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Microfluidics for extracellular vesicle separation and mimetic synthesis: Recent advances and future perspectives

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ABSTRACT

Extracellular vesicles (EVs) play a critical role in the regulation of various biological processes and pathologies, and have significant utility as potential diagnostic biomarkers and drug delivery systems. That said, conventional methods for EV manipulation and analysis suffer from several drawbacks, including low yield and/or purity, complexity and high cost. For these reasons, there has been growing interest in the development of microfluidic-based tools for fast and efficient EV processing. Herein, we first highlight some of the most interesting recent advances in microfluidic technologies for the separation of EVs, as well as the synthesis of EV mimetics for drug delivery applications. We then discuss the advantages and disadvantages of currently available technologies and provide opinion on some of the most important future challenges and areas of application.

1. Introduction

Extracellular vesicles (EVs) are lipid bilayer-delimited species that are liberated from a cell but, unlike a cell, cannot replicate. These cell-derived membranous structures can be broadly separated into two primary groups: exosomes (between 50 and 150 nm in size) that originate from intraluminal vesicles (ILVs) generated within endosomal-derived multivesicular bodies (MVBs), and microvesicles (between 50 and 500 nm in size) that are shed from the plasma membrane (Fig. 1) [1]. In contrast to other membrane budding events involved in intracellular trafficking, these vesicles are generated by budding from the cytosol, thereby allowing both specific and non-specific loading of cytoplasmic cargo into their lumen and membranes. Several studies have demonstrated that a diversity of EVs (with respect to RNA and protein cargos) can be detected in a range of biofluids (including blood, saliva, urine, breast milk, cerebrospinal fluid, amniotic fluid and ascites), opening the exciting possibility that they could be used as biomarkers reporting human health and disease [2,3]. Indeed, *in vitro* [4] and *in vivo* [5] experiments have shown that EV can shuttle functional proteins, and RNAs-encoded messages to neighbouring cells, thereby not only opening new channels for cell-to-cell communication, but also allowing the design of new therapeutic strategies, notably by engineering EVs for delivering RNA therapeutics. For example, modification of EV-associated RNA profiles has been associated with autoimmune diseases [6], asthma [7], cardiovascular diseases [8], cancers [9], and infectious diseases [10]. However, these promising applications are still hindered by significant gaps in our current understanding of the molecular and cellular pathways underlying EV biogenesis and cargo encapsulation. Conventional methods for EV isolation suffer from difficulties associated with purification, complex multi-step workflows, and high associated costs [11]. Moreover, batch-to-batch variations when engineering EVs for clinical applications also challenge the scaling-up production process [12]. However, recent advances in microfluidic technologies, able to precisely manipulate fluids on the microscale, have begun to provide promising and powerful solutions to the aforementioned issues. Indeed, looking to the future, microfluidic tools may offer a route towards rapid and efficient EV manipulation that is inaccessible to current methods. In this brief perspective, we highlight recent progress in microfluidic-based techniques for EV separation, and discuss how such tools can be used for the controllable creation of EV mimetics as drug delivery systems (DDSs). Importantly, we also consider some of the key advantages and disadvantages of currently available platforms and provide a personal opinion on some of the most important challenges and future areas of application.

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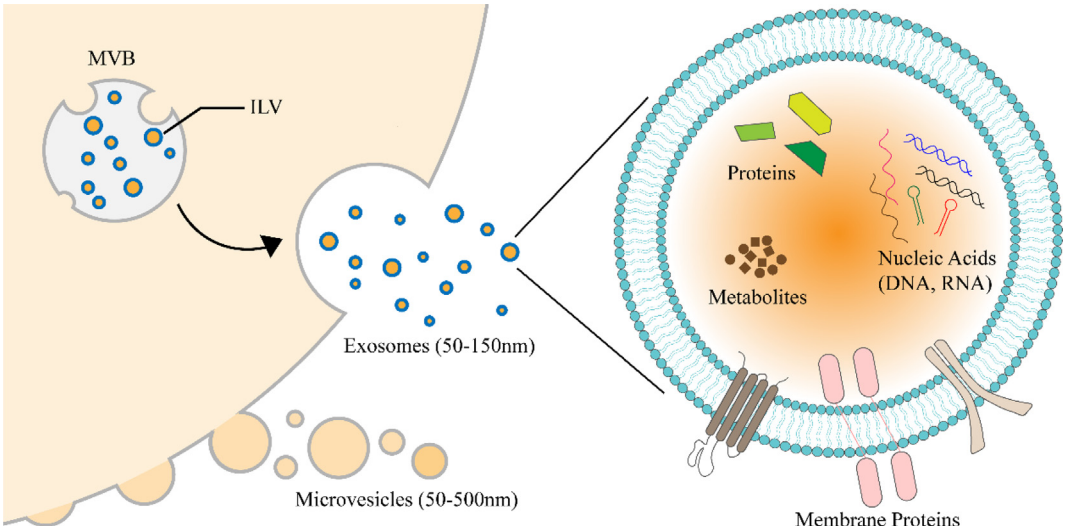

Fig. 1. Biogenesis and release of EVs from cells. Exosomes, derived from ILVs generated within MVBs, are released via fusion events between MVBs with the plasma membrane. Microvesicles are directly formed through budding of the plasma membrane. EVs encapsulate functional cargos in their membranes and lumen, including nucleic acids, proteins, lipids and metabolites, which can have functional impacts upon EVs uptake and act as circulating biomarkers in biofluids.

2. Microfluidic separation of EVs

Ever since Manz and co-workers presented the first chip-based devices for electrophoretic separations in 1992 [13], much activity has leveraged the dramatic gains in performance that are engendered through system downsizing. Whilst much early work was focused on the development of microfluidic systems for the separation of molecular-scale species, most notably DNA and proteins [14,15], significant activity has also been devoted to the separation and isolation of micro-scale and nanoscale entities in a rapid, selective and continuous fashion [16]. In this regard, recent years have seen a significant interest in developing microfluidic platforms able to process, separate and isolate EVs. Microfluidic tools for EV separation can generally be classified as being either "size-based" or "immunoaffinity-based" in their action. The proceeding discussion highlights representative examples of such technologies, with the interested reader being directed to more extensive commentaries elsewhere [17,18].

2.1. Size-based separation

2.1.1. Passive separation

2.1.1.1. Viscoelastic microfluidics. Viscoelastic separations in microfluidic channels leverage variations in the strength of the elastic lift force, F_e, acting on particles of different size [19]. For example, by adding poly(oxyethylene) to both sheath and sample flows, Liu and co-workers directly separated EVs from fetal bovine serum, achieving 96% purity and 93.6% recovery of small EVs (sEVs, < 200 nm) at a volumetric throughput of 200 μL/h [20]. Additionally, a microfluidic platform utilizing a co-flow of a Newtonian sheath fluid and a viscoelastic sample fluid (containing EVs and λ-DNA), was used to fractionate three different EV subtypes (30–200 nm, 200–1000 nm and > 1000 nm) from a cell culture medium [21]. The fraction purity and recovery for each sub-population was in excess of 90% and 89%, respectively, but with a relatively low volumetric throughput of 50 μL/h. More recently, Asghari et al. reported a sheathless oscillatory viscoelastic microfluidic platform, able to differentially focus both small (< 120 nm) and large EVs (> 1 μm), as well as synthetic nanoparticles as small as 20 nm (Fig. 2a) [22]. Unlike continuous viscoelastic microfluidic platforms, which are limited by reduced channel lengths, oscillatory flows mimic extended distances through the high-speed switching of the flow direction. The combination of viscoelastic flows and oscillatory flows suppresses Brownian motion, provides access to high flow velocities and improves focusing efficiencies. Importantly, the basic method has significant potential in the separation of much smaller biological nanocarriers (such as exomeres) [23], as well as the isolation of EVs from smaller lipoprotein carriers [24].

2.1.1.2. Filtration. Microfluidic systems incorporating nanoporous membranes have been successfully used to separate EVs from complex biological media and biofluids. For example, Davies and co-workers presented a microfluidic device containing porous polymer monolith membranes, prepared in situ, which act to filter EVs with diameters below 500 nm from whole blood [25]. To enhance filtration efficiencies, electrokinetic flows were adopted to eliminate blockages caused by the passage of proteins through the membrane. Moreover, Cho et al. utilized an electric field across a 30 nm pore size dialysis membrane to drive away free proteins and simultaneously capture EVs from plasma [26]. This approach yielded EV recoveries of up to 65% and removal of 83.6% of protein within 30 min. To enhance filtration purity, Liu and colleagues developed a microfluidic device composed of five membranes with different pore sizes (200, 100, 80, 50 and 30 nm) to fractionate EVs from plasma and urine samples at volumetric throughputs as high as 5 mL/h (Fig. 2b) [27]. Interestingly, Wang and co-workers replaced such a membrane with a ciliated (silicon nanowire) micropillar array for the selective trapping of 40–100 nm diameter lipid vesicles[28], with captured vesicles being subsequently released by nanowire dissolution. However, the fabrication of such micropillar arrays is a relatively complex and niche procedure.

2.1.1.3. Deterministic lateral displacement. Deterministic lateral displacement (DLD) is a passive microfluidic separation technique introduced by Austin and co-workers in 2004 [29]. DLD devices leverage bespoke pillar arrays to generate fluid bifurcations within a hydrodynamic flow. Particle separation at low Reynolds numbers is realized by controlling the relative width of the generated streamlines with respect to the size of the species under investigation. Due to its passive nature, simplicity of operation and exquisite separation performance, DLD holds significant promise as a tool for separating and isolating nanoscale species. In brief, DLD devices integrate a pillar array to separate microscale or nanoscale species around a critical cut-off diameter, D_C. Species larger than D_C will adopt a "bumping" motion and displace laterally across the array, whilst particles with a diameter smaller than D_C will move along the streamlines in a "zig-zag" fashion.

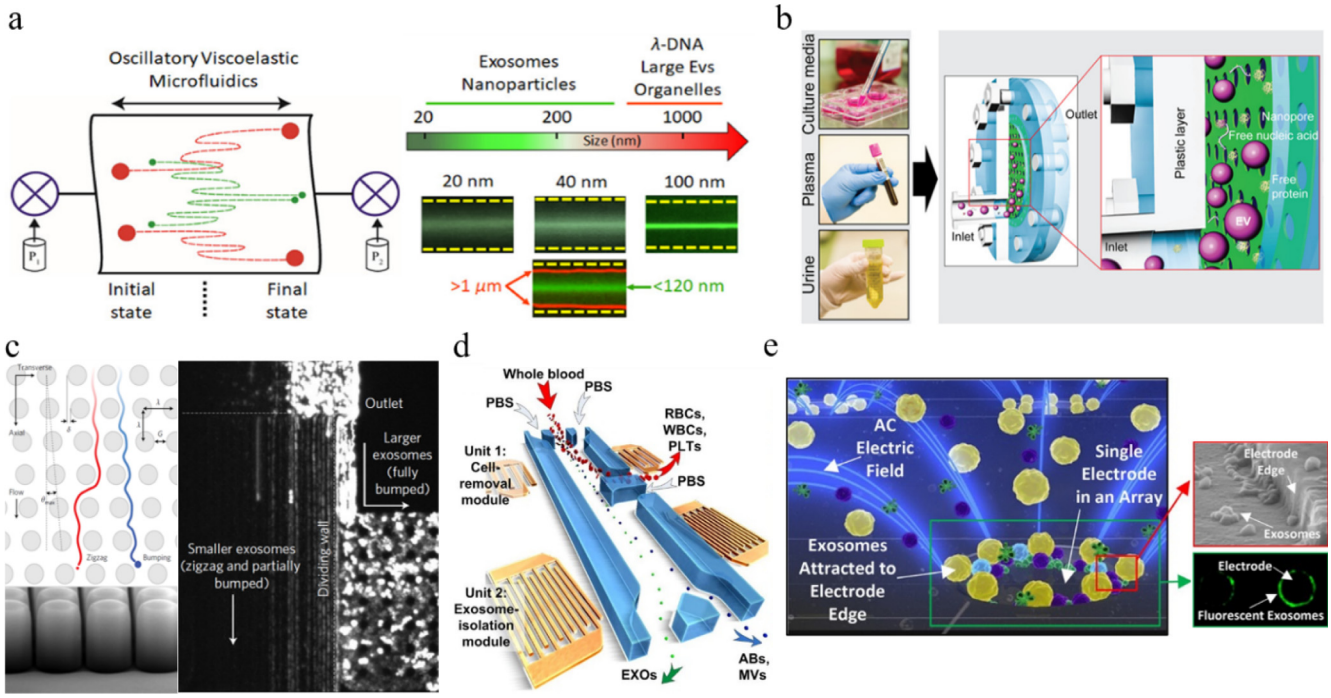

Fig. 2. Sized-based microfluidic methods for EV separation. (a) Oscillatory viscoelastic microfluidics for separation and focusing of nanoscale species. Reprinted with permission from ref. [22] Copyright 2020, American Chemical Society. (b) Nanoporous filter for fractionation of EV subgroups with different sizes. Reprinted with permission from ref. [27] Copyright 2017, American Chemical Society. (c) Nano-DLD for EV separation using a nanopillar array. Reprinted with permission from ref. [31] Copyright 2016, Springer Nature. (d) Acoustofluidic module for separation of EVs from whole blood. Reprinted with permission from ref. [35] Copyright 2017, National Academy of Sciences. (e) Alternating current electrokinetic chip for separation of EVs through an array of circular microelectrodes. Reprinted with permission from ref. [38] Copyright 2017, American Chemical Society.

In this way, Santana and co-workers presented a DLD device possessing a D_C value of approximately 250 nm, which was able to separate sEVs from a heterogeneous EV sample pre-purified from a cell culture medium [30]. Moreover, Wunsch and co-workers used nano-DLD arrays with gap sizes ranging from 25 to 235 nm to sort nanoparticles with diameters between 20 and 110 nm [31]. In addition, the authors were able to separate sEVs (using a pillar array with a 235 nm gap size and a D_C of 100 nm) from an EV sample derived from human urine (Fig. 2c). Despite success in isolating EVs, the separation throughput of this platform was remarkably low (between 6 and 12 nL/h). More recently, Smith and co-workers reported a nano-DLD chip able to separate EVs from both serum and urine, integrating 1024 parallel arrays [32]. Although this design led to a remarkable increase in throughput (up to approximately 1 mL/h), the recovery remained unacceptably low (50%).

2.1.2. Active separation

2.1.2.1. Acoustophoresis. Acoustophoretic systems employ the primary acoustic radiation force, which is proportional to the cube of the particle radius, to achieve EV separation based on differences in both size and acoustic properties (such as the acoustic contrast factor) [33]. To this end, and through use of a pair of interdigitated transducers (IDTs), Lee and co-workers presented on-chip separations of sEVs from an EV sample pre-purified from a cell culture medium, as well as medium-sized EVs (< 450 nm) from a red blood cell population [34]. Huang and associates additionally exploited IDT-based acoustofluidic device for a range of EV separations [35,36]. For example, they recently described a modular acoustofluidic platform able to separate sEVs from undiluted blood samples, with a throughput of 240 μL/h [35]. Critically, this platform was able to achieve both high purity (98.4%) and efficient recovery (82.4%) of sEVs from a mixture of two pre-purified EV sub-populations (exosomes and microvesicles) derived from primary human trophoblasts (Fig. 2d). In a follow-up publication, the authors also demonstrated the efficient separation of EVs and lipoproteins based on differences in their acoustic properties; an outstanding achievement when considering the similarity of the component size distributions [36].

2.1.2.2. Dielectrophoresis. Dielectrophoresis (DEP) is a versatile technique that leverages variations in DEP force (as a function of species size) to manipulate and discriminate different polarized particles in fluid suspensions exposed to non-uniform electric fields [37]. The effective use of DEP to separate and isolate EVs necessitates the generation of electric fields with high spatial gradients, since the DEP force is proportional to the gradient of the electric field squared. To this end, Ibsen and co-workers successfully used an alternating current electrokinetic microarray device to rapidly isolate and recover glioblastoma EVs from human plasma samples, based on differences in their dielectric properties [38]. Significantly, since the entire extraction process takes less than 30 min, subsequent immunofluorescence detection of exosomal protein biomarkers could also integrated within a chip-based format (Fig. 2e). More recently, the same authors reported a rapid pancreatic cancer diagnostic involving the capture and analysis of EVs from raw biofluids (such as whole blood, plasma or serum) [39]. Unfortunately, this platform, although powerful, is unsuitable for long-term continuous in-flow EV separation, since the electrodes are gradually covered by EVs, causing a deterioration in separation efficiency. Zhao et al. also reported a DEP geometry that allows for the continuous and high-resolution separation of synthetic nanoparticles [40]. Here, DEP forces are generated by applying a small voltage difference across a microchannel via a large orifice (several hundred microns wide) on one side of the channel wall and a smaller orifice (several hundred nanometers wide) on the opposite channel wall. Such an arrangement is potentially very useful for separating EVs. However, it is worth noting that in most DEP platforms the generation of strong electric field gradients requires the

application of relatively high voltages that can harm biological entities *via* Joule heating [37].

2.2. Immunoaffinity-based separation

Unlike the aforementioned size-based separation methods, immunoaffinity-based techniques utilize antibodies to capture EVs, and thus can provide high specificity towards a particular EV population. Such an approach can be implemented through the use of surface-modified microstructures or functionalized beads.

2.2.1. Surface-modified microstructures

In a pioneering study, Chen et al. employed herringbone groves decorated with biotinylated CD63 antibodies to isolate EVs from serum and cell culture media [41]. Here, RNAs from channel surface-bound EVs were subsequently extracted and analysed, showcasing the potential of this method as a point-of-care tool for the diagnosis of cancers. In addition, Kanwar and co-workers reported a microfluidic device (christened the ExoChip) composed of alternately positioned circular chambers functionalized with CD63 antibodies and connected by straight narrow channels [42]. Such a fluidic geometry allows for extended sample retention times and enhanced EV capture efficiencies. Furthermore, Kang et al. achieved both the capture and release of EVs through use of a chemically cleavable linker between the channel surface and antibody capture molecules [43]. To effect improvements in sensitivity, Zhang and co-workers designed a microchannel containing Y-shaped microposts coated with graphene oxide and polydopamine (GO/PDA) [44]. This functionalization provides numerous reactive sites for antibody immobilization, and is thus highly useful in enhancing the EV capture process (Fig. 3a). However, the no-slip boundary condition at the liquid–solid interface can significantly reduce the interaction between particles and microstructures [45]. To overcome this boundary problem, the authors adopted a microfluidic platform containing self-assembled nanoporous herringbone features made from micro- or nano-silica colloids [46]. This structure enabled efficient drainage of the fluid at the boundary layer and greatly reduced near-surface hydrodynamic resistance, thus allowing the isolation of low-concentration tumour-associated EVs.

2.2.2. Functionalized beads

When compared to the functionalized microstructures described previously, beads that can be decorated with EVs are more easily manipulated, and thus allow complex downstream analysis of captured species. In this regard, He and co-workers have reported an integrated platform combining immunomagnetic EV capture, lysis and enzyme-linked immunosorbent assay (ELISA)-based detection directly from plasma [47]. Their method demonstrated a two-magnitude increase in sensitivity when compared to commercial ELISA kits. Additionally, Ko and colleagues designed a multichannel microfluidic system comprising nanoporous membranes coated with a magnetic material able to trap EVs attached to superparamagnetic nanoparticles [48]. Subsequently, mRNA from these EVs was analysed for disease classification using a machine learning algorithm. In a similar vein, Shao and co-workers recently presented a complex microfluidic platform (termed immunomagnetic exosomal mRNA (iMER) analysis), which enables immunomagnetic selection, RNA collection, real-time reverse transcription and qPCR, for the evaluation of drug efficacy (Fig. 3b) [49]. Finally, Xu and associates developed a two-stage microfluidic platform for the isolation and analysis of EVs [50]. Here, Y-shaped microposts were used to create anisotropic flows and enhance interactions between beads and EVs.

3. Synthesis of EV mimetics for drug delivery

Conventional approaches for engineering EVs as DDSs rely either on the engineering of parent cells from which EVs are secreted or the direct engineering of purified EVs [51]. Since the degree of EV heterogeneity exhibited by a single cell remains unknown, it is difficult to exploit distinctive EV transport properties for drug delivery applications, as the isolation and characterization of specific subtypes is far from simple. Additionally, EV properties will vary between cell batches and passage numbers, making standardized therapies difficult to manufacture on a large scale. Moreover, the low yields associated with EV isolation has limited large-scale production for clinical applications. This has resulted in a growing interest in producing artificial EV mimetics as DDSs. In this regard, microfluidic systems provide highly controllable and reproducible environments to facilitate scaled-up production at low cost. In general terms, microfluidic approaches for the synthesis of EV mimetics can be classified as being either top-down or bottom-up in nature.

3.1. Bottom-up synthesis of fully artificial EV mimetics

Bottom-up methods aim at creating EV mimetics, or lipid nanoparticles (LNPs), by combining nanoscale components to form larger structures. Such hyperstructures (liposomes) consist of proteins, nuclei acids and/or drugs. A number of microfluidic strategies can be used to produce these EV mimetics, including hydrodynamic focusing, microvortices, chaotic mixing and droplet formation.

3.1.1. Hydrodynamic focusing

Hydrodynamic focusing involves the use of a sheath flow (normally water) to focus a central fluid stream containing lipids dissolved in an organic solvent to form LNPs. The size of the formed LNPs can easily be tailored by changing the flow rate ratio between the sheath and central flow. For example, Jahn et al. first showed how a T-shaped microfluidic geometry could be used to generate LNPs of controllable size between 100 and 300 nm [52]. In addition, Majedi and co-workers used a similar T-junction geometry to encapsulate paclitaxel (a common chemotherapy medication) into LNPs with an efficiency over 95% (Fig. 4a)

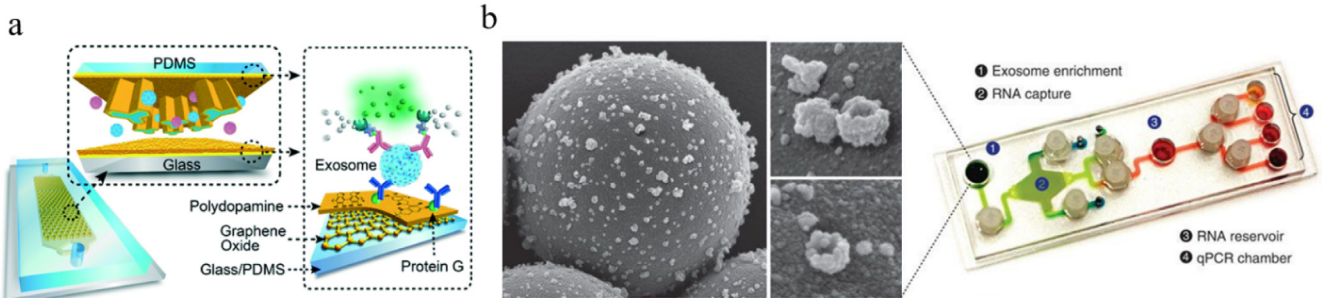

Fig. 3. Immunoaffinity-based microfluidic methods for EV separation. (a) Immunocapture of EVs by Y-shaped microposts coated with GO/PDA nanostructures. Reprinted with permission from ref. [44] Copyright 2016, The Royal Society of Chemistry. (b) Integrated iMER platform for immunocapture of EVs and downstream quantification of exosomal mRNA. Reprinted with permission from ref. [49] Copyright 2015, Springer Nature.

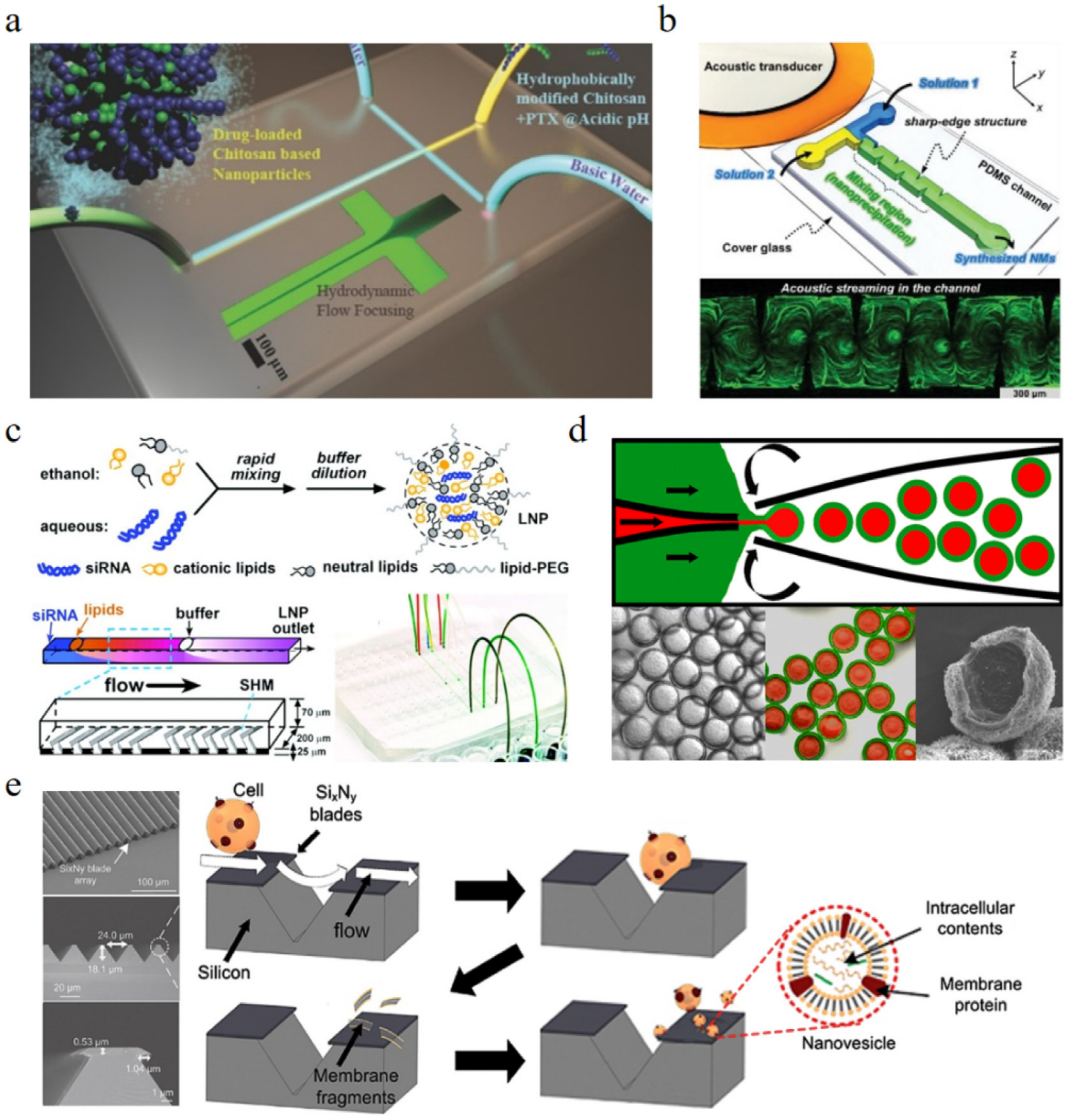

Fig. 4. Microfluidic-based methods for synthesis of EV mimetics as DDSs. (a) Hydrodynamic focusing method for production of paclitaxel-loaded LNPs. Reprinted with permission from ref. [53] Copyright 2013, John Wiley and Sons. (b) Acoustic wave-induced microvortices for nanoparticle synthesis. Reprinted with permission from ref. [58] Copyright 2019, John Wiley and Sons. (c) A series of staggered herringbone micromixers for generation of siRNA-loaded LNPs. Reprinted with permission from ref. [60] Copyright 2012, American Chemical Society. (d) Droplet-based microfluidics for synthesis of biodegradable core–shell carriers. Reprinted with permission from ref. [62] Copyright 2013, American Chemical Society. (e) Top-down approach for generation of LNPs via silicon nitride blades. Reprinted with permission from ref. [65] Copyright 2015, Elsevier.

[53]. Finally, using a 3D printed flow focusing microfluidic geometry (with an extremely high aspect ratio), Chen and co-workers realised LNP production at rates up to 0.24 g/h, with an average LNP diameter around 100 nm [54].

3.1.2. Microvortices

At slightly elevated Reynolds numbers, microvortices can be generated within microfluidic systems and utilized for LNP formation. Using a three-inlet microfluidic platform to generate symmetric microvortices, Kim and associates successfully synthesized lipid-polymer hybrid nanoparticles at a throughput of 3 g/h, with sizes ranging from 30 nm to 170 nm as determined by variations in the Reynolds number [55]. Subsequently, doxorubicin and gold nanocrystals were encapsulated into these nanoparticles and used as a cancer therapy in mice [56]. In a related study, different species, including the small molecule drug simvastatin and gold, iron oxide and quantum dot nanocrystals, were introduced into the high-density lipoprotein [57]. This highlights the potential applications of this technology in drug delivery and medical imaging. More recently, Huang and co-workers reported an acoustofluidic device, which generates counter-rotating vortices to rapidly mix reagents across the channel width and incorporate DNA into LNPs, with significantly improved reproducibility compared to bulk vortex mixing (Fig. 4b) [58].

3.1.3. Chaotic mixing

Chaotic mixing is one of the most widely used microfluidic methods for LNP production. For example, Zhigaltsev et al. successfully generated doxorubicin-loaded LNPs with diameters between 20 and 50 nm using a staggered herringbone micromixer [59]. Chen et al. also reported a similar microfluidic mixer to incorporate siRNA into LNPs (Fig. 4c) [60]. Such siRNA-loaded LNPs were then successfully used in gene silencing experiments. In a related study, a different mixing geometry based on a baffle mixer, was successfully used to produce pH-sensitive siRNA loaded LNPs, with sizes ranging from 20 to 100 nm [61].

3.1.4. Droplet microfluidics

Droplet-based microfluidic systems have become ubiquitous tools in biological research and unsurprisingly have been used to good effect in the robust generation of homogeneous microparticles. To this end, Windbergs et al. produced core–shell emulsions by encapsulating a hydrophilic agent (doxorubicin hydrochloride) within the aqueous core and a hydrophobic agent (paclitaxel) in the lipid shell (Fig. 4d) [62]. In addition, Kantak et al. were able to generate more complex micron-sized multilayer particles, demonstrating the potential for multiplexed drug delivery [63].

3.2. Top-down cell membrane-derived EV mimetics

Top-down approaches typically use nanometer-sized structures to break apart cells into EV mimetics. For example, Jo and co-workers used an array of hydrophilic microfluidic channels to extrude cells and produce EVs mimetics with an average diameter of 100 nm for mRNA delivery [64]. Yoon et al. used silicon nitride blades to generate nanovesicles incorporated with polystyrene latex beads (Fig. 4e) [65]. Significantly, the generated EV mimetics were able to deliver the encapsulated beads across the plasma membrane of recipient cells, whereas bare beads were unable to penetrate the plasma membrane of recipient cells. However, top-down synthesised EV mimetics must be further purified via several centrifugation steps to remove cell debris, a time-consuming and lossy procedure. Currently, there are only a few reports describing microfluidic technologies for producing cell membrane-derived EV mimetics. Nevertheless, since the cell membrane is an excellent source of EV mimetics, it is highly likely that new techniques will soon be developed to produce EV mimetics from cell membranes.

4. Challenges and perspectives

We have highlighted some of the most important microfluidic technologies that have been used for the isolation of EVs and the production of EV mimetics. Although much progress has been made within a short period of time, it is clear that no single microfluidic EV manipulation technique is a panacea. Indeed, when assessing the current status of microfluidic research for EV studies, it is evident that the scientific literature contains a wide variety of technologies and protocols that will be useful in some but not all applications.

Technologically, it is clear that the development of new and enhanced separation and detection components is needed to improve both the temporal and spatial resolution associated with EV processing. The analytical throughput of microfluidic-based EV separation systems still remains lower than gold-standard bulk methods, and most microfluidic separation techniques cannot be easily parallelized to address such throughput limitations. For example, acoustofluidic devices equipped with IDT components are relatively bulky and difficult to parallelize. Additionally, due to the highly heterogeneous nature of EVs, the ability to isolate EVs of user-defined sizes (in high purity) remains a tedious procedure. Although, for instance, microfluidic EV isolation platforms based on membrane filtration [25] or nanopillars [28] circumvent the issues of tedious processing (associated with conventional EV isolation methods), they are inherently prone to blockage and challenging to fabricate. In contrast, microfluidic platforms utilizing viscoelastic flows (e.g. oscillatory viscoelastic microfluidics) for passive and high-resolution EV separation can be simply fabricated and multiplexed in a facile manner. We envision that such a separation method will play an important role in sequestering EV subgroups. Moreover, to reveal EV population heterogeneity, high-throughput single EV detection methods will need to be developed to screen multiple EV subtypes. Accordingly, there exists significant demand for microfluidic platforms able to isolate, process and analyse EVs in a sensitive, efficient and high-throughput manner. To date, many EV detection techniques with outstanding limits of detection have been developed. These include fluorescence detection [42,46,66], colorimetric detection [67,68], electrochemical detection [50,69] and surface plasmon resonance [70]. However, only a small number are capable of single EV detection [66], which is essential when analysing highly heterogeneous EV populations. Indeed, the development of new materials, nanofabrication techniques and optical spectroscopies will certainly advance our ability to detect single EVs within microfluidic environments. Additionally, high-throughput single EV detection methods will produce large amounts of data, which can be potentially combined with machine-learning techniques to assist in EV analysis. We envisage that the combination of single EV detection with machine learning analysis and single EV sorting techniques will soon become a research topic of much interest, enhancing the understanding of EV biology and its clinical utility.

On a more general level, the continued development of microfluidic technologies will undoubtedly facilitate the integration of EV isolation, detection and analysis within monolithic devices, and in turn minimize cross-contamination, simplify workflows and reduce the need for manual interventions. Such developments will also lead to faster EV assays, and significant decreases in required sample volumes. Despite these advantages, microfluidic tools for EV isolation remain rare [11]. This is in large part due to the many challenges faced when developing integrated platforms capable of multistep operations. Indeed, many existing microfluidic devices for EV isolation provide high purity, fast recovery times and low production costs, but are prohibitively complex, especially when performing multistep processes. For instance, active separation techniques utilize applied fields (most commonly, magnetic, electrical, optical and acoustic) [16,17], with functional operations requiring complex control architectures [47,49]. Accordingly, an exciting avenue for research in the short-medium term will be the use of passive viscoelastic and/or inertial manipulation methods and downstream label free EV characterization. Indeed, the development of such microfluidic technologies is likely necessary in bringing diagnostic, therapeutic and prognostic tools into clinical settings.

Microfluidic systems able to robustly produce EV mimetics for drug delivery applications are still at an early stage of development. Although EV mimetics have a similar structure to cell-derived EVs, the bottom-up generation of EV mimetics always involves the addition of chemical components, which are likely to cause biocompatibility issues. In addition, these bottom-up synthesised EV mimetic membranes typically lack functional proteins for endocytosis, which will affect the drug delivery efficiency into recipient cells. Whilst top-down methods can better leverage plasma membrane proteins, it is far more difficult to control which parts of cells can be incorporated into EV mimetics during membrane fragment self-assembly. That said, both EV mimetic synthesis approaches can benefit from the aforementioned separation techniques when post purification is needed. Indeed, EV mimetics currently produced using microfluidic systems are simply not of a quality for use in therapeutic interventions or clinical applications. More research is required to better understand the cellular uptake process, targeting efficiency, stability, *in vivo* kinetics and potential side effects of EV mimetics. To accelerate such research and the design of EV mimetics, organ-on-a-chip devices with an advantage mimic *in vivo* physiological microenvironments are likely to be crucial in evaluating EV mimetic cytotoxicity.

Finally, and from a device standpoint, microfluidic devices made of elastomers (most notably polydimethylsiloxane) are typically intended for single use applications. In many cases their fabrication can be complex enough to potentially affect their wide spread adoption in biological experimentation. In this regard, the lack of fabrication methods that are inexpensive, robust, scalable and simple to use is troublesome and may prevent mainstream biology laboratories from adopting microfluidic tools. However, in contrast to conventional EV manipulation methods (that require large volumes of consumables, bulky equipment and complex workflows) the cost of a “microfluidic experiment” is almost always significantly lower. Improvements in scalable manufacturing processes will undoubtedly further lower

associated costs and open up the possibilities for point-of-care diagnostics and point-of-use therapeutics.

To conclude, we hope that this brief analysis has highlighted the significant role that microfluidic tools have played, and will continue to play, in EV research. On a more general level, EVs will undoubtedly become increasingly powerful tools in understanding and treating a wide variety of diseases. We expect that in the short-medium term, microfluidic systems combined with viscoelastic fluids, optics, plasmonics and machine learning tools will provide a rich palette of opportunities for automated and high-throughput EV research, with commercial microfluidic platforms for EV analysis becoming *de rigueur* in the biology lab.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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