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1 **Recent electrokinetic strategies for isolation, enrichment and separation of extracellular**  
2 **vesicles**

3

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19 exosomes, isolation.

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26 **Abstract**

27 Extracellular vesicles (EVs) are a family of cell-derived membrane vesicles that are present in  
28 almost all body fluids. EVs have gained significant interest over the last decades as mediators  
29 of key functions in numerous patho-physiological condition (clearance, signalling, trophic  
30 support, cargo delivery) and as potential prognostic or diagnostic biomarkers. The  
31 endogenous delivery capacities of these nanometric entities also hold a high potential as  
32 engineered drug nanocarriers for clinical and pharmaceutical applications, especially for  
33 targeted therapies. Nevertheless, knowledge about the features of individual EVs  
34 (composition, physical and chemical characteristics) is still at the infancy because of the  
35 technical challenges to purify and analyze the various subpopulations of EVs. In this review,  
36 a comprehensive overview of electrokinetically driven methods for isolation, enrichment and  
37 characterization of EVs is presented. This review covers new trends of analytical science  
38 (over 7 years up till 2020), serving for high-quality EVs production, isolation, analysis and  
39 quality control, which are expected to provide powerful and complementary alternatives to the  
40 conventional and recently emerged approaches such as microfluidics. We critically discuss  
41 here the pros and cons of the different instrumental and methodological developments for  
42 electrokinetic strategies applied to EVs.

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51 **1. Introduction**

52 Extracellular vesicles (EVs) cover a family of heterogeneous particles delimited by a  
53 phospholipid bilayer and secreted by all types of cells, with a size ranging from around 30 nm  
54 up to a few micrometers. Based on existing knowledge of their biogenesis, EVs can be  
55 narrowly classified into two major groups: exosomes and microvesicles (see Fig 1 for EVs  
56 formation and secretion). However, these generic terms might mean different things  
57 depending on the research field [1]. For such reason, as endorsed by International Society of  
58 Extracellular Vesicles, “extracellular vesicles” is used as an umbrella term to represent all  
59 types of cell-secreted vesicles [2]. It is now well established that EVs are implicated in a wide  
60 range of physiological processes, such as immune response, tissue repair, inflammation and  
61 neuronal functions [3-5], as well as in different pathological processes, like diabetes, liver  
62 disease, neurodegenerative diseases and cancer progression [6-8]. As EVs and their  
63 composition reflects the patho-physiological state of their producing cells, they provide a new  
64 source of biomarkers for diagnostic and prognostic applications [9-12]. Their capacities to  
65 deliver encapsulated materials in a targeted way open new avenues to harness them as  
66 biocompatible drug nanovesicles in targeted therapies [13-16]. Depending on their cellular  
67 origin and state, EVs are highly miscellaneous in size, cargo and membrane composition [17].  
68 Even though the past decades have witnessed an exponential development in the field of EVs,  
69 there is still a lack of widely accepted specific markers to characterize the different  
70 subpopulations of EVs. Moreover, guidelines for isolation, enrichment and separation of EVs  
71 samples are still facing difficulties to properly exclude contaminants and separate EV  
72 subpopulations. In this context, further investigations are required to improve isolation,  
73 enrichment and separation of extracellular vesicles in order to fully understand their intrinsic  
74 properties and to confirm their great potential in clinical applications.

75

76 EV isolation and enrichment is a prerequisite before their clinical use as diagnostic biomarker.  
77 A sufficiently high yield and good reproducibility of EVs purification / isolation process is  
78 required to get a representative batch of the whole EVs population and avoid unacceptably  
79 high batch-to-batch variations with evident failure at identification and validation of relevant  
80 biomarkers for instance. During the last years, many reviews have been dedicated to  
81 summarize and compare different technologies for isolation of EVs [18-26]. The traditional  
82 methods used for EV isolation exploit a variety of EVs properties, such as their density,  
83 shape, size, specific interaction with solvents and surface proteins (see Table 1 for comparison  
84 of the most commonly used EV isolation methods). Typical batch-wise isolation techniques  
85 include ultracentrifugation (UC), gradient ultracentrifugation, ultrafiltration, polymer co-  
86 precipitation, size-exclusion liquid chromatography (SEC), field flow fractionation and  
87 immunoaffinity. Among them, ultracentrifugation is the most widely used primary isolation  
88 method across all EVs applications (> 80 %) [27]. However, this approach has many  
89 disadvantages such as bulky and expensive equipment, time-consuming procedure,  
90 contamination by protein aggregates and other particles, as well as high-volume sampling. A  
91 comparison study on EVs isolation yields from human serum achieved with some of these  
92 conventional methods revealed the highest numbers of total isolated EVs for polymer-based  
93 precipitation and size-exclusion chromatography methods (*i.e.*  $5.6 \times 10^{10}$  and  $4.2 \times 10^{10}$   
94 particles / mL according to NTA analysis, respectively) [28]. These were followed by  
95 ultracentrifugation and density gradient ultracentrifugation approaches that led to the  
96 obtention of EVs concentrations of between  $3.8 \times 10^9$  and  $9.5 \times 10^9$  particles / mL [28]. In  
97 parallel, microfluidic technologies have been recently exploited for EVs isolation, making use  
98 of both physical and biochemical properties of EVs at microscale level. Microfluidics offers a  
99 tremendous potential by minimizing the sample volume, cost and reagent consumption,  
100 performing the reactions faster and conducting multiple assays in small devices

101 simultaneously. These microfluidic systems bring also new functionalities and employ a wide  
102 range of EVs isolation approaches such as microporous filtration, immunoaffinity, trapping on  
103 nanowires, together with the use of acoustic, electrophoretic and electromagnetic sorting  
104 mechanisms [18, 19, 29-33].

105

106 Once EVs isolation has been achieved, it is also extremely challenging to verify the identity  
107 of EVs and monitor their quantity and quality. Until now, EVs characterization by multiple  
108 and complementary techniques has to be carried out to evaluate the performance of the  
109 isolation methods and to give an insight into the EV nature [21, 22, 34, 35]. Microscopy-  
110 based methods (transmission electron microscopy TEM, atomic force microscopy AFM),  
111 dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), single EV analysis  
112 (SEA) method and tuneable resistive pulse sensing (TRPS) are among the most widely used  
113 to measure the physical characteristics of EVs. These methods and their inherent advantages  
114 and drawbacks for EVs detection / characterization are summarized in Table 2. Besides these  
115 techniques, conventional protein analysis techniques, such as Western blotting, enzyme-  
116 linked immunosorbent assay (ELISA) and mass spectrometry (MS), provide high-throughput  
117 mapping of EVs' membrane and intra-vesicular proteins. EVs also contain nucleic acids  
118 which can be successfully examined by conventional extractions such as phenol-based  
119 method and spin column techniques. This led to the development of DNA/RNA-detection-  
120 based analysis tools for EVs, notably polymerase chain reaction with real-time fluorescence  
121 measurements (RT-PCR) or more recent technologies such as Droplet PCR, Ion-Exchange  
122 Nano-detector and localized surface plasmon resonance (LSPR) based assay [21]. Among all  
123 conventional methods used for EV characterization, Western blotting (74 %), single particle  
124 tracking (72 %, mainly with NTA) and electron microscopy (60 %) are among the most  
125 widely used [27]. Microfluidic-based technologies have also evolved in the field of EV

126 detection, in which capture and characterization of EVs are combined in one integrated  
127 platform [29, 31, 32, 36]. These emerging approaches are expected to provide fast and  
128 sensitive measurement with little sample consumption.

129  
130 In a related context, EVs have been considered as nanoparticles (NPs) since they are within  
131 the same size range. Among analytical methods commonly used for NP characterization,  
132 electrokinetic approaches have emerged as simple and powerful techniques that are  
133 complementary to other spectroscopic, microscopic and optical ones, providing  
134 supplementary information on NPs' electrophoretic mobility,  $\zeta$ -potential, particle size and  
135 biomolecular interactions [37-40]. Indeed, microscale electrophoresis relying on  
136 electrophoretic migrations of charged species under a high electric field, with its notable  
137 advantages of high separation efficiency, limited consumption of sample/chemicals and high  
138 potential for miniaturization and integration, has been widely used for high-resolution  
139 separation, isolation as well as preconcentration of NPs. With a similar viewpoint,  
140 electrokinetic strategies are expected to provide a powerful tool / alternative to conventional  
141 methods for EVs production, analysis and quality control. By application of a (high) voltage,  
142 the negatively charged EVs can migrate at different mobilities according to their size, charge  
143 and shape. The electroosmotic flow (EOF), which is the bulk liquid motion normally occurred  
144 under a high voltage over a charged support, typically silica capillaries possessing negative  
145 surface charges, can also be used to tune the apparent mobilities of EVs. This mechanism can  
146 be exploited for either i) isolation of EVs (via a selective membrane for instance) from other  
147 untargeted entities (e.g. proteins, cell membrane debris) or ii) separation of EVs over a  
148 microchannel (capillary or microchip) (see Fig. 2 for schematic illustration). Both non-  
149 uniform electric fields (for dielectrophoresis) and uniform ones (for microscale  
150 electrophoresis), as well as different variants of electrophoresis (for instance isotachophoresis,

151 capillary zone electrophoresis etc.) can be used for these purposes. While both electrokinetic  
152 separation and isolation techniques rely on an electrical field to drive EVs, the migration  
153 pattern and geometric design of the system would determine the respective purpose (typically  
154 micro-bored channels for EVs microscale separation and chambers separated by membranes  
155 or porous supports for EVs isolation). Normally, high electrical fields (more than 300 V/cm)  
156 are required for the former whereas lower ones are employed for the latter. Herein, we report  
157 on the first comprehensive overview of different electrokinetic strategies for isolation,  
158 separation and quantification of EVs. These strategies that exploit the electrokinetic migration  
159 of EVs are expected to possess advantages over conventional approaches in term of high  
160 resolution for EVs separation, high purity for EVs isolation, high potential for miniaturization  
161 and integration, as well as high versatility (i.e. EVs isolation, separation and characterization  
162 using an electrical field). Different instrumental and methodological developments for such  
163 purposes as well as their positive features and limitations are discussed. There have been  
164 almost 35 research articles on this topic, with more than one-third released in the last two  
165 years (2019-2020), confirming the recent interest of our research community in this emerging  
166 approach to provide a reliable and accurate tool for purifying, characterizing and quantifying  
167 EVs.

168

## 169 **2. A glance at EVs characteristics**

170 It is widely accepted that the EVs size, contents and membrane composition are highly  
171 heterogeneous [5]. This heterogeneity relates partially to their origin and the patho-  
172 physiological state of the producing cells and will impact their different functionalities [41,  
173 42]. Yet this heterogeneity is also dependent on various other factors. EVs indeed can vary  
174 widely in terms of their composition and may carry specific sets of proteins, lipids or RNA  
175 species that then determine their fate and functions [5, 43]. While the accumulating data in the



176 past few years indicated that all EVs possess a negative surface charge due to their negatively  
177 charged phospholipid membrane, EVs from different origins may exhibit different zeta  
178 potentials, varying from -10 to -40 mV [25]. The EVs concentrations also vary significantly  
179 from one biofluid to another, from the range of  $10^7$  particles / mL for cerebrospinal fluid  
180 (CSF) up to  $10^8$  - $10^9$  particles / mL for plasma and blood [25]. In addition, the number of EVs  
181 released also changes depending on the physiological state of the parent cell and the  
182 microenvironment conditions [44]. Similar to the behavior of cells, EVs are capable of  
183 responding to changes in the microenvironment that surrounds them, resulting in EVs shape,  
184 size and zeta potential variations in different buffer conditions [44, 45]. Zijlstra and Di Vizio  
185 recently provided evidence that the heterogeneity of EVs is not only driven by size variation,  
186 but also defined by their diverse proteomic profiles, N-Glycosylation patterns, lipidic, RNA  
187 and DNA profiles [46]. All these data therefore suggest that while at first sight both EVs and  
188 NPs were thought to share similar size and charge characteristics, a deeper investigation  
189 revealed that EVs are much more complex than NPs and may behave differently from NPs  
190 under an electric field [47].

191

192 Lysis and/or characteristic modification of EVs can also occur, requiring a careful and  
193 restricted selection of suspension buffer composition and ionic strength during isolation and  
194 analysis [48-50]. This suggests that numerous parameters must be considered when handling  
195 the EVs, and high attention must be paid to maintain the native properties of EVs during  
196 isolation, purification, characterization and quantification. It is well known that the  
197 dependency of NPs' electrophoretic mobilities on their size, charge, charge-to-size ratio and  
198 shape can be determined only under very specific conditions [51-54]. Various parameters,  
199 notably background electrolyte (BGE) ionic strength, pH and composition as well as applied  
200 electric fields were found to have impacts on NPs' electrophoretic mobilities [55]. The

201 situation is even more constrained for EVs. The fact that EVs from different origins probably  
202 have different shapes and also different proteins / biomolecules on their surface and that they  
203 are prone to lysis or shape-deformed renders it very difficult to establish rules for the sole  
204 dependency of EVs electrophoretic migration behavior on charge and/or size-based  
205 parameters. The application of a high electric field, as suggested by Jones *et al.* and d'Orlyé *et*  
206 *al.* when dealing with NPs [52, 56], may not be applicable for EVs because it would provoke  
207 EVs lysis. With the present state of the art of EV purification technologies, it is still not  
208 readily possible to obtain EVs with very low degree of polydispersity and narrow size  
209 distribution [47], whereas this is already possible for NPs synthesis. All these features imply  
210 that the solid background already acquired for NPs should be used with care when applying to  
211 EVs, and consideration of the difference between EVs' and NPs' behaviors should be taken  
212 into account when developing electrokinetic strategies for EVs production and  
213 characterization.

214

### 215 **3. Electrokinetic isolation and enrichment of EVs**

216 The utility of an electric field to isolate and manipulate EVs has been recently exploited as  
217 contact-free particle sorting mechanism within different developed microdevices. A summary  
218 of electrokinetic approaches for EVs isolation and enrichment is shown in **Table 3**. The  
219 electrophoretic sorting of EVs is based on different electric field intensity and charge-to-mass  
220 ratios of the EVs, without the need of pumps or other moving parts. Some of the devices  
221 presented here have proved the advantage of electrophoresis in EVs isolation via a filtration  
222 mode. In this sense, Davies *et al.* demonstrated direct isolation of EVs from mouse's whole  
223 blood [57]. In this system, they designed a porous polymer monolith in the center of an open  
224 cross flow channel to act as a membrane filter. This nano-pored monolithic membrane,  
225 playing the role of a size exclusion filter, was prepared *in situ* and was integrated into a

226 poly(methyl methacrylate) micro-system. To render the monolithic membrane compatible  
227 with the extraction of vesicles, pore size was tuned to fit EVs sizes by changing the ratio of  
228 porogenic solvent to monomer solution. Then, direct current (DC) electrophoresis with a bias  
229 voltage of 10V (corresponding to an electric field of 6.25 V/cm) was activated with the  
230 electric field applied perpendicular to the flow direction to propel the EVs across the  
231 membrane and drive them in the opposite direction to the positively charged interfering large  
232 debris and proteins, thus avoiding the clogging that occurred when simple pressure-driven  
233 mode is employed (see Fig 3 for comparison between pressure-driven and electrokinetically-  
234 driven EVs isolation). In this configuration, sample and collection streams were performed at  
235 a relatively high flowrate (2  $\mu\text{L}/\text{min}$ ) to prevent larger particles and cells in the bulk stream  
236 from retaining onto the membrane. EVs extraction from 240  $\mu\text{L}$  of blood could be completed  
237 within 2 hours using this system. A 10-fold better purity of isolated EVs (approaching the  
238 purity offered by ultracentrifugation) was achieved when compared to that obtained with the  
239 pressure-driven mode. Moreover, the electrophoresis mode demonstrated the capacity to  
240 remove most of the soluble proteins from mouse blood, similarly to conventional  
241 ultracentrifugation. Nevertheless, the EVs recovery with this electrokinetic mode was  
242 unsatisfactory, with only 2% of EVs recovered. In another microdevice isolation system, Cho  
243 et al. applied a higher electric field across a porous dialysis membrane with an appropriate  
244 pore size (30 nm) to selectively capture EVs on the membrane from mouse blood plasma  
245 while facilitating protein migration through the membrane [58]. In this isolation system, two  
246 electrodes and two membranes between electrodes are juxtaposed. Three flow channels were  
247 arranged between electrodes and membranes, one for sample solution, one for cathodic buffer,  
248 and the other for anodic buffer. EVs and other particles (e.g., proteins) move horizontally in  
249 the sample flow confined between the membranes, and simultaneously migrate vertically  
250 under the applied voltage. As EVs and most of the proteins in the plasma (e.g., albumin) have

251 negative surface charges, they move toward the anode and encounter the membrane, whereas  
252 particles with neutral or positive charges (e.g., histidine, lysine, non-polar lipids) move with  
253 the sample flow or are driven toward the cathode by electrostatic attraction. When the  
254 negatively charged particles arrive at the membrane pores, only small species (i.e., proteins)  
255 can pass through the membrane to enter the buffer channel and are consequently flushed out.  
256 In contrast, particles  $> 30$  nm (i.e., EVs) accumulate on the membrane, and the attached EVs  
257 are then washed and collected by pipetting with phosphate buffered saline (PBS). The  
258 membrane can be regenerated after each EVs collection for repeated use. Based on NTA  
259 measurements, this method permitted a 65% EVs recovery rate in approximately 30  
260 minutes. According to RNA analysis and characteristic EV protein (CD63), this device led to  
261 less impurities than commercial polyethylene glycol (PEG) precipitation method (84 % vs 68  
262 %, respectively). However, EVs isolated using this technique were less pure than those  
263 isolated by conventional ultracentrifugation (98 %).

264

265 In a related context, Mogi et al. proposed in 2018 the use of an electrical filtration device  
266 utilizing an ion-depletion zone in a microchannel to concentrate EVs from the culture media  
267 of four types of cell lines [59]. The approach is called ion concentration polarization (ICP).  
268 Readers can refer to a recent review for the theoretical background and extended applications  
269 of this electrokinetic concentration mode on chips utilizing ICP [60]. The ion-depletion  
270 zone, from which all positively and negatively charged particles are excluded, was generated  
271 by applying a voltage between two microchannels, i.e. main and ground (GND) channels,  
272 bridged by a cation-exchange membrane. The application of a voltage caused cations around  
273 the bridged area of the high-voltage side in the main channel to be drawn into the low-voltage  
274 zone of the GND one, resulting in an increase in the anions' amount relative to the cations'  
275 one. This led to an instantaneous expulsion of anions by electrostatic repulsion to re-establish

276 the electro-neutral situation, thereby forming an ion-depletion zone. As long as the  
277 electrostatic force required to generate the ion-depletion zone is still stronger than the driving  
278 force needed to move particles into the zone, the ion-depletion zone works as an intangible  
279 barrier to prevent any charged particles from entering in this zone. As a result, any negatively  
280 charged particles (more specifically EVs) passing through this zone by a pressure-driven flow  
281 are pushed away to the other side of the microchannel for preconcentration / collection (see  
282 Fig 4). They demonstrated that the ion-depletion zone method caused less damage to EVs  
283 when compared to standard ultracentrifugation isolation process. The EV recovery yields  
284 achieved for ICP and ultracentrifugation isolation were 98 % and 57 %, respectively. Further  
285 evidence is of course needed to certify the potential of the former for EVs concentration and  
286 separation from clinical samples. Interestingly, the ion depletion feature was also exploited in  
287 the same year by Marczak *et al.* These authors designed a different electrophoresis assisted  
288 device for EVs isolation that uses a cation-selective membrane to produce a transverse local  
289 electric field [61]. Under this electric field, the EVs pumped with a syringe into the microchip  
290 were carried into a perpendicular channel containing agarose gel, which filtered undesirable  
291 cell debris, and finally got concentrated near the membrane surface. When tested with cell  
292 culture media and blood sample spiked with EVs isolated with a commercial kit, this device  
293 was able to isolate between 60% and 80% of the incoming EVs within 20 min. This system  
294 demonstrated a superior recovery yield compared to the conventional techniques of  
295 ultracentrifugation (6 %) and polymer-based precipitation (ExoQuick, 30 %). Furthermore,  
296 the use of negatively charged membranes allowed prevention of membrane pore clogging by  
297 repelling the exosomes, whereas the utilization of the external ion concentration polarization  
298 phenomenon enriched these target entities. Later, Cheung *et al.* exploited the use of ion-  
299 selective membranes as well, by printing them directly on a PMDS microchannel [62]. This  
300 system incurred surface binding-based or passive trapping mechanism near the conductive

301 polymer membrane, allowing to simultaneously preconcentrate and capture EVs. The buffer  
302 solution was loaded into the cathodic reservoir and allowed to flow through the channel,  
303 whereas the EV sample was loaded into the anodic reservoir. Once the voltage was applied  
304 (electric field of 45 V/cm), the flow generated by the electroosmotic flow (EOF) pushed the  
305 EVs towards the cathode reservoir in front of the printed membrane. This ICP-based  
306 electrokinetic concentrator was demonstrated to preconcentrate and capture EVs from human  
307 breast cancer cell lines by increasing the EVs concentration by ~100-fold within 30 min. The  
308 limit of detection achieved was  $5 \times 10^7$  particles / mL, which is better by two orders of  
309 magnitude than those obtained with a microfluidic approach without ICP. Their fabrication  
310 strategy that decoupled the ion-selective membrane from the substrate allowed the flexibility  
311 of either using an antibody-immobilized substrate to increase the EVs capture specificity or a  
312 glass substrate with passive microtraps to capture EVs in a highly efficient manner. However,  
313 once again, the EVs samples tested with this system came from batches extracted using a  
314 commercial kit. Direct application of this device to untreated clinical samples has yet to be  
315 demonstrated.

316

317 Diverted from DC electrophoresis driven mechanism, a nonuniform electric field was  
318 combined with immunoaffinity strategy for isolation of EVs [63]. Vaidyanathan et al.  
319 developed a microfluidic device containing a long array of asymmetric functionalized  
320 electrode pairs within three individual microfluidic channels. The use of an alternating current  
321 across the electrodes generated flow vortices that both increased collisions between target  
322 EVs and functionalized electrodes and reduced nonspecific adsorption of weakly bound  
323 molecules from the electrode surface. Detection and quantification of EVs spiked in PBS and  
324 breast cancer patient serum were performed both visually and via absorbance measurements  
325 from colorimetric solutions. A 3-fold enhancement in detection sensitivity compared to a

326 pressure-driven system (via a syringe pump) was reported. On the other hand, considering that  
327 it is closer to an immunoaffinity based method, it could only capture EVs expressing a given  
328 specific antigen, potentially excluding other EVs subpopulations concurrently present in a  
329 biofluid.

330

331 At a glance, electrokinetic strategies exhibit relatively higher EVs recovery yields (varying  
332 between 60 - 98 %, depending on the working principle) compared to those obtained with  
333 conventional precipitation (30 %) and ultracentrifugation (6 % - 57 %, depending on testing  
334 conditions). Better purities are also achieved with electrokinetic approaches. Among all  
335 approaches presented, the electrophoretic isolation on porous membranes and ICP-based gel  
336 electrophoresis offered the highest EV isolation yield, which is far superior than those of  
337 conventional methods. This technique [61], however, may come with a penalty of large size  
338 distribution up to 400 nm. On the other hand, much narrower size distribution was achieved  
339 with the ICP system [59], but further tests using clinical samples need to be performed in  
340 terms of EVs recovery and purity compared to conventional approaches. Note that a fair and  
341 systematic comparison of EVs isolation performances between electrokinetic strategies,  
342 microfluidic ones and the established methods (i.e. ultracentrifugation, polymer precipitation,  
343 membrane filtration, size exclusion chromatography SEC and membrane affinity) could not  
344 be made at the actual stage, as electrokinetic and microfluidic approaches are still under  
345 development and considered as emerging trends. They are therefore not included yet in  
346 different systematic performance evaluation / comparison studies. To have a rough estimation,  
347 readers can nevertheless glean to a recent study by Tian et al. for information on the EVs  
348 isolation performance of different established methods [64]. Starting from a same platelet-free  
349 plasma sample, ultracentrifugation is first ranked for the purity of isolated EVs (78 %),  
350 followed by SEC (28 %) and polymer precipitation (5 - 18 %). On the contrary, the yields

351 provided by polymer precipitation kits (83-89 %) are much higher than those obtained with  
352 SEC (65 %) and ultracentrifugation (2 %). The presence of electrokinetic strategies in such  
353 comparison study is expected soon to appear.

354

#### 355 **4. Electrokinetic separation and quantification of EVs**

##### 356 ***4.1. Dielectrophoresis of EVs***

357 The reported electrokinetic strategies for separation and quantification of EVs are summarized  
358 in **table 4**. Dielectrophoresis (DEP) was the first approach developed for such purpose. DEP  
359 is the motion mechanism of an electrically polarizable particle that occurs in the presence of a  
360 non-uniform electric field [65]. Upon generation of a non-uniform electric field, the  
361 movement of charged particles driven by the dielectric force depends on their size and  
362 dielectric properties, the electrical properties of the suspending solution as well as the  
363 frequency and intensity of the applied electric field. DEP was employed to manipulate EVs  
364 after successful application with microparticles, nanoparticles and cells [66-68]. The DEP  
365 separation force generated by the alternating current electrokinetic (ACE) microarray chip  
366 device was first demonstrated in 2017 by Ibsen et al. for rapid isolation and recovery of  
367 glioblastoma EVs spiked in human plasma in less than 30 min [69]. The setup of this system  
368 is illustrated in Fig. 5. On the ACE chip, the EVs were attracted to the high-field regions  
369 around the microelectrode edges, while cells or larger particles in the samples were  
370 concentrated into the DEP low-field areas between the microelectrodes. The attracted EVs  
371 were either observed under SEM and detected by fluorescent staining after washing away the  
372 excess plasma or eluted from the chip and collected for further analysis. Nevertheless, protein  
373 aggregates and other cellular debris were undesirably collected as well. The same ACE  
374 procedure was then applied for capture and analysis of EVs from whole blood, plasma and  
375 serum of pancreatic ductal adenocarcinoma (PDAC) patients [70]. Two EV associated



376 proteins of interest, glypican-1 and CD63, were subsequently detected and measured using  
377 immunofluorescence-based methods to distinguish between PDAC patients from healthy  
378 controls with 99% sensitivity and 82% specificity. However, it was not clear whether the high  
379 levels of glypican-1 and CD63 were caused by elevated protein-to-exosome ratio or increased  
380 number of exosomes. In another study, Shi et al. reported a dielectrophoretic approach to  
381 design an insulator-based dielectrophoresis (iDEP) to capture EVs at the proximity of a glass  
382 nanopipette tip using a DC field [71]. The instrumental setup is shown in Fig. 6, in which a  
383 nanopipette was inserted into the opening of a chamber and connected with platinum  
384 electrodes. This device was based on the application of negative voltage polarity at the base of  
385 the pipette and induction of a strong non-uniform electric field that created a DEP force near  
386 the pipette region. The EVs trapping was controlled by three dominant forces including  
387 dielectrophoretic, electrophoretic and electroosmotic ones. The separation performance was  
388 first confirmed with fluorescence intensity measurements using fluorescently labelled  
389 artificial liposomes in PBS solution to simulate EVs capture. The separation capability of this  
390 iDEP device was further demonstrated in an extended setup connecting four micropipettes in  
391 parallel on a single chip, to carry out the extraction of EVs within 20 minutes from 200  $\mu$ L of  
392 undiluted human plasma, serum or saliva [72]. According to NTA results, this method showed  
393 two orders of magnitude greater yields when compared to the differential centrifugation  
394 method, with a particle size distribution ranging from 50 nm to 150 nm covering most of the  
395 small and large EV subpopulations. This yield improvement came with a penalty of larger  
396 initial sample volumes needed. Ayala-Mar et al. then designed another direct  
397 current–insulator-based dielectrophoretic (DC-iDEP) system not only for EVs capture but  
398 also for EVs separation and collection based on their size [73, 74]. This device is composed of  
399 a microchannel having two different electrically insulating post sections, each with different  
400 gap space (15  $\mu$ m and 10  $\mu$ m). Adjacent to these two sections, side channels with recovery

401 reservoirs are arranged to allow collection of the separated sample. When DC voltages are  
402 applied, the EVs are dragged by the EOF across the main channel. As a result, large EVs are  
403 captured in the first post array, while smaller size EVs are trapped in the second post array.  
404 DLS measurements of two recovered fractions demonstrated successful separation of two  
405 different EV sizes of approximately 120 nm and 75 nm in only 20 s. Furthermore, such a  
406 tool was able to concentrate and separate EVs from previously extracted EV samples. Chen et  
407 al. then gave an account of the development of a fast DEP method to isolate EVs with higher  
408 yield (>87%) and higher purity than conventional ultracentrifugation from plasma [75]. This  
409 DEP chip consisted of interdigitated electrodes covered with poly-HEMA hydrogel to reduce  
410 their degradation by the contact with high conductivity samples. Under positive DEP force,  
411 the EVs were successfully attracted and concentrated near the edges of the microelectrodes.  
412 The DEP step was followed by *in situ* lysis of EVs for identification of miRNAs levels in  
413 lung cancer patients. In addition, the authors demonstrated with DEP chips the possibility of  
414 *in situ* siRNA loading by electroporation of EVs that could be exploited for drug delivery  
415 and/or gene therapy. To conclude this section, the dielectrophoretic-based methods presented  
416 here demonstrate label-free, rapid and high-throughput separation of EVs. However, in our  
417 opinion, only few devices proved to be able to concentrate and separate EVs from complex  
418 biological mixtures. Pre-treatment of biological samples was always needed as forefront of  
419 these DEP approaches. Integration of upstream sample processing to downstream DEP-based  
420 separation of EVs should be envisaged and constitutes probably attractive perspectives to  
421 improve the performance of these systems in the future.

422

#### 423 ***4.2. Microscale electrophoresis of EVs***

424 During the past decade, microscale electrophoresis has been explored to provide analysis and  
425 characterization of EVs and potentially distinguish EV subtypes based on their electrophoretic

426 migration in a microchannel under a high intensity electric field. The first electrokinetic  
427 approach coupled with light-scattering-based detection was reported in 2013 by Ichiki's group  
428 for tracking EVs and obtaining their zeta potential, using an on-chip microcapillary  
429 electrophoresis ( $\mu$ CE) system coupled with a laser dark-field microscope [76-79]. A typical  
430 setup is shown in Fig. 7. This  $\mu$ CE consisted in a microfluidic channel in which EVs flow  
431 under application of DC voltage and afterwards they are tracked thanks to a laser dark-field  
432 microscope. This system enabled individual EVs visualization in a dark field by detecting the  
433 scattered laser light and their zeta potential was deduced from the measured electrophoretic  
434 mobility, revealing a strong correlation between the EVs zeta potential and their cells of  
435 origin. Furthermore, the authors developed an on-chip immunoelectrophoresis method to  
436 profile the protein expression of individual EVs, detecting their shifts in the electrophoretic  
437 velocity which resulted from the binding of EVs to specific antibody markers. This system  
438 was expected to be a valuable tool for sensitive profiling of EVs of tumor origin and therefore  
439 for early cancer diagnosis. Nevertheless, specific purpose-made instrumentation and manual  
440 operation are required for this new application, hindering its high throughput potential.

441  
442 Diverted from the microchip setup, Lan et al. reported the first result of a capillary  
443 electrophoresis (CE) method applied for urinary EVs [80]. The authors investigated the  
444 potential of CE for separation of two kinds of EVs exhibiting different densities and obtained  
445 by sucrose gradient ultracentrifugation. However, it was not clear if the peaks detected from  
446 the CE analysis of low- and high- density EVs were either a result of different EVs size or  
447 different charge characteristics related to their different origins. No concrete information  
448 could be withdrawn from this work, due to (at least partially) the non-optimized conditions  
449 employed for CE analysis of EVs. More recently, two capillary electrophoresis methods have  
450 been proposed for the identification and quantification of EVs after their isolation. Piotrowska

451 et al. demonstrated a CE-UV method for qualitative and quantitative analysis of bacterial EVs  
452 [81]. To maintain EVs stability during CE analysis under electric field, the authors employed  
453 large buffering counter-ions. Thanks to this configuration, they obtained satisfactory  
454 separation of EVs from other non-target entities / molecules without any sample pretreatment  
455 in about 15 minutes, using the optimized BGE composed of 50 mM 1,3-  
456 Bis(tris(hydroxymethyl)methylamino)propane (BTP) and 75 mM glycine (pH 9.5) and  
457 application of an electric field of 333 V/cm. This method coupled with UV detection at 200  
458 and 230 nm enabled the identification and quantification of EVs in the samples featuring  
459 protein concentration down to 0.17 mg/mL. Although this method enabled to quantify the  
460 number of vesicles in isolates, the linear correlation achieved was far from optimal ( $R^2 =$   
461 0.81) due to the presence of co-isolated impurities and the lack of a standard for method  
462 validation. Furthermore, the ability of this CE-UV method to separate and distinguish EVs  
463 from different origins was not investigated. In a parallel work, Morani et al. proposed a CE  
464 method with a laser-induced fluorescent (LIF) detection for the electrokinetic profiling of EVs  
465 from different sources (animal and human fluids) [47]. In this approach, EVs were first  
466 rendered fluorescent via an intra-membrane labeling. The CE-LIF separation of labelled EVs  
467 from the residual fluorophore was performed under an electric field of 500 V/cm. A new BGE  
468 was proposed for such purpose, employing large and weakly charged molecules (i.e.  
469 Tris(hydroxymethyl)aminomethane and N-Cyclohexyl-2-aminoethanesulfonic acid) at  
470 extremely high concentrations to avoid / minimize adsorption of EVs onto the silica capillary  
471 wall and lysis of EVs during electrophoresis. To avoid aggregation of EVs, buffer substitution  
472 of the analyzed samples was performed to avoid the high conductivity of the PBS matrix of  
473 the samples. A protocol overview and a typical electropherogram are shown in Fig. 8. Unlike  
474 UV detection, LIF detection offered better sensitivity achieving a detection limit of  
475 approximately  $8 \times 10^9$  EVs / mL (i.e. 5-fold better than the CE-UV approach) that is

476 sufficient for this method to be used for quality control of EVs batches isolated with different  
477 conventional EV isolation techniques. Despite the additional fluorescent labelling step to  
478 render the EVs detectable with a LIF detection, no morphology modifications of EVs were  
479 produced according to DLS and NTA data. This CE-LIF method allowed to reveal different  
480 profiles and electrophoretic mobilities for isolated EVs from different origins. However,  
481 further characterization study is needed to prove whether such differences are size-dependent  
482 or due to different surface composition. Different from the intra-membrane labelling strategy  
483 for CE-LIF of EVs, Tani and Kaneta have very recently proposed an alternative for LIF-based  
484 quantification of exosomes, using indirect capillary electrophoresis immunoassay (CEIA) of  
485 exosomal membrane protein, CD63 [82]. In this method, reactions between the exosomes and  
486 a fluorescently labeled anti-CD63 antibody were optimized to form a CD63 complex  
487 localized on the surface of exosomes, followed by removal of the exosome-antibody complex  
488 by centrifugation prior to CE-LIF analysis of the supernatant containing the free fluorescent  
489 antibody. The diminution of peak areas was proportional to the increase in EVs' amount when  
490 the initial fluorescent antibody's concentration was kept constant. Thus, the concentration of  
491 the exosomal membrane's CD63 could then be estimated based on the slope of the linear  
492 relationship. This "indirect" method that measures the concentration of the fluorescent  
493 antibody allows straightforward quantification of exosomes without possible interference of  
494 unwanted peaks. Nevertheless, no information on the electrokinetic profile reflecting the size /  
495 charge distribution and eventual presence of EVs subpopulations can be provided this way.  
496 Detection bias can also occur if membrane debris are co-present in the EVs sample.

497

#### 498 ***4.3. Other related techniques***

499 In addition to the electrokinetic strategies listed above, other techniques have also been  
500 investigated. Petersen et al., for instance, presented a cyclical electric field flow fractionation

501 (Cy-EI-FFF) approach to separate EVs based on their size and charge [45]. Basically,  
502 application of an AC voltage produces a strong electric field perpendicular to the direction of  
503 flow, which enables EVs separation within a channel, due to their different electrophoretic  
504 mobilities. This strategy showed changes in electrophoretic mobilities when similarly sized  
505 polystyrene particles, melanoma exosomes sourced from a common human A375 melanoma  
506 cell line or synthetic liposome-based exosome mimetics were substituted in different buffers  
507 (i.e. deionized water, PBS, trehalose and isopropyl alcohol) having different salt  
508 concentrations. Therefore, EVs could be separated depending on their different charge in  
509 different buffers. In another work, Al Ahmad et al. reported an electrical detection and  
510 quantification of EVs via capacitance-voltage measurements [83]. The authors compared  
511 three types of EVs and demonstrated the identification of EVs according to their  
512 corresponding self-capacitance. However, the employed equipment in this work enabled only  
513 to estimate the EVs count. This could be overcome in the future by implementation of more  
514 precise micro/nano-based capacitance analysers. Recently, Akbarinejad proposed a new  
515 approach relying on a low electric field and an electrochemically switchable substrate for the  
516 fast, selective, nondestructive, and efficient capture and release of EVs [84]. In this system,  
517 using gold-sulfur (Au-S) covalent bonding, the electrospun substrates were functionalized  
518 with SH-terminated aptamer probes that are selective to EV surface proteins. The specific  
519 aptamer–EV interactions allows efficient capture of EVs and easy removal of the  
520 nonspecifically bound material through washing steps. The trapped EVs were then released  
521 into fresh PBS at a high efficiency (> 92 %) by applying a potential of  $-1.2$  V for 5 min for  
522 cathodic cleavage of the Au–S bond. This approach was demonstrated for capture, enrichment  
523 and release of EVs derived from primary human dermal fibroblast (HDFa) and breast cancer  
524 (MCF-7) cell lines with low nonspecific adsorption.

525

526 A high resolution particle-to-particle measurement technique, developed by Trau et al. for  
527 nanoparticle characterization [85, 86] and commercialized under a trade name of qNano –  
528 Izon Science [87], has recently been adapted for measurements of EVs' size, volume and  
529 surface charge. This elastic pore sensor, employing the tunable resistive pulse sensing (TRPS)  
530 principle is composed of conically shaped, size-tunable pores that are fabricated by  
531 puncturing a polyurethane thermoplastic membrane with a tungsten needle. Stretching or  
532 relaxing the elastic membrane allow the dimensions of the conical pore to be tuned to the size  
533 range of the target particles. When a voltage is applied across the conical pore, its shape  
534 results in a resistance gradient. TRPS measures nanoparticles suspended in electrolytes on a  
535 particle-by-particle basis as they pass through a nanopore. Sample particles are driven through  
536 the nanopore via a combination of pressure and voltage. Each particle causes a resistive pulse  
537 or “blockade” signal that is detected and measured. The blockade magnitude is directly  
538 proportional to the volume of each particle. The blockade duration is dependent on the  
539 velocity and surface charge of the particle, and blockade frequency is used to determine  
540 particle concentration. Applications of this technology have been extended recently to  
541 measure physiochemical parameters (size, concentration and charge) of EVs and  
542 nanomedicines with high precision.

543

544 When positioning the electrokinetic and related strategies for EVs analysis together with other  
545 commonly used methods, the electrokinetic ones would have the advantage of high resolution.  
546 Nevertheless, they are still mostly at research stage and require for most of them further  
547 improvement of measurement reproducibility. To bring a fair view to readers, the limitations  
548 of other established techniques are also mentioned, including limited resolution and  
549 sensitivity for NTA and DLS, low throughput for TEM and limited multi-parameter capability  
550 for flow cytometer. Depending on the measurement requirements and available infrastructure,

551 a single instrument or a combination of techniques is generally used to zoom into target EVs.  
552 Electrokinetic and related strategies, while still needing to further mature in the near future,  
553 could be included in the list of the methods of choice for this purpose.

554

#### 555 **4. Conclusion remarks and perspectives**

556 The potential of EVs as a source of prognostic or diagnostic biomarkers but also as drug  
557 nanocarriers or gene therapy tools has attracted significant attention over the recent years.  
558 However, many technical challenges should be taken into account when dealing with such  
559 vesicles, notably their high heterogeneity, their small sizes, their fragility and also the  
560 tendency to aggregate or lyse. To date, ultracentrifugation (highest EV purity, lowest EV  
561 yield), commercial kits of polymer precipitation (highest EV yield, lowest EV purity) and  
562 SEC (compromise between purity and yield) are still the methods of choice for EVs isolation.  
563 For EV analysis, NTA, DLS, flow cytometry and TEM are the most widely used techniques.  
564 Electrokinetic strategies, together with microfluidic ones, bring not only new functionalities  
565 but also more possibilities of integration. They constitute an emerging trend that is expected  
566 soon to be ranked among the list of preferred methods for EVs isolation and analysis.

567 As reviewed here, there are different electrokinetically driven methods for isolation,  
568 preconcentration and separation of EVs. Most of them are still at the stage of research  
569 development (with an exception for TRPS). Application of such strategies on clinical samples  
570 and systematic comparison of their performances to those of established techniques still need  
571 to be investigated. Although some of electrokinetic approaches demonstrated their ability to  
572 distinguish subpopulation of EVs, results are highly variable according to how samples are  
573 handled or collected, storage conditions and on the measurement itself. Further improvement  
574 of the measurement reproducibility and precision is prerequisite. Although the current  
575 electrokinetic methods are unable to fully combine purification, enrichment, separation and



576 detection of EVs, they are individually capable of carrying out these steps quite efficiently as  
577 discrete modules. Thus, we expect in the future the emergence of integrated electrokinetic  
578 systems into a single platform to perform all these steps from highly complex biofluids. This  
579 is indeed a positive feature (*i.e.* performing EVs isolation and analysis with a high degree of  
580 automation, integration and ease of use) that electrokinetic strategies can address and make  
581 them afterwards superior to more conventional methods. All these developments require  
582 however the use of high-quality EV standards with narrow size distribution and homogeneous  
583 population to reduce discrepancies and enable unbiased devices comparison. The generation  
584 of recombinant EVs as a biological reference may open the door for well-characterized EV  
585 standards [88], and should be used as the starting material for further development of  
586 electrokinetic strategies for EVs isolation and analysis.

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594 Biosystèmes) – University Paris Saclay.

595 The authors have declared no conflict of interest.

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602 **Table 1:** Summary of the most commonly used EVs isolation techniques and their advantages

603 / disadvantages.

Method	Advantages	Disadvantages
Ultracentrifugation	<ul style="list-style-type: none"><li>• Most commonly used</li><li>• High purity by density gradient centrifugation</li></ul>	<ul style="list-style-type: none"><li>• High equipment cost</li><li>• Time consuming</li><li>• Large sample volume needed</li><li>• Potential vesicle aggregation and mechanical damage</li><li>• Low yield</li></ul>
Ultrafiltration	<ul style="list-style-type: none"><li>• Low equipment cost</li><li>• Quick and simple</li><li>• Possibility of processing many samples simultaneously</li></ul>	<ul style="list-style-type: none"><li>• Possible loss of vesicles due to clogging</li><li>• Potential vesicle deterioration and deformation</li></ul>
Polymer-based precipitation	<ul style="list-style-type: none"><li>• Low cost</li><li>• No special equipment required</li><li>• High yield</li></ul>	<ul style="list-style-type: none"><li>• Co-precipitation of protein aggregates and polymeric contaminants</li><li>• Low purity</li><li>• Required clean-up steps</li></ul>
Size-exclusion chromatography	<ul style="list-style-type: none"><li>• Keep native structure of vesicles</li><li>• High purity</li><li>• Good reproducibility</li></ul>	<ul style="list-style-type: none"><li>• Limitation on sample volume</li><li>• Long run time</li><li>• Specific columns required</li></ul>
Immunoaffinity capture	<ul style="list-style-type: none"><li>• High purity</li><li>• High efficiency for specific EV subtypes</li></ul>	<ul style="list-style-type: none"><li>• High reagent cost</li><li>• Extra step for elution may damage EVs' native structure</li><li>• Antibody cross-reactivity</li><li>• Low yield</li></ul>

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610 **Table 2:** Summary of commonly used EVs characterization techniques and their advantages /  
 611 disadvantages.

<b>Method</b>	<b>Advantages</b>	<b>Disadvantages</b>
Transmission electron microscopy (TEM)	<ul style="list-style-type: none"> <li>• Direct assessment of morphology and size</li> <li>• Powerful magnification and resolution</li> </ul>	<ul style="list-style-type: none"> <li>• Laborious sample preparation</li> <li>• Time consuming</li> <li>• Expensive equipment</li> </ul>
Dynamic light scattering (DLS)	<ul style="list-style-type: none"> <li>• Fast</li> <li>• No need for sample preparation</li> </ul>	<ul style="list-style-type: none"> <li>• Inaccurate with polydisperse and size-heterogeneous samples</li> </ul>
Nanoparticle tracking analysis (NTA)	<ul style="list-style-type: none"> <li>• Ability to analyse normal and fluorescent samples</li> <li>• Approximate particle counts</li> </ul>	<ul style="list-style-type: none"> <li>• Contamination caused by diluents during the preparation of samples</li> <li>• Results are operator-dependent</li> </ul>
Flow cytometry	<ul style="list-style-type: none"> <li>• Single EV molecular characterization</li> <li>• Quantitative and qualitative</li> </ul>	<ul style="list-style-type: none"> <li>• Detection limit is flow dependent</li> <li>• Aggregates or debris can give false results</li> <li>• Technical expertise required</li> </ul>
Tunable resistive pulse sensing (TRPS)	<ul style="list-style-type: none"> <li>• Particle by particle measurement of size and charge</li> </ul>	<ul style="list-style-type: none"> <li>• Difficult to select the correct nanopore setup</li> <li>• Possible pore clogging</li> </ul>

		<ul style="list-style-type: none"> <li>• Less reproducible</li> </ul>
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612

613 **Table 3:** Electrokinetic strategies for isolation and enrichment of EVs

EV isolation approach	Sample type	Recovery yield (%)	Isolation throughput (μL/min)	Isolated size (nm)	Ref
Electrophoresis-driven filtration	Mouse whole blood	1.5	2	150	[57]
Electrophoretic isolation on porous membrane	Mouse plasma	65	20	~ 10 - 400	[58]
Ion-depletion zone	Cell culture filtered EVs	98	1	not communicated	[59]
Ion concentration polarization (ICP)-based gel electrophoresis	Cell culture supernatant, blood serum	60 - 80	2.5 - 3.5	≤ 150	[61]
Ion concentration polarization (ICP)-based electrokinetic concentrator	Cell culture supernatant	not communicated	1	~ 50 - 75	[62]
alternating current electrohydrodynamic (ac-EHD) induced nanoshearing	Exosome pellets from breast and prostate cancer cell lines	not communicated	not communicated	30–350	[63]

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634 **Table 4:** Electrokinetic strategies for separation and quantification of EVs

EV analysis approach	Sample type	Detection limit	Calibration range	Operation time	Isolated size (nm)	Concentration (particles / mL)	Ref
Alternating current electrokinetic (ACE) microarray chip	glioblastoma EVs purified from cell culture and spiked in human plasma	not communicated	not communicated	< 30 min	~ 50 – 150 (UC)	~ 1–10 × 10 <sup>9</sup> (loaded into the system)	[69]
Alternating current electrokinetic (ACE) microarray chip	EVs from whole blood, plasma and serum of pancreatic ductal adenocarcinoma (PDAC) patients	not communicated	not communicated	< 30 min	~ 20 - 500	not communicated	[70]
Nanopipette insulator-based dielectrophoresis (iDEP) device	EVs extracted from plasma of healthy donors and resuspended in PBS	not communicated	not communicated	~ 2 min (only for entrapment)	not communicated	not communicated	[71]
Nanopipette insulator-based dielectrophoresis (iDEP) device (four pipettes in parallel)	EVs from undiluted human plasma, serum and saliva	not communicated	not communicated	20 min (10 min entrapment and 10 min collection)	50 – 150	1.01 × 10 <sup>12</sup> (detected after isolation)	[72]
Direct current–insulator-based dielectrophoretic (DC-iDEP) system	EVs purified from human breast adenocarcinoma cells	not communicated	not communicated	20 s (for entrapment and separation)	~ 75 (section 2); ~ 120 (section 1)	2.1 × 10 <sup>10</sup> (loaded into the system)	[73, 74]
Dielectrophoresis (DEP) chip	EVs isolated from plasma of lung cancer patients	not communicated	not communicated	30 min	~ 50 - 250	7.13 ± 0.86 × 10 <sup>10</sup> (detected after isolation)	[75]
On-chip microcapillary electrophoresis (μCE) coupled with a laser dark-field microscope	EVs extracted from six types of human cell	not communicated	not communicated	not communicated	not communicated	not communicated	[76]
On-chip microcapillary electrophoresis (μCE) coupled with a laser dark-field microscope	EVs extracted from normal and cancer prostate cells	not communicated	not communicated	not communicated	not communicated	not communicated	[77]
On-chip microcapillary electrophoresis (μCE) coupled with a laser dark-field microscope + immunoelectrophoresis	EVs collected from culture supernatant of human breast cancer cells and from mouse blood	not communicated	not communicated	not communicated	~ 50 – 450 (UC)	1.19×10 <sup>12</sup> (loaded into the system)	[78]
Capillary electrophoresis (CE) coupled with UV-	EVs isolated from human urine	not communicated	not communicated	~ 4 h (separation)	80 – 120 (low density EVs);	not communicated	[80]

visible detector					40 – 100 (heavy density EVs) (Sucrose gradient UC)		
Capillary electrophoresis (CE) coupled with UV-visible detector	EVs isolated from <i>Pectobacterium betavascolorum</i> strain	protein concentration down to 0.17 mg/mL		< 15 min (separation)	< 200 (differential centrifugation + filtration)	not communicated	[81]
Capillary electrophoresis coupled with laser-induced fluorescent (LIF) detection	EVs isolated from bovine milk, pony plasma/serum and human plasma	$8 \times 10^9$ EVs / mL	$1.22 \times 10^{10}$ to $1.20 \times 10^{11}$ EVs / mL	< 30 min (separation)	< 200 (sucrose gradient UC, size exclusion chromatography, monolithic affinity chromatography)		[47]
Cyclical electrical field flow fractionation (Cy-EI-FFF)	EVs purified from human melanoma cells	not communicated	not communicated	not communicated	~ 120 (radius) (differential centrifugation)	not communicated	[45]
Capacitance-voltage measurements	EVs isolated from human embryonic kidney cell line and small cell lung cancer cell line	not communicated	not communicated	10 min	90 – 110 (ExoQuick-TC kit)	$> 1 \times 10^6$	[83]

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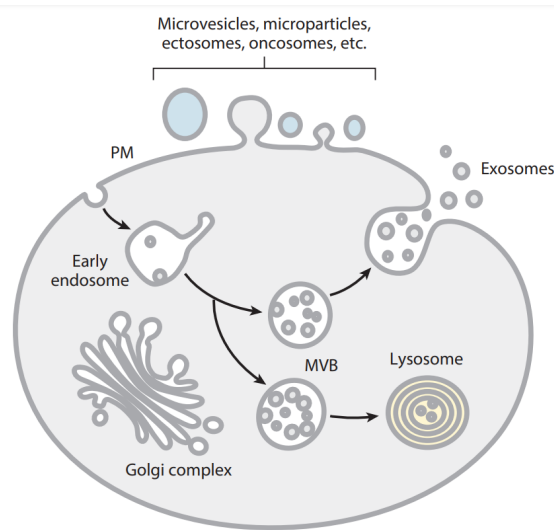
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647 **Figure captions:**

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649 Fig. 1. *Schematic representation of the different types of membrane vesicles released by*  
650 *cells, either by direct budding from the plasma membrane (PM) or by fusion of*  
651 *internal multivesicular compartments (MVB) with the PM. Reprinted from [89] with*  
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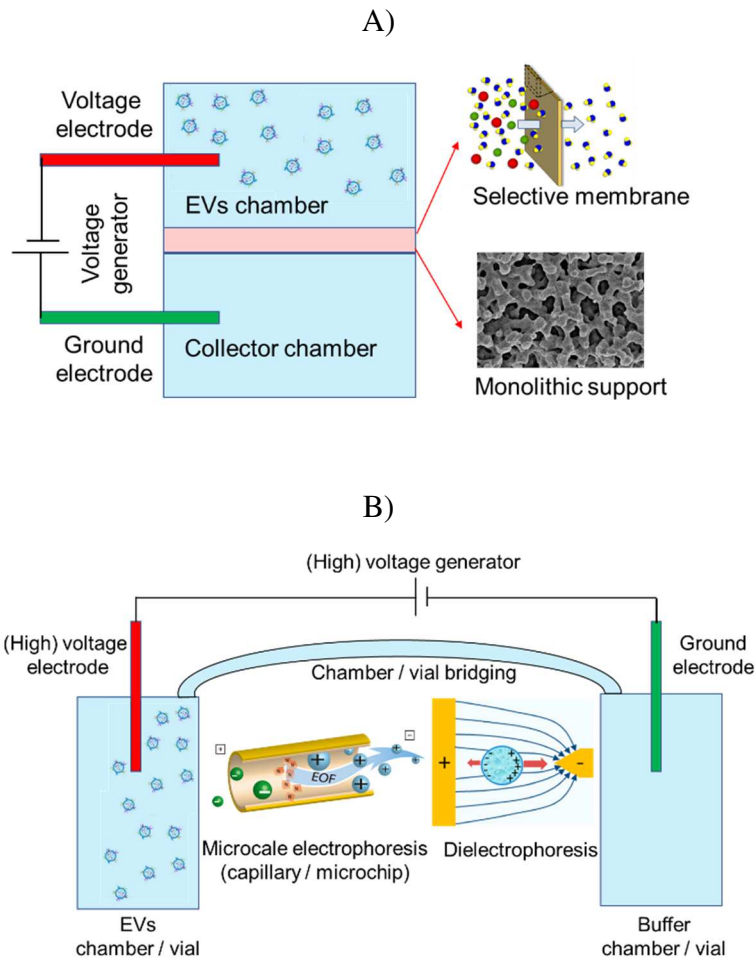
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670 Fig. 2. Schematic drawings for electrokinetically driven methods for (A) EVs isolation; and  
671 (B) EVs separation and characterization.

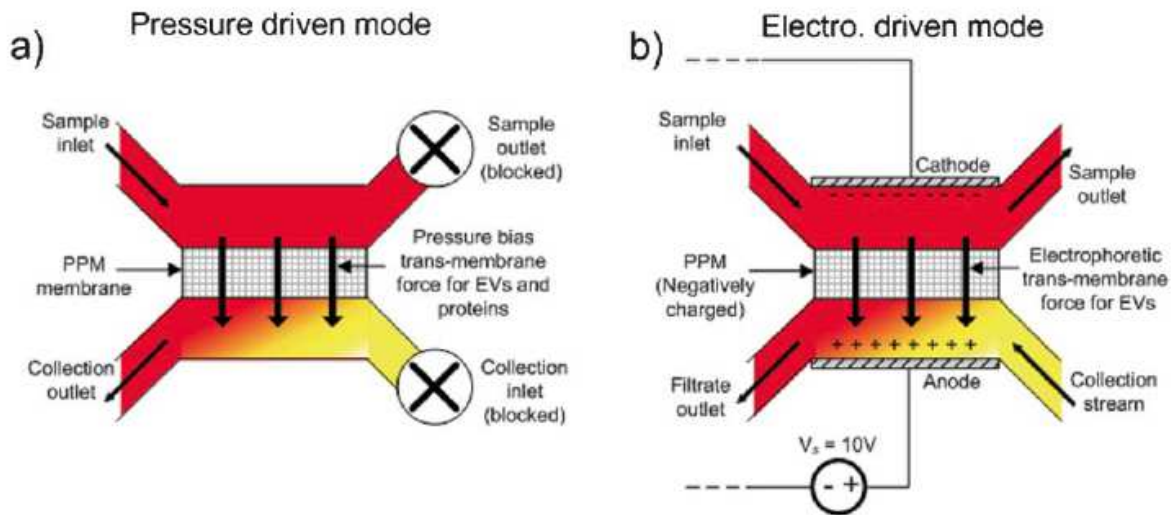
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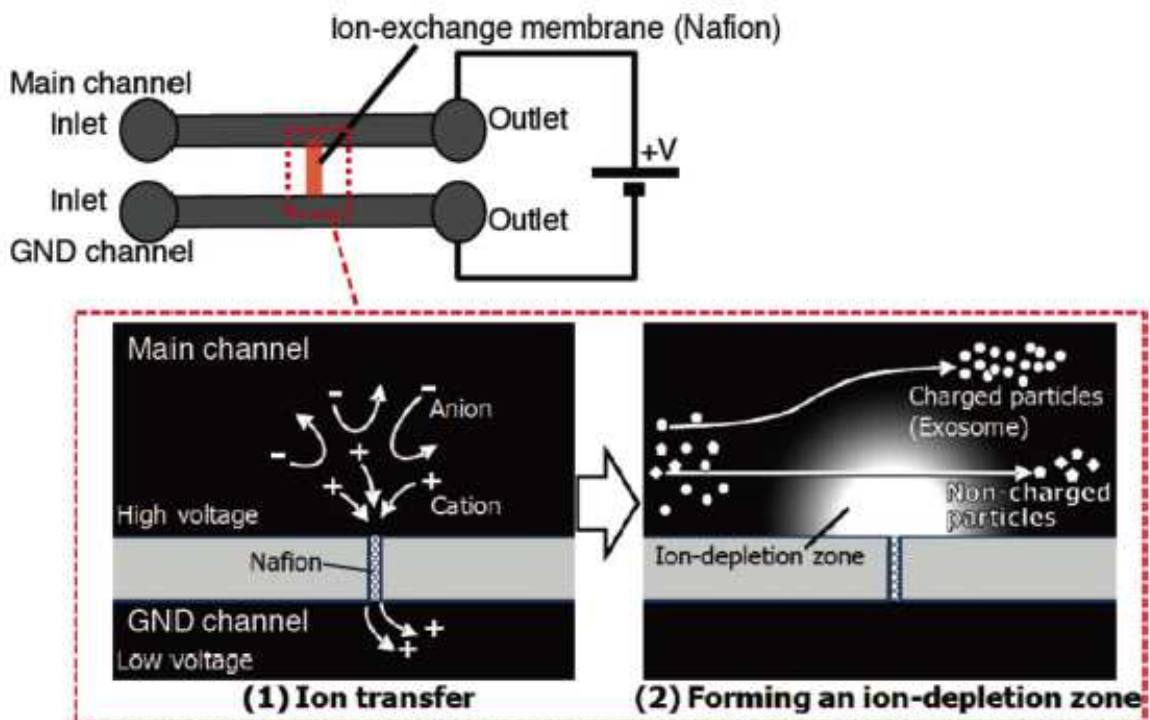


691 Fig. 3. (a) Schematic drawing of pressure-driven filtration procedure. Inlet pressure bias  
692 drives particles through the membrane into the collection chamber. After injection  
693 of the blood sample, the filtrate is collected by injecting one chamber volume of  
694 PBS through the collection inlet to eject the permeated EVs. "X" indicates an  
695 outlet blocked by sealing the attached plastic tubing. (b) Schematic of  
696 electrophoresis-driven filtration. Bias voltage is applied across the porous polymer  
697 monolith (PPM) membrane so that negatively charged vesicles experience a  
698 transmembrane driving force. Filtration is performed under syringe pump driven  
699 flow. 10 V is applied over an electrode separation distance of 1.5 cm for an electric  
700 field strength of 6.7 V/cm. Reprinted from [57] with permission. Copyright (2012)  
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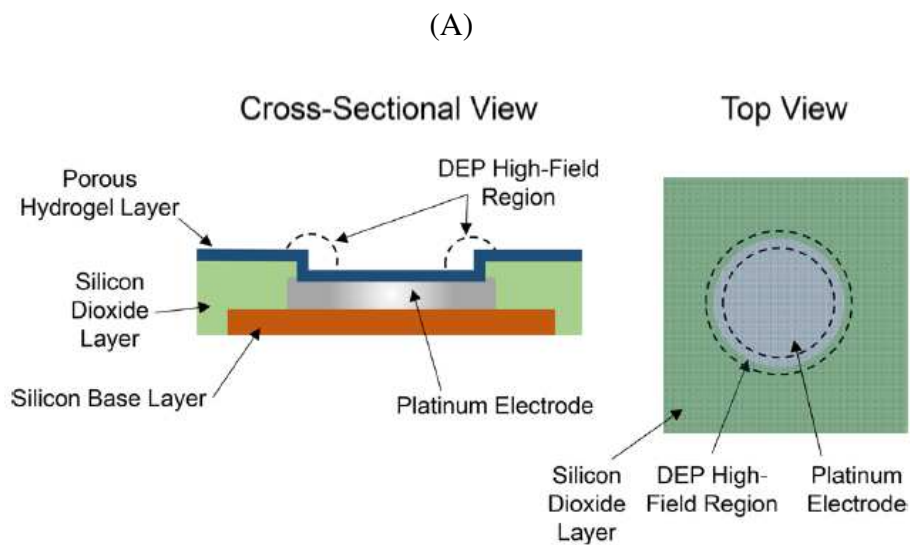
712 Fig. 4. Schematic drawing of the generation of an ion-depletion zone for EVs  
713 preconcentration. An ion-depletion zone is formed by applying a voltage between  
714 two microchannels bridged by an ion-exchange membrane. (1) Cations that are  
715 drawn into and anions are pushed away from the edge of the Nafion membrane on  
716 the high-voltage side by an electrostatic force. (2) The generated ion-depletion zone  
717 is used as an intangible barrier against the entry of charged particles. A Nafion  
718 pattern intersects the Main and the GND channels. The inlet and outlet ports of the  
719 channels are in contact with the electrodes to apply an electric potential difference  
720 between the two microchannels. Reprinted from [59] with permission. Copyright  
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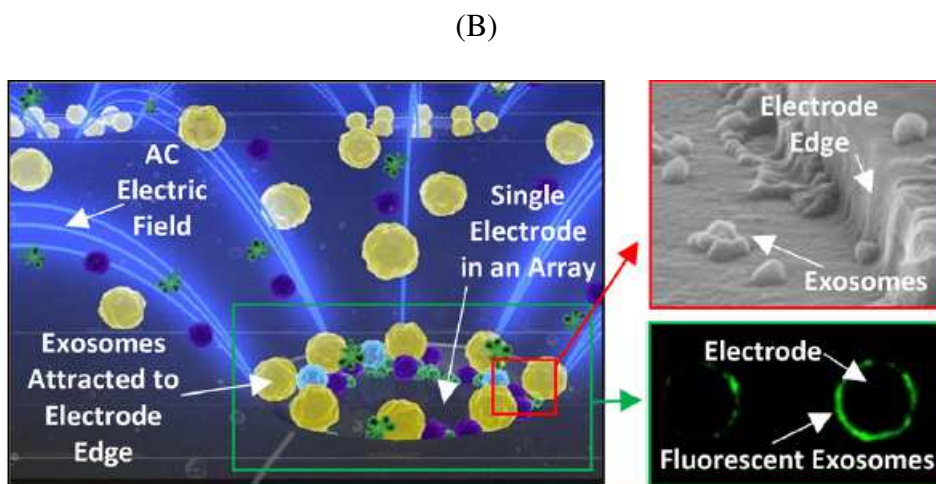
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730 Fig. 5. Schematic drawing (A) as well as simulated and captured photos (B) of the  
731 microelectrode array chip showing the cross-sectional and top views of a single  
732 electrode. Over 1000 electrodes can be set in a single device. The DEP high-field  
733 regions where particles are collected are shown within the dotted lines. The darker  
734 color of the silicon dioxide layer and electrode in the top view represents the  
735 overlying transparent porous hydrogel layer. Reprinted from [69] with permission.  
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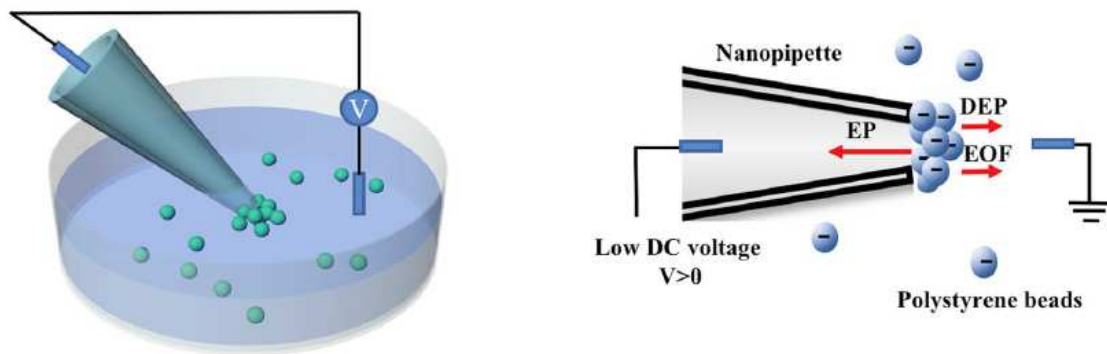


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746 Fig. 6. (A) Schematic drawing of the nanopipette DEP device for entrapment of the  
747 particles suspended in the solution. (B) The overall setup. The ionic current across  
748 the pipette and the trajectory of the particles were simultaneously recorded. DEP  
749 stands for dielectrophoresis; EP and EOF stand for electrophoresis and  
750 electroosmotic flow, respectively. Reprinted from [71] with permission. Copyright  
751 (2018) Nature.

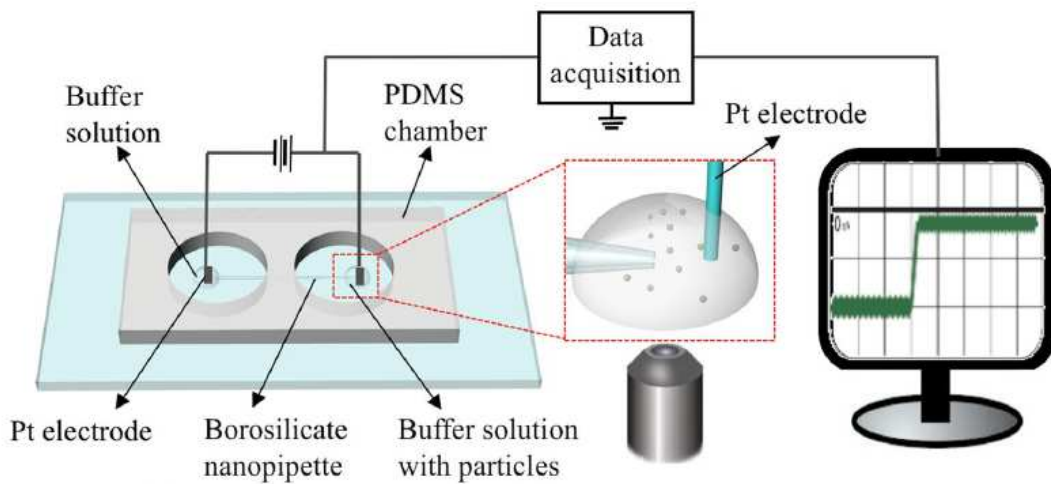
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(B)

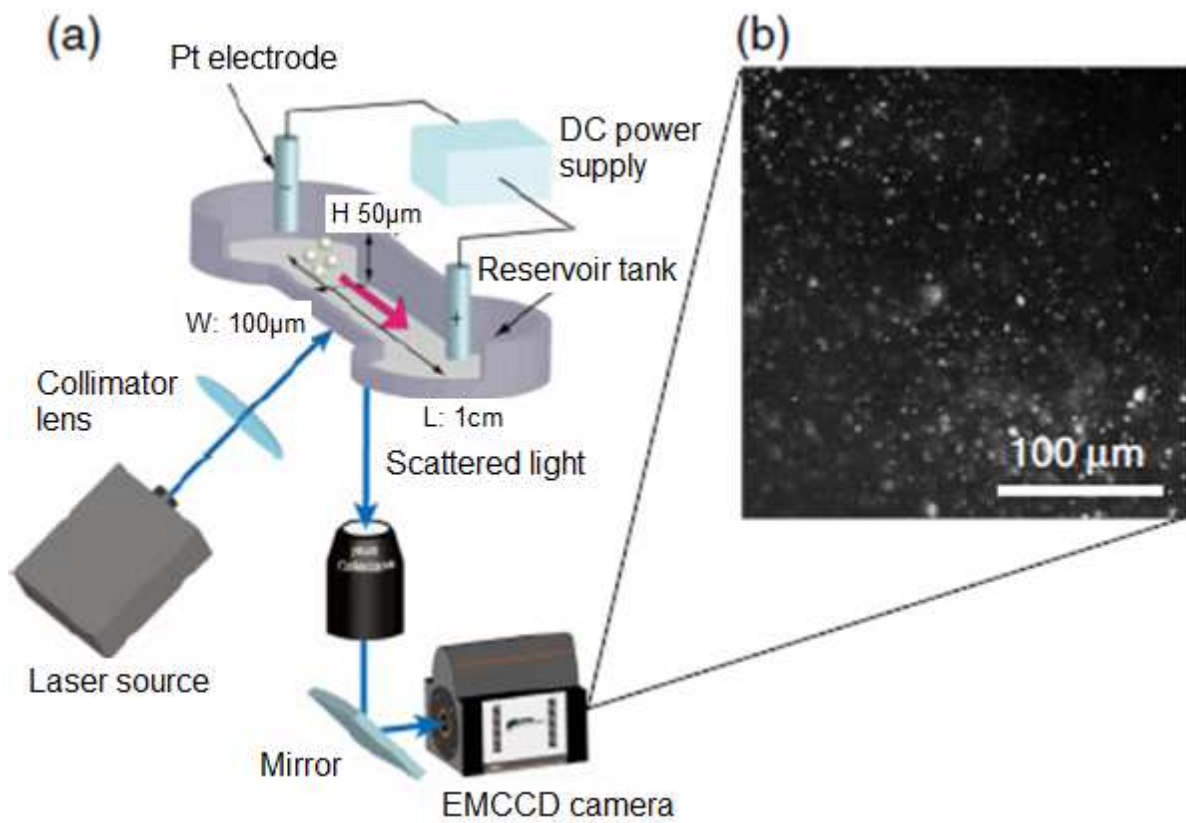


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763 Fig. 7. (a) Schematic drawing of the on-chip  $\mu$ CE system with a laser dark-field  
764 microscope. The system consists of a  $\mu$ CE chip, a pair of platinum electrodes, a DC  
765 power supply, a laser source, an inverted microscope, and an EM-CCD camera.  
766 The  $\mu$ CE chip equipped with a microfluidic channel with small reservoir tanks on  
767 both ends and the platinum electrodes were mounted on the inverted microscope.  
768 (b) Dark-field image of exosomes. Reprinted from [76] with permission. Copyright  
769 (2013) IOPScience.

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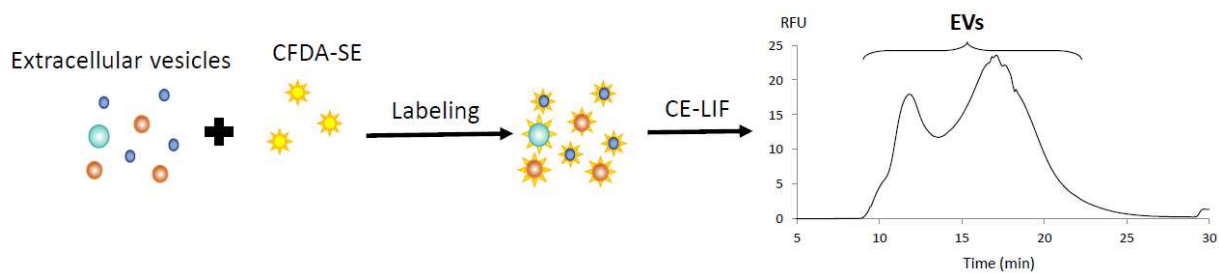
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778 Fig. 8. *Work flow of the CE-LIF method for separation and quantification of fluorescent*  
779 *EVs via intra-membrane labelling approach. CFDA-SE: 5-(and-6)-*  
780 *Carboxyfluorescein diacetate succinimidyl ester dye. Reprinted from [47] with*  
781 *permission. Copyright (2020) Elsevier.*  
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