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► To cite this version:

Sameh Obeid, Joseph Chamieh, Thanh Duc Mai, Marco Morani, Melissa Reyre, et al.. Fast, simple and calibration-free size characterization and quality control of extracellular vesicles using capillary Taylor dispersion analysis. *Journal of Chromatography A*, 2023, 1705, pp.464189. 10.1016/j.chroma.2023.464189 . hal-04176156

HAL Id: hal-04176156

<https://hal.science/hal-04176156v1>

Submitted on 2 Aug 2023

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Fast, simple and calibration-free size characterization and quality control of extracellular vesicles using capillary Taylor dispersion analysis

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Abstract

This study reports the development of a Taylor Dispersion Analysis (TDA) method for the size characterization of Extracellular Vesicles (EVs), which are highly heterogeneous nanoscale cell-derived vesicles (30-1000 nm). Here, we showed that TDA, conducted in uncoated fused silica capillary (50 μm i.d.) using a conventional Capillary Electrophoresis instrument, is able to provide absolute sizing (requiring no calibration) of bovine milk-derived EVs in a small sample volume (~ 7 nL) and over their entire size range, even the smallest ones (< 70 nm) not accessible via other techniques that provide nanoparticle sizing in suspension. TDA size measurements were repeatable (RSD $< 10\%$) and the average EV sizes were found in the range of 120-210 nm, in very good agreement with those measured with Nanoparticle Tracking Analysis, commonly used for EV characterization. TDA allowed quantitative estimation of EVs for concentrations $\geq 2 \times 10^{11}$ EVs/mL. Furthermore, TDA was able to detect minor changes in EV size (i.e. by ~ 25 nm upon interaction with specific anti-CD9 antibodies of ~ 150 kDa), and to highlight the impact of extraction methods (i.e. milk pretreatment: freezing, acid precipitation or centrifugation; the type of size-exclusion chromatography column) and of fluorescent labeling (i.e. intravesicular or surface labeling) on the isolated EV population size. In parallel to EV sizing, TDA allowed to detect molecular contaminants (average sizes ~ 1 -13 nm) present within the sample, rendering this method a valuable tool to assess the quality and quantity of EV isolates.

Keywords: *Taylor Dispersion Analysis, Extracellular Vesicles, Size characterization, Quality control, Capillary Electrophoresis*

32 **1. Introduction**

33 Extracellular Vesicles (EVs) are a heterogeneous group of cell-derived membrane-bound
34 vesicles, with sizes ranging between 30-1000 nm (typically <500 nm), released by both
35 eukaryotic and prokaryotic cells [1–3]. EVs are currently classified into three major groups
36 based on their biogenesis mode: Exosomes, microvesicles and apoptotic bodies. Unlike
37 apoptotic bodies, exosomes and microvesicles are secreted by healthy cells. However, due to
38 the difficulties in assigning an EV to a particular biogenesis pathway, the generic term EVs is
39 now widely used for all lipid bilayer-delimited particles and naturally released from cells [4].
40 EVs play a crucial role in intercellular communication as they facilitate the transfer of proteins,
41 lipids and nucleic acids between cells under physiological and pathological conditions. Over
42 the past decade, the interest in studying EVs has been growing since these vesicles are
43 regarded as promising diagnostic and prognostic biomarkers as well as new therapeutic agents
44 or drug delivery nanosystems [3,5,6].

45 A variety of well-established techniques and approaches are being commonly used for the
46 detection and characterization of EVs, including microscopic methods (e.g. electron
47 microscopy, atomic force microscopy), dynamic light scattering (DLS), nanoparticle tracking
48 analysis (NTA), tunable resistive pulse sensing (TRPS) and flow cytometry [7–9]. Novel
49 analytical methods are also being developed and optimized to study EVs such as imaging flow
50 cytometry, interferometric plasmonic imaging and various microfluidic-based technologies
51 [10,11]. However, the accurate assessment of nanometric EVs is hampered by technical
52 challenges related to EV's inherent properties [12], notably their high degree of heterogeneity
53 in biochemical composition due to different cellular origin and their complex biogenesis [3].
54 Furthermore, the contaminants present in EV samples (e.g. protein complexes/aggregates and
55 lipoproteins), which may overlap in size, density and/or compositions with EVs, often interfere
56 with their functional and physical characterization [13]. This comes from the fact that
57 currently used isolation methods, which affect directly the yields, purity and type of isolated
58 EVs, lack standardization [7,14]. Due to these challenges, the methods used for EV
59 characterization are still limited in terms of size detection range, accuracy, sampling
60 representativeness and/or reproducibility. This is further complicated by the lack of reference
61 materials that can be reliably used to calibrate EV analysis techniques (e.g. optical techniques
62 such as flow cytometry and NTA) and standardize measurements between labs [15,16]. Thus,
63 powerful techniques allowing rapid and accurate physical characterization and particularly

64 sizing of EVs in suspension, over their entire size range, with minimal sample consumption and
65 calibration requirements, are still in great demand.

66 Here, we propose a new alternative technique for the sizing of EVs and the assessment of their
67 purity using Taylor Dispersion Analysis (TDA). This technique, which is based on Poiseuille
68 laminar flow-induced dispersion [17], is a simple and rapid method for the determination of
69 the weight-/number- averaged hydrodynamic radii. It is also an absolute method requiring no
70 calibration nor prior knowledge of the sample concentration. TDA is applicable to any kind of
71 analyte of virtually any size ranging from angstrom to sub-micrometer, with much less bias
72 from the presence of large particles or aggregates compared to DLS [18]. In addition, any kind
73 of sample matrix or medium can be employed for this analysis. The elution profile of the
74 analytes can be detected using various detection modes such as UV absorbance, fluorescence,
75 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), backscattering interferometry and
76 photochemical oxidation [18–21]. These features make TDA a promising tool for studying
77 heterogeneous (in size and composition) particles such as EVs. Furthermore, TDA can be
78 conducted in narrow fused silica capillaries (*typically* ~50 μm i.d.) and using a conventional
79 Capillary Electrophoresis (CE) instrument, presenting several advantages such as a very small
80 sample volume consumption (i.e. ~10 nL per injection) and a high degree of automation. While
81 TDA has been applied to a broad range of analytes from small molecules [22,23] to nano-scale
82 particles of various sizes up to 250 nm [24–28], to date, determining the diffusion coefficients
83 of EVs by TDA, and thus their mean hydrodynamic size, has never been explored.

84 Herein, we optimized TDA conditions coupled to UV and laser-induced fluorescent (LIF)
85 detection to investigate the size of bovine milk-derived EVs using fused silica capillaries. TDA
86 measurement sensitivity and accuracy were also evaluated. The developed TDA-UV method
87 was employed to explore the impact of milk pretreatments and isolation methods on the size
88 and purity of the isolated EVs. In parallel, we evaluated the efficacy of TDA coupled to LIF
89 detection to provide specific sizing of EVs after labeling with an intravesicular fluorescent dye
90 (i.e. CFDA-SE) or FITC-conjugated antibody directed against an EV membrane tetraspanin. This
91 work demonstrates the potential of TDA for the size characterization and quality assessment
92 of EVs, while providing also quantitative estimation of isolated EVs.

93 **2. Materials and methods**

94 **2.1. Chemical and reagents**

95 Dulbecco's phosphate buffered saline (PBS), sodium dodecyl sulfate (SDS, 98.5% (GC)) and
96 Protease Inhibitors Cocktail (cOmplete™, Mini, Roche) were purchased from Merck (Saint
97 Quentin Fallavier, France). Sodium hydroxide (NaOH, 1M) was obtained from VWR
98 (Fontenay-sous-Bois, France). Buffers were prepared with deionized water purified with a
99 Direct - Q3 UV purification system (Millipore, Milford, MA, USA). Coomassie (Bradford)
100 Protein Assay Kit, Bovine Serum Albumin (BSA), Vybrant™ CFDA-SE Cell Tracer Kit (dye 5-
101 (and-6)-Carboxyfluorescein diacetate succinimidyl ester), unconjugated- and FITC-
102 conjugated mouse anti-bovine CD9 monoclonal antibodies (MM2/57) were purchased
103 from ThermoFisher Scientific (Illkrich, France). EV samples were isolated from bovine milk
104 or whey protein concentrate (WPC), using different protocols described below. The WPC
105 samples were kindly provided by the Dairy Goat Co-operative (N.Z.) (DGC, Ltd, Hamilton
106 New Zealand).

107 **2.2. Isolation of EVs from fresh and frozen bovine milk**

108 EVs were prepared from fresh bovine milk samples (BM), or frozen BM (f-BM) in the
109 presence of proteases inhibitors. Whole bovine milk samples (50 mL) were centrifuged at
110 3000 xg for 30 min at 4°C (Allegra X-15R, Beckman Coulter, France) to separate fat globules,
111 cells and cell debris from skimmed milk. The major milk proteins (i.e. caseins) were
112 subsequently discarded from skimmed milk either by acid precipitation (AP) or by
113 *centrifugation (C)* prior to EV isolation from the whey portion of milk using size exclusion
114 chromatography.

115 **2.2.1. Preparation of whey by acid precipitation of major milk proteins (caseins)**

116 Acid precipitation of major milk proteins was done by adding 10 % acetic acid to the milk
117 samples and by incubation at 37°C for 10 min and then continued by addition of 5 mL of
118 1M sodium acetate, and incubation for 10 min at RT. This was followed by centrifugation
119 at 1500 xg, 4°C for 15 min and filtration of supernatant using vacuum-driven filtration
120 system Millipore Steritop, 0.22 µm. The whey supernatants were concentrated by
121 centrifugation at 4000 xg and 20°C using Amicon 100 kDa centrifugal filter units (Merck
122 Millipore).

123 **2.2.2. Preparation of whey by pelleting of major milk proteins (caseins) by** 124 **centrifugation**

125 Whey was prepared using two different centrifugation protocols. Briefly, the initial volume
126 of 45 mL of skimmed milk was distributed in 1 and 2 mL aliquots. One or two milliliters of

127 skimmed bovine milk were centrifugated at a mid-force of 21,500 xg for 35 min (protocol
128 C1) or 50 min (protocol C2), at 4°C, respectively. Sequential filtration of both whey
129 preparations using 0.45 & 0.22 µm membranes (Sartorius Minisart) was performed prior to
130 concentration step by Amicon-15 100 kDa filter units up to 6 mL of final volume (Merck
131 Millipore).

132 **2.2.3. Isolation of EVs from whey using size exclusion chromatography (SEC)**

133 Five hundred µl of a whey sample, obtained from fresh or frozen BM by acid precipitation
134 (BM-AP or f-BM-AP), or from frozen milk by centrifugation (f-BM-C), was loaded onto a qEV
135 original SEC 70 nm (qEV70) or qEV original SEC 35 nm (qEV35) columns (Izon Science, New
136 Zealand), previously washed and equilibrated with PBS, and EV populations were
137 fractionated. Fraction collection (0.4 mL per fraction) was carried out immediately using
138 PBS as the elution buffer. The selected elution fractions (1-3) were pooled. Several washing
139 steps with PBS were applied to obtain highly pure EV fractions and were subsequently
140 concentrated using 100 kDa Amicon centrifugal filter units (Merck Millipore).

141 **2.3. Isolation of EVs from bovine whey protein concentrate (WPC)**

142 WPC was spray dried, soluble milk protein manufactured from fresh New Zealand cheese
143 whey using an ultra-filtration process. The protein content of WPC was 80.3 g of total whey
144 proteins per 100 g of dried powder. Bovine WPC was reconstituted in triplicate according
145 to instructions of Dairy Goat Cooperative (DGC). Briefly, 10 grams of whey protein
146 concentrate was resolved in 100 mL of warm water (30 °C). The final volume of 20 ml of
147 reconstituted whey was used for pre-treatment stage. The same methodology for pre-
148 treatment by acid precipitation and EV isolation was used as described previously for
149 isolation of EVs from the whey fraction of bovine milk using SEC approach, except the
150 loaded volume. After acid precipitation of the milk samples, 100 µl of pre-treated whey was
151 loaded onto a qEV original SEC 70 column (Izon Science, New Zealand) previously washed
152 and equilibrated with PBS and EV populations were fractionated. Fraction collection
153 (0.5 mL per fraction) was carried out immediately using PBS as elution buffer. The selected
154 elution fractions were pooled as described previously (Section 2.2.3) and were
155 subsequently concentrated using 100 kDa Amicon centrifugal filter units (Merck Millipore).

156 **2.4. Characterization of EV preparations**

157 To validate the preparations of enriched EVs from BM or WPC, the absence of EVs in the
158 eluents and filtrates was verified, serving to control the purification efficiency. Once the

159 purification was completed, the isolated populations were characterized by Bradford assay,
160 by Transmission Electron Microscopy (TEM), with negative staining by uranyl acetate, and
161 by Nanoparticle Tracking Analysis (NTA).

162 **2.4.1. EV protein concentration determination**

163 One microliter of each EV sample was used to measure the protein concentration by
164 Bradford assay. A standard linear curve was set up using BSA. Measurements were done in
165 triplicate.

166 **2.4.2. Nanoparticle tracking analysis of EVs**

167 EV size distribution and particle concentration were determined with NTA Zetaview
168 (Particle Metrix, Germany) system. All experiments were carried out with pre-diluted
169 samples in PBS, leading to particle concentration within the 10^7 - 10^9 particles/mL range for
170 optimal analysis. The Zetaview system (Particle Metrix) is equipped with a 488 nm laser.
171 NTA analysis was performed in triplicate for each sample. Each measurement was
172 performed on 11 different positions within the sample cell with following specifications and
173 analysis parameters: sensitivity 80, shutter 100, Max Area 1000, Min Area 10, Min
174 Brightness 20. The results were validated while obtaining at least 1000 valid tracks for each
175 run. For data capture and analysis, the NTA software version 8.05.05 SP2 (Particle Metrix)
176 was used. Data represent the mean size \pm standard deviation (SD) of triplicate
177 measurements.

178 **2.4.3. Transmission electron microscopy (TEM) of EV isolates**

179 The EVs were analyzed after deposition of 4 μ l of suspension on a copper grid covered with
180 a carbon film for 5 mins, then stained with 1% uranyl acetate after absorption of the
181 contrasting liquid excess. The grids were observed on a Hitachi HT7700 transmission
182 electron microscope at 80 kV (Elexience - France). Images were acquired using an AMT
183 camera.

184 **2.5. Taylor Dispersion Analysis of EVs**

185 The TDA-UV and TDA-LIF experiments were carried out using Sciex MDQ CE systems (Sciex
186 Separation, Brea, CA) equipped either with UV absorption detector (200nm) or a solid-state
187 laser induced fluorescence detector ($\lambda_{\text{excitation}}$: 488 nm, $\lambda_{\text{emission}}$: 520 nm) positioned at 50.2
188 cm from the inlet of the capillary. The TDA-UV and TDA-LIF measurements were performed
189 at 20°C and 25°C, respectively. Uncoated fused silica capillaries (CM Scientific, Silsden, UK)

190 with I.D. of 50 μm , O.D. of 375 mm, effective length (L_{eff}) of 50.2 cm and total length (L_{tot})
 191 of 60 cm were used. The capillaries were preconditioned by successive flushing at 1.4 bar
 192 with water for 10 min, 1 M NaOH for 10 min and then water for 10 min. The EV samples
 193 were injected hydrodynamically from the inlet end by applying a pressure of 28 mbar for 9
 194 s, corresponding to a volume of ~ 7 nL. The mobilization pressure was kept constant at 28
 195 mbar. PBS 1X was used as the mobilization buffer. The injected EV concentrations were
 196 estimated based on NTA. All EV samples were diluted in PBS 1X at the desired concentration
 197 prior to their analysis. The measurements were performed in triplicate. Taylorgrams were
 198 recorded using Karat 32 software and then exported into an Excel file for subsequent data
 199 processing, where the temporal variance of the elution profile was quantified by peak
 200 integration. The latter is the result of the peak broadening due to Taylor dispersion.
 201 Consequently, the diffusion coefficient D ($\text{m}^2 \text{s}^{-1}$) and the hydrodynamic radius R_h (m) are
 202 determined using Eq. (1) and Eq. (2), respectively:

$$203 \quad D = \frac{R_c^2 t_0}{24\sigma^2} \quad (1)$$

$$204 \quad R_h = \frac{k_B T}{6\pi\eta D} \quad (2)$$

205 where R_c is the capillary radius (m), t_0 is the average elution time (s), σ^2 is the temporal
 206 variance of the peak (s^2), k_B is the Boltzmann constant (J K^{-1}), T the temperature (K) and η
 207 the viscosity of the carrier liquid (Pa s). It is noteworthy that Eq. (1) is valid when the peak
 208 appearance time t_0 is higher than the characteristic diffusion time of the solute on a
 209 distance equal to the capillary radius as verified by Eq. (3) [29,30]:

$$210 \quad \tau = \frac{D t_0}{R_c^2} \geq 1.25 \quad (3)$$

211 where τ is an adimensional characteristic time. Axial diffusion should also be negligible
 212 compared to convection as verified by Eq. (4) [29,30]:

$$213 \quad P_e = \frac{u R_c}{D} \geq 40 \quad (4)$$

214 where P_e is the Peclet number and u is the linear mobile phase velocity (m/s).

215 In the case of a sample mixture containing n different components of individual diffusion
 216 coefficient D_i , the Taylorgram $S(t)$ can be expressed as a sum of n individual Gaussian
 217 contributions $S_i(t)$, all centered at the same elution time t_0 :

218
$$S(t) = \sum_{i=1}^n S_i(t) = \sum_{i=1}^n \frac{A_i}{\sigma_i \sqrt{2\pi}} e^{-\frac{1(t-t_0)^2}{2\sigma_i^2}} \quad (5)$$

219 where A_i is a coefficient that is proportional to the concentration in species i and that
220 depends on the response coefficient of the species i , at the specific detection wavelength.
221 The diffusion coefficient of the species i is directly related to the standard deviation σ_i
222 according to equation (1). In order to obtain information about the size distribution of the
223 species in the mixture from the Taylorgram $S(t)$ a direct curve fitting of the elution profile
224 with the sum of n Gaussian curves according to equation (5), was applied when the total
225 number of species, n , is limited ($n \leq 4$). The curve fitting was conducted using the Least
226 Significant Difference method and the “GRG nonlinear” algorithm in Microsoft Excel. The
227 n -Gaussian fit was performed only on the left half of the elution peak to avoid any impact
228 of eventual peak tailing on the size measurement. Repeatability of TDA measurements was
229 quantified by calculating the relative standard deviation (RSD) on R_h values. The data
230 represent mean size \pm SD.

231 **2.6. Labeling of EVs**

232 **2.6.1. With antibodies**

233 For labeling of EVs with a specific antibody directed against CD9 tetraspanin, 10 μ L of EVs
234 (at 2×10^{12} EVs/mL) were incubated with the anti-CD9 monoclonal antibody (MM2/57), at a
235 final concentration of 2 μ g/mL, for 1 h at RT. Labeling EVs with anti-CD9 conjugated to FITC
236 was done by incubation of EVs with the fluorescent antibody (final concentration of 4
237 μ g/mL), for 1 h at RT. Fluorescently labeled EVs were then washed twice with PBS, to
238 remove free antibodies, by centrifugation at 10 000 xg using a Nanosep centrifugal tube
239 with 300K MWCO Omega™ membrane (Pall laboratory). The estimation of the final labeled
240 EV concentrations was based on the initial EV concentrations (i.e. before labeling),
241 determined by NTA, and taking into account the final volume recovered after the washing
242 steps.

243 **2.6.2. With a fluorescent dye**

244 The fluorescent labeling of EVs was implemented using the 5-(and-6)-Carboxyfluorescein
245 diacetate succinimidyl ester (CFDA-SE) dye according to the protocol described previously
246 [31]. Briefly, a 10 mM stock solution of the dye was first prepared in DMSO following the
247 manufacturer’s instructions. Prior to the labeling, the DMSO stock solution was diluted to
248 200 μ M in PBS. EVs were incubated with CFDA-SE (50:50, v/v) for 2 h in the dark, at RT.

249 Labeled EVs were then washed twice with PBS, to remove residual free CFDA-SE, by
250 centrifugation at 12000 x g using a Microcon-30 kDa centrifugal filter unit (Millipore).

251 **2.7. Statistical analysis**

252 Data were analyzed with GraphPad Prism 9 (CA, USA). Unpaired two-tailed Student's t-test
253 was used to compare two different samples measured by the same technique (TDA or NTA)
254 and results obtained for the same sample by both techniques. Moreover, one-way ANOVA
255 with Tukey's multiple-comparison test was used to evaluate whether the mean EV size,
256 determined by TDA, changes as function of the injected concentration. Data were
257 considered significantly different at p value ≤ 0.05 (> 0.05 equals not significant (ns), ≤ 0.05
258 *, ≤ 0.01 **, ≤ 0.001 ***).

259 **3. Results and discussion**

260 **3.1. Optimization and validation of TDA method for measurement of EV sizes and** 261 **concentrations**

262 Operating conditions in TDA (mobilization pressure, capillary nature and dimensions) were
263 carefully selected to remain in the Taylor regime (i.e. equations (3) and (4) were fulfilled),
264 for all solutes having a hydrodynamic diameter (D_h) ranging from 1 to about 300 nm
265 (Chamieh et al., 2017). TDA-UV was first conducted on EVs isolated from fresh bovine Milk
266 (BM), which was pretreated by acid precipitation and purified using a qEV35 SEC column
267 (BM-AP-qEV35). The selected mobilization buffer was 1x PBS and all analyzed samples were
268 diluted also in that buffer. The elution peak appeared relatively symmetric indicating
269 minimal adsorption of analytes onto the silica capillary wall under those conditions (Fig. 1-
270 A). The peak was best fitted with the sum of two Gaussians due to the presence of UV
271 absorbing small molecules within the EV sample, appearing as a small peak at the top of
272 the UV signal. The major population, contributing to 94 ± 1 % of the total UV absorbance
273 signal, represents EVs and was shown to exhibit an average hydrodynamic diameter of
274 189.9 ± 5.4 nm. This value is in good agreement with the results obtained by NTA for this
275 sample, which indicated an average EV size of 178.7 ± 6.7 nm (Fig. 1-B, Table 1). The
276 smallest size population revealed with TDA showed a mean size of 1.22 ± 0.04 nm and
277 represented only 6 ± 1 % of the total UV signal. This population is probably constituted by
278 residual small contaminants co-isolated with EVs, such as β -lactoglobulin, the main whey
279 protein with a hydrodynamic size of ~ 1.2 nm [32], which was particularly found to be present
280 in BM-derived EV samples prepared by acid precipitation when analyzed by mass
281 spectrometry (data not shown). Hence, in addition to EV size characterization, TDA offers

282 the advantage to detect the presence of molecular contaminants within the sample, which
283 are below the size limit of NTA of 70 nm [33].

284 To assess the performance of TDA-UV for measurement of EV size and concentrations, the
285 experiments were performed with EVs at different concentrations (from 2×10^{11} to 2×10^{13}
286 EVs/mL) and with triplicate analyses. Size measurements were highly repeatable for EV
287 concentrations over 5×10^{11} EVs/mL, with RSD ranging from 2.4 to 7.6% (Fig. 1-A, C).
288 Moreover, the average EV size measured by TDA was similar (~ 191 nm; $p > 0.05$) regardless
289 of the injected EV concentration (Fig. 1-C). The TDA measurement repeatability was similar
290 to that achieved with NTA in this work (0.5-4%) and close to that reported by others in the
291 literature (0.8-7.8 %) [33]. The EV peak areas exhibited a clear linear relationship (R^2 of
292 0.998) with the corresponding injected EV concentrations in the range of 2×10^{11} - 8×10^{12}
293 EVs/mL (Fig. 1-D). These results indicate that TDA-UV allows precise EV size measurement
294 for concentrations $\geq 5 \times 10^{11}$ EVs/mL and can also be used for the determination of EV
295 concentrations using a linear calibration curve established with standard EVs owing similar
296 characteristics (i.e. origin, purity..).

297 To evaluate whether TDA is able to discriminate slight changes in EV size, we measured the
298 size of an EV sample before and after incubation with a specific bovine anti-CD9 antibody,
299 which is supposed to bind to CD9 tetraspanin, a membrane protein expressed on the
300 surface of bovine milk-EVs [34]. This analysis was done on EVs isolated from frozen BM,
301 pretreated by acid precipitation, using qEV70 column (f-BM-AP-qEV70). The mean size of
302 EVs was found to increase from 210.1 ± 3.3 nm to 234.7 ± 25.4 nm after incubation of EVs
303 with anti-CD9 antibodies (Fig. 2-A). A similar tendency was observed by NTA (Fig. 2-B). Even
304 if values determined before and after incubation with the antibody did not significantly
305 differ, the observed size increase is in accordance with the association of EVs with IgG
306 monoclonal antibodies (~ 150 KDa) having a hydrodynamic size of ~ 10 - 13 nm [35–37]. This
307 shows the accuracy of TDA and its capability to detect minor changes in EV sizes.

308 **3.2. Application of TDA-UV to monitor the impact of isolation methods on EVs size**

309 The developed and validated TDA-UV method was then employed to determine the sizes
310 of different BM-derived EVs, prepared using different isolation protocols, in order to
311 investigate the effect of pre-analytical variables (i.e. milk pretreatment and SEC columns
312 used for their isolation) on the size heterogeneity and purity of extracted EVs.

313 **3.2.1. Impact of the freezing of bovine milk prior to EV isolation**

314 First, we compared the sizes of EVs prepared from fresh BM (BM-AP-qEV35) or frozen BM
315 (f-BM-AP-qEV35) but using same extraction protocol. The mean sizes determined by TDA
316 for both EV samples were similar ($p > 0.05$), with values of 189.9 ± 5.4 nm and 194.0 ± 7.8
317 nm for EVs isolated from fresh and frozen BM, respectively (Fig. 3-A, Table 1). In both
318 samples, the peak area corresponding to EVs represented more than 90 % of the total UV
319 signal, while the remaining signal corresponded to residual small molecules of ~ 1 nm, which
320 suggests similar low levels of protein contaminants, mainly β -lactoglobulin, within these
321 samples. NTA analysis was in accordance with TDA showing no significant impact of the
322 freezing of milk on the sizes of isolated EVs but did not point out the presence of protein
323 contaminants (Fig. 3-A).

324 **3.2.2. Impact of the type of SEC purification columns used to isolate EVs**

325 The TDA-UV method was then used to evaluate the impact of the SEC purification columns
326 on EV sizes. The mean size determined by TDA for EVs isolated from f-BM-AP using qEV70,
327 of 210.1 ± 3.3 nm, was shown to be significantly higher ($p < 0.05$) than that obtained for EVs
328 isolated using qEV35 column (194.0 ± 7.8 nm) (Fig. 3-A). However, the level of small
329 contaminants (~ 1 nm) was similar (between 9 - 12%) in both samples (Table 1). Although
330 qEV70 SEC column has an optimum recovery range shifted toward larger EVs (i.e. 70-1000
331 nm) compared to that of qEV35 (i.e. 35-350 nm), TDA showed only a slight increase of the
332 mean EV size when isolated using qEV70. These results suggest that large EVs (> 350 nm)
333 are rare in these samples and do not significantly influence the mean EV size when using
334 qEV70 for their isolation. This can be explained by the $0.22 \mu\text{m}$ filtration step included in
335 the EV isolation protocol (see section 2.2.1). Unlike TDA, NTA measurements showed no
336 significant difference ($p > 0.05$) in EV sizes among the samples prepared using qEV70 (i.e.
337 172.0 ± 0.9 nm) or using qEV35 (i.e. 176.7 ± 7.0 nm) (Fig. 3-A, Table 1). This may be due to
338 the important sample dilution required by NTA that could further minimize the influence
339 of the low abundant large EVs on the measured average EV size.

340 **3.2.3. Impact of the milk method used to discard the major milk proteins**

341 The TDA-UV method was used to verify the purity of EV samples with different abundance
342 of contaminating milk proteins. The EV sample obtained from milk pretreated by acid
343 precipitation (f-BM-AP) using qEV70, was considered as sufficiently pure (88% of the total
344 UV signal) (Fig. 3-B, Table 1). TDA measurements were then conducted on two samples
345 obtained from f-BM that were pretreated mechanically using two different centrifugation

346 protocols (f-BM-C1 or C2), in order to discard the major milk proteins (caseins), prior to EV
347 isolation from whey fractions using qEV70 column. Both samples were expected to exhibit
348 higher protein contamination compared to the sample prepared by acid precipitation. This
349 was evidenced by the 3 to 11-fold higher ratios of protein concentration/EV count
350 measured by BCA assay and NTA for f-BM-C1 & C2, respectively. The Taylorgrams recorded
351 from both samples showed an asymmetric broadening of the elution peaks (Fig. 3-B)
352 resulting probably from the interaction between analytes within the sample and the
353 capillary wall [38]. Furthermore, in agreement with the high ratios of protein to EVs
354 determined for f-BM-C1 & C2, TDA analysis showed that molecular contaminants in both
355 samples were very abundant (i.e. ~50 % and ~100 % of the total UV signal, respectively)
356 compared to f-BM-AP (i.e. ~12%; Table 1). In addition, the average size of these
357 contaminants was found higher in f-BM-C1 & C2 (2.8 and 12.7 nm, respectively) compared
358 to f-BM-AP (1.1 nm). This is consistent with mass spectrometry analysis that showed
359 abundance of various whey proteins (e.g. lactoferrin and bovine serum albumin), larger
360 than β -lactoglobulin, in such samples (data not shown). TEM images were in excellent
361 agreement with TDA showing the presence of more contaminant protein traces (electron
362 dense smudges) in the samples isolated using centrifugation (Fig. 3-C), particularly in f-BM-
363 C2 where EV imaging was hindered by the contaminants. The largest size population
364 revealed by TDA in f-BM-C1 samples, which is attributed to EVs, showed a mean size of 95.4
365 ± 21.5 nm (Fig. 3-A), which is significantly smaller than that measured by TDA for EVs in f-
366 BM-AP (i.e. 210.1 ± 3.3 nm). This can be explained by the presence of small sized protein
367 aggregates, resulted from the aggregation propensity of the highly abundant proteins
368 within the f-BM-C1 & C2 samples, that bias the analysis and lead to a shift of EV size toward
369 smaller sizes.

370 **3.2.4. EV size and purity in a WPC preparation**

371 Finally, TDA analysis of EVs isolated from WPC using qEV70 SEC column showed a mean EV
372 size of 121.5 ± 11.9 nm (Fig. 3-A), which contributed to ~91 % of the UV signal (Table 1).
373 Small contaminants of 1.3 ± 0.4 nm were present at low proportion (i.e. ~9 %), similar to
374 the results observed for EV samples isolated from BM treated by acid precipitation.
375 However, compared to these latter, EVs isolated from WPC showed significantly smaller
376 size as measured by TDA and NTA (Fig. 3-A, Table 1).

377 If most of the sizes determined for the different EV samples by TDA are relatively similar to
378 those obtained by NTA, the results obtained from f-BM-C1 and f-BM-C2 samples, which
379 exhibit high protein contamination, were particularly different between both techniques.
380 These discrepancies arise from the difference in their working principles. NTA determines
381 the Brownian motions of single particles, and subsequently their sizes, based on the light
382 scattered by the particles, and thus has a restricted resolution for small particles and
383 present a lower size limit of ~ 70 nm [33]. Thus, NTA is likely not influenced by the presence
384 of high content of protein contaminants in the analyzed sample. In contrast, TDA
385 determines the diffusion coefficient of analytes based on their dispersion under Poiseuille-
386 like flow, allowing measurement of a wide range of sizes (from angstrom to
387 submicrometer) [28]. These results show the potential of TDA as complementary technique
388 allowing to evaluate the purity of EV samples by displaying the abundance of contaminating
389 proteins. The purity (i.e. relative peak area of EVs among the total UV signal) of the different
390 EV samples evaluated by TDA was repeatable (RSD of 0.3 - 6.7 %) for concentrations \geq
391 5×10^{11} EVs/mL (Table 1).

392 **3.3. TDA-LIF for monitoring the size of labeled EVs**

393 The TDA method was then used with LIF detection to check whether we could use an
394 alternative mode of detection. EVs were labeled with either CFDA-SE, an intravesicular dye,
395 or through their surface markers, using an α -CD9 antibody conjugated to FITC.

396 TDA-LIF analysis of EVs labeled with CFDA-SE showed three subpopulations with mean sizes
397 of 283.2 ± 33.5 nm, 38.0 ± 15.1 nm and 1.33 ± 0.03 nm, representing ~ 72 %, 13 % and 15 %
398 of the total signal, respectively (Fig. 4). If the largest subpopulation (283.2 nm) corresponds
399 to the labeled EVs, the one with a mean diameter of 38.0 nm is most likely very small EVs
400 and/or fluorescent EV debris (i.e. conjugated to CFSE) that may result from the labeling
401 process. The smallest size of 1.33 ± 0.03 nm represents the free dye that spontaneously
402 hydrolyze and turn fluorescent. This was confirmed by TDA tests on free CFDA-SE (data not
403 shown). To understand the size increase found for EVs after labeling with CFDA-SE (i.e.
404 283.2 ± 33.5 nm), compared to unlabeled EVs (i.e. 189 ± 5.4 nm, determined by TDA-UV),
405 we applied the same washing protocol used to remove free CDFA to unlabeled EVs
406 (centrifugation through a 30 kDa filter). We also observed a similar significant increase in
407 the size of unlabeled EVs. This washing step is therefore responsible for that size increase

408 which may be due to EV aggregation and/or a tendency of the smallest EVs to adsorb onto
409 the filter membrane.

410 The TDA-LIF detection was then used to evaluate the size of EVs labeled with anti-CD9
411 conjugated to FITC. Surprisingly, the size of labeled-EVs was found to be significantly
412 smaller (163.6 ± 12.4 nm) than that determined by TDA-UV for the same EVs before labeling
413 (189.9 ± 5.4 nm) (Fig. 4-B). This may be because TDA-LIF provide specific sizing of the EV
414 subpopulation expressing CD9 tetraspanin. This latter protein marker was indeed found to
415 be enriched in small EVs [39,40], which explains this shift toward smaller sizes measured
416 by TDA despite the association of EVs with anti-CD9 antibodies. Furthermore, TDA revealed
417 the presence of small contaminants of 5.8 ± 0.4 nm within the samples, suggesting the
418 presence of residual FITC-conjugated antibodies and free FITC following EVs labeling.

419 TDA-LIF size measurements were also repeatable with RSDs in the range of 0.8-11.8 %.
420 These results show the capacity of TDA-LIF to monitor the changes in EV size after different
421 fluorescent labeling processes.

422 **4. Conclusion**

423 From our findings, we propose TDA as new method for the size characterization of
424 heterogeneous EVs. TDA is an absolute method, requiring no size calibration, allowing to
425 measure the hydrodynamic sizes of analytes within a small sample volume. This is of
426 particular interest as the scientific community working on EVs is struggling to find relevant
427 reference materials to calibrate and standardize EV analysis techniques. TDA could
428 characterize the size of bovine milk-EVs, in their sample matrix, over their whole size range.
429 Although TDA is capable to detect small EVs < 50 nm, which are below the lower limit of
430 NTA, both techniques were shown to determine similar sizes at least for relatively low
431 polydisperse and non-contaminated samples, such as bovine EVs used in our study.

432 Furthermore, TDA allows quantitative assessment of EVs and molecular contaminants (e.g.
433 proteins) present in the samples. This makes TDA method very useful tool for the
434 estimation of the purity of EV isolates (i.e. ratio of EVs to contaminating proteins), which is
435 particularly important for subsequent use of EVs. Thus, TDA can be considered as a tool for
436 simple, accurate and straightforward quality control of EV samples, which is currently
437 addressed using approaches combining electron microscopy and western Blot that are
438 labor intensive, time consuming, unsuited to routine daily use and lack accuracy [41]. Here,
439 TDA was successfully applied to investigate the impact of pre-analytical variables on the

440 size heterogeneity and purity of isolated bovine EVs (Table 1). Our results showed that
 441 freezing of bovine milk prior to EV isolation seems to have no impact on the size of isolated
 442 EVs. On the other hand, the type of SEC purification column (qEV35 Vs qEV70) used in
 443 the isolation process had only a slight influence on the size of the resulting BM-derived EVs.
 444 Furthermore, acid precipitation was shown to be more efficient in removing milk proteins
 445 compared to centrifugation, resulting in EV preparations with significant higher purity.

446 Moreover, the TDA-LIF approach was shown to allow to monitor the changes in EV size
 447 after fluorescent labeling and also to distinguish EV sub-population based on their surface
 448 protein. This method has therefore a great potential to be used as a complementary
 449 approach for the size, quality characterization as well as the quantification of EVs. The
 450 integration of an online electrokinetic preconcentration of EVs in capillary at the forefront
 451 of TDA analysis, is currently being investigated in order to improve the sensitivity/LOD of
 452 this method.

453 **Table 1.** Summary of the results obtained by TDA and NTA on the different bovine milk-
 454 derived EV preparations. AP: acid precipitation; C1, C2: centrifugation protocols 1 or 2.

Sample	Frozen/fresh bovine milk	Protocol used to discard major milk proteins	SEC column	NTA (nm)	TDA	
					EV size (nm)	EV purity (Relative peak area of EVs among total UV signal)
BM-AP	Fresh	AP	qEV35	178.7 ± 6.7	189.9 ± 5.4	94.3 ± 0.8 %
f-BM-AP	Frozen	AP	qEV35	176.7 ± 7.0	194.0 ± 7.8	91.1 ± 1.0 %
f-BM-AP	Frozen	AP	qEV70	172.0 ± 0.9	210.1 ± 3.3	88.0 ± 2.6 %
f-BM-C1	Frozen	C1	qEV70	179.6 ± 0.7	95.4 ± 21.5	50.3 ± 2.0 %
f-BM-C2	Frozen	C2	qEV70	179.2 ± 2.6	*	< 50 %
WPC	Whey powder	AP	qEV70	146.1 ± 3.7	121.5 ± 11.9	91.1 ± 2.2 %

455 (*) EV size determination was hindered by the high abundance of contaminants.

456 **Declaration of competing interest**

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

459 **CRediT authorship contribution statement**

460 **Sameh Obeid:** Visualization, Methodology, Investigation, Data curation, Formal analysis,
461 Writing – original draft. **Joseph Chamieh:** Data curation, Visualization, Writing - review &
462 editing. **Thanh Duc Mai:** Conceptualization, Supervision, Visualization, Writing - review &
463 editing. **Marco Morani:** Methodology, Preliminary investigation, Formal analysis. **Melissa**
464 **Reyre:** Formal analysis, Data curation. **Zuzana Krupova:** Funding Acquisition, Investigation,
465 Formal analysis, Data curation, Writing - review & editing. **Pierre Defrenaix:** Funding
466 Acquisition, Project administration. **Hervé Cottet:** Data curation, Visualization, Writing -
467 review & editing. **Myriam Taverna:** Conceptualization, Project administration, Funding
468 Acquisition, Supervision, Validation, Writing - review & editing.

469 **Data availability**

470 Data will be made available on request.

471 **Acknowledgements**

472 This project was funded by “France Relance” (France recovery plan, contract 254315) and
473 has received financial support from the Centre National de la Recherche Scientifique (CNRS)
474 through the MITI program (Mission pour les Initiatives Transverses et Interdisciplinaires,
475 2021-2022). The authors thank Christine Longin, from University Paris-Saclay, INRAE,
476 AgroParisTech, GABI (Jouy-en-Josas, France) for TEM imaging. We would like to thank
477 Sophie Gallier, from the Dairy Goat Co-operative (N.Z.) (DGC, Ltd, Hamilton New Zealand)
478 for providing the WPC samples.

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Figure captions

Fig. 1. Size characterization by TDA (A, C, D) or by NTA (B) of EVs isolated from fresh BM-AP using qEVoriginal SEC 35 nm column. (A) Taylorgrams representing three repetitions obtained by injecting EVs at 2×10^{12} EVs/mL. Two-Gaussian fit shown in dotted black line, was required due to the presence of small contaminants within the sample appearing as small peak on the top of the signal corresponding to EVs. Taylorgrams were aligned on the x-scale for better visual comparison. (B) NTA profile with the average EV size obtained. (C) TDA measurement repeatability determined at different EV injected concentrations. (D) Mean peak area, corresponding to EV contribution, plotted as function of injected EV concentration. Data represent mean \pm SD of triplicate measurements. BM: bovine milk; AP: acid precipitation.

Fig. 2. Size characterization obtained by TDA (A) or NTA (B) for EVs before and after incubation with specific α -CD9 antibody. EVs were isolated from f-BM-AP using qEVoriginal SEC 70 nm. Data represent mean \pm SD of triplicate measurements. f-BM: frozen bovine milk; AP: acid precipitation. Taylorgrams in (A) were aligned on the x-scale for better visual comparison.

Fig. 3. Size measured using TDA or NTA for the different BM-derived EV samples prepared using different protocols (A). All TDA analyses were performed by injecting EVs at 2×10^{12} EVs/mL. Data represent mean \pm SD of triplicate measurements. (B) Representative Taylorgrams and (C) TEM images of EVs isolated from frozen bovine milk (f-BM), pretreated either by acid precipitation (AP) or by two different centrifugation protocols (i.e. C1 or C2), using qEV70 SEC column. White arrows indicate EVs. High electron-dense -black- patches in TEM micrographs correspond to protein dense regions. Taylorgrams in (B) were aligned on the x-scale for better visual comparison.

Fig. 4. TDA-LIF of fluorescently labeled EVs. (A) Taylorgrams obtained from EVs labeled by CFDA-SE or anti-CD9 conjugated to FITC. EVs were isolated from fresh BM-AP using qEVoriginal SEC 35 nm column prior to labeling. Taylorgrams were aligned on the x-scale for better visual comparison. (B) EV hydrodynamic diameter obtained by TDA-UV, for unlabeled EVs, or by TDA-LIF for fluorescently labeled-EVs. Data represent mean \pm SD of triplicate measurements.

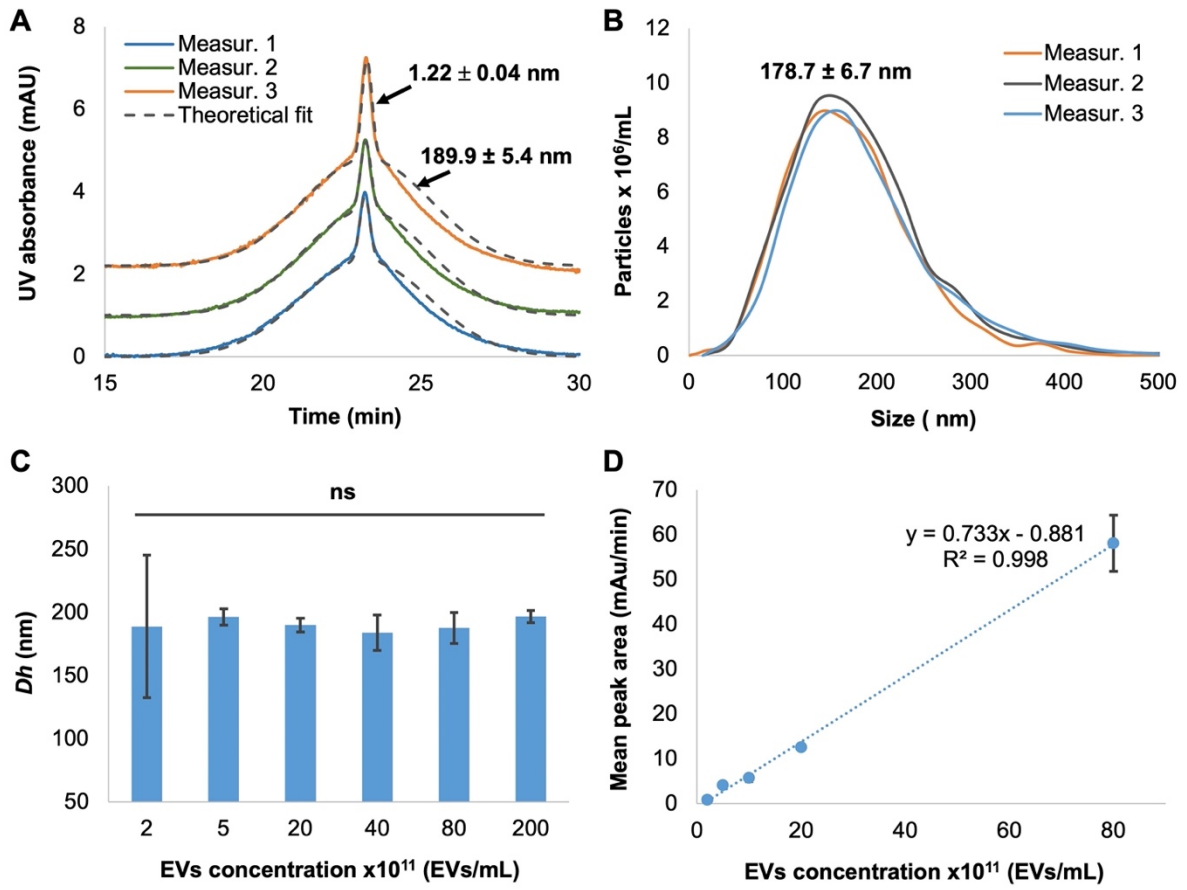


Fig.1

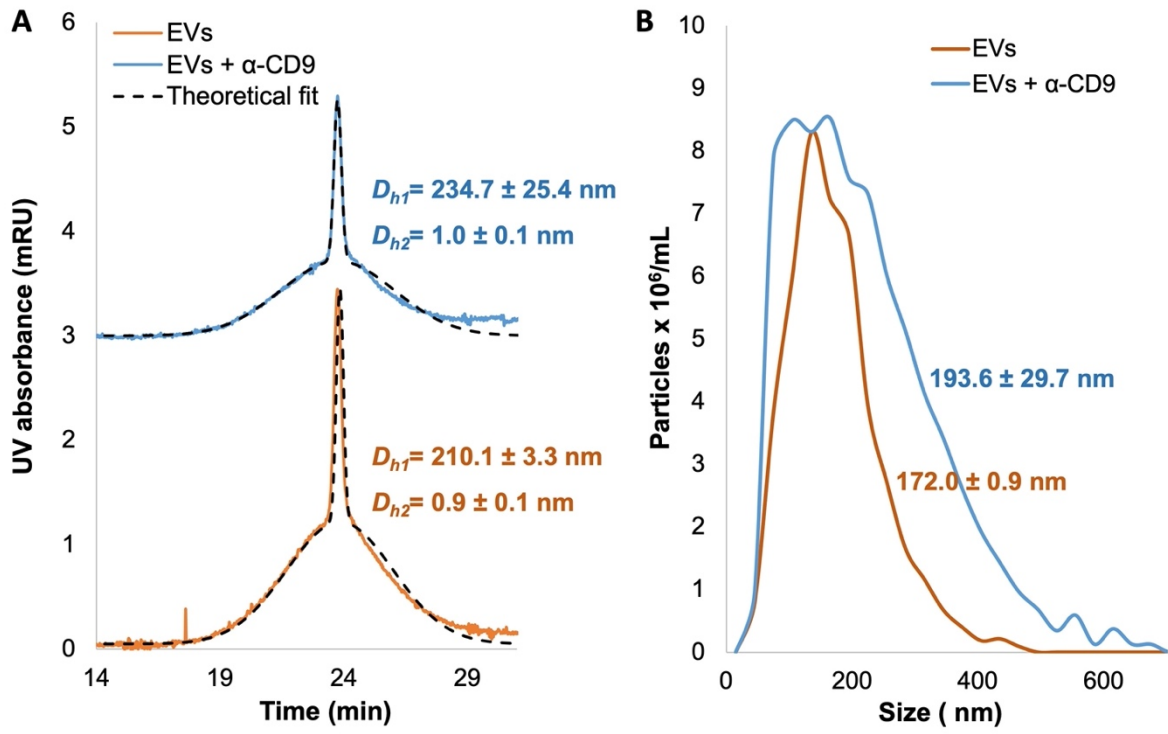


Fig.2

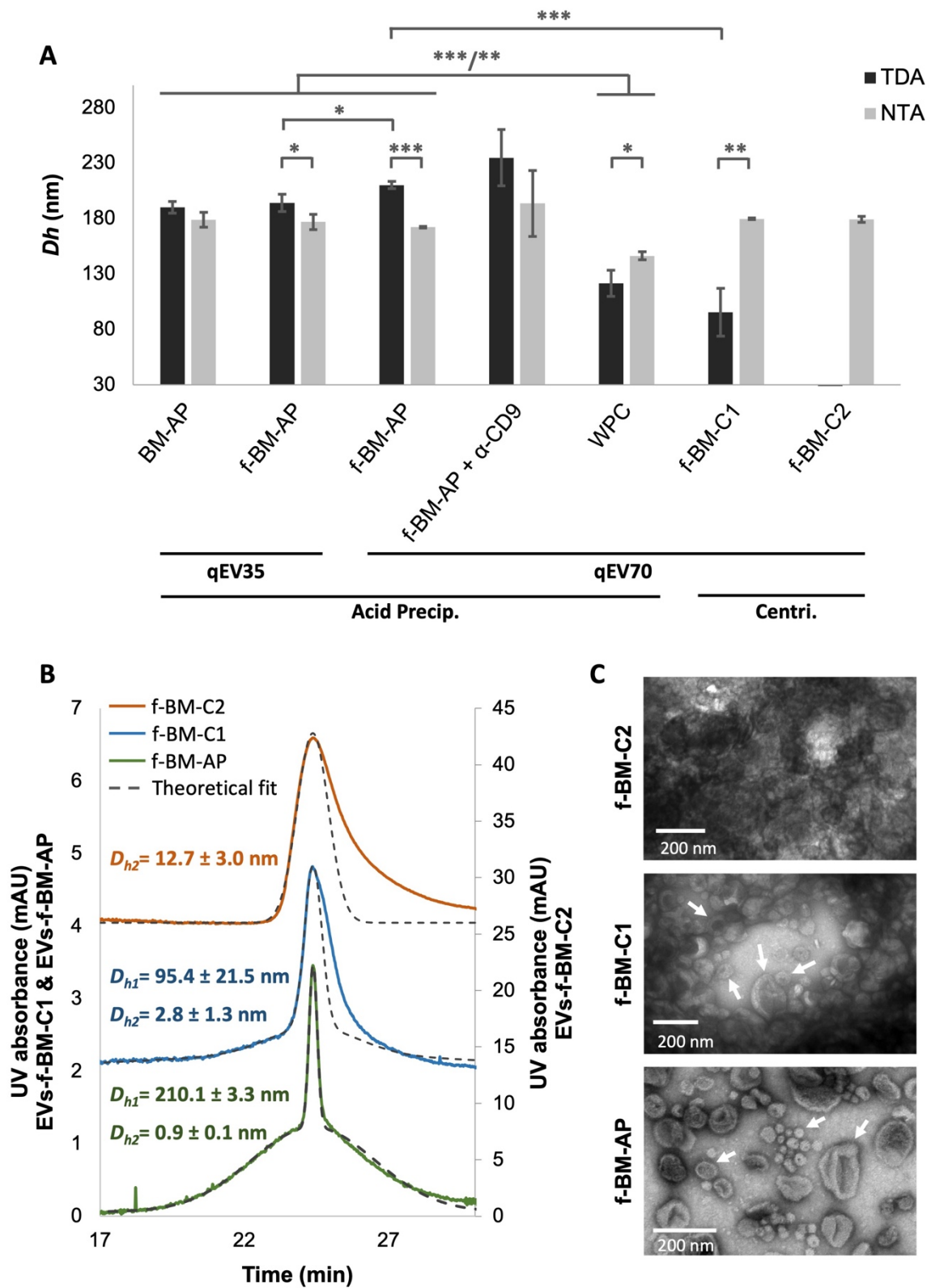


Fig.3

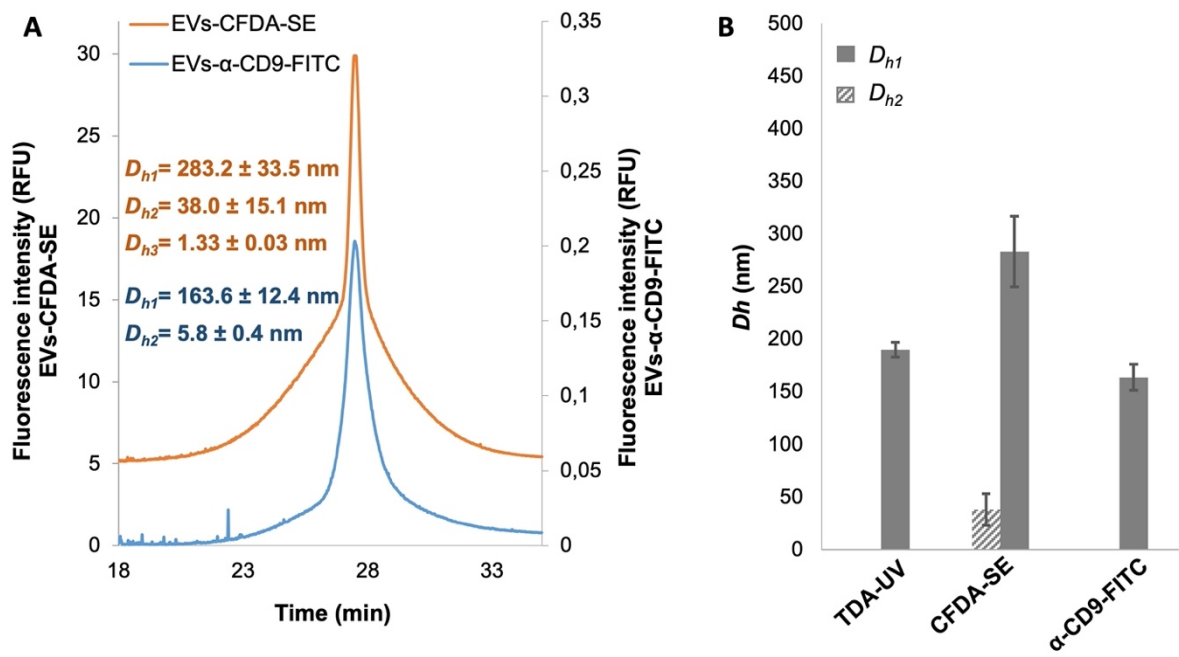


Fig.4