ROYAL SOCIETY OF CHEMISTRY View Article Online
DOI: 10.1039/C8LC00246K

Lab on a Chip

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Microfluidic Engineering of Exosomes: Editing Cellular Messages for Precision Therapeutics

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Studying extracellular vesicle (EV), particularly exosomes, is holding great promise, yet technically challenging in defining such small and molecularly diverse nanovesicles. With intrinsic molecular payload and biodegradability, molecular engineering of exosomes opens new avenues for mediating cellular responses and developing novel nano-delivery systems in precision therapeutics. Microfluidic lab-on-chip technology is taking pivotal roles in such emerging field. This review examines scientific advancements of microfluidic technology for engineering exosomes and assesses future applications and perspectives in developing precision therapeutics, which could serve the community by identifying potential new research areas or technologies that are urgently needed in precision therapeutics.

Introduction

Since the 2013 Nobel Prize in Medicine for the discovery of vesicles, substantial scientific interests have been devoted to a sub-group of vesicles called exosomes. Exosome-based precision medicine for cancer diagnosis and prognosis gains substantial attention and holds great promise[1-2]. However, studying exosomes is extremely challenging, due to enormous technical difficulties in defining and analyzing such small and molecularly diverse nanovesicles[3-5].

In this review, we discuss exosomes through the term of "extracellular vesicle", in order to give a clear elucidation. Extracellular vesicle (EV) is a loose term, which typically describes three types of vesicles: exosomes, microvesicles, and apoptotic bodies.[6] The major differences between these three vesicles are their cellular origins and molecular pathways. As illustrated in Fig. 1, the formation of exosomes begins with the creation of endosomes as intracellular vesicles.[7] Inward invagination occurs at the endosomal membrane, which creates small membrane-bound intraluminal vesicles (ILVs). At this point, the endosome is referred to as a multivesicular body (MVBs). The MVBs then follow one of two paths to exit: the lysosomal pathway or the secretory pathway.[8-9] In the lysosomal pathway, the MVB releases its contents into a lysosome for degradation. In the secretory pathway, the MVB fuses with the plasma membrane and secretes its contents into the extracellular space, which are now referred to as exosomes and range in size between 30-150 nm.

In contrast in Fig.1, microvesicles are formed directly through outward budding of the plasma membrane and can range from around 50 nm to 1 μm in diameter. In comparison to exosomes and microvesicles, apoptotic bodies fragmented from apoptotic cells are currently of little interest for therapeutic applications and will not be discussed in this perspective review.

Both exosomes and microvesicles contain a lipid bilayer and protein content derived from their parent cells.[10] However, due to their different biogenesis, the lipid bilayer of exosomes contains lipid types from both the plasma membrane and the Golgi apparatus, while microvesicles contain lipid types from the plasma membrane only[6]. The protein content of EVs reflects the presence of proteins in the parent cell at the time of formation[9]. As a result, both exosomes and microvesicles contain biomolecules specific to parent cellular function and status, such as, but not limited to, cytosolic proteins (tubulin, actin, actin-binding proteins), signal transduction proteins (protein kinases, 14-3-3, heterotrimeric G proteins), nucleic acids, metabolic enzymes (peroxidases, pyruvate, lipid kinases, enolase 1),

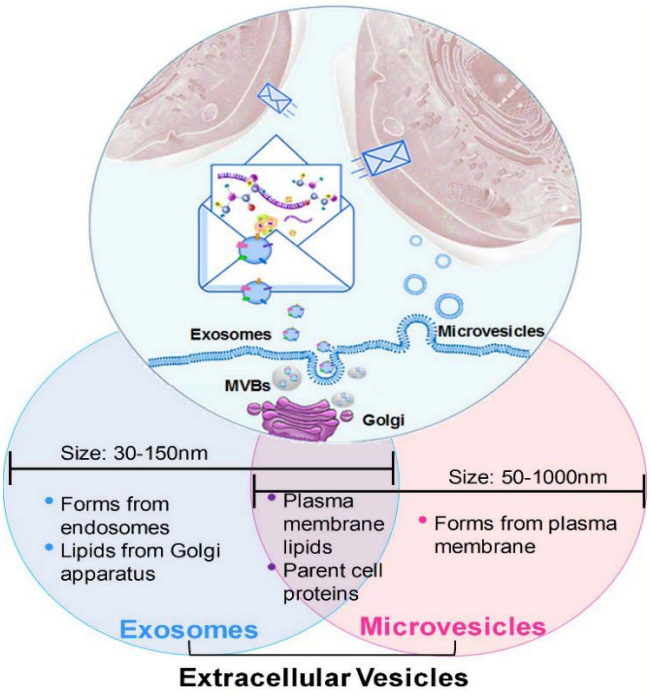

Fig. 1. Illustration of exosome biogenesis and comparison to microvesicles.

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DOI: 10.1039/C8LC00246K

tetraspanins, and heat shock proteins (HSP70, HSP90).[11-13] However, during the formation of ILVs, specific proteins and nucleic acids are sorted into the cytosolic interior and membrane of exosomes, making their surface and contents slightly different than microvesicles.[14] Although many studies have attempted to identify biomarkers specific only to exosomes, these studies collectively struggle to find such specific markers, likely due to the difficulties in completely isolating exosomes from microvesicles, and the heterogeneity of exosome subtypes found from parent cells. Certain markers, such as tetraspanins CD63, CD81, CD9, and CD45, FLOT1, Alix, HSP70, TFRC, and TSG101, have been used to detect the presence of exosomes. [15-17] Unfortunately, there are no good solutions to precisely differentiate exosomes from microvesicles, nor subtypes of exosomes secreted in variable cellular status. Current exosomes are pooled from a large population of cells, and the understanding of exosome biology completely stems from these ensemble-average measurements of exosome properties. There is still a long path for the clear elucidation of biogenesis, consistent classification of exosome subpopulations, and a good understanding of their molecular packaging.

Nevertheless, exosomes, sometimes so-called EVs, have been observed to play a vital role in communication, delivery, and mediation of diseases, without the need of cell-cell contact.[6, 9, 18-24] Upon their release from the parent cell, EVs can either bind to local cells, the extracellular matrix, or enter bodily fluids such as blood or cerebrospinal fluid.[9, 22] Such movement allows EVs to deliver important contents and signals to cells both locally and distant. In fact, upon injection of marked EVs into the bloodstream, they were found to be delivered to tissues around the body within minutes,[19] making them one of the fastest delivery vehicles. Meanwhile, markers on the EV surface act as targeting mechanisms, allowing them to bind to targeted cell types for mediating the exchange of genetic information and signal transductions.[9, 24] Three mechanisms have been proposed for interpreting the cellular uptake of exosomes: 1) Endocytotic mechanisms which engulf the exosome into the cell; 2) Fusion with the cell membrane directly for the release of contents into cytoplasm; 3) Receptor-ligand type interactions for signaling internalization.[6-7, 22-23];

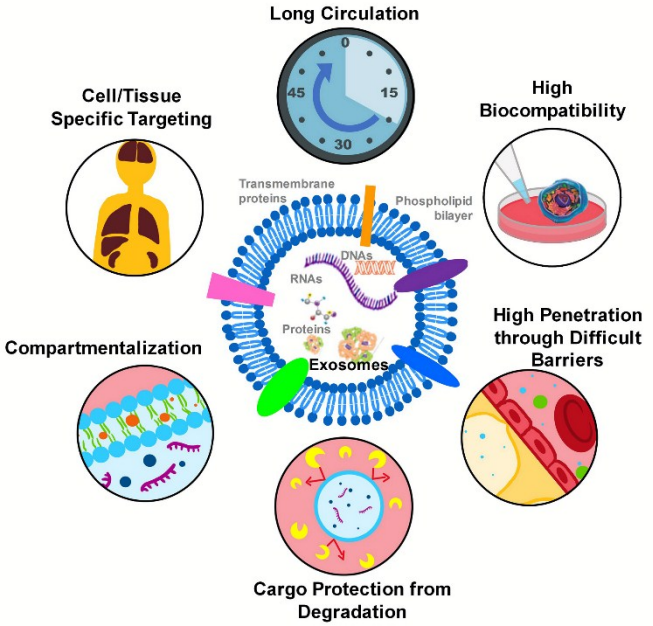

Fig. 2. Illustration of exosome structures and delivery advancements.

Due to EVs' large quantity in many bodily fluids and enclosure of a group of proteins and RNA representatives to their parent cells, EVs are very promising for precision medicine in diagnosing disease and potentially replacing invasive procedures such as biopsies.[20, 22, 25-26] Even more impressive is the expansive potential of EVs as a therapeutic delivery vehicle. It has been found that altered surface molecules on exosomes can avoid circulation clearance, such as blocking the scavenger receptor class A family (SR-A) to decrease liver clearance,[24] compared to natural exosomes without alteration undergoing rapid clearance through the kidneys, liver, spleen, and lungs.[6, 9,24] Additionally, the small size and slight negative charge of exosomes allow them to avoid clearance through the reticuloendothelial system (RES) and, in turn, decrease renal clearance[10, 23] and result in a longer circulation time at therapeutic sites. Future research on molecular engineering of exosomes could lead to even more tailored clearance routes.[27] Many studies have shown that engineering surface molecules on exosomes allows specific tissue targeting,[6, 19, 28-29] which opens a new avenue for speeding up precision therapeutics. Cancer immunotherapy could benefit the most from such engineered molecular targeting mechanisms, as it increases the accuracy of drug delivery and decreases systemic toxic effects of therapy. EVs have a natural tendency to accumulate in solid tumors due to the high penetration and enhanced retention in the dense tissue microenvironment. The abnormally formed blood vessels and surrounding compromised lymphatic system around tumor tissues also delay the efficient drainage of EVs, leading to the accumulation.[23-24] Another advantage of using EVs as a delivery vehicle is their ability to load both hydrophobic and hydrophilic contents, either in the interior or in the lipid bilayer.[22-23] The bilayer membrane effectively protects the cargo and prevents it from enzymatic degradation during circulation. [6, 23] Multiple signaling molecules and co-stimulating factors can be loaded at the same time for delivering into specific cell types.[22, 30] EVs have also demonstrated the ability to easily cross tissue boundaries and spread into deep tissues[10] such as the blood-brain barrier[6, 23, 31-32] and the blood-tumor barrier[22], which have traditionally been a challenge in delivery. EVs are highly biocompatible, biodegradable, stable, and exhibit low immunogenicity,[6, 23-24, 29, 33-34] and have been shown to aid regeneration,[19, 35-36] induce stem cell differentiation[35, 37] and specific immune responses.[33, 38] All of these traits, as summarized in Fig. 2, make EVs a promising therapeutic delivery device. This review focuses on the state-of-the-art approaches for engineering EVs or exosomes, as well as the important and innovative roles that microfluidic lab-on-chip technology can play in unlocking the power of EVs and exosomes.

Advances in Engineering Exosomes

Bioengineered exosomes as emerging immunotherapeutics gain substantial attention in developing a new generation of cancer vaccines[38-43], which have shown fascinating results in pre-clinical studies and early-phase clinical trials[39-40, 44-45] with increased stability, solubility, and bioavailability.[46] A recent phase-II trial which evaluated IFN-DC-derived exosomes loaded with MHC I/II restricted cancer antigens as maintenance immunotherapy for non-small cell

lung cancer patients[47] have shown the capability for promoting T cell and natural killer (NK) cell-based immune responses in patients.[41] Several Phase II clinical studies have recently been initiated as well for treating malignant ascites and pleural effusion using tumor cell-derived vesicles loaded with chemotherapeutic drugs (ClinicalTrials.gov). Exosome encapsulated drugs have proved to be valuable in addressing multiple clinical issues such as therapeutic resistance and the toxicity to central nervous system.[48-49] Exosomes are also very promising for gene therapies because they naturally perform horizontal transfer of genetic information to specific recipient cells in a pathophysiological contest, and preserve the functionality of genetic cargos.[50-52] The different surface markers on exosomes which are varied from types of parent cells could influence functional therapy.

Generally, there are two broad strategies for exosome engineering: 1) manipulation of parent cells either through genetic or metabolic engineering; 2) functionalization of purified exosomes using surface molecular engineering and membrane permeabilization.[53] We summarize the latest engineering strategies for modifying and reconstructing exosomes employed in drug delivery and cancer immunotherapy.

Surface Engineering

There are multiple strategies developed recently for molecular surface engineering exosomes, as illustrated in Fig. 3 with their advantages and drawbacks. Surface display technology via donor cell manipulation has been applied to modify exosome surface structures in targeted drug delivery.[54-59] Genetic modification of parent cells is a popular method to display potent proteins or peptides on the surface of exosome membranes.[59] In a study by Tian *et. al*, tumor-specific targeting was achieved by transfecting immature dendritic cells (DCs) for expressing Lamp2b exosomal membrane proteins fused with the breast cancer cell-specific iRGD peptide. Over 60% exosomes secreted from engineered DCs displayed Lamp2b on their surface. In addition, by growing parent cells in modified medium containing 40 μg/mL biotin-functionalized DSPE,[60] almost 100% exosomes can inherit biotinylated membrane from parent cells. Manipulating donor cells leads to the expression of markers on the exosome surface in high efficiency. However, the transfection efficiency is not consistent, which is highly dependent on RNA species. For instance, in a study by Kooijmans *et al.*, only 15-25% exosomes displayed anti-epidermal growth factor receptor (EGFR) nanobodies on their membrane via transfection of Neuro2A cells.[61]

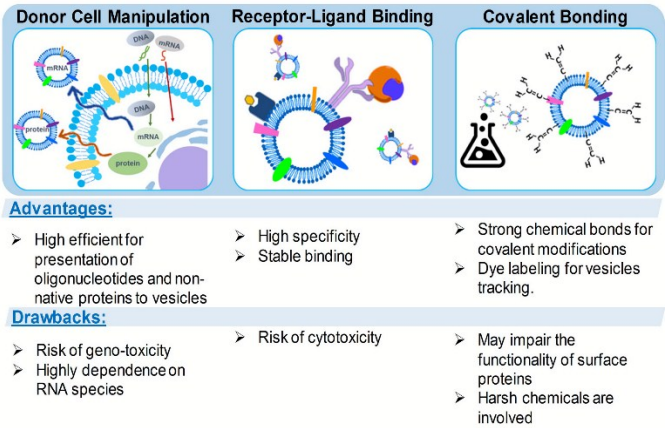

Fig. 3. Illustration of strategies for surface engineering exosomes.

Receptor-ligand binding can also be used for modifying the exosome surface. Qi *et al.* reported a technique by anchoring superparamagnetic nanoparticles onto reticulocytes-derived exosome surfaces through transferrin–transferrin receptor interactions,[62] which yielded superparamagnetic drug delivery for targeting diseased cells via responding to an external magnetic field. Alternatively, anchoring binding groups on exosome surfaces can be managed via transgene expression in parent cells. In a recent study, murine melanoma B16BL6 cells were transfected with a plasmid vector encoding streptavidin and lactadherin to obtain SAV-LA modified exosomes, which enabled the introduction of radioactive labeling of exosomes subsequently via streptavidin-biotin binding.[63] Maguire *et al.* introduced a method employing specific binding between transgenic biotin-acceptor peptides on the surface of exosome with biotinylated magnetic nanoparticles.[64] The receptor-ligand binding approach offers an effective way for exosome surface reconstruction. More importantly, this approach is highly specific for activating or eliminating signaling pathways associated with exosomal surface membrane proteins and receptors.

Covalent bonding has also been investigated for exosome surface engineering. Covalent bonds typically have bond energies in the range of 200-900 kJ mol^{-1}, which is much stronger than noncovalent interactions (cf. 2-13 kJ mol^{-1}). Unlike cells, exosomes are nonliving entities. Thus, the bioconjugation and "click chemistry" reactions can be introduced without concern of impairing biological activity.[65] Smyth *et al*. applied alkyne-based cross-linking reactions and successfully attached Azide-Fluor 545 fluorescent molecules to the surface of mouse 4T1 breast cancer cell-derived exosomes.[66] So far, conjugation chemistry reported no effect on the size of exosomes, nor any changes in adherence or internalization with recipient cells. However, chemically engineered exosomes are expected to affect bio-distribution. Efforts have been made to improve tracking of exosomes *in vivo* for studying bio-distribution.[67]

Membrane Permeabilization Mediated Cargo Loading

Due to the lipid membrane bilayer of EVs and exosomes, the hydrophobic drugs can be passively loaded via hydrophobic binding during the incubation. One successful example is for anti-inflammatory and antioxidant treatment using exosomes carrying curcumin, which is difficult to deliver in aqueous solutions traditionally.[68] Sun *et al.* developed curcumin-loaded mouse lymphoma EL-4 exosomes via direct incubation allowing membrane hydrophobic binding.[46] Haney *et al.* loaded a tetramer protein (250 kDa) into monocyte-derived exosomes by incubation at room temperature for 18 h.[69] However, the nonspecific hydrophobic binding between cargos and exosomes makes passive loading suffer from long incubation times and low loading capacity.

For delivery of hydrophilic compounds, such as RNA, the hydrophilicity actually prevents passive loading through the hydrophobic lipid bilayer membrane. Therefore, membrane permeabilization strategies adapted from the liposome field, including electroporation, sonication, direct transfection, and saponin permeabilization, have been developed for exosome cargo loading as compared in Fig. 4.[70] These methods are termed as active loading and all require the disruption of the exosome membrane, but they differ in terms of loading scalability and product quality.

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By creating small transient pores in the lipid bilayer membrane via the electrical field induced cross membrane potential, electroporation has been widely employed for cell transfection since 1980s.[71-72] Adapted in 2012, electroporation was successfully applied to load siRNA into exosomes by Alvarez-Erviti and colleagues.[73, 74 75] Subsequent systemic administration of engineered exosomes in mice showed the inhibition of Beta-Site APP-Cleaving Enzyme and protein expression in the brain. Walhgren *et al.* electroporated siRNA to plasma exosomes[76] for delivering to human monocytes and lymphocytes. Electroporation for exosome cargo loading minimizes perturbation of sensitive exosome components (e.g., ligands and receptors) without introducing additional chemicals. So far, no Joule heating-induced thermal damage was observed to membrane components, which is believed to be due to the application of instant electrical pulses in the millisecond range.[74]

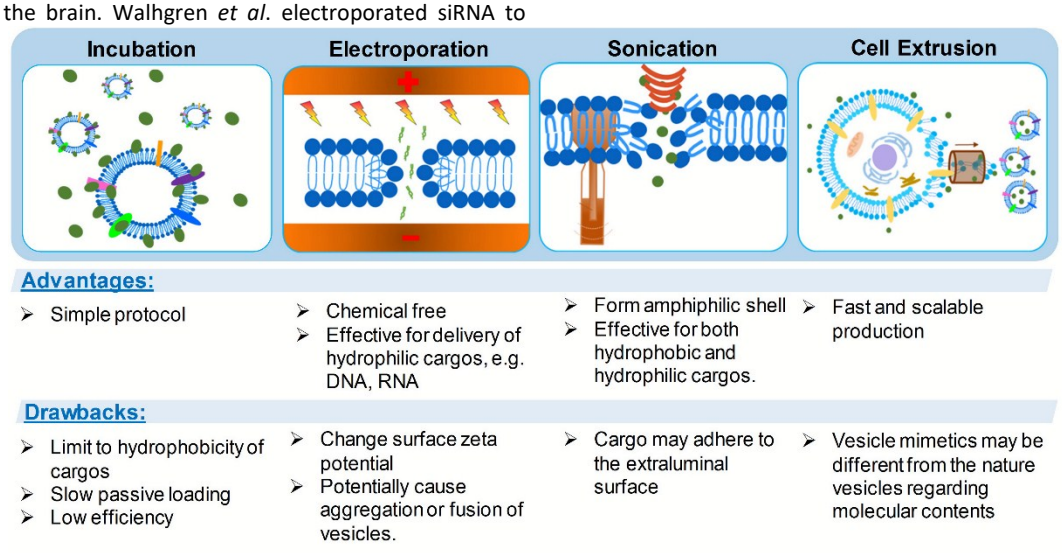

Fig. 4 Illustration of strategies for membrane permeabilization mediated cargo loading of exosomes.

Other commonly applied active cargo-loading methods for exosomes are sonication, freeze-thaw cycles, and incubation with membrane permeabilizers.[77-78] Haney *et al.* investigated several loading methods in order to load exosomes with the antioxidant enzyme catalase, including simple incubation at room temperature, saponin-mediated permeabilization, sonication, freeze-thaw cycles, and extrusion.[69] It was observed that reformed exosomes upon sonication, electroporation, as well as saponin, resulted in high loading efficiency, sustained release, and catalase preservation against degradation. However, sonication and extrusion-derived vesicles showed significant size increase via nanoparticle tracking analysis. Unfortunately, there has not been much investigation regarding the influence of these different methods on the delivery bioactivity of reconstructed exosomes and is worth exploring in the future.

Exosome mimetics are a type of vesicles produced by extruding donor cells through membrane filters with 100-400 nm pore size.[70] The vesicles are fabricated artificially by breaking up cells and then reforming the contents into exosome mimetics. This extrusion method can produce exosomes as high as 100-fold in quantity when compared to exosomes naturally released by cells.[79-81] By subjecting mammalian cells with doxorubicin to extrusion through a serial filtering device (e.g., pore sizes 10, 5 and 1 μm), Jang *et al.* generated high quantities of mimetic exosomes carrying sheltered drug.

Table 1. Summary of variable applications of engineered exosomes in precision therapeutics.

	Surface Engineering			Membrane Permeabilization			
	Cell Manipulation	Affinity Binding	Covalent bonding	Incubation	Electroporation	Sonication	Cell Extrusion (Vesicle Mimetics)
Exosome source	**Cell culture:** imDC,[29] HepG2[60], DCs from 57BL/6 mice[82], Neuro2A[61], MCA101[83]	**Cell culture:** Hela[84], HepG2[60] **Blood**[62]	**Cell culture:** 4T1 cell line[66], B16F10 cells (AHA-integrated exosomes)[85]	**Cell culture:** EL-4[46], U-87 MG[86], Raw 264.7[48, 87], U87[88] **Bovine milk**[89-90]	**Cell culture:** imDC[29], CRL 6475[91], DC from C57BL/6 mice[82], HEK293T[92], **Human plasma**[76]	**Cell culture:** Raw 64.7[48, 87]	**Cell suspension:** Raw 264.7[87], ES-D3[93], U937[79] **Grapefruit**[94]
Cargo type	iRGD peptide[29], biotin[60], Lamp2b[82], GPI linked nanobody[61], Chicken egg ovalbumin[83]	Lipofectamine LTX[84], Transferrin-conjugated superparamagnetic nanoparticle clusters[62], Avidin[60]	Azide-Fluor 545[66], DBCO-Cy3[85]	Curcumin[46, 89], rhodamine[86], paclitaxel[48, 86], doxorubicin[86], catalase[87], withaferin[89], anthocyanidin[89], paclitaxel[89], docetaxel[89], paclitaxel[90], hydrophobic siRNA[88]	Doxorubicin[29], Superparamagnetic iron oxide nanoparticles[91], siRNA[76, 82], dsDNA[92]	catalase[87], paclitaxel[48]	Catalase[87], Polystyrene beads[93], Doxorubicin[79], Inflammatory related receptor enriched plasma membrane[94]
Efficiency (%) or binding capacity	60%[29], 100%[60], Not reported[82-83], 15-25%[61]	Not reported[62, 84], 91%[60]	~1.5 alkyne groups in 150 kDa exosomal protein[66], 790 nM in 1 mg/mL exosome[85]	2.9 g in 1 g exosomes[46], 0.008-0.1 g in 1 g exosomes[86], 4.9%[87], 10-40%[89], 18.5% with saponion[90], 30%[88], 1.4%[48]	20%[29], 0.5 μg iron particles per μg exosome protein[91], 25%[82], 27%[76], 2%[92]	26.1%[87], 28%[48]	22.2%[87], 60%[93], 0.052-0.332 g in 1 g exosomes[79], 83%[94]

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The sheltered Doxorubicin can traffic to tumor tissue and reduce tumor growth.[79] Compared to the free drug, a 20-fold lower amount of drug was needed via exosome mimetics delivery for reducing tumor growth to the same extent. However, Fuhrmann *et al.* observed that harsh mechanical forces used in extrusion may cause the alteration of zeta potential on the surface of the exosomal membrane. Cytotoxicity was observed in the cell uptake experiment using extruded exosomes carrying porphyrin from MDA-MB231 breast cancer cells.[95] Although these observations were speculated for attributing to intensive extrusion process, further characterization of exosome mimetics is necessary.

Currently, the successful applications of exosomes in therapeutics are entirely dependent on the capacity of cargo loading. Another effective approach to load therapeutic nucleic acid cargos into exosomes is the modification of parent cells, i.e. through genetic engineering or medication with cytotoxic drugs. Variable therapeutic cargos, including small molecule compounds, proteins, and nucleic acid drugs, have been studied for loading into exosomes via different methods and techniques as summarized in Table 1. As research continues to progress, more side effects have been observed for several types of engineered exosomes. For instance, harsh electroporation conditions may trigger the aggregation of exosomes, and change their morphological characteristics.[96-98] The harsh extrusion conditions were reported to alter the zeta potential of the exosomes, which could cause cytotoxicity.[95] Consequently, surface-engineering of exosomes is widely accepted as a better alternative to liposomes and exosome mimetics.[58-59, 99]. Presently, there are still many challenges and pitfalls in this research field. As shown in Table 1, the cargo loading efficiency is still quite low (~ 30%) among all the approaches. In addition, exosomes released from cells are usually in a limited quantity with dynamic molecular contents. The technologies for quality control and mass production of exosomes are desperately needed to achieve fast, high-throughput, and highly-efficient cargo loading. Such concerns are currently being addressed by researchers employing microfluidic platforms.

Microfluidic Engineering of Exosomes

Molecular engineering offers the alluring prospect for making exosomes as versatile therapeutics beyond their native functions. The approaches for modifying exosomes are often adapted from well-established cell manipulation technologies, such as electroporation, sonication, incubation, etc. Exosomes are significantly smaller than cells, which results in a higher degree of membrane curvature with less surface area, and more difficult conditions for transfection. Microfluidic systems overcome many drawbacks of benchtop systems because they are intrinsically customizable, automatable, scalable, and capable of highly-efficient mass transport. In fact, microfluidic lab-on-chip technology has been proven as a highly effective method for triggering the fast growth of exosome research.[25, 100-104] Although the development is still at an early stage, microfluidic technology has proven its superior performance in isolation, molecular analysis, and detection[105-109]. As the advances of microfluidic technology, the high throughput analysis of EVs and exosomes has been achieved up to 100 μL/min, while multiple on-chip detection systems have been developed with detection limits as low as ~50 exosomes per μL.[25, 102], In addition to the significant contribution of microfluidic isolation and molecular analysis of EVs and exosomes, microfluidic engineering of exosomes has also emerged in recent years. Due to the relatively short history of EVs and exosomes being discovered, characterized, and utilized in therapeutics, only a few reports employing microfluidic technology for engineering exosomes, and the full potential and capability have not yet been well explored. In this section, we review the advances of microfluidic technology for engineering EVs and exosomes, which is the area we anticipate growing exponentially in the next five years. The future perspectives and pitfalls in precision therapeutics will be discussed as well.

Microfluidic Extrusion for Engineering Exosome Mimetics

Park's group has initiated the microfluidic extrusion method to generate exosome mimetics. In the first report in 2014, Jo *et al.* developed constriction microchannels with small dimensions for mechanically breaking down cells into mimetic exosomes.[110] The microfluidic device was shown in Fig 5a with a length of 100-400 μm and a width of 3 to 7 μm. When cells were squeezed into the microchannel by a syringe pump, the membranes were elongated due to the resistant shear force on the surface of the microchannel. As a result, the elongated lipid bilayer was broken and re-assembled as small nanoparticles thermodynamically. These exosome mimetics were fabricated endogenously using living embryonic stem cells containing mRNA, intracellular proteins, and plasma membrane

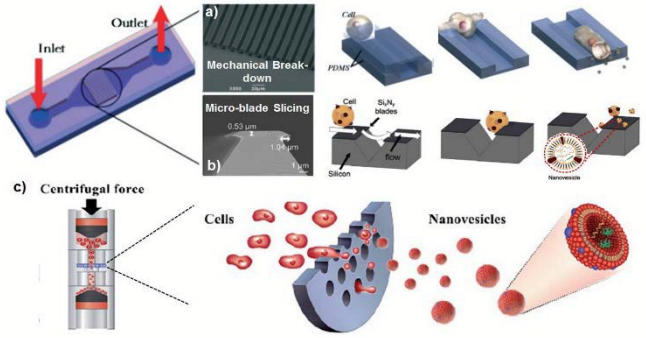

Fig. 5 The microfluidic extrusion method for fabricating exosome mimetics from donor cells. (a) Mechanical elongation and cellular breaking down into exosome mimetics. Adapted from Ref. 110 with permission from the Royal Society of Chemistry. (b) Micro-blade slicing of cells. Adapted from Ref. 111, with permission from Elsevier copyright 2015. (c) Micro-sized pore squeezing of cells into exosome mimetics. Figures were adapted from Ref. 113 with permission from the Royal Society of Chemistry.

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DOI: 10.1039/C8LC00214K

proteins at the stage of re-assembling. The size of formed mimetic exosomes was controllable and found to be dependent on the microchannel geometry (e.g., cross-section area and length) in a range of 60-120 nm. The successful cellular uptake of exosome mimetics encapsulated with cytosols staining was demonstrated. The similar delivery ability of engineered exosome mimetics was found as the naturally secreted exosomes, by comparing exogenous genes in the recipient cells. Using conventional soft-lithography and polydimethylsiloxane (PDMS) device molding, this microfluidic approach is facile and scalable, with a high flow throughput of 6.5 μL/min. However, the measured total amount of RNAs and proteins indicates that exosome mimetics were generated from about one-fifth of donor cells. Such small production may be partially due to cell clogging at the entrance of microchannels and adhering to the inside surface of the microchannel. Nevertheless, it holds promise for large-scale mimetic exosome production and modification.

An improved microfluidic device was reported by Park's group subsequently, which produces exosome mimetics by slicing living cell membrane with micro-fabricated silicon nitride blades (500 nm-thick), as shown in Fig. 5b.[111] The device fabrication used conventional bulk silicon fabrication processes and soft lithography. The fascinating part of this device is the patterning of cantilever-blades by growing a 100 nm-thick silicon oxide layer and subsequently depositing a 500 nm-thick Si_xN_y. The pattern of Si_xN_y is designable by using AZ photoresist and lithography, followed by inductively-coupled plasma reaction-ion-etching (RIE). The dry etching removes the Si_xN_y layer and the silicon oxide layer for exposing silicon substrate. Living cells entered into the flow are subjected to slicing by touching the sharp edge of silicon blade. The sliced cell fragments can re-assemble into exosome mimetics due to the minimization of free energy of lipid bilayers. The high throughput production of exosome mimetics was achieved at ~1.50×10^{10} vesicles with a particle size of ~100-300 nm per million cells. The number of produced vesicles is ~100 times higher than the number of exosomes secreted from the same number of cells.[112] Under an encapsulating test, ~30% of fluorescent beads were enveloped during cell fragment re-assembly. Compared to previous cell disruption methods enabled by mechanical shear forces,[110, 113] both the protein content and the number of vesicles were significantly higher using microfluidic cell slicing.

In a subsequent study, Jo *et al.* introduced a micro-filter device consisting of a polycarbonate filter with micro-sized pores, by utilizing the cell extrusion principle in micro-scale. Combined with a common centrifuge, the large-scale generation of cell-derived exosome mimetics can be achieved in high automation and efficiency.[113] As illustrated in Fig. 5C, exosome mimetics were directly produced by fragmenting cells during centrifugation (1×10^8 cells at 2000 rpm), due to the shear force and elongation of cells while passing through hydrophilic, micro-sized pores. The quantity of exosome mimetics produced by this centrifuge micro-device could be 250-fold higher than that from naturally secreted exosomes. Most importantly, the intracellular molecular contents were 2-fold higher compared to exosomes naturally secreted.

Microfluidic Surface Engineering of Living-cell Derived Exosomes

Compared to microfluidic extrusion approaches for randomly packaging and re-assembly, surface engineering is more promising for producing less impaired exosomes secreted from living cells. Although production throughput needs to be enhanced, microfluidic surface engineering of exosomes is an excellent approach for studying exosome packaging mechanisms, biogenesis, and understanding delivery signaling pathways. It has been shown that parent cells possess a sorting mechanism for guiding a selective subset of microRNAs to be loaded into exosomes, and a few proteins (e.g., Y-box protein 1[114], RNA-Binding Protein SYNCRIP[115]) may be involved in this sorting process[116]. Such evidence suggests that specific molecular sorting into exosomes may be a mechanism for long distance exporting and signal transduction[14, 117]. In contrast, the delivery bioactivity of exosome mimetics may require further validation for comparing naturally secreted exosomes with sorting mechanism.

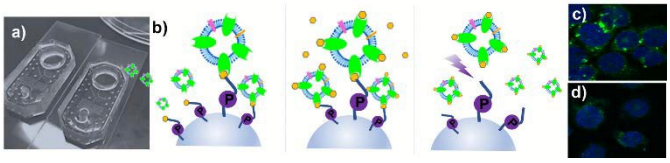

Fig. 6 Microfluidic surface engineering of living-cell derived exosomes. (a) 3D-molded PDMS microfluidic chip integrated with on-chip cell culture and streamlined surface engineering of culture derived exosomes. (b) Illustration of surface engineering process for exosome capture, surface binding, and photo-release. (c) Antigen-presenting cell uptake of gp-100 tumor peptide surface modified exosomes, compared with cellular uptake of native exosomes without surface engineering (d). The cellular nucleus was stained with DAPI in blue and exosomes were stained with PKH67 in green.

Compared to benchtop surface engineering approaches, microfluidic technology offers tremendous advancements. Due to the nature of micro-scale, mass transfer is much more efficient and able to achieve thousand-fold enhancements for fluids mixing, specific molecular binding, and transport.[118] The functional integration also allows multiple processing steps to be automated in one device for high throughput and scale up. Our research group recently introduced 3D printing technology for building microfluidic devices which adds the third-dimensional control over 3D microstructures for enhancing the mixing capability of microfluidics[119]. We devised a 3D-molded PDMS microfluidic chip integrated with on-chip cell culture and streamlined surface engineering of culture-derived exosomes. Fig. 6a shows the PDMS chip with integrated functionalities, including cell culture, exosomes capture, and surface engineering.[120] Current microfluidic technology has been well developed for exosome isolation and molecular analysis[25]. However, the processed exosomes are either in small quantities or bound to solid surfaces/particles, and unable to stay intact for downstream therapeutic preparations. In order to overcome such challenges, we introduced the photo-cleavable linker functionalized on magnetic nanoparticles for selectively capturing MHC-1 positive exosomes secreted from the on-chip culture (Fig. 6b). The immunogenic exosomes derived from dendritic cells carry an intrinsic payload of MHC class I and II molecules and other co-stimulatory molecules for mediating immune responses. Our microfluidic cell culture system allows the surface engineering of cultured immunogenic exosomes (MHC I+) with tumor antigenic peptides, meanwhile, the functional exosomes can be photo-released at downstream for immunity stimulation. We have tested the cellular uptake of engineered exosomes by antigen presenting cells, which showed much-improved internalization capability compared to non-engineered exosomes as shown in Fig. 6c

(engineered exosomes) and 6d (native exosomes), which is promising for developing novel cancer vaccines and delivery in immunotherapy.

Applications and Future Perspectives

Microfluidic technology has shown unique capability for speeding up exosome research towards precision medicine. However, the enormous potential has not been fully exploited yet. We envision that substantial research in investigating exosomes can be facilitated by using microfluidic technology. For instance, microfluidic platforms have been introduced in cell electroporation for the past two decades,[121] which will have great adaptability for electroporating extracellular vesicles and exosomes. Although it has not been reported so far for transfecting exosomes via microfluidic electroporation approach, several unmatched capabilities offered by microfluidic electroporation is obvious, including 1) Precisely controlled electric field and electric pulse in spatial and temporary for high-efficiency electroporation; 2) Microscale dimension allows low potential difference which greatly reduces side effects often seen by benchtop approach; 3) The pH variation and Joule heating caused by electric fields can be minimized in microscale; 4) The high functional integration, high throughput, and scale up are amenable and straightforward.

Precise liquid handling and mixing via microfluidic approach is another promising feature for engineering nanovesicles and exosomes. Laminar flow is a typical flow profile in microfluidic devices dominated by molecular diffusion, which can be precisely controlled for achieving high-efficient mass transport between streams with different species.[122] Efficient mixing within microfluidic channels has been proven to improve reaction rate,[123] and can be integrated with variable sample processing and molecular analysis.[122, 124-127] Our research group developed an ExoSearch chip with a serpentine microfluidic mixer previously to enhance immunomagnetic bead-based exosome isolation and detection.[105] This high-efficient mass transport, as well as a high surface to volume ratio of microstructures perfectly meet the needs of surface engineering, either for delivering hydrophobic therapeutics to exosome membrane or facilitating affinity binding reactions.

Exosomes are increasingly being recognized as contributing factors in many diseases, and their potential as therapeutics holds substantial promise for developing exciting strategies in drug delivery and cancer immunotherapy.[128] Thus, bioengineering of exosomes is becoming more and more important. Traditional benchtop methods for exosome modifications will continue to play a significant role in the future. However, as microfluidic technology develops, we believe microfluidic approaches will eventually replace benchtop methods for engineering exosomes in speeding up precision therapeutics. With the advances of on-chip cell culture technologies,[129-131] a fully integrated microfluidic system, including cell culture, exosomes isolation and engineering, as well as exosome-mediated therapeutic delivery will be an essential research direction for understanding exosome biogenesis fundamentally, and seeking novel therapeutic strategies.

Exosomes are secreted from all living cells and can be harvested from a variety of sources, including cow milk, plant, bacterium, and variable human bodily fluids as shown in Fig. 7. Thus, the heterogeneous subtypes and complicated molecular contents presented in exosomes pose a daunting challenge for precision engineering and processing of exosomes. In fact, microfluidic technology can play a unique role in solving this challenge by integrating with sophisticated sample preparation functionalities, such as sorting, filtration, subtyping and molecular probing. The high

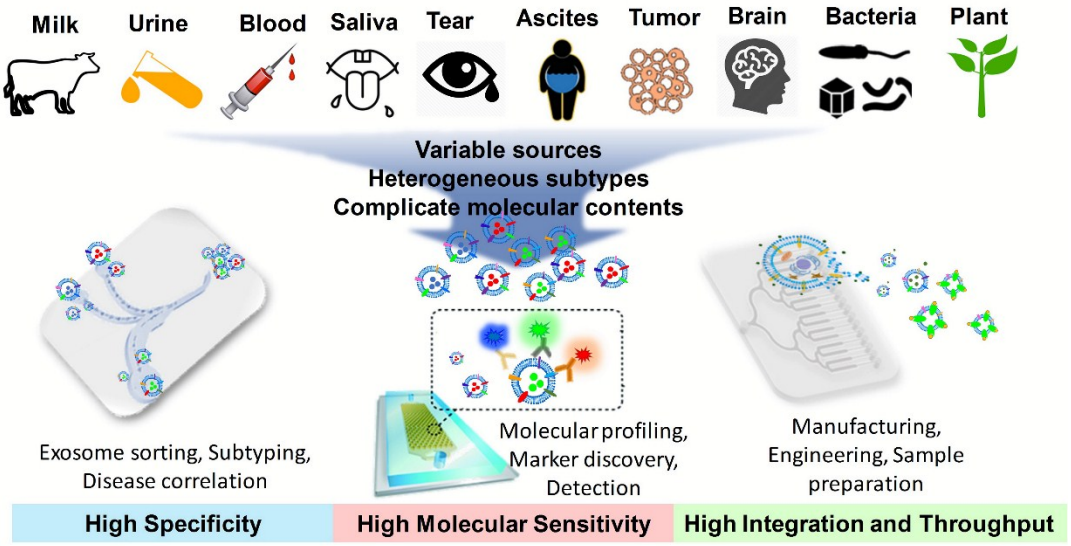

Fig. 7 Illustration of the "power" of microfluidic technology in exosome research for speeding up precision medicine.

specificity and high sensitivity offered by microfluidic approach would also contribute to high-resolution manipulation of exosomes for clinical applications. Presently, the biggest challenge for microfluidic engineering of exosomes still lies in the processing volume that is required for meeting large-scale therapeutic demands. Although there has been an attempt at large-scale production of exosome mimetics via microfluidic cell extrusion, the molecular contents of engineered exosomes still need to be well characterized for meeting the desired bioactivity. Thus, interfacing high throughput cell culture and combining continuous flow processing are necessary. Considering that substantial quantities of exosomes are needed to achieve a therapeutic effect,[132] highly scalable approaches for the

mass production of therapeutic exosomes will be emerging for future precision therapeutics but has not been explored yet. In addition, there is also a continuous need to deal with the manufacturing, storage, and administration of therapeutic exosomes, which has not been well standardized. Nevertheless, the potential therapeutic values of EVs and exosomes have been increasingly promising. As a versatile tool, microfluidic technology is expected to fully unlock the potential of these diverse nanovesicles in the near future for speeding up precision medicine.

Author contributions

In the authorship, Dr. Qingfu Zhu and Mikala Heon contribute to the original draft and preparation of review manuscript, Zheng Zhao contributes to the data collection, and Dr. Mei He contributes to the conceptualization, review and editing, and supervision.

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

Our work is funded by USDA-NIFA 2017-67021-26600, NIH NIGMS P20 GM103418, and NIH NIGMS P20 GM103638.

References

1. Hodson, R., Precision medicine. *Nature* **2016,** *537* (7619), S49.
2. Mora, E. M.; Alvarez-Cubela, S.; Oltra, E., Biobanking of Exosomes in the Era of Precision Medicine: Are We There Yet? *Int J Mol Sci* **2015,** *17* (1).
3. Wu, M.; Ouyang, Y.; Wang, Z.; Zhang, R.; Huang, P. H.; Chen, C.; Li, H.; Li, P.; Quinn, D.; Dao, M.; Suresh, S.; Sadovsky, Y.; Huang, T. J., Isolation of exosomes from whole blood by integrating acoustics and microfluidics. *Proc Natl Acad Sci U S A* **2017,** *114* (40), 10584-10589.
4. Yamashita, T.; Takahashi, Y.; Nishikawa, M.; Takakura, Y., Effect of exosome isolation methods on physicochemical properties of exosomes and clearance of exosomes from the blood circulation. *Eur J Pharm Biopharm* **2016,** *98*, 1-8.
5. Zeringer, E.; Barta, T.; Li, M.; Vlassov, A. V., Strategies for isolation of exosomes. *Cold Spring Harb Protoc* **2015,** *2015* (4), 319-23.
6. Barile, L.; Vassalli, G., Exosomes: Therapy delivery tools and biomarkers of diseases. *Pharmacology & Therapeutics* **2017,** *174* (Supplement C), 63-78.
7. Tkach, M.; Théry, C., Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. *Cell* **2016,** *164* (6), 1226-1232.
8. Kowal, J.; Tkach, M.; Thery, C., Biogenesis and secretion of exosomes. *Curr Opin Cell Biol* **2014,** *29*, 116-25.
9. Robbins, P. D.; Morelli, A. E., Regulation of immune responses by extracellular vesicles. *Nature Reviews Immunology* **2014,** *14*, 195.
10. Luan, X.; Sansanaphongpricha, K.; Myers, I.; Chen, H.; Yuan, H.; Sun, D., Engineering exosomes as refined biological nanoplatforms for drug delivery. *Acta Pharmacologica Sinica* **2017,** *38*, 754.
11. Thery, C.; Zitvogel, L.; Amigorena, S., Exosomes: composition, biogenesis and function. *Nat Rev Immunol* **2002,** *2* (8), 569-79.
12. Akers, J. C.; Gonda, D.; Kim, R.; Carter, B. S.; Chen, C. C., Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *J Neurooncol* **2013,** *113* (1), 1-11.
13. Colombo, M.; Raposo, G.; Thery, C., Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol* **2014,** *30*, 255-89.
14. Stoorvogel, W., Resolving sorting mechanisms into exosomes. *Cell Res* **2015,** *25* (5), 531-2.
15. Soo, C. Y.; Song, Y.; Zheng, Y.; Campbell, E. C.; Riches, A. C.; Gunn-Moore, F.; Powis, S. J., Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. *Immunology* **2012,** *136* (2), 192-197.
16. Kalra, H.; Adda, C. G.; Liem, M.; Ang, C.-S.; Mechler, A.; Simpson, R. J.; Hulett, M. D.; Mathivanan, S., Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. *PROTEOMICS* **2013,** *13* (22), 3354-3364.
17. Dragovic, R. A.; Collett, G. P.; Hole, P.; Ferguson, D. J. P.; Redman, C. W.; Sargent, I. L.; Tannetta, D. S., Isolation of syncytiotrophoblast microvesicles and exosomes and their characterisation by multicolour flow cytometry and fluorescence Nanoparticle Tracking Analysis. *Methods* **2015,** *87*, 64-74.
18. Hurwitz, S. N.; Conlon, M. M.; Rider, M. A.; Brownstein, N. C.; Meckes, D. G., Nanoparticle analysis sheds budding insights into genetic drivers of extracellular vesicle biogenesis. *Journal of Extracellular Vesicles* **2016,** *5* (1), 31295.
19. Teixeira, J. H.; Silva, A. M.; Almeida, M. I.; Barbosa, M. A.; Santos, S. G., Circulating extracellular vesicles: Their role in tissue repair and regeneration. *Transfusion and Apheresis Science* **2016,** *55* (1), 53-61.
20. Fuhrmann, G.; Herrmann, I. K.; Stevens, M. M., Cell-derived vesicles for drug therapy and diagnostics: Opportunities and challenges. *Nano Today* **2015,** *10* (3), 397-409.
21. Gehrmann, U.; Näslund, T. I.; Hiltbrunner, S.; Larssen, P.; Gabrielsson, S., Harnessing the exosome-induced immune response for cancer immunotherapy. *Seminars in Cancer Biology* **2014,** *28* (Supplement C), 58-67.
22. Tang, X.-J.; Sun, X.-Y.; Huang, K.-M.; Zhang, L.; Yang, Z.-S.; Zou, D.-D.; Wang, B.; Warnock, G. L.; Dai, L.-J.; Luo, J., Therapeutic potential of CAR-T cell-derived exosomes: a cell-free modality for targeted cancer therapy. *Oncotarget* **2015,** *6* (42), 44179-44190.
23. Ren, J.; He, W.; Zheng, L.; Duan, H., From structures to functions: insights into exosomes as promising drug delivery vehicles. *Biomaterials Science* **2016,** *4* (6), 910-921.
24. Wang, J.; Zheng, Y.; Zhao, M., Exosome-Based Cancer Therapy: Implication for Targeting Cancer Stem Cells. *Frontiers in Pharmacology* **2017,** *7* (533).
25. He, M.; Zeng, Y., Microfluidic Exosome Analysis toward Liquid Biopsy for Cancer. *J Lab Autom* **2016,** *21* (4), 599-608.
26. Contreras-Naranjo, J. C.; Wu, H. J.; Ugaz, V. M., Microfluidics for exosome isolation and analysis: enabling liquid biopsy for personalized medicine. *Lab Chip* **2017,** *17* (21), 3558-3577.
27. Parodi, A.; Molinaro, R.; Sushnitha, M.; Evangelopoulos, M.; Martinez, J. O.; Arrighetti, N.; Corbo, C.; Tasciotti, E., Bio-inspired

engineering of cell- and virus-like nanoparticles for drug delivery. *Biomaterials* **2017,** *147*, 155-168.

28. Xitong, D.; Xiaorong, Z., Targeted therapeutic delivery using engineered exosomes and its applications in cardiovascular diseases. *Gene* **2016,** *575* (2, Part 2), 377-384.

29. Tian, Y.; Li, S.; Song, J.; Ji, T.; Zhu, M.; Anderson, G. J.; Wei, J.; Nie, G., A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. *Biomaterials* **2014,** *35* (7), 2383-2390.

30. Tian, X.; Zhu, M.; Nie, G., How can nanotechnology help membrane vesicle-based cancer immunotherapy development? *Human Vaccines & Immunotherapeutics* **2013,** *9* (1), 222-225.

31. Sterzenbach, U.; Putz, U.; Low, L.-H.; Silke, J.; Tan, S.-S.; Howitt, J., Engineered Exosomes as Vehicles for Biologically Active Proteins. *Molecular Therapy* **2017,** *25* (6), 1269-1278.

32. Gilligan, K. E.; Dwyer, R. M., Engineering Exosomes for Cancer Therapy. *International Journal of Molecular Sciences* **2017,** *18* (6), 1-12.

33. Tan, A.; De La Peña, H.; Seifalian, A. M., The application of exosomes as a nanoscale cancer vaccine. *International Journal of Nanomedicine* **2010,** *5*, 889-900.

34. Chen, H.; Zeng, Y.; Liu, W.; Zhao, S.; Wu, J.; Du, Y., Multifaceted applications of nanomaterials in cell engineering and therapy. *Biotechnology Advances* **2013,** *31* (5), 638-653.

35. Huang, C.-C.; Narayanan, R.; Alapati, S.; Ravindran, S., Exosomes as biomimetic tools for stem cell differentiation: Applications in dental pulp tissue regeneration. *Biomaterials* **2016,** *111* (Supplement C), 103-115.

36. Su, S.-A.; Xie, Y.; Fu, Z.; Wang, Y.; Wang, J.-A.; Xiang, M., Emerging role of exosome-mediated intercellular communication in vascular remodeling. *Oncotarget* **2017,** *8* (15), 25700-25712.

37. Jin, Y.; Lee, J. S.; Min, S.; Park, H.-J.; Kang, T. J.; Cho, S.-W., Bioengineered Extracellular Membranous Nanovesicles for Efficient Small-Interfering RNA Delivery: Versatile Platforms for Stem Cell Engineering and In Vivo Delivery. *Advanced Functional Materials* **2016,** *26* (32), 5804-5817.

38. Morishita, M.; Takahashi, Y.; Matsumoto, A.; Nishikawa, M.; Takakura, Y., Exosome-based tumor antigens–adjuvant co-delivery utilizing genetically engineered tumor cell-derived exosomes with immunostimulatory CpG DNA. *Biomaterials* **2016,** *111* (Supplement C), 55-65.

39. Syn, N. L.; Wang, L.; Chow, E. K.; Lim, C. T.; Goh, B. C., Exosomes in Cancer Nanomedicine and Immunotherapy: Prospects and Challenges. *Trends Biotechnol* **2017,** *35* (7), 665-676.

40. Bell, B. M.; Kirk, I. D.; Hiltbrunner, S.; Gabrielsson, S.; Bultema, J. J., Designer exosomes as next-generation cancer immunotherapy. *Nanomedicine* **2016,** *12* (1), 163-9.

41. Pitt, J. M.; André, F.; Amigorena, S.; Soria, J.-C.; Eggermont, A.; Kroemer, G.; Zitvogel, L., Dendritic cell–derived exosomes for cancer therapy. *The Journal of Clinical Investigation* **2016,** *126* (4), 1224-1232.

42. Tian, H.; Li, W., Dendritic cell-derived exosomes for cancer immunotherapy: hope and challenges. *Ann Transl Med* **2017,** *5* (10), 221.

43. Viaud, S.; Théry, C.; Ploix, S.; Tursz, T.; Lapierre, V.; Lantz, O.; Zitvogel, L.; Chaput, N., Dendritic Cell-Derived Exosomes for Cancer Immunotherapy: What's Next? *Cancer Research* **2010,** *70* (4), 1281.

44. Tran, T. H.; Mattheolabakis, G.; Aldawsari, H.; Amiji, M., Exosomes as nanocarriers for immunotherapy of cancer and inflammatory diseases. *Clin Immunol* **2015,** *160* (1), 46-58.

45. Greening, D. W.; Gopal, S. K.; Xu, R.; Simpson, R. J.; Chen, W., Exosomes and their roles in immune regulation and cancer. *Semin Cell Dev Biol* **2015,** *40*, 72-81.

46. Sun, D.; Zhuang, X.; Xiang, X.; Liu, Y.; Zhang, S.; Liu, C.; Barnes, S.; Grizzle, W.; Miller, D.; Zhang, H.-G., A Novel Nanoparticle Drug Delivery System: The Anti-inflammatory Activity of Curcumin Is Enhanced When Encapsulated in Exosomes. *Molecular Therapy* **2010,** *18* (9), 1606-1614.

47. Besse, B.; Charrier, M.; Lapierre, V.; Dansin, E.; Lantz, O.; Planchard, D.; Le Chevalier, T.; Livartoski, A.; Barlesi, F.; Laplanche, A.; Ploix, S.; Vimond, N.; Peguillet, I.; Théry, C.; Lacroix, L.; Zoernig, I.; Dhodapkar, K.; Dhodapkar, M.; Viaud, S.; Soria, J.-C.; Reiners, K. S.; Pogge von Strandmann, E.; Vély, F.; Rusakiewicz, S.; Eggermont, A.; Pitt, J. M.; Zitvogel, L.; Chaput, N., Dendritic cell-derived exosomes as maintenance immunotherapy after first line chemotherapy in NSCLC. *Oncoimmunology* **2016,** *5* (4), e1071008.

48. Kim, M. S.; Haney, M. J.; Zhao, Y.; Mahajan, V.; Deygen, I.; Klyachko, N. L.; Inskoe, E.; Piroyan, A.; Sokolsky, M.; Okolie, O.; Hingtgen, S. D.; Kabanov, A. V.; Batrakova, E. V., Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells. *Nanomedicine: Nanotechnology, Biology and Medicine 12* (3), 655-664.

49. Heusermann, W.; Hean, J.; Trojer, D.; Steib, E.; von Bueren, S.; Graff-Meyer, A.; Genoud, C.; Martin, K.; Pizzato, N.; Voshol, J.; Morrissey, D. V.; Andaloussi, S. E. L.; Wood, M. J.; Meisner-Kober, N. C., Exosomes surf on filopodia to enter cells at endocytic hot spots, traffic within endosomes, and are targeted to the ER. *The Journal of Cell Biology* **2016,** *213* (2), 173.

50. Melo, Sonia A.; Sugimoto, H.; O'Connell, Joyce T.; Kato, N.; Villanueva, A.; Vidal, A.; Qiu, L.; Vitkin, E.; Perelman, Lev T.; Melo, Carlos A.; Lucci, A.; Ivan, C.; Calin, George A.; Kalluri, R., Cancer Exosomes Perform Cell-Independent MicroRNA Biogenesis and Promote Tumorigenesis. *Cancer Cell 26* (5), 707-721.

51. Mizrak, A.; Bolukbasi, M. F.; Ozdener, G. B.; Brenner, G. J.; Madlener, S.; Erkan, E. P.; Strobel, T.; Breakefield, X. O.; Saydam, O., Genetically engineered microvesicles carrying suicide mRNA/protein inhibit schwannoma tumor growth. *Mol Ther* **2013,** *21* (1), 101-8.

52. Stewart, M. P.; Sharei, A.; Ding, X.; Sahay, G.; Langer, R.; Jensen, K. F., In vitro and ex vivo strategies for intracellular delivery. *Nature* **2016,** *538*, 183.

53. Armstrong, J. P. K.; Holme, M. N.; Stevens, M. M., Re-Engineering Extracellular Vesicles as Smart Nanoscale Therapeutics. *ACS Nano* **2017,** *11* (1), 69-83.

54. El-Andaloussi, S.; Lee, Y.; Lakhal-Littleton, S.; Li, J.; Seow, Y.; Gardiner, C.; Alvarez-Erviti, L.; Sargent, I. L.; Wood, M. J. A., Exosome-mediated delivery of siRNA in vitro and in vivo. *Nature Protocols* **2012,** *7*, 2112.

55. Kuate, S.; Cinatl, J.; Doerr, H. W.; Überla, K., Exosomal vaccines containing the S protein of the SARS coronavirus induce high levels of neutralizing antibodies. *Virology* **2007,** *362* (1), 26-37.

56. Yim, N.; Ryu, S.-W.; Choi, K.; Lee, K. R.; Lee, S.; Choi, H.; Kim, J.; Shaker, M. R.; Sun, W.; Park, J.-H.; Kim, D.; Do Heo, W.; Choi, C.,

Exosome engineering for efficient intracellular delivery of soluble proteins using optically reversible protein–protein interaction module. *Nature Communications* **2016,** *7*, 12277.

57. Stickney, Z.; Losacco, J.; McDevitt, S.; Zhang, Z.; Lu, B., Development of exosome surface display technology in living human cells. *Biochemical and Biophysical Research Communications* **2016,** *472* (1), 53-59.

58. Hood, J. L., Post isolation modification of exosomes for nanomedicine applications. *Nanomedicine* **2016,** *11* (13), 1745-1756.

59. Kutralam-Muniasamy, G.; Perez-Guevara, F., Recombinant surface engineering to enhance and expand the potential of biologically produced nanoparticles: A review. *Process Biochemistry* **2017,** *59* (Part A), 4-17.

60. Wang, J.; Li, W.; Zhang, L.; Ban, L.; Chen, P.; Du, W.; Feng, X.; Liu, B. F., Chemically Edited Exosomes with Dual Ligand Purified by Microfluidic Device for Active Targeted Drug Delivery to Tumor Cells. *ACS Appl Mater Interfaces* **2017,** *9* (33), 27441-27452.

61. Kooijmans, S. A. A.; Aleza, C. G.; Roffler, S. R.; van Solinge, W. W.; Vader, P.; Schiffelers, R. M., Display of GPI-anchored anti-EGFR nanobodies on extracellular vesicles promotes tumour cell targeting. *Journal of Extracellular Vesicles* **2016,** *5* (1), 31053.

62. Qi, H.; Liu, C.; Long, L.; Ren, Y.; Zhang, S.; Chang, X.; Qian, X.; Jia, H.; Zhao, J.; Sun, J.; Hou, X.; Yuan, X.; Kang, C., Blood Exosomes Endowed with Magnetic and Targeting Properties for Cancer Therapy. *ACS Nano* **2016,** *10* (3), 3323-3333.

63. Morishita, M.; Takahashi, Y.; Nishikawa, M.; Ariizumi, R.; Takakura, Y., Enhanced Class I Tumor Antigen Presentation via Cytosolic Delivery of Exosomal Cargos by Tumor-Cell-Derived Exosomes Displaying a pH-Sensitive Fusogenic Peptide. *Molecular Pharmaceutics* **2017,** *14* (11), 4079-4086.

64. Maguire, C. A.; Balaj, L.; Sivaraman, S.; Crommentuijn, M. H. W.; Ericsson, M.; Mincheva-Nilsson, L.; Baranov, V.; Gianni, D.; Tannous, B. A.; Sena-Esteves, M.; Breakefield, X. O.; Skog, J., Microvesicle-associated AAV Vector as a Novel Gene Delivery System. *Molecular Therapy* **2012,** *20* (5), 960-971.

65. Oude Blenke, E.; Klaasse, G.; Merten, H.; Plückthun, A.; Mastrobattista, E.; Martin, N. I., Liposome functionalization with copper-free "click chemistry". *Journal of Controlled Release* **2015,** *202* (Supplement C), 14-20.

66. Smyth, T.; Petrova, K.; Payton, N. M.; Persaud, I.; Redzic, J. S.; Graner, M. W.; Smith-Jones, P.; Anchordoquy, T. J., Surface Functionalization of Exosomes Using Click Chemistry. *Bioconjugate Chemistry* **2014,** *25* (10), 1777-1784.

67. Takahashi, Y.; Nishikawa, M.; Shinotsuka, H.; Matsui, Y.; Ohara, S.; Imai, T.; Takakura, Y., Visualization and in vivo tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection. *J Biotechnol* **2013,** *165* (2), 77-84.

68. Gupta, S. C.; Patchva, S.; Aggarwal, B. B., Therapeutic roles of curcumin: lessons learned from clinical trials. *AAPS J* **2013,** *15* (1), 195-218.

69. Haney, M. J.; Klyachko, N. L.; Zhao, Y.; Gupta, R.; Plotnikova, E. G.; He, Z.; Patel, T.; Piroyan, A.; Sokolsky, M.; Kabanov, A. V.; Batrakova, E. V., Exosomes as drug delivery vehicles for Parkinson's disease therapy. *Journal of Controlled Release* **2015,** *207* (Supplement C), 18-30.

70. Sutaria, D. S.; Badawi, M.; Phelps, M. A.; Schmittgen, T. D., Achieving the Promise of Therapeutic Extracellular Vesicles: The Devil is in Details of Therapeutic Loading. *Pharm Res* **2017,** *34* (5), 1053-1066.

71. Sardesai, N. Y.; Weiner, D. B., Electroporation delivery of DNA vaccines: prospects for success. *Curr Opin Immunol* **2011,** *23* (3), 421-9.

72. Impellizeri, J. A.; Ciliberto, G.; Aurisicchio, L., Electro-gene-transfer as a new tool for cancer immunotherapy in animals. *Vet Comp Oncol* **2014,** *12* (4), 310-8.

73. Wahlgren, J.; Statello, L.; Skogberg, G.; Telemo, E.; Valadi, H., Delivery of Small Interfering RNAs to Cells via Exosomes. In *SiRNA Delivery Methods: Methods and Protocols*, Shum, K.; Rossi, J., Eds. Springer New York: New York, NY, 2016; pp 105-125.

74. Weaver, J. C., Electroporation: A general phenomenon for manipulating cells and tissues. *Journal of Cellular Biochemistry* **1993,** *51* (4), 426-435.

75. Alvarez-Erviti, L.; Seow, Y.; Yin, H.; Betts, C.; Lakhal, S.; Wood, M. J. A., Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nature Biotechnology* **2011,** *29*, 341.

76. Wahlgren, J.; Karlson, T. D. L.; Brisslert, M.; Vaziri Sani, F.; Telemo, E.; Sunnerhagen, P.; Valadi, H., Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes. *Nucleic Acids Research* **2012,** *40* (17), e130-e130.

77. Luan, X.; Sansanaphongpricha, K.; Myers, I.; Chen, H.; Yuan, H.; Sun, D., Engineering exosomes as refined biological nanoplatforms for drug delivery. *Acta Pharmacol Sin* **2017,** *38* (6), 754-763.

78. Vader, P.; Mol, E. A.; Pasterkamp, G.; Schiffelers, R. M., Extracellular vesicles for drug delivery. *Adv Drug Deliv Rev* **2016,** *106* (Pt A), 148-156.

79. Jang, S. C.; Kim, O. Y.; Yoon, C. M.; Choi, D.-S.; Roh, T.-Y.; Park, J.; Nilsson, J.; Lötvall, J.; Kim, Y.-K.; Gho, Y. S., Bioinspired Exosome-Mimetic Nanovesicles for Targeted Delivery of Chemotherapeutics to Malignant Tumors. *ACS Nano* **2013,** *7* (9), 7698-7710.

80. Jang, S. C.; Gho, Y. S., Could bioengineered exosome-mimetic nanovesicles be an efficient strategy for the delivery of chemotherapeutics? *Nanomedicine* **2014,** *9* (2), 177-180.

81. Lunavat, T. R.; Jang, S. C.; Nilsson, L.; Park, H. T.; Repiska, G.; Lässer, C.; Nilsson, J. A.; Gho, Y. S.; Lötvall, J., RNAi delivery by exosome-mimetic nanovesicles – Implications for targeting c-Myc in cancer. *Biomaterials* **2016,** *102* (Supplement C), 231-238.

82. Alvarez-Erviti, L.; Seow, Y. Q.; Yin, H. F.; Betts, C.; Lakhal, S.; Wood, M. J. A., Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* **2011,** *29* (4), 341-U179.

83. Zeelenberg, I. S.; Ostrowski, M.; Krumeich, S.; Bobrie, A.; Jancic, C.; Boissonnas, A.; Delcayre, A.; Le Pecq, J. B.; Combadiere, B.; Amigorena, S.; Thery, C., Targeting tumor antigens to secreted membrane vesicles in vivo induces efficient antitumor immune responses. *Cancer Res* **2008,** *68* (4), 1228-35.

84. Nakase, I.; Futaki, S., Combined treatment with a pH-sensitive fusogenic peptide and cationic lipids achieves enhanced cytosolic delivery of exosomes. *Sci Rep* **2015,** *5*, 10112.

85. Wang, M.; Altinoglu, S.; Takeda, Y. S.; Xu, Q., Integrating Protein Engineering and Bioorthogonal Click Conjugation for Extracellular Vesicle Modulation and Intracellular Delivery. *PLOS ONE* **2015,** *10* (11), e0141860.

86. Yang, T.; Martin, P.; Fogarty, B.; Brown, A.; Schurman, K.; Phipps, R.; Yin, V. P.; Lockman, P.; Bai, S., Exosome delivered anticancer drugs

across the blood-brain barrier for brain cancer therapy in Danio rerio. *Pharm Res* **2015,** *32* (6), 2003-14.
87. Haney, M. J.; Klyachko, N. L.; Zhao, Y.; Gupta, R.; Plotnikova, E. G.; He, Z.; Patel, T.; Piroyan, A.; Sokolsky, M.; Kabanov, A. V.; Batrakova, E. V., Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J Control Release* **2015,** *207*, 18-30.
88. Didiot, M.-C.; Hall, L. M.; Coles, A. H.; Haraszti, R. A.; Godinho, B. M. D. C.; Chase, K.; Sapp, E.; Ly, S.; Alterman, J. F.; Hassler, M. R.; Echeverria, D.; Raj, L.; Morrissey, D. V.; DiFiglia, M.; Aronin, N.; Khvorova, A., Exosome-mediated Delivery of Hydrophobically Modified siRNA for Huntingtin mRNA Silencing. *Molecular Therapy* **2016,** *24* (10), 1836-1847.
89. Munagala, R.; Aqil, F.; Jeyabalan, J.; Gupta, R. C., Bovine milk-derived exosomes for drug delivery. *Cancer Lett* **2016,** *371* (1), 48-61.
90. Agrawal, A. K.; Aqil, F.; Jeyabalan, J.; Spencer, W. A.; Beck, J.; Gachuki, B. W.; Alhakeem, S. S.; Oben, K.; Munagala, R.; Bondada, S.; Gupta, R. C., Milk-derived exosomes for oral delivery of paclitaxel. *Nanomedicine: Nanotechnology, Biology and Medicine* **2017,** *13* (5), 1627-1636.
91. Hu, L.; Wickline, S. A.; Hood, J. L., Magnetic resonance imaging of melanoma exosomes in lymph nodes. *Magn Reson Med* **2014**.
92. Lamichhane, T. N.; Raiker, R. S.; Jay, S. M., Exogenous DNA Loading into Extracellular Vesicles via Electroporation is Size-Dependent and Enables Limited Gene Delivery. *Mol Pharm* **2015,** *12* (10), 3650-7.
93. Yoon, J.; Jo, W.; Jeong, D.; Kim, J.; Jeong, H.; Park, J., Generation of nanovesicles with sliced cellular membrane fragments for exogenous material delivery. *Biomaterials* **2015,** *59*, 12-20.
94. Wang, Q.; Ren, Y.; Mu, J.; Egilmez, N. K.; Zhuang, X.; Deng, Z.; Zhang, L.; Yan, J.; Miller, D.; Zhang, H.-G., Grapefruit-Derived Nanovectors Use an Activated Leukocyte Trafficking Pathway to Deliver Therapeutic Agents to Inflammatory Tumor Sites. *Cancer Research* **2015,** *75* (12), 2520.
95. Fuhrmann, G.; Serio, A.; Mazo, M.; Nair, R.; Stevens, M. M., Active loading into extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins. *Journal of Controlled Release* **2015,** *205* (Supplement C), 35-44.
96. Hood, J. L.; Scott, M. J.; Wickline, S. A., Maximizing exosome colloidal stability following electroporation. *Analytical Biochemistry* **2014,** *448* (Supplement C), 41-49.
97. Johnsen, K. B.; Gudbergsson, J. M.; Skov, M. N.; Christiansen, G.; Gurevich, L.; Moos, T.; Duroux, M., Evaluation of electroporation-induced adverse effects on adipose-derived stem cell exosomes. *Cytotechnology* **2016,** *68* (5), 2125-38.
98. Kooijmans, S. A. A.; Stremersch, S.; Braeckmans, K.; de Smedt, S. C.; Hendrix, A.; Wood, M. J. A.; Schiffelers, R. M.; Raemdonck, K.; Vader, P., Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles. *Journal of Controlled Release* **2013,** *172* (1), 229-238.
99. van der Meel, R.; Fens, M. H. A. M.; Vader, P.; van Solinge, W. W.; Eniola-Adefeso, O.; Schiffelers, R. M., Extracellular vesicles as drug delivery systems: Lessons from the liposome field. *Journal of Controlled Release* **2014,** *195* (Supplement C), 72-85.
100. Liga, A.; Vliegenthart, A. D. B.; Oosthuyzen, W.; Dear, J. W.; Kersaudy-Kerhoas, M., Exosome isolation: a microfluidic road-map. *Lab on a Chip* **2015,** *15* (11), 2388-2394.
101. Yang, F.; Liao, X.; Tian, Y.; Li, G., Exosome separation using microfluidic systems: size-based, immunoaffinity-based and dynamic methodologies. *Biotechnology Journal* **2017,** *12* (4), 1600699-n/a.
102. Contreras-Naranjo, J. C.; Wu, H.-J.; Ugaz, V. M., Microfluidics for exosome isolation and analysis: enabling liquid biopsy for personalized medicine. *Lab on a Chip* **2017,** *17* (21), 3558-3577.
103. Salafi, T.; Zeming, K. K.; Zhang, Y., Advancements in microfluidics for nanoparticle separation. *Lab on a Chip* **2017,** *17* (1), 11-33.
104. Gholizadeh, S.; Shehata Draz, M.; Zarghooni, M.; Sanati-Nezhad, A.; Ghavami, S.; Shafiee, H.; Akbari, M., Microfluidic approaches for isolation, detection, and characterization of extracellular vesicles: Current status and future directions. *Biosens Bioelectron* **2017,** *91*, 588-605.
105. Zhao, Z.; Yang, Y.; Zeng, Y.; He, M., A microfluidic ExoSearch chip for multiplexed exosome detection towards blood-based ovarian cancer diagnosis. *Lab Chip* **2016,** *16* (3), 489-96.
106. Zhang, P.; He, M.; Zeng, Y., Ultrasensitive microfluidic analysis of circulating exosomes using a nanostructured graphene oxide/polydopamine coating. *Lab Chip* **2016,** *16* (16), 3033-42.
107. Shao, H.; Chung, J.; Balaj, L.; Charest, A.; Bigner, D. D.; Carter, B. S.; Hochberg, F. H.; Breakefield, X. O.; Weissleder, R.; Lee, H., Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy. *Nat Med* **2012,** *18* (12), 1835-40.
108. Im, H.; Shao, H.; Park, Y. I.; Peterson, V. M.; Castro, C. M.; Weissleder, R.; Lee, H., Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. *Nat Biotechnol* **2014,** *32* (5), 490-5.
109. Im, H.; Lee, K.; Weissleder, R.; Lee, H.; Castro, C. M., Novel nanosensing technologies for exosome detection and profiling. *Lab Chip* **2017,** *17* (17), 2892-2898.
110. Jo, W.; Jeong, D.; Kim, J.; Cho, S.; Jang, S. C.; Han, C.; Kang, J. Y.; Gho, Y. S.; Park, J., Microfluidic fabrication of cell-derived nanovesicles as endogenous RNA carriers. *Lab on a Chip* **2014,** *14* (7), 1261-1269.
111. Yoon, J.; Jo, W.; Jeong, D.; Kim, J.; Jeong, H.; Park, J., Generation of nanovesicles with sliced cellular membrane fragments for exogenous material delivery. *Biomaterials* **2015,** *59* (Supplement C), 12-20.
112. Théry, C.; Amigorena, S.; Raposo, G.; Clayton, A., Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids. In *Current Protocols in Cell Biology*, John Wiley & Sons, Inc.: 2001.
113. Jo, W.; Kim, J.; Yoon, J.; Jeong, D.; Cho, S.; Jeong, H.; Yoon, Y. J.; Kim, S. C.; Gho, Y. S.; Park, J., Large-scale generation of cell-derived nanovesicles. *Nanoscale* **2014,** *6* (20), 12056-12064.
114. Shurtleff, M. J.; Temoche-Diaz, M. M.; Karfilis, K. V.; Ri, S.; Schekman, R., Y-box protein 1 is required to sort microRNAs into exosomes in cells and in a cell-free reaction. *Elife* **2016,** *5*.
115. Santangelo, L.; Giurato, G.; Cicchini, C.; Montaldo, C.; Mancone, C.; Tarallo, R.; Battistelli, C.; Alonzi, T.; Weisz, A.; Tripodi, M., The RNA-Binding Protein SYNCRIP Is a Component of the Hepatocyte Exosomal Machinery Controlling MicroRNA Sorting. *Cell Rep* **2016,** *17* (3), 799-808.
116. Zhang, J.; Li, S.; Li, L.; Li, M.; Guo, C.; Yao, J.; Mi, S., Exosome and exosomal microRNA: trafficking, sorting, and function. *Genomics Proteomics Bioinformatics* **2015,** *13* (1), 17-24.

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DOI: 10.1039/C8LC00246K

117. Villarroya-Beltri, C.; Baixauli, F.; Gutierrez-Vazquez, C.; Sanchez-Madrid, F.; Mittelbrunn, M., Sorting it out: regulation of exosome loading. *Semin Cancer Biol* **2014,** *28*, 3-13.
118. Kuo, J. S.; Chiu, D. T., Controlling mass transport in microfluidic devices. *Annu Rev Anal Chem (Palo Alto Calif)* **2011,** *4*, 275-96.
119. Plevniak, K.; Campbell, M.; Myers, T.; Hodges, A.; He, M., 3D printed auto-mixing chip enables rapid smartphone diagnosis of anemia. *Biomicrofluidics* **2016,** *10* (5), 054113.
120. Z. Zhao, J. S., M. He, On-chip harvesting and photo-release of immunogenic extracellular vesicles for cancer immunotherapy. *Proceedings of the 21st International Conference on Miniaturized Systems for Chemistry and Life Sciences* **2017**, 898-899.
121. Wang, S. N.; Lee, L. J., Micro-/nanofluidics based cell electroporation. *Biomicrofluidics* **2013,** *7* (1).
122. Lee, C.-Y.; Chang, C.-L.; Wang, Y.-N.; Fu, L.-M., Microfluidic Mixing: A Review. *International Journal of Molecular Sciences* **2011,** *12* (5), 3263.
123. Seong, G. H.; Crooks, R. M., Efficient Mixing and Reactions within Microfluidic Channels Using Microbead-Supported Catalysts. *Journal of the American Chemical Society* **2002,** *124* (45), 13360-13361.
124. Ward, K.; Fan, Z. H., Mixing in microfluidic devices and enhancement methods. *J Micromech Microeng* **2015,** *25* (9).
125. Suh, Y. K.; Kang, S., A Review on Mixing in Microfluidics. *Micromachines* **2010,** *1* (3), 82.
126. Johnson, T. J.; Ross, D.; Locascio, L. E., Rapid Microfluidic Mixing. *Analytical Chemistry* **2002,** *74* (1), 45-51.
127. Kevin, W.; Fan, Z. H., Mixing in microfluidic devices and enhancement methods. *Journal of Micromechanics and Microengineering* **2015,** *25* (9), 094001.
128. Perkel, J. M., Membrane messengers: Extracellular vesicles. *Science* **2016,** *352* (6291), 1349-1351.
129. Halldorsson, S.; Lucumi, E.; Gómez-Sjöberg, R.; Fleming, R. M. T., Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices. *Biosensors and Bioelectronics* **2015,** *63* (Supplement C), 218-231.
130. Yang, Y.; Leong, K. W., Chapter 1 - Microfluidic Cell Culture Platforms with Embedded Nanoscale Features. In *Microfluidic Cell Culture Systems*, William Andrew Publishing: Oxford, 2013; pp 3-26.
131. Xiao, Y.; Zhang, B.; Hsieh, A.; Thavandiran, N.; Martin, C.; Radisic, M., Chapter 12 - Microfluidic Cell Culture Techniques. In *Microfluidic Cell Culture Systems*, William Andrew Publishing: Oxford, 2013; pp 303-321.
132. Wilhelm, S.; Tavares, A. J.; Dai, Q.; Ohta, S.; Audet, J.; Dvorak, H. F.; Chan, W. C. W., Analysis of nanoparticle delivery to tumours. *Nature Reviews Materials* **2016,** *1*, 16014.

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DOI: 10.1039/C8LC00246K

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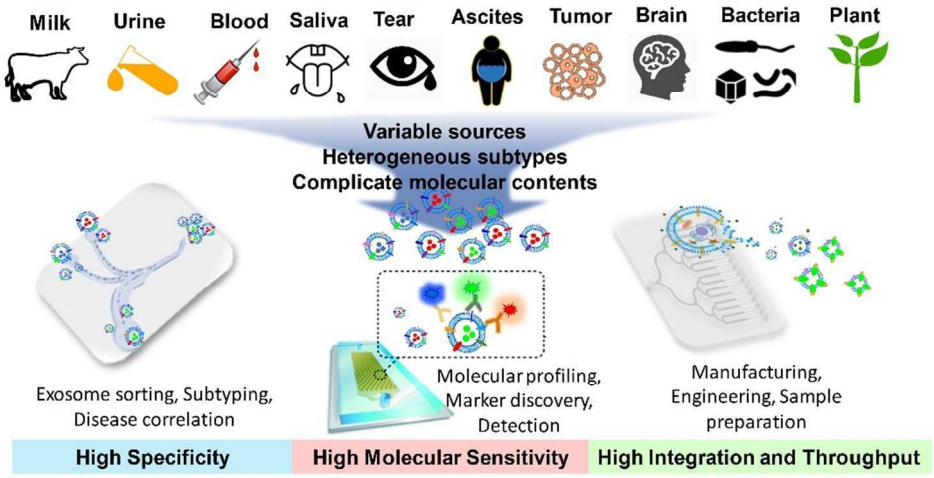

This review examines scientific advancements of microfluidic technology for engineering exosomes and assesses future applications and perspectives in developing precision therapeutics, which could serve the community by identifying potential new research areas or technologies that are urgently needed in precision therapeutics.