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Elsevier B.V.
2021-01-11

Liangsupree, T, Multia, E & Riekkola, M-L 2021, 'Modern isolation and separation techniques for extracellular vesicles', Journal of Chromatography. A, vol. 1636, 461773. https://doi.org/10.1016/j.chro

http://hdl.handle.net/10138/326689
10.1016/j.chroma.2020.461773

Journal of Chromatography A 1636 (2021) 461773

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Modern isolation and separation techniques for extracellular vesicles

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ARTICLE INFO

Article history:
Received 30 September 2020
Revised 26 November 2020
Accepted 28 November 2020
Available online 3 December 2020

Keywords:
Extracellular vesicles
Isolation
Separation
Size-based
Charge-based
Affinity-based

ABSTRACT

Extracellular vesicles (EVs) are heterogenous membrane-bound vesicles released from various origins. EVs play a crucial role in cellular communication and mediate several physiological and pathological processes, highlighting their potential therapeutic and diagnostic applications. Due to the rapid increase in interests and needs to elucidate EV properties and functions, numerous isolation and separation approaches for EVs have been developed to overcome limitations of conventional techniques, such as ultracentrifugation. This review focuses on recently emerging and modern EV isolation and separation techniques, including size-, charge-, and affinity-based techniques while excluding ultracentrifugation and precipitation-based techniques due to their multiple limitations. The advantages and drawbacks of each technique are discussed together with insights into their applications. Emerging approaches all share similar features in terms of being time-effective, easy-to-operate, and capable of providing EVs with suitable and desirable purity and integrity for applications of interest. Combination and hyphenation of techniques have been used for EV isolation and separation to yield EVs with the best quality. The most recent development using an automated on-line system including selective affinity-based trapping unit and asymmetrical flow field-flow fractionation allows reliable isolation and fractionation of EV subpopulations from human plasma.

1. Introduction

Extracellular vesicles (EVs) are a group of diverse membrane-bound nanosized particles having a size range of 30-5000 nm. Exosomes (50-150 nm), a subtype of EVs, are originated from endosomal origin but may share similar size and density with ectosomal EVs, such as microvesicles (MVs), making differentiation of EV subclasses highly challenging. In addition, other plasma components, such as lipoproteins (high-density lipoprotein (HDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), very low-density lipoprotein (VLDL), and chylomicrons) and viruses, share similar sizes and densities with EVs (Fig. 1), complicating selective size- or density-based EV isolation [1]. Exosomes and EVs in general have shown potential in biomarker discovery for diagnostics of various diseases [2]. For diagnostic purposes, high purity isolates are not necessary, while the high yield of isolation is more important. However, well-defined, intact, and pure EV subpopulations could serve therapeutic purposes (e.g., in drug delivery, immunotherapy, or cellular reprogramming). Since EVs take part in cellular communication by transferring nucleic acids (e.g., RNAs) and metabolites from cells to cells, the role of subpopulations in intercellular communication needs to be further studied. These challenges require modern isolation and separation techniques to uncover how different cell origins or states affect the EV composition. This is particularly crucial in tumor progression since carcinogenic materials are carried by EVs, making them attractive as biomarkers for non-invasive diagnosis and prognosis of the stage of cancer [1,3]. To stop the disease progression, even the removal of tumor-derived EVs has been suggested [4]. Discovering new EV subpopulations, such as exomeres [5], have lately revealed that additional effort is needed on improving isolation and characterization techniques.

Currently, popular techniques include ultracentrifugation (UC), size-exclusion chromatography (SEC), ultrafiltration (UF), precipitation, and immunoaffinity (IA) capture, while e.g., asymmetric flow field-flow fractionation (AsFlFFF/AF4) has been an emerging technique used to fractionate EV subpopulations, such as exomeres and exosomes. Other emerging techniques are microfluidics and combined multi-step methods. A combination of methods is often the best option for EV isolation since diverse origin, complex nature, and heterogeneity of EVs require sophisticated isolation approaches [6]. In addition, conventional isolation techniques (e.g., UC) limit scaling-up of EV production due to intra- and inter-batch variations in EV preparations [6]. Many of the current technologies are also time-intensive, require tedious manual labor, and are prone to lipoprotein and other protein contamination. For

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https://doi.org/10.1016/j.chroma.2020.461773

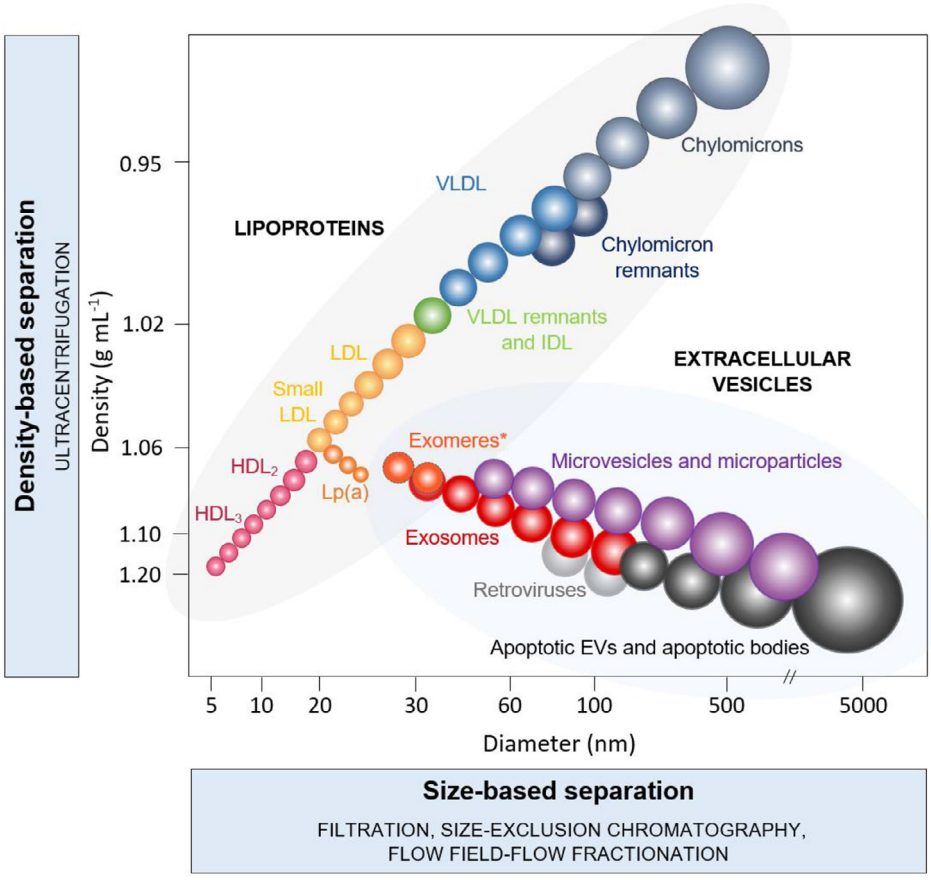

Fig. 1. Extracellular vesicles (EVs) and lipoprotein subtypes (HDL: high-density lipoprotein, LDL: low-density lipoprotein, Lp(a): lipoprotein(a), IDL: intermediate-density lipoprotein, and VLDL: very-low-density lipoprotein) and their physical characteristics. The density of exomeres has not yet been determined experimentally. Information used to generate this figure was acquired from [8–10].

therapeutic purposes, EV isolation and separation technologies should be scalable, more automated, selective to specific subpopulations of EVs, and able to handle complex biological fluids [6,7].

This review gives a comprehensive overview of the current status of the techniques applicable for isolation, separation, and fractionation of subpopulations of EVs from prokaryotes and eukaryotes excluding ultracentrifugation and precipitation-based techniques due to their above-mentioned limitations. The emphasis is given to modern and most recently developed techniques that take advantage of size, charge, and affinity for the separation of different EV subpopulations.

2. Modern EV isolation and separation techniques

In this review, modern isolation and separation techniques in the field of EVs are divided into three categories based on the isolation principle, namely size-, charge-, and affinity-based techniques. Although UC is still the most commonly used technique in 2020 [11], there has been a significant increase in SEC applications since 2010 as shown in Fig. 2. Affinity-based techniques as well as filter-based techniques have also started to become more popular in the last six years. The utilization of newly emerging techniques in the EV field, such as flow field-flow fractionation, ion-exchange, as well as electrophoresis and dielectrophoresis has been increasing since 2016.

2.1. Size-based techniques

2.1.1. Size-exclusion chromatography

Size exclusion chromatography (SEC) is a well-established technique used for macromolecule separation based on their molecular size or hydrodynamic volumes [12]. A typical SEC system consists of a porous stationary phase for chromatographic separation with or without coupling to a pump for elution as shown in Fig. 3A. SEC has been used for the isolation of EVs from a large variety of sample matrices from both prokaryotes and eukaryotes, including cell culture-derived samples, blood-based samples (plasma and serum) [13–17], urine [18,19], milk [20], saliva [21], nasal lavage [22], synovial fluid [23], cerebrospinal fluids [24,25], ascites [26–29], and tear [21] (Table 1). Diluted samples, especially urine and cell culture-derived samples, are often concentrated or subjected to soluble contaminant removal using filtration-based techniques prior to injection to the column [30]. The commonly used stationary phase materials for EV isolation and separation are cross-linked agarose beads (commercially named as Sepharose® (CL-2B and CL-4B) and Sephacryl® S-400) as summarized in Table 1. Agarose beads are highly scalable and flexible and thus adjustable to meet sample requirements. Various studies have also utilized qEV SEC columns available in different bed volumes and size exclusion limits (intra-pore sizes). Sepharose® CL-4B with a size exclusion limit of 42 nm [31] was found to be more suitable for separating EVs from protein contaminants, such as albumin, compared to Sepharose® CL-2B [32–34] with an exclusion limit of 75 nm [31]. An improved resolution was also obtained with a larger column bed volume (1 mL vs. 10 mL) [34]. In addition, Arntz et al. isolated plasma-derived EVs using two Sepharose® CL-2B SEC columns having the same stacking volume but different column lengths (56 mm vs. 222 mm) [35]. They discovered that by using the longer column, protein and immunoglobulin contamination was reduced by 90%, while the EV particle size distribution and yields remained the same [35].

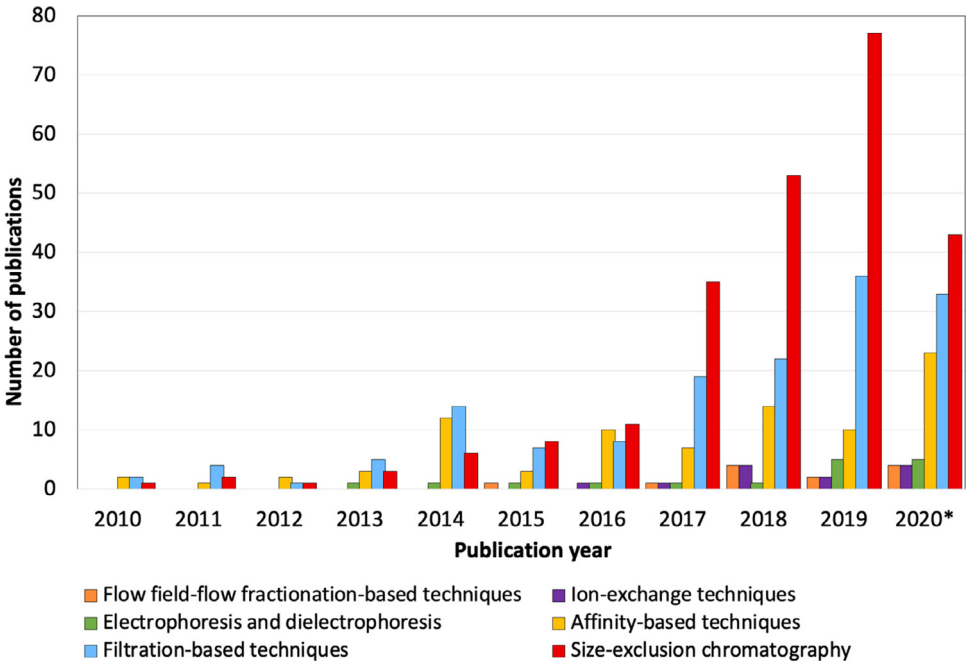

Fig. 2. Modern EV isolation, separation, and fractionation techniques based on the number of published articles per year (*as of September 2020).

Table 1
Size-exclusion chromatography for EV isolation and separation.

Column	Sample matrix	Reference
IZON® qEV column	Cell culture	[55]
	Plasma or serum	[14,15]
	Urine	[46,56]
	Saliva and tear fluid	[21,57]
	Milk	[20,58]
	Lymph node and spleen	[59]
	Uterine flush	[13,60]
	Seminal plasma and brain	[61]
	Induced pluripotent stem cell-derived neurons	[62]
	Stromal vascular fraction	[63]
	Nasal lavages	[22]
	Skin sections and dermal intestinal fluid	[64]
Sepharose® CL-2B column	Cell culture	[65,66]
	Plasma or serum	[67,68]
	Urine	[69,70]
	Cerebrospinal fluid	[24,25]
	Peritoneal dialysis effluent	[71,72]
	Saliva	[73]
	Stool	[74]
Sepharose® CL-4B column	Cell culture	[75]
	Plasma	[33,43]
Sephacryl® S-400 column	Cell culture	[76,77]
	Plasma or serum	[33,78]
Sephacryl® S-500 column	Milk	[79]
	Synovial fluid	[23]
Sephacryl® S-1000 column	Seminal fluid	[80]
Superdex® 200 column	Cell culture	[81,82]
	Seminal plasma	[83]
	Serum	[84]

SEC is commonly used to isolate heterogeneous populations of EVs, while the separation of distinct EV subpopulations remains a challenge due to limited resolution. However, recently, the separation of urinary exosome sub-populations, including small (36±6 nm), medium (60±10 nm), and large exosomes (102±16 nm), was achieved using two-dimensional SEC including two SEC columns. The first column having a smaller pore size (50 nm) was used to pre-isolate exosomes, and the second column with a larger pore size (200 nm) further separated the collected exosomes into three different size ranges with satisfactory resolution [18].

Several studies have also demonstrated the superiority of SEC over conventional EV separation techniques, such as UC and precipitation with chemical agents. For instance, since high-density lipoproteins (HDLs) and EVs share a similar density range, EVs isolated from plasma by density gradient UC, a separation technique based solely on density difference, were co-isolated with HDLs [36], while with SEC this issue was not encountered due to HDLs being smaller than EVs (Fig. 1). Moreover, SEC successfully removed unwanted serum albumin that is present in EV isolates [33,37]. Studies also showed that SEC yielded cell culture- and plasma-derived EVs with better functionality compared to UC [38–40]. The recovery of plasma-derived EVs isolated by differential UC was also negatively affected by the highly viscous nature of plasma [41]. These findings proved that UC was not suitable for EV isolation from plasma. SEC also yielded higher-quality cell culture-derived EVs, with less protein contamination, and less compositional and structural alteration compared to EV isolation using precipitating agents, such as polyethylene glycol and PRotein Organic Solvent PRecipitation [42]. It is also worthy to note that the commonly used SEC mobile phase, phosphate buffered saline (PBS) (pH 7.4), preserves EV functionality and integrity.

Although SEC has been effective in removing the majority of HDLs and relatively small proteins from plasma, its major drawback is co-isolation with other lipoproteins, including chylomicrons, VLDL, VLDL remnants (e.g., IDL), and LDL, sharing the same size range with EVs [17] (Fig. 1). In addition, it was found that SEC isolated relatively smaller EVs than UC, resulting in different proteomes [17]. Self-packing of SEC can also be tedious and decrease the reliability of the method.

Over 40% of studies have also combined SEC with other techniques to overcome SEC limitations as well as to improve EV purity. For instance, anion exchange chromatography has been used to selectively remove lipoproteins after SEC separation [43]. Other studies have also shown that the combination of SEC with other techniques, such as UC and UF, improves EV quality [44–48]. Therefore, the choice of combined separation techniques needs careful thinking to guarantee the best results.

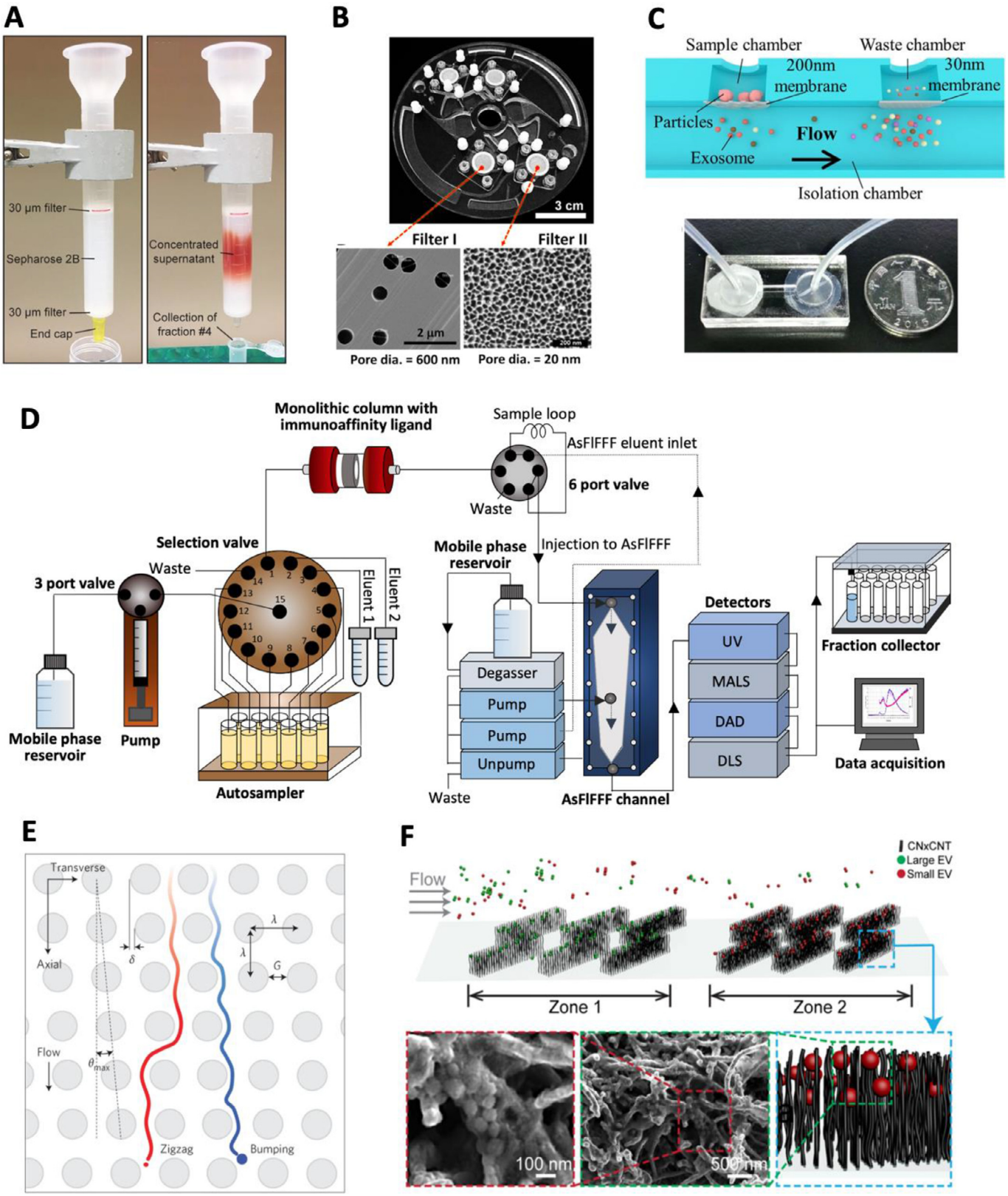

Fig. 3. Size-based EV isolation, separation, and fractionation methods: A) Mini-SEC column for tumor-derived exosome isolation from cell culture media. Reprinted with permission from [49], copyright 2019 Elsevier. B) Exodisc for urinary EV isolation. Reprinted with permission from [50], copyright 2017 American Chemical Society. C) Double-filtration microfluidic device for isolation of urinary EVs. Reprinted with permission from [51], copyright 2017 Springer Nature (Creative Commons Attribution CC BY license). D) Isolation of EV subpopulations from human plasma by immunoaffinity chromatography coupled to asymmetrical flow field-flow fractionation. Reprinted with permission from [52], copyright 2020, American Chemical Society (Creative Commons Attribution CC BY license). E) Lateral displacement arrays for exosome separation. Reprinted with permission from [53], copyright 2016 Springer Nature. F) Three-dimensional carbon nanotube arrays for size-based EV capture from cell culture. Reprinted with permission from [54], copyright 2020 American Chemical Society.

Table 2
Filtration-based techniques for EV isolation.

Technique	Separation system	Molecular weight cut-off (MWCO) or pore size	Sample matrix	Reference
Centrifugal ultrafiltration (UF)	Centrifugal filter unit	10 kDa MWCO	Urine	[46,97]
			Cell culture	[98,99]
		30 kDa MWCO	Cell culture	[92]
			Stromal vascular fraction	[63]
		50 kDa MWCO	Cell culture	[92]
			Plasma	[34]
		100 kDa MWCO	Urine	[19,46,97]
			Cell culture	[92,100]
			Plasma or serum	[74,101]
			Stool	[74]
			Semen	[61]
			Cerebrospinal fluid	[102]
			Peritoneal dialysis effluent	[103]
			Bronchoalveolar lavage fluid	[93]
			Saliva	[104]
		300 kDa MWCO	Serum	[95]
		500 kDa MWCO	Cell culture	[105]
Tangential flow filtration (TFF)	Membrane filter	10 kDa MWCO	Cell culture	[106]
	Hollow fiber membrane	100 kDa MWCO	Cell culture	[107,108]
	Membrane filter	300 kDa MWCO	Cell culture	[109,110]
	UF membrane filter capsule	500 kDa MWCO	Cell culture	[111]
	Filter	750 kDa MWCO	Cell culture	[87]
Exodisc	Track-etched polycarbonate (PC) membrane and anodic aluminum oxide (AAO) membrane	600 nm (PC membrane) and 20 nm (AAO membrane) pore sizes	Cell culture	[50]
ExoTIC (exosome total isolation chip)	Track-etched PC and a polyethersulfone (PES) filter	30 nm or 50 nm (PC) and 200 nm (PES) pore sizes	Plasma, urine, and lavage	[90]
Hydrostatic filtration dialysis (HFD)	Cellulose membrane	1000 kDa MWCO	Urine	[112,113]
			Cell culture	[113]
Integrated double-filtration microfluidic device	Whatman® Nuclepore™ track-etched membranes	0.2 μm and 30 nm pore sizes	Urine	[51]

2.1.2. Filtration

Filtration is another popular size-based separation technique used for EV purification. In this review, the focus was made on modern and more automated filtration techniques thus excluding the applications of conventional filters. Ultrafiltration (UF) utilizes membranes with defined molecular weight cut-off (MWCO) ranging from 10-100 kDa for most studies (Table 2). UF has been widely used to isolate EVs from relatively dilute samples, such as urine and cell cultures. UF devices generally consist of a UF membrane inserted in a container. The filtration is often performed using centrifugal UF which is advantageous in being a relatively simple and easy-to-use technique and offers faster EV isolation compared to UC [85]. However, many of the UF methods suffer from protein contaminants and EV losses to the membrane [3,86] as well as from morphological changes of EVs that have not been studied in detail. In addition to centrifugal UF, tangential flow filtration (TFF), also known as cross-flow filtration, is a UF technique widely used for EV isolation and separation. In TFF, a stream of fluids containing EVs flow tangentially by moving across the UF membrane (hollow fiber membrane) but not directly through the membrane. Molecules smaller than MWCO travel through the membrane and are discarded, while molecules larger than the cut-off level, such as EVs, remain on the membrane and are recirculated and concentrated [87,88]. TFF is more beneficial compared to conventional filters in which the fluid flows directly through the membrane, which often results in cake formation that clogs the pores [88]. In comparison to SEC, TFF also concentrates EVs, while SEC dilutes the isolates [87], making TFF suitable for large-scale EV isolation from diluted samples. A special filtration technique, hydrostatic filtration dialysis (HFD) [89], where 1000 kDa cellulose ester dialysis membranes are employed, has been developed to isolate EVs from urine. HFD does not require centrifugation steps like many other UF methods and exhibits low EV losses.

Specialized filtration devices have been developed utilizing track-etched membranes (30, 50, 200, and 600 nm pore sizes) for fast and size-selective isolation of EVs [50,51,90] (Figs. 3B and 3C). With a sequential centrifugal UF, it is possible to isolate exosomes from cell culture media of human colon carcinoma cell organoids [91]. This method utilizes sequentially hydrophilic polyvinylidene difluoride membranes with 0.65, 0.45, 0.22, and 0.1 μm pores. The addition of filtration steps increased the purity of the isolates but reduced the yields [3]. Although UF alone might be sufficient for EV isolation in some cases, additional purity and selectivity can be achieved by combining different techniques with UF. For instance, centrifugal UF has been extensively used in combination with SEC to isolate EVs from cell culture media, urine, and bronchoalveolar lavage fluid [92–94]. UF in combination with AsFlFFF on the other hand has been used to isolate exosomes from a more complex biofluid, human serum [95]. An even more sophisticated combination of three different methods (UF, SEC, and AsFlFFF) has been developed to study urinary EVs from human urine samples [96].

2.1.3. Flow field-flow fractionation

Flow field-flow fractionation (FFF) is an emerging size-based fractionation technique for EV separation, with asymmetrical flow field-flow fractionation (AsFlFFF or AF4) being the most popular FFF subtechnique used. The separation of macromolecules in AsFlFFF is based on their diffusion coefficients. An AsFlFFF channel contains two plates separated by a spacer with a permeable membrane at the bottom (accumulation wall) [114]. Parabolic flow profile carries EVs from the channel to detectors. Size-based fractionation is achieved by a perpendicular cross-flow to a parabolic flow profile that separates EVs based on their size on the accumulation wall [114]. The cross-flow can be modified and optimized even between runs to enhance separation efficiency, making AsFlFFF more flexible compared to SEC. In AsFlFFF, small EVs remain further from the bottom of the channel due to their higher diffusion coefficient,

Table 3
Flow field-flow fractionation-based techniques for EV isolation and fractionation.

Technique	Separation channel material	Sample matrix	Reference
Immunoaffinity chromatography - asymmetrical flow field-flow fractionation (IAC-AsFlFFF)	350 µm spacer, 10 kDa regenerated cellulose membrane	Plasma	[52]
Frit-inlet AsFlFFF	350 µm spacer, 10 kDa regenerated cellulose membrane	EV isolated from serum	[95]
AsFlFFF	190 μm spacer, 10 kDa regenerated cellulose membrane	Exosomes isolated by UC from urine	[117]
	350 µm spacer, 10 kDa regenerated cellulose membrane	UF and SEC purified EVs from urine	[96]
		Plasma and serum	[118]
		Immunoaffinity purified EVs from plasma	[115,116]
	490 µm spacer, 10 kDa regenerated cellulose membrane	Small EVs from ultracentrifuged cell cultures	[5]
	190, 250, 350, and 480 μm spacers, 10 kDa regenerated cellulose membrane	Lyophilized exosome standard purified from cell culture	[114]
	350 and 490 µm spacer, regenerated cellulose and polyethersulfone	Cell culture	[119]
	350 µm spacer, 30 kDa nadir cellulose mambrane	Cell culture	[120]
		Lyophilized exosome standard purified from cell culture	[121]

while larger EVs stay closer to the accumulation wall [114]. In normal mode, the parabolic flow profile carries smaller EVs to the detectors first followed by larger ones as opposed to SEC, where larger particles arrive first. In combination with light scattering detectors, AsFlFFF can provide accurate information on EV size distribution, size morphology, and aggregation or agglomeration states. Coupled with a fraction collector, EV subpopulations can also be collected for additional studies. One of the advantages of AsFlFFF for EV separation is gentle fractionation since there are no shear forces from the stationary phase deteriorating the particles as in the case of SEC. In addition, AsFlFFF allows buffer exchange with the EV formulation buffer, being important especially in potential therapeutic applications of fractionated EV subpopulations. However, fractionated EVs obtained from both AsFlFFF and SEC are diluted and may require an extra pre-concentration step prior to further studies. In addition, to avoid self-association and overloading effects, only small quantities of the sample can be injected. This can limit the applications when large volumes of the sample need to be processed.

The most commonly used accumulation wall membrane for fractionation of EVs has been a 10 kDa regenerated cellulose membrane with 350 µm spacer (Table 3). Pre-concentration of urine or cell culture media samples in most cases has been done with UC. For example, frit-inlet AsFlFFF has been used to separate exosomes from HDLs and LDLs in human serum samples pre-isolated by UF, UC, or exosome isolation kit [95]. Above all, AsFlFFF has been especially important for the separation of subpopulations of EVs, resulting in the separation of new EV subpopulation called exomeres from small and large exosomes from ultracentrifuged cell-line culture media first reported by Zhang et al. [5]. Following this work, $CD9^+$ and $CD61^+$ EVs were pre-isolated from human plasma using immunoaffinity chromatography (IAC) with monolithic columns immobilized with monoclonal antibodies against CD9 and CD61. The isolates were further fractionated by AsFlFFF, resulting in EV fractions having the size range of exomeres as well as small and large exosomes without lipoprotein contaminants that are often found in UC and SEC isolates [115,116]. The pre-isolation method also allowed the isolation of EVs in higher yields compared to UC and SEC based on capillary electrophoresis-laser-induced fluorescence (CE-LIF) signal intensities of fluorescently labeled EVs [115]. The IAC was afterward successfully integrated on-line to AsFlFFF for automated isolation and fractionation of $CD9^+$ and $CD61^+$ EVs [52]. The IAC-AsFlFFF system (Fig. 3D) provided highly reliable and reproducible isolation and fractionation (RSD 2.9-4.2%) and could process up to 18 plasma samples per day in an automated fashion. The system enabled for the first time reproducible study of amino acids and glucose found in subpopulations of $CD9^+$ and $CD61^+$ EVs in the size range of exosomes and exomeres [52].

2.1.4. Other size-based techniques

Other modern techniques include deterministic lateral displacement (DLD) pillar arrays as summarized in Table 4. DLD is a microfluidic technique that separates EVs based on their trajectories in a pillar array. Particles smaller than the set DLD critical diameter will follow a zigzag mode, while larger particles travel in a bumping or displacement mode (Fig. 2E), resulting in separation based on their size differences [53]. DLD pillar arrays with 235 nm nanopillar gap have been used to separate exosomes in the size range of 20 to 110 nm [53]. In another study, integrated nanoDLD arrays showed a superior yield (approximately 50%) of EVs from urine and plasma, compared with UC, density gradient UC, SEC, and precipitation-based isolation [122]. DLD pilar arrays have also been exploited to detect BD21 vesicles with exosome-specific beads coated with antibodies [123]. Even electroosmotic flow-driven DLD micro- and nanopillar array chips have been recently used to separate and enrich EVs from breast cancer cells (MDA-MB-231) based on their size [124]. In addition to DLD pillar arrays, carbon nanotube arrays have been developed for size-based, label-free, and rapid EV separation from glial cells (Fig. 2F) [54].

Separation of adenocarcinomic human alveolar basal epithelial cell-derived EVs based on fluid dynamics and particle size discrimination has been reported by Liu et al. [125]. This microfluidic system utilized viscoelasticity for exosome isolation and gave recoveries of over 80% and purity of more than 90%. It also had a higher processing speed compared to DLD pilar arrays. Sheathless oscillatory viscoelastic microfluidics system [126] has been utilized for EV focusing and separation from bovine milk and cultured cell-derived EVs. In addition, the λ-DNA mediated viscoelastic microfluidic system has been developed to separate EV subpopulations [127].

2.2. Charge-based techniques

2.2.1. Ion-exchange techniques

Separation of EVs using ion-exchange techniques, such as chromatography and metal-affinity based systems exploits the interactions between negatively charged EV membrane components whose charges have been determined by zeta potential [128] and an anion exchanger with positively charged functional groups or cations. In general, bound EVs can be released by introducing high salt concentrations to increase the ionic strength of the buffer to promote the desorption of EVs from positively charged media. Anion-exchange chromatography (AIEC) has recently been used

Table 4
Other size-based techniques for EV isolation.

Technique	Separation system	Sample matrix	Reference
Deterministic lateral displacement (DLD) pillar array	Nanopillar chips	BD21 vesicles	[123]
		Exosomes isolated from urine	[53]
Electroosmotic flow-driven DLD pillar array		Cell culture	[124]
NanoDLD pillar array		Serum and urine EVs	[122]
Oscillatory viscoelastic microfluidic system	Microfluidics	Small EVs produced by in vitro cultured cells and EVs from bovine milk	[126]
Viscoelasticity-based microfluidic system		Cell culture	[125]
λ-DNA mediated viscoelastic microfluidic system		EVs isolated from cell culture by differential centrifugation	[127]

Table 5
Ion-exchange based techniques for EV isolation and separation.

Technique	Separation system	Sample matrix	Reference
Anion-exchange chromatography (AIEC)	Anion exchange column	Cell culture	[138]
	Column packed with anion exchange resin	Cell culture	[131]
	Column packed with anion exchange resin (quaternary ammonium)	Cell culture	[99,139–141]
	Diethylaminoethyl cellulose Sephadex® A-50	Amniotic fluid	[132]
	Monolithic quaternary amine column	Cell culture	[129,142]
Anion exchange	Anion-exchange magnetic beads	Cell culture and plasma	[133]
Nickel-based isolation	Anion-exchange agarose beads (Ni^{2+})	Cell culture and plasma	[134]
Cation- and anion-exchange chromatography	Column packed with either cation exchange resin (sulfoxyethyl) or anion exchange resin	Cell culture	[130]

for the isolation of EVs from cell culture [129–131] and amniotic fluid [132], while anion-exchange magnetic beads have been used for human plasma [133] (Table 5). A comparative study by Heath et al. demonstrated the applicability of the AIEC technique using a monolithic column with quaternary amine functionality (strong anion exchanger) for the isolation of cell-derived EVs [129]. The method required shorter isolation time (under 3 h for 1 L of cell culture supernatant) compared to UC and yielded intact EVs with higher purity compared to TFF. In addition, EV isolation from amniotic fluids was also possible using diethylaminoethyl cellulose resin (weak anion exchanger) as reported by Kosanović et al. [132]. The AIEC required optimization of separation and elution conditions, including concentration and volume of the salt gradient. For instance, it was found that a higher concentration of NaCl (1 M vs 0.2 M) was best for larger cell-derived EVs [132]. In addition, differences in glycoprotein compositions of EVs also affected separation in ion-exchange chromatography [132]. Despite the versatility and scalability of AIEC, its applications in EV research as an individual technique is mostly limited to cell culture so far. More complex biological matrices, such as blood and plasma, are challenging for ion-exchange methods due to the presence of other charged biomolecules in high amounts. The method can, however, complete other EV isolation techniques to remove viral contaminants and other proteins from the isolate to increase EV purity.

In addition to AIEC, nickel-functionalized agarose beads have also been used to capture a heterogeneous population of EVs from human plasma via electrostatic interaction in a similar way as in AIEC [134] (Fig. 4A). Interestingly, without manipulating ionic strength to release bound EVs, the EVs did not experience significant osmotic stress [135] since PBS (pH 7.4) with chelating agents (EDTA and citric acid) was used to release EVs while maintaining their integrity and stability. With this system, EVs of the large size range (50–700 nm) were isolated in addition to co-isolating exosomes, apoptotic bodies, and microvesicles [134]. Thus, this method alone was insufficient for the separation of different EV subpopulations.

2.2.2. Electrophoresis and dielectrophoresis

Electrophoresis and dielectrophoresis (DEP) are relatively new additions to the charge-based separation of EVs (Table 6) (Figs. 4B and 4C). In electrophoresis, EVs and their subpopulations can be separated based on their electrophoretic mobilities [115], giving additional information on characteristics of charged EVs that is not possible with size- and density-based techniques. With the DEP, it is possible to gain even deeper information on the properties of both charged and non-charged EVs, and it has also been successfully used in microfluidic format [143]. The on-chip electrophoresis separation of breast cancer cell-derived EVs and exosomes has been achieved with an electric field of 50 V/cm [136,144,145] (Fig. 4B). A combination of 300 kDa dialysis bag and electrophoresis has been utilized to isolate lemon-derived EVs [146]. In a proof of concept study, *Pectobacterium* sp. derived EVs were separated from other macromolecular aggregates by CE with 10 kV in normal polarity mode [147]. The authors also hypothesized that subpopulations of EVs could be studied with CE, which was proved in a study by Morani et al. [115], where EVs from different sources were separated with CE-LIF. Bovine milk-derived EVs were pre-isolated with sucrose gradient UC, pony plasma/serum and human plasma-derived EVs with SEC, and human plasma with monolithic IAC. The study demonstrated for the first time that CE based separation of EV subpopulations in the size range of exomeres and exosomes is possible due to different electrophoretic mobilities of the subpopulations. In addition, agarose gel electrophoresis has been used for high-efficiency EV separation from lipoproteins in human plasma with 100V for 2 h [148].

Alternating current electrokinetic (ACE) microarray chip devices have been successfully utilized to isolate and recover exosomes and EVs from blood, serum, plasma, and cell culture media in less than 30 min [149–152]. The isolations were achieved with an alternating current of 14 V and 15 kHz for 10 min. Direct current–insulator-based dielectrophoresis (iDEP) was successful in exosome separation from EVs purified from human breast adenocarcinoma (MCF-7) cells [153] as well as from human plasma and serum [154]. DEP has been also used to isolate and study EVs from human non-small cell lung cancer cells [133] and pancreatic cancer cell culture-derived EVs [155].

2.2.3. Other charge-based techniques

Other charge-based techniques have also been used for EV isolation and separation. Ion concentration polarization is a technique that could simultaneously isolate and preconcentrate exosomes [157]. The chip was packed with agarose gel and ion-selective membrane and could concentrate exosomes by a factor of 15 every 10 min. The recovery rate was 60-80%, which

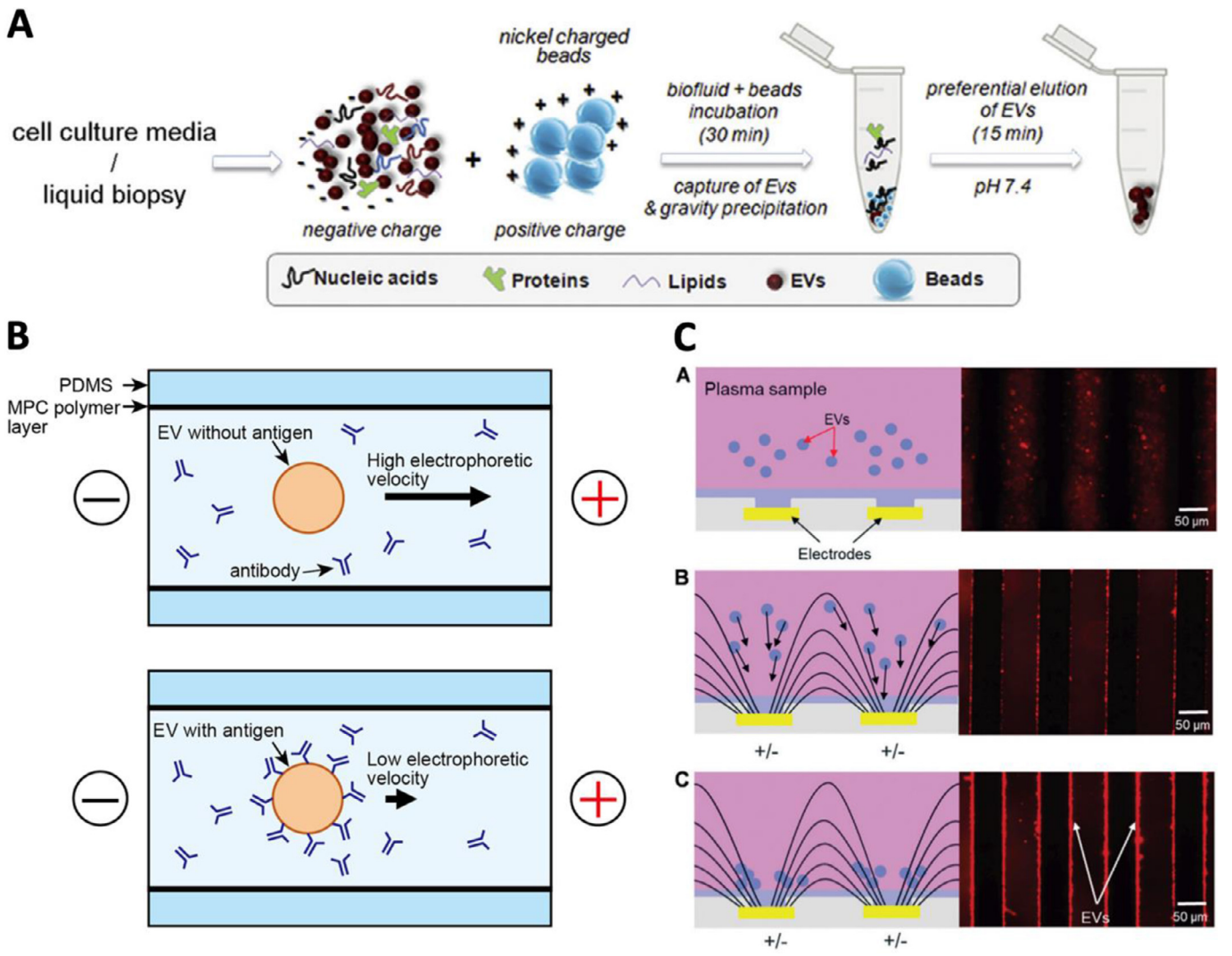

Fig. 4. Charge-based techniques for EV isolation: A) Nickel-based ion-exchange method for EV capture from human plasma. Reprinted with permission from [134], copyright 2019 Elsevier. B) On-chip electrophoresis for the isolation of breast cancer cell-derived EVs. Reprinted with permission from [136], copyright 2015, open access (Creative Commons Attribution license). C) Dielectrophoretic platform for isolation of EVs from human non-small cell lung cancer cells (A549). Reprinted with permission from [137], copyright 2019 Royal Society of Chemistry.

Table 6
Electrophoresis and dielectrophoresis (DEP) based techniques for EV separation and isolation.

Technique	Separation parameters	Sample matrix	Reference
Alternating current electrokinetic (ACE) microarray chip device	Alternating current 14 V, 15 kHz for 10 min	UC-isolated EVs from cell cultures Blood, serum, and plasma Plasma	[151] [150] [149,152]
Agarose gel electrophoresis	2 h at 100 V	Plasma	[148]
Capillary electrophoresis	Fused silica capillary (I.D. of 50 μm, O.D. of 375 μm, effective length (L_{eff}) of 50.2 cm, and total length (L_{tot}) of 60.2 cm) under 25 kV (normal polarity)	Pre-isolated plasma, milk, and plasma/serum-derived EVs	[115]
Capillary zone electrophoresis	Uncoated fused silica capillaries (50 µm i.d. x 30.2 cm total capillary length) under constant voltage of 10 kV (normal polarity)	*Pectobacterium* sp.	[147]
Direct current–insulator-based dielectrophoresis (DEP)	Media conductivity 1.6 S/m, with 0.01-1.5 mHz field frequency at 50 kHz increments for 30s Sinusoidal wave 2-10 kHz and amplitude 6 Vp-p or 4 kHz and amplitude 2-10 Vp-p Direct current for 20 min (10 V/cm) Direct current of 1500-2000 V for 20s (main channel) and 200 V for 1 min (side channels)	Cell culture-derived EVs isolated with differential UC Cell culture Plasma and serum Cell culture purified with total exosome isolation reagent	[155] [137] [154] [153]
Electrophoresis with dialysis	300 kDa dialysis bag, 300 mA for 30 min and after changing electrophoretic direction additional 150 min	Lemon	[146]
Electrophoretic migration through porous membrane	Track-etched polycarbonate (PCTE) 30 nm membranes, voltage 0–200V, flow rate 10–80 L/min	UC isolated EVs from the whole blood of mice	[156]
On-chip immunoelectrophoresis	An electric field of 50 V/cm	Cell culture	[136]
On-chip microcapillary electrophoresis			[144,145]

Table 7
Affinity-based EV isolation and separation techniques.

Separation system	Affinity ligand	Sample matrix	Affinity type	Reference
Magnetic beads	Anti-EpCAM antibody	Ascites	Immunocapture	[26,27]
	Anti-ICAM-1 monoclonal antibody	Cell culture		[202]
	Anti-CD9, anti-CD63, and anti-CD81 antibodies	Cell culture		[203]
	Anti-CD34 and anti-CD61 antibodies	Cell culture and plasma		[204]
	Anti-CSPG4 monoclonal antibody	Plasma		[205]
	Anti-prostate-specific membrane antigen antibody	Plasma		[168]
	Anti-ceramide antibody	Serum		[206]
Silica nanospring	TG97 (inhibitor of prostate-specific membrane antigen enzymatic activity)	Cell culture		[181]
Agarose resin	Anti-human CD171 (L1CAM) antibody	Plasma		[170]
Magnetic beads	Anti-epidermal growth factor receptor (EGFR), anti-EGFRvIII, and anti-CD9 antibodies	Cell culture	Microfluidic immunocapture	[207]
Polymeric monolithic disks	Anti-human-CD9 and anti-human-CD61 monoclonal antibodies	Plasma	Immunoaffinity chromatography	[52,115,116]
Magnetic beads	CD63-aptamer	Plasma and cell culture	Affinity capture	[183]
Magnetic beads	Vn96 peptide	Cell culture		[185]
ME^{TM} kit	Vn96 peptide	Plasma		[17]
Magnetic beads	T-cell immunoglobulin domain and mucin domain-containing protein 4 (Tim4)	Cell culture		[190]
Magnetic beads	Tim4	Plasma		[208]
Magnetic beads	*Solanum tuberosum* (potato) lectin	Urine		[192]
Agarose gel column	Concanavalin A (ConA)- and wheat germ agglutinin (WGA)-reactive glycans	Semen		[193]
Magnetic beads	Annexin A5	Plasma, serum, and ascites		[28]
Agarose beads	Heparin	Cell culture		[184]
Sepharose and HiTrap Heparin HP column	Heparin and ConA	Serum	Affinity chromatography	[84]

was significantly higher than conventional methods (6% for UC and 30% for ExoQuick^{TM}). Cyclical electrical field-flow fractionation (cyclical ElFFF) is another electrophoretic mobility-based separation technique that has been recently utilized to separate purified melanoma exosomes [158] and small and medium-size EVs from plasma and glioblastoma cells [159].

2.3. Affinity-based techniques

Besides charge-, density-, and size-based isolation techniques, affinity-based isolation is among the most popular techniques used for EV isolation (Fig. 2). Affinity-based approaches utilize highly selective and specific interactions between proteins or receptors found on the EV membrane and their corresponding ligands, such as antibodies. To develop an affinity-based isolation technique, the ligands are immobilized or conjugated onto/into a variety of solid media, such as magnetic beads or polymeric materials (e.g., agarose beads and monolithic columns) (Table 7). Based on isolation mechanisms, affinity-based isolation can be categorized into two groups, namely affinity chromatography and immunocapture, while affinity ligands can be further divided into bioaffinity ligands (e.g., antibodies, peptides, and transmembrane proteins) and heparin. The most widely used affinity-based technique for EV isolation relies on immunoaffinity capture by employing antibodies against EV surface proteins. In general, the antibodies are covalently coupled to magnetic beads via biotinylation. The most commonly used antibodies target tetraspanin proteins commonly enriched on exosome and EV surfaces, such as CD9, CD63, and CD81 [160,161]. One of the major advantages of immunoaffinity, in addition to the selective isolation of EVs in general, is the possibility to isolate EVs originating from different cell types. For instance, epithelial cell adhesion molecule (EpCAM) or CD326, a type of glycoprotein, has been found to be overexpressed in different cancer cells, including lung, stomach, colon, prostate, and ovarian cancer [162–165]. This discovery has led to the use of anti-EpCAM conjugated magnetic beads to target and isolate tumor-derived EVs [162,166]. In addition to anti-EpCAM, Sharma et al. used a monoclonal antibody having specificity toward a peptide of melanoma cells but not healthy cells to capture melanoma cell-derived exosomes from human plasma [167]. Other antibodies have also been used for the isolation of specific EVs, including anti-A33 (colon epithelial cell-specific exosomes) [166], anti-prostate-specific membrane antigen (PSMA) (prostate-derived EVs) [168], anti-CD105 (endothelial cell-specific exosomes) [169], anti-CD171 (L1CAM) (neuron-derived EVs) [170], anti-CD34 (endothelial progenitor cell-specific exosomes) [169], anti-CD61 [115,116] (Fig. 5A) and anti-CD41 [169] (platelet-specific exosomes), and anti-CD235a (erythrocyte-specific exosomes) [169].

In general, bound EVs are either eluted from the beads using appropriate reagents, or alternatively, bead-EV complexes can be directly used for further analysis (e.g., immunoassay). For instance, Zhao et al. have developed a microfluidic device (ExoSearch chip) for continuous exosome isolation and detection from human plasma using magnetic beads conjugated with three antibodies against common exosomal markers (CD9, CD81, and CD63) for immunocapture and fluorescence-labeled tumor markers (CA-125, EpCAM, and CD24) for probing [171] (Fig. 5B). Other microfluidic devices utilizing immunocapture-based isolation methods have also been reported [172] (Fig. 5C). Besides exosomes, microvesicle-specific markers, annexins (e.g., annexin V and annexin A1), a family of phospholipid-binding and membrane-binding protein [173,174], have also been used for the isolation of microvesicles and as a negative control in exosome isolation [169].

Among different solid supports, immunoaffinity chromatography with monolithic materials has been extensively used to isolate and separate biomacromolecules, including lipoproteins [175] and extracellular vesicles [115,116,176]. For instance, immunoaffinity chromatography methods using polymeric monolithic disk columns were developed in our laboratory to isolate CD9^{+} and CD61^{+} (platelet-derived EVs) from human plasma [52,115,116] (Fig. 5A). The methods could easily isolate intact CD9^{+} and CD61^{+} EVs from plasma in under 30 min in a single step, and the yielded EVs could readily be used in further analysis. In addition, the monolithic disk was integrated on-line to AsFlFFF for automated iso-

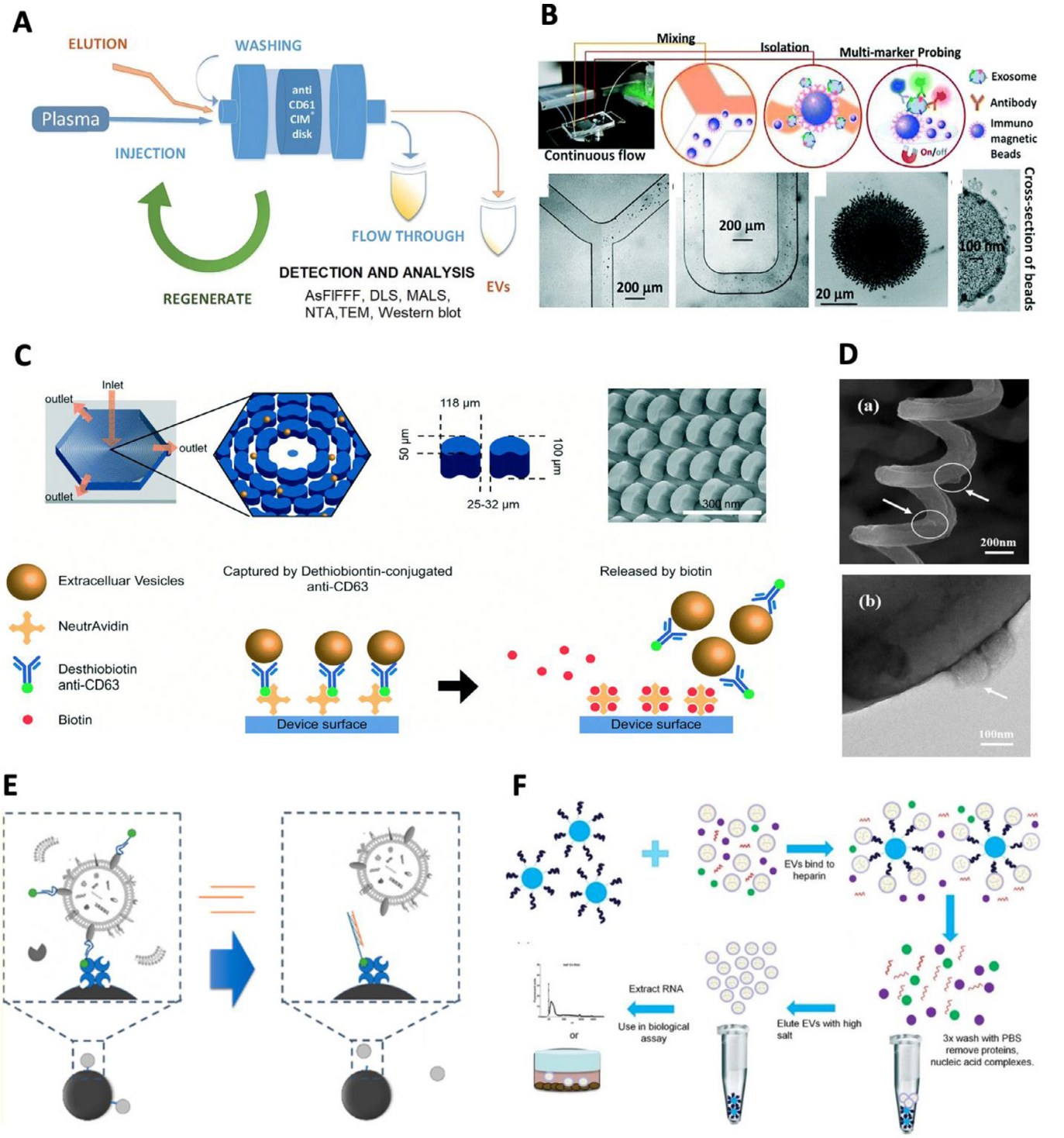

Fig. 5. Affinity-based EV isolation systems: A) Immunoaffinity monolithic disk column for CD61⁺ EV isolation from plasma. Reprinted with permission from [116], copyright 2019 Elsevier. B) Microfluidic device (ExoSearch chip) for exosome isolation and detection from plasma using anti-CD9, CD81, and CD63 antibodies conjugated beads. Reprinted with permission from [171], copyright 2016 Royal Society of Chemistry (Creative Commons Attribution-NonCommercial 3.0 Unported Licence). C) Microfluidic device (OncoBean chip) for EV capture using the anti-CD63 antibody. Reprinted with permission from [172], copyright 2020 Royal Society of Chemistry. D) Silica nanospring with TG97 ligand for exosome capture (arrows indicating the exosomes). Reprinted by permission from [181], copyright 2017 Springer Nature. E) Capture and non-destructive release of EVs using aptamer-based magnetic isolation. Reprinted with permission from [183], copyright 2019 American Chemical Society. F) Heparin-coated magnetic beads for EV isolation from cell culture. Reprinted with permission from [184], copyright 2015, Springer Nature, open access (Creative Commons Attribution CC BY license).

lation and fractionation of CD9⁺ and CD61⁺ EV subpopulations [52]. The IAC-AsFlFFF system could isolate CD9⁺ and CD61⁺ EVs and fractionate them to their subpopulations (size range of exomeres and exosomes) up to 18 plasma samples per day. The monolithic disks immobilized with antibodies could also be reused multiple times [116]. Monolithic columns have several advantages over particulate-based stationary supports for the separation of large biomolecules, including low back pressure and convective mass transfer rather than diffusive transport, enabling high flow rates and short separation times due to large intercon-

nected pores [177,178]. In particular, polymer-based monoliths can withstand alkaline pH conditions required for elution or desorption of bound particles unlike silica-based particles and monoliths. The polymer-based monoliths can also be directly coupled to other separation methods or detection systems for further analyses, making them attractive solid supports for EV isolation. Besides magnetic beads and monolithic supports, other solid media used for EV isolation have also been reported, such as magnetic nanowires [179], paper (cellulose) [180], and nanospring [181] (Fig. 5D).

In addition to immunoaffinity-based approaches, aptamers have been used to capture EVs with high affinity and specificity. Aptamers are single-stranded DNA or RNA oligonucleotides having three-dimensional structure developed using systematic evolution of ligands by exponential enrichment (SELEX) technology, enabling the selection of EV-specific aptamers, and thereby usually referred to as chemical antibodies [182]. Recently, Zhang et al. [183] used magnetic beads conjugated with DNA aptamers specific for CD63 and MUC1 (tumor biomarker) for EV capture from human plasma and cancer cell culture [183] (Fig. 5E). The group demonstrated the non-destructive release of bound EVs from the aptamers by introducing a complementary sequence to hybridize with the aptamer, resulting in the aptamer breakage and the release of intact EVs. This made the aptamer-based isolation more advantageous than antibody-conjugated magnetic beads due to elution issues of EVs encountered with magnetic beads. The yielded EVs from the aptamer-based method were more uniform and of a narrower size range in comparison to EVs isolated using UC [183]. Although aptamers are believed to bind EVs with high specificity, they have not been widely used for EV isolation but mostly for analysis and detection [127].

Venceremin (Vn), a specific class of peptides, was found to specifically bind to heat shock proteins (HSPs) and thus has been used as an affinity ligand to capture HSP-containing EVs [185]. Ghosh et al. developed an affinity pull-down method using Vn96-coated magnetic beads to capture EVs from cell culture, urine, and plasma samples that yielded similar properties for the EVs compared to those isolated using UC [185]. In addition, a commercial kit, ME^{TM} (microvesicle enrichment kit), utilizing the same principle was also used for the isolation of clinically-feasible urinary [186] and cancer cell-derived EVs [187]. Nevertheless, a comparative proteome study by Askeland et al. found that EVs isolated from healthy platelet-poor plasma using the ME^{TM} kit contained low EV specific proteins (low EV yield) and a substantial amount of non-EV components, while lipoprotein contamination was rather small [17]. Moreover, the reproducibility and consistency of the method were lower compared to high-speed centrifugation and SEC, suggesting a need for method improvement [17]. These findings suggested that the quality of EVs yielded by Vn96 peptide affinity pull-down methods was highly dependent on the sample type.

A transmembrane protein, namely T-cell immunoglobulin domain and mucin domain-containing protein 4 (Tim4 or Timd4), binds strongly to phosphatidylserine exposed on apoptotic cells, exosomes, and microvesicles [188,189] and therefore could potentially be used as an affinity ligand for EV isolation. Nakai et al. developed an immunoaffinity capture using Tim4-Fc immobilized magnetic beads for the isolation of EVs from cell culture, serum, and urine [190]. The EVs isolated using this method were of higher purity without detectable protein contaminants compared to UC and precipitation methods. The elution of EVs from Tim4 was also possible through the use of Ca^{2+} chelators since the Tim4-EV binding was Ca^{2+}-dependent [190]. However, since phosphatidylserine is present in many EV subpopulations, the resulting EVs isolated using the Tim4-based method can also contain not only exosomes, but also apoptotic bodies.

Lectins, non-immune-system proteins having specificity towards carbohydrate residues [177], such as glycans, enriched on EV surface, have also been used for EV isolation. For instance, Yamamoto et al. developed a method using agglutinin prepared from the freshwater cyanobacterium *Oscillatoria Agardhii* (OAA) as affinity ligand immobilized on magnetic beads to capture cancer cell-derived EVs [191]. Plant-derived lectins were also used for EV isolation, such as *Solanum tuberosum* (potato) (STR) lectin for affinity capture of urinary EVs [192] as well as concanavalin A (ConA) and wheat germ agglutinin (WGA) for affinity chromatographic separation of seminal prostasomes [193]. Above all, lectins have been mostly used for identification and detection of EVs, such as glycosylation or glycan profiling of EV surface using array systems [194–196], and lectin binding assay [197], while not many lectin-based methods have been developed for EV isolation.

In addition to bioaffinity-based isolation, other affinity ligands have also been exploited. For instance, heparin was found to inhibit the uptake of brain tumor cell-derived EVs into recipient cells, suggesting possible binding of heparin to EV surface [198]. Studies have shown that heparin exhibits interactions with EV surface components, including annexin, zymogen, HSPs, and histone [184,199]. This finding led to the development of heparin-affinity based methods for EV isolation from cell culture [184], plasma [184,200], and serum [84] (Fig. 5F). Nevertheless, it is known that heparin also binds other biomolecules, such as LDLs [201]. Therefore, additional purification or sample pretreatment steps are required to avoid lipoprotein contamination.

Advantages of affinity-based approaches for EV isolation and separation include fast process, simple instrument, easy operation, and most importantly, high specificity and selectivity for EVs of interest, making them highly attractive and suitable for clinical applications and diagnostic purposes. Moreover, due to high specificity, low non-specific binding can be achieved with immunoaffinity-based methods. Many preparative methods can be directly coupled with on-line detection and analysis systems [52]. Drawbacks of these methods include costly ligands (e.g., antibodies) and several optimization parameters required for efficient isolation, such as elution process capable of maintaining EV integrity, number of magnetic beads, and specificity of ligands toward antigens. In addition, immunocapture efficiently isolates EVs from cell culture but usually not from more complex matrices, such as plasma, due to multiple competitive binding sites, resulting in low yield. The elution of bound EVs from bead-based methods is also often troublesome [3]. However successful elution of intact EVs and their subpopulations (exomeres and exosomes) have been shown in studies with antibodies immobilized in the monolithic columns [52,115,116].

2.4. Other techniques

In addition to the previously mentioned techniques, EVs have also been reported to be isolated with other techniques. For instance, hydrophobic interaction chromatography (HIC) has been used for the isolation of exosomes from exosome-spiked human plasma, exosome-spiked human urine, and cell line using poly(ethylene terephthalate) (PET) capillary-channeled polymer (C-CP) [209–211]. This technique uses PET with weakly ionized surfaces to bind with the hydrophobic surface of exosomes. The exosomes adsorbed on the surface were then desorbed using gradient elution. Unfortunately, this technique is most probably not applicable for all kinds of samples, such as biological fluids, due to the presence of other hydrophobic molecules than EVs in a large amount. Microfluidic platforms are also among new emerging techniques of EV isolation due to their small size, automation, and only small sample volume required. Different isolation principles have been used in microfluidic platforms, such as immuno-

capture. In addition, acoustic trapping microfluidic devices have been reported for EV isolation from cell culture media, urine, and plasma [212–215]. The acoustic trapping technology utilizes ultrasonic wave scattering to capture EVs. The devices work by trapping seeding particles, such as polystyrene beads, with EVs via acoustic forces, resulting in the formation of clusters of EVs and the particles depending on their size, density, and compressibility. These clusters are then washed and released upon deactivation of ultrasound [214]. The method has yielded EVs comparable to those isolated using UC [213] and only required a volume as low as 12.5 µL [215]. Magnetic nanowires (Fe/Au) have also been used to isolate tumor-derived EVs from cancer cells by internalizing the nanowires into EVs located in cells of interest (e.g., cancer cells) and using a magnetic stand to attract and isolate EVs [216,217]. Yield and size distribution were comparable to those obtained by UC and a commercial kit [217].

3. Concluding remarks

A growing interest in EV research and the drawbacks of conventional ultracentrifugation and precipitation-based methods for EV isolation have led to a noticeable increase in the development of numerous more advanced EV isolation techniques to obtain EVs of high quality for further analysis in order to elucidate their properties, functions, cargoes, and potentials for various applications. Currently, there are no “one-size-fits-all” standalone techniques for EV isolation. General criteria for efficient isolation techniques include being fast, automated, simple, and suitable for further applications as well as being capable of isolating EVs with satisfactory quality and yield. Since different techniques and methods offer both advantages and disadvantages and have different principles for isolation and separation, combinations of techniques, such as SEC with filtration-based methods, have been a common trend in numerous publications in order to obtain best suited EVs for desired applications. As the EV field is still rapidly evolving, there is an increasing need to study, for instance, EV subpopulations as well as their functions and properties. Immunoaffinity methods are highly advantageous for the selective isolation of EVs from different cellular origins, while other affinity-based techniques can be used to isolate EVs of specific properties. Hyphenation of these methods to size-based techniques, such as AsFlFFF or AF4, can further provide separation of EV subpopulations based on size, as was shown with automated on-line IAC-AsFlFFF system. In addition, microfluidic platforms utilizing different isolation principles have also been developed in order to overcome other limitations, such as the need of large sample volumes. Various microfluidic platforms also offer both isolation and detection possibilities, allowing simultaneous isolation and analysis of EVs.

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.

Acknowledgement

Financial support was provided by the Research Council for Natural Sciences and Engineering, Academy of Finland (grant number 1311369).

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