties of exosomes and their internalization by cells were not significantly affected by the click chemistry modification.

In a more recent study Smyth *et al*. investigated the biodistribution of the exosomes in comparison with liposomal formulations which are prepared with exosomal lipid extracts. They used fluorescent dye (1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindotricarbocyanine Iodide) (DIR) labeling or ^{111}In radiolabeling for *in vivo* biodistribution tracking and applied the formulations on different tumor bearing murine species (BALB/c, NU/J and NOD.CB17-Prkdcscid/J). It has been observed that after intravenous administration neither exosomes nor liposomes are able to accumulate significantly in the tumors. The main sites of distribution were liver and spleen followed by kidneys and lungs. After intratumoral administration, however, analysis of the excised tumors revealed that exosomes stay associated with the tumors to a significantly greater degree than liposomes. So, it was concluded that unmodified exosomes are not useful as a tumor-specific delivery system for systemic administration, but may rather be preferable for intratumoral delivery (88).

The knowledge about development of targeted EV-based delivery systems is based on limited research papers. This strategy mainly involves usage of cell strains engineered with special plasmid vectors that encode fusion proteins. These fusion proteins consist of an EV transmembrane domain and a targeting domain, as in the case with luciferase labeling. The first study for targeted EV preparation and testing is that of Alvarez-Erviti and coworkers (76). They isolated dendritic cells from mice and genetically engineered them to express neurospecific RVG peptide fused to exosomal membrane protein; Lamp2b. Exosomes produced by these cells expressed RVG peptide on their surface which provided active targeting to neuronal cells in the brain. siRNA loaded to the neuronal-targeted RVG-exosomes shoved specific gene silencing activity on different parts of the brain and haven't induced nonspecific knockdown in the liver and other organs as was the case with naked siRNAs (76). Similarly, Tian *et al*. engineered mouse immature dendritic cells with iRGD-Lamp2b fusion protein (87). This ensured production of exosomes capable of targeting αv integrin-expressing cancer cells. Compared to free drug, doxorubicin loaded to iRGD-targeted exosomes showed higher *in vitro* activity on MDA-MB-231 cancer cells. Besides, targeted exosomal doxorubicin exhibited higher *in vivo* antitumor activity in BALB/c nude mice. Significantly lower cardiac toxicity, a serious side effect of doxorubicin treatment, is observed with the targeted exosomal doxorubicin (87).

Ohno *et al*. engineered human embryonic kidney cells (HEK293) to express the transmembrane domain of platelet-derived growth factor receptor fused either to the epidermal growth factor receptor-binding peptide (GE11) or epidermal growth factor (EGF) (117). By this way they achieved targeting of miRNA-loaded EVs to EGFR overexpressing breast cancer cells (HCC70). In histological experiments researchers didn't find any organ damage in treated animals *in vivo*.

One challenge with the biodistribution and targeting studies of EVs is the incorporation of dyes, or other reporter molecules on the surface of EVs. Their presence on the membranes of EVs may lead to unpredicted reactions with the

immune system subsequently leading to rapid elimination by the RES. Additionally it is not certain whether the signal observed is originating from the EV-associated dye, or from free dye molecules (124). At cellular level, although the dye appears localized in a given body region, it is not known to what extent it internalizes in the cytoplasm of individual cells in this region. Therefore, both the effect of the labeling technique on the biodistribution of EVs, and the internalization property of the reporter molecules at cellular level should be investigated in more detail.

From the research conducted on biodistribution of EVs, it appears that following *in vivo* administration unmodified EVs tend to localize mainly in the reticuloendothelial system (RES). This, in fact, may not only be a result of the possible immune reactions with the labeling molecules, but also due to the fact that the majority of EVs used in these studies are obtained from the culture medium of commercially available or previously established cell lines. In this instance the EVs are not originating from the animals they are going to be administered to. So, it is likely that these EVs could carry antigenic proteins on their surface, and thus, are recognized by the RES of the animals. As to a recent report it was demonstrated that the biodistribution of EVs is dependent on the source of producer cells, the route of administration, and the presence of targeting ligands on the surface of EVs (124). Good examples about the difference between the localization of exosomes in syngeneic and allogeneic animals are also provided (76,124,129). Although it is concluded that there is no great difference between the biodistribution of EVs from individuals with distinct genetics, it appears that allogeneic and heterologous exosomes are more rapidly and extensively eliminated by the RES. Yet there are no real studies conducted with analogous EVs. It will be beneficial to conduct more studies that comparatively investigate the biodistribution pattern of analogous, syngeneic, allogeneic, and heterologous EVs in experimental animal models to determine if analogous EVs provide more satisfactory results such as escaping the RES and the immune system. The real question, what would be the possible fate of autologous (self) exosomes after intravenous administration remains unanswered. Table 4 summarizes recent biodistribution and targeting strategies on EV drug delivery. The table also provides a comparisaon of the sources of EV-producer cells and the experimental animal model used.

On the other hand, as the readily isolated and characterized cell lines provide the chance of straightforward applications, the establishment of primary cell culture from living animals in order to produce EVs with analogous character can be quite time-consuming and require special conditions like aseptic working area.

Another important issue which may concern not only the targeting ability, but also the labeling of EVs was pointed to be the degradation of targeting molecules on the surface of EVs during biogenesis which has recently been put on focus by Hung and Leonard (130). As transfection of producer cell lines appears the most relevant way of providing reporter/targeting ability to exosomes, this condition represents a challenge to overcome. It was shown that glycosylation is able to protect degradation of fusion proteins on EVs' surface and enhances the targeting potential (130).

While yet much research is needed to prove if there is a real benefit of autologous and syngeneic EVs over allogeneic ones in respect to their biodistribution and targeting ability, vesicles derived from prokaryotes, namely; bacterial outer membrane vesicles (OMVs) are also tested as targeted drug delivery vehicles for cancer therapy (119). Researchers used *Escherichia coli* strain with reduced endotoxicity to human cells and engineered the strain to express HER2 (human epidermal growth factor receptor-2) on the outer cell membrane which are later localized on released OMVs. Intravenous injection of these HER2 targeted OMVs resulted in targeted gene silencing and induced significant regression of tumor weight in a BALB/c nude mice xenografted with HER2 overexpressing HCC-1954 cells (119). No evidence for non-specific side effects was reported with OMVs *in vivo*.

CONCLUSIONS

The ability of EVs to incorporate intracellular substances and to transfer them efficiently to other cells, either endocrinally or paracrinally inspired scientists to introduce them in the field of drug and gene delivery. The advantage of EVs as drug delivery systems is mainly hidden in their feature to be of eobiotic origin, which means that they are composed of the same constituents of the organism to which they are going to be administered. They can be produced by virtually any cell type. Collecting cells from patients themselves and using these cells for production of self-derived nanosized vesicles for therapeutic molecule delivery could provide highly

biocompatible and minimally toxic delivery nanovehicles.

Table 4. Summary of labeling methods and targeting strategies with extracellular vesicles.

Reference	EV source	Cell line origin	Administered organism	Distribution tracking / visualization method	Targeting ligand
(84)	EL-C cells	C57BL/6n mouse lymphoma	C57BL/6j mice	IRDye-800CW fluorescent dye	-
(88)	4T1 cells	BALB/c fC3H mouse mammary gland tumor	BALB/c mice, NOD.CB17-Prkdcscid/j mice, Nude mice	DiR fluorescent dye	-
	PC3 and MCF7 cell lines	Human prostate and breast adenocarcinoma	Nude mice	^{111}In radiolabeling	-
(91)	Raw 264.7 cells	BALB/c mouse macrophages	C57BL/6 mice	Dil fluorescent dye	-
(123)	Mesenchymal stem cells	Human	CD1 nude mice	DiD fluorescent dye	-
(124)	Dendritic cells	C57BL/6 mouse bone marrow	NMRI mice	DiR fluorescent dye	RVG peptide
	Mesenchymal stem cells	Human bone marrow aspirates	NMRI mice	DiR fluorescent dye	-
	HEK293T cells	Human embryonic kidney	NMRI or C57BL/6 mice	DiR fluorescent dye or EGFP	-
	C2C12 cells	C3H mouse muscle tissue	NMRI mice	DiR fluorescent dye	-
	B16-F10 cells	C57BL/6j mouse melanoma	NMRI mice	DiR fluorescent dye	-
	OLN-93 cells	Rat oligodendrocytes	NMRI mice	DiR fluorescent dye	-
(125)	B16BL6 cells	C57BL/6 mouse melanona	BALB/c	^{125}I radiolabeling	-
(126)	B16BL6 cells	C57BL/6 mouse melanoma	C57BL/6 and BALB/c mice	*Gaussia* luciferase or PKH26	-
(127)	HEK293T cells	Human embryonic kidney	Nude mice	*Gaussia* luciferase or biotin-fused *Gaussia* luciferase	
(128)	4T1 cells	BALB/c fC3H mouse mammary gland tumor	*in vitro* tested	Azide-Fluor 545	-
(76)	Dendritic cells	C57BL/6 mouse bone marrow	C57BL/6 or BALB/c	na	RVG peptide or MSP peptide
(87)	Dendritic cells	Immature mouse	BALB/c nude mice	DiR fluorescent dye	iRGD peptide
(117)	HEK239 cells	Human embryonic kidney	RAG2$^{-/-}$ mice	DiR fluorescent dye	GE11 peptide
(119)	msbB mutant W3110 *E. coli*	K12-derived *E. coli* strain	BALB/c nude mice	Cy5.5-labeled siRNA	affi$_{HER2}$ peptide

na – not available

May be the most important hallmark of these self-derived, nanosized vesicles is their tremendous convenience for developing personalized nanomedicines.

As the field of extracellular vesicle research continuously produces advanced knowledge for their role in biological pathways, cell-cell communications, pathogenesis, disease progression, biomarker development etc., much work is yet to be performed to further understand their potential for drug delivery purposes. Firstly, although there is a substantial amount of knowledge concerning the biogenesis of EVs, exact mechanisms of their formation and release still remain to be clarified. While the post-isolation loading can be investigated in a way similar to the liposome research, little is known about the basic phenomena lying behind drug, protein or genetic material sorting into EVs during their biogenesis.

Secondly, compared to other conventional nanoparticle types, the difficulties opposing the development of a reproducible and calibrated method of EV production appear to be the greatest technical challenge for EVs in the field of therapeutics delivery. The producer cells, cell culture conditions, isolation and characterization techniques all need to be improved in order to obtain a reproducible and calibrated method of production of EVs with GMP compliance. They also cannot be subjected to procedures like homogenization and particle size reduction.

Thirdly, together with the assumptions for their great compatibility as self-derived vesicles, the safety concept of extracellular vesicles should also be clarified in more detail. For example some reports imply that possible future use of EVs as drug delivery vehicles should be accompanied by considerations of kidney health in order to avoid unwanted accumulation of drugs and consequent side effects. Once their safety aspect is clarified, these tiny self-derived nano-sized vesicles would boost the efficacy of many small molecule drugs and biopharmaceuticals.

In conclusion, drug and gene delivery through biologically derived nanovesicles is becoming more and more attractive subject for the medicine. According to the literature reports extracellular vesicles are expected to be less toxic, and more compatible with the host immune system than liposomes and other nanoparticles with synthetic origin. Furthermore, the great opportunity to isolate specific cells from a host organism and use them for production of extracellular vesicles with targeting moieties will pave the way for development of personalized nanomedicines of the future.

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