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1 **Preventing the impact of solute adsorption in Taylor Dispersion Analysis:**
2 **application to protein and lipid nanoparticle analysis**

3
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9
10 **Abstract**

11 Taylor Dispersion Analysis (TDA) allows diffusion coefficient (D) or hydrodynamic radius (R_h) determina-
12 tion on a wide range of size between angstroms and about 300 nm. However, solute adsorption phenomena
13 can affect the repeatability and reproducibility of TDA. Several numerical studies addressed the theoretical
14 impact of solute adsorption in TDA, but very few experimental studies focus on this topic and no experi-
15 mental methodologies were proposed so far to reduce the impact of adsorption in TDA. In this work, an
16 experimental protocol, called plug-in-front TDA, consisting of adding the solute in the eluent at a lower
17 concentration compared to the injected sample, was proposed to strongly limit the impact of adsorption on
18 the R_h determination. This protocol was suggested based on the evidence that adsorption / desorption
19 phenomena impacting narrow bore fused silica TDA in aqueous conditions are typically slow processes
20 that can be counteracted by saturating the interaction sites during the experiments. Successful applications
21 to proteins and mRNA lipid nanoparticles (LNP) in vaccine against Covid 19 and protein analysis were re-
22 ported. TDA of proteins in conditions of strong interactions with the capillary surface was possible using
23 the plug-in-front methodology. We anticipate that such experimental methodology will greatly help the
24 experimentalist for implementing TDA in various applications.

26 **Keywords:** Taylor dispersion analysis, adsorption, lipid nanoparticles, hydrodynamic radius.

27

28 **1. Introduction**

29 Taylor Dispersion Analysis (TDA) is a powerful technique used to determine hydrodynamic
30 radii (R_h) and diffusion coefficient (D) of numerous compounds [1]. Based on the pioneer work of
31 Taylor [2,3] and Aris [4], R_h and D can be determined by analyzing the peak broadening of a solute
32 plug in a laminar Poiseuille flow [3,4]. Firstly applied to gaseous solutes [5], determination of these
33 characteristics was developed in aqueous media for small molecules such as ethanol, acetone or
34 benzene and its derivatives [6–8]. At the end of the last century, a regain of interest in TDA was
35 observed with the work of Bello et al. [9] who highlighted the use of capillary electrophoresis (CE)
36 instrumentation to speed-up, miniaturize and automatize TDA in typically 50 μm i.d. capillaries.

37 Since TDA is an absolute method (no calibration is needed) with low sample consumption
38 and a large range of size (from angstrom to about 300 nm in diameter) [10], it offers numerous
39 advantages compared to other techniques such as dynamic light scattering (DLS) or diffusion or-
40 dered spectroscopy NMR (DOSY NMR) [11,12]. Indeed, TDA is not sensitive to the presence of
41 dusts or large aggregates contained in the sample, contrary to what is observed in scattering tech-
42 niques. From simple molecules [1,13,14] to (bio)(macro)molecules [15–18], as well as lipid nano-
43 particles [19–21], TDA is nowadays a recognized straightforward sizing method applicable to a
44 huge variety of solutes.

45 Analytical conditions must be carefully chosen to correctly perform TDA. First, the solute
46 must be prepared in the exact same buffer as the one used as the mobile phase to avoid any mis-
47 match buffer signal perturbation [22]. The Peclet number Pe and the average elution time t_0 that
48 depend on the capillary length, the mobilization pressure, and the solute size, must be controlled
49 to remain in the conditions of validity of TDA (see theoretical background section for more de-

50 tails) [3]. It exists also a maximum sizing limit for TDA above which hydrodynamic chromatog-
51 raphy occurs and can contributes to the peak dispersion [23]. It was also shown that for coiled
52 capillaries with capillary i.d. higher than 100 μm , radial mixing of the solute may occur at high
53 mobilization pressure. This phenomenon dramatically affects the TDA analysis because radial mix-
54 ing generates thinner peaks leading to larger diffusion coefficient values [24,25].

55 TDA relies on the assumption of no physicochemical interaction between the solute and the
56 capillary wall. In 1958, Golay theoretically studied the impact of retention on the peak broadening
57 of a solute in an open tube the wall of which is coated with a thin retentive layer [26]. He derived a
58 long-time asymptotic expression of the analyte dispersion coefficient, D_{disp} , (in $\text{m}^2.\text{s}^{-1}$)

$$59 \quad D_{disp} = \frac{D}{1+k} + \frac{1+6k+11k^2}{(1+k)^3} \frac{R_c^2 u^2}{48D} \quad (1)$$

60 where D is the analyte molecular diffusion coefficient in the carrier fluid (in $\text{m}^2.\text{s}^{-1}$), R_c is the capil-
61 lary radius (in m), u the carrier fluid average velocity (in $\text{m}.\text{s}^{-1}$), and k the retention factor [26]. The
62 Taylor-Aris result is recovered by setting $k = 0$ in this equation. The first term reflects the axial
63 molecular diffusion, the second the Taylor contribution (mass transfer in the carrier fluid). An ad-
64 ditional term accounting for the diffusional mass transfer within the stationary retentive layer can
65 be added [26] but is often found negligible owing to the very small thickness of this layer and is
66 not of concern in our case. Eq. (1) relies on instantaneous equilibrium at the interface between the
67 fluid and stationary phases. Recognizing that equilibrium is not always reached at the interface,
68 Aris [4], then Khan [27], extended the analysis by adding, to the right hand side of Eq. (1), a third
69 term reflecting interfacial resistance and depending on the sorption rate constants. However, be-
70 cause the formulation of their kinetic model involved the thickness of the retentive layer, it is not
71 directly applicable to the situation encountered in TDA capillaries which generally do not contain
72 such a layer. The contribution of the mass transfer resistance due to adsorption on the wall was

73 obtained by Giddings using either the stochastic theory [28] or the nonequilibrium theory of
74 chromatography [29], so that the dispersion coefficient can be expressed as

$$75 \quad D_{disp} = \frac{D}{1+k} + \frac{1+6k+11k^2}{(1+k)^3} \frac{R_c^2 u^2}{48D} + \frac{k}{(1+k)^3} \frac{u^2}{k_d} \quad (2)$$

76 with $k=k_a/k_d$, k_a and k_d being the adsorption and desorption rates (in s^{-1}), respectively. For adsorp-
77 tion on heterogeneous surfaces, $1/k_d$ in the last term of this equation should be replaced by $\langle \tau_a \rangle$,
78 the mean duration of a step spent by a molecule in the adsorbed state on the capillary wall [30].

79 Several theoretical models based on different approaches have been developed to account
80 for the influence of solute interaction with the wall to the dispersion coefficient (or, equivalently,
81 to the plate height). A summary of these works can be found in the introduction of the article by
82 Hlushkou et al. [31]. Eq. (2) is retrieved when general models are applied to conditions pertaining
83 in TDA (steady Poiseuille flow in a tube without irreversible adsorption or reaction on the tube
84 wall) [32–37]. Using a random walk particle-tracking technique, Hlushkou et al. performed 3-D
85 simulations of advective-diffusive solute transport in the carrier flow in a tube combined with a
86 stochastic approach of the sorption process [31]. The finiteness of the adsorption rate is account-
87 ed for by using a fixed value of the probability that a solute molecule is adsorbed when it hits the
88 wall. The value of the retention factor is obtained by proper adjustment of the adsorption proba-
89 bility and the mean adsorption time $\langle \tau_a \rangle$. Whatever the flow velocity and the retention factor, they
90 obtained perfect fit of the dispersion coefficient according to Eq. (1) (Golay equation) for an ad-
91 sorption probability of 1, which corresponds to very fast sorption rates (equilibrium assumption).
92 For a slow interfacial transfer (low value of the adsorption probability), their results fit well with
93 Eq. (2) in the case of an exponential distribution of the duration of the adsorption step. However, if
94 this distribution is uniform (all adsorption steps have then the same duration τ_a), the dispersion
95 coefficient is lower and fits with a modification of Eq. (2) in which the last term is divided by 2.
96 This clearly shows that the dispersion depends on the microscopic details of the adsorption pro-

97 cess, in agreement with the general stochastic theory of chromatography which predicts that the
98 temporal variance of a solute peak depends on the variance of the number of adsorption steps and
99 on that of the duration of an adsorption step [38,39]. The peak temporal variance is thus lower
100 when the latter vanishes.

101 It should be noted that the derivation of Eqs (1) and (2) relies on a first order kinetic model,
102 hence on a linear adsorption isotherm. Madras et al. investigated the influence of non-equilibrium
103 dynamic adsorption between the solute and the capillary inner surface on the peak shape via nu-
104 merical simulation using a Langmuir isotherm [40]. They observed deviations from Gaussian peak
105 shapes depending on the kinetic rate constants and on the number of active sites on the wall, in
106 agreement with the findings of TDA experiments on phenanthrene in a supercritical fluid per-
107 formed by Smith et al. [41] and Hamilton [42].

108 Eqs. (1) and (2) give the asymptotic long-time expressions of the dispersion coefficient. If the
109 capillary is not long enough, the peak elution profile may be strongly distorted and sometimes
110 bimodal. This was observed by experiments and/or simulations on short tubes either without ad-
111 sorption [43–45] or with adsorption [46–48]. Analytical solutions to the system of mass balance
112 equations controlling the migration of a solute in a straight conduit with reversible and/or irre-
113 versible adsorption have been recently obtained [49,50]. They allow to get a highly detailed view
114 of the complexity of the evolution of the solute concentration distribution at all positions within
115 the capillary as a function of time. Especially, the instantaneous mean zone velocity and dispersion
116 coefficient are obtained as the growth rates of the first moment and of half of the second central
117 moment of the cross-sectional averaged distribution of the solute concentration along the tube,
118 respectively. It is found that, depending on the values of the adsorption and desorption rates, the
119 zone velocity and dispersion coefficient may, during a short period, be negative. The classical Tay-
120 lor regime is asymptotically recovered at long times. The influence of the initial condition used to
121 release the solute in the capillary has been investigated [49–51].

122 In this work, we study experimentally the adsorption of model small molecules and proteins.
123 To limit the impact of slow desorption kinetics phenomena on TDA measurements, we propose a
124 new methodological protocol. Applications to the sizing of lipid nanoparticles (LNP) used in
125 mRNA vaccines are demonstrated.

126

127 **2. Experimental section**

128 **2.1. Chemicals and Materials.** 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES, \geq
129 99.5% purity), 2-(N-morpholino)ethane sulfonic acid (MES, \geq 99.5% purity),
130 tris(hydroxymethyl)aminomethane (Tris, \geq 99.5% purity) and sodium tetraborate decahydrate
131 (Borax \geq 99.5% purity) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). So-
132 dium hydroxide (NaOH, 98 % purity) and hydrochloric acid (fuming, \geq 37%) were purchased from
133 Fluka (Saint-Quentin-Fallavier, France). Acetonitrile (ACN, HPLC grade) was purchased from Carlo
134 Erba (Val de Reuil, France). Acetic acid (99.99% purity) was supplied by VWR Chemicals (Rosny-
135 sous-Bois, France). Pfizer vaccine COMIRNATY™ Original/Omicron BA. 4-5 (lot number: GJ7181,
136 with an expiration date in July 2023) against Covid 19 was generously given by Pharmacie des 4
137 Seigneurs (Montpellier, France) and kept at -80°C .

138 Toluene (analytical reagent grade) was purchased from Fisher Scientific (Loughborough,
139 Leicestershire, UK). 1-Methyl-4-(1-methylethyl) benzene (*p*-cymene, purity $<$ 98%) was pur-
140 chased from Parfum Cosmetic World (Grasse, France). Cyclo(Ala-Ala) peptide was purchased from
141 Bachem (Bubendorf, Switzerland). Triphenylphosphine (99% purity), Myoglobin (Myo, from eq-
142 uine skeletal muscle, 95% purity) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier,
143 France). All aqueous solutions were prepared using deionized water (18 M Ω) delivered by a Syn-
144 ergy UV water purification system (Millipore, Fontenay sous Bois, France).

145

146 **2.2. Eluent and sample preparation.** 2 M acetic acid (pH 2.2, $\eta = 1.12$ cp) was prepared by dilu-
147 tion of acetic acid with deionized water (12.8 g for 100 mL). (50/10 mM) MES/NaOH buffer (pH
148 5.8, $\eta = 0.99$ cp) was prepared by mixing 1.95 g of MES with 1 mL of NaOH 1M in 100 mL volumet-
149 ric flask. (20/10 mM) HEPES-NaOH buffer (pH 7.0, $\eta = 0.96$ cp) was prepared by mixing HEPES
150 (0.477 g for 100 mL) with sodium hydroxide (10 mL of a 1M NaOH solution to a 100 mL volumet-
151 ric flask). 10 mM sodium tetraborate decahydrate buffer (pH 9.1, $\eta = 0.98$ cp) was prepared by
152 dissolving 0.191 g of Borax powder ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$) in a 10 mL volumetric flask. ACN/HEPES
153 solution was prepared by mixing pure ACN with 40 mM HEPES / 10 mM NaOH pH 7.4 buffer with
154 a ratio of 70/30 (w/w). The viscosity of this solution was measured at 0.39 cp. (20/17 mM) Tris-
155 HCl buffer (pH 7.4, $\eta = 0.92$ cp) was prepared by mixing 0.24 g of Tris with 1.7 g of 1 M HCl in a
156 100 mL volumetric flask.

157 Cyclo(Ala-Ala), toluene, p-cymene and triphenylphosphine were diluted in ACN/HEPES solu-
158 tion at a concentration of 10 gL^{-1} , 6 gL^{-1} , 8 gL^{-1} and 0.7 gL^{-1} , respectively. Myoglobin (Myo) powder
159 was diluted either in 2 M acetic acid, or in MES/NaOH buffer, or in HEPES/NaOH buffer, or in sodi-
160 um tetraborate decahydrate buffer at a concentration of 2 gL^{-1} , and stored at 5°C . The sample was
161 diluted twice to obtain the desired concentration (1 gL^{-1}) for TDA analysis. TDA of LNP was per-
162 formed by injecting LNP solution without dilution. DLS analysis of LNP was performed by diluting
163 $60 \mu\text{L}$ of LNP solution with 1 mL of filtered ($0.02 \mu\text{m}$ filter) Tris-HCl buffer (20/17 mM).

164 For protein plug-in-front eluent preparation, myoglobin solution (named S_0 , at 2 gL^{-1}) was di-
165 luted to the desired concentration (i.e., from 0.05 gL^{-1} up to 0.5 gL^{-1} for eluent preparation (S_2))
166 and to 1 gL^{-1} for the analyzed solution (S_1) using the pH 5.8 or 7.0 previously prepared background
167 electrolytes.

168 For LNP plug-in-front preparation, $10 \mu\text{L}$ of LNP solution were mixed with $190 \mu\text{L}$ of (20/17
169 mM) Tris-HCl buffer (dilution factor 20) for the preparation of S_2 solution.

170 **2.3. Taylor Dispersion Analysis Experiments.** TDA experiment was performed on a 7100 Ag-
171 ilent system (Agilent Technology, Santa Clara, USA), piloted with ChemStation, using 50 μm i.d.
172 capillaries. Fused silica capillaries (50 μm i.d., 375 μm o.d.) were purchased from Polymicro Tech-
173 nologies (Phoenix, AZ, USA). Coated DB-1 and DB-17 capillaries (50 μm i.d.) were from Agilent
174 Technology (Santa Clara, USA). Fused silica capillaries were conditioned by flushing NaOH 1 M
175 solution for 5 min at 1 bar, followed by a 5 min flush of pure water at 1 bar. Coated capillaries were
176 used after a simple flush with the eluent. For TDA in the case of fast desorption kinetics, fused sili-
177 ca capillaries and DB-1 and DB-17 coated capillaries were 80 cm long (71 cm to the detector). For
178 the rest of the study, fused silica capillaries were 40 or 60 cm long (31.5 and 51.5 cm to the detec-
179 tor, respectively).

180 For protein analysis, the presaturation protocol on 40 cm long fused silica capillaries con-
181 sists in flushing the capillary with the sample containing the analyte (for 10 min at 100 mbar, 5
182 capillary volumes) before analysis. After presaturation, the capillary was flushed with the back-
183 ground electrolyte (BGE) without protein for 1 min at 1 bar (5 capillary volume) followed by a
184 plug injection (30 mbar 4 s) and mobilization with the same BGE.

185 For protein plug-in-front experiments, the capillary was flushed with S_2 solution containing
186 diluted proteins for 200 s at 100 mbar for the 40 cm long capillary (corresponding to 1 capillary
187 volume). The analyzed protein solution (S_1) was injected at 30 mbar for 4 s and mobilized with S_2
188 solution. Between each run, the capillary was flushed with the BGE without protein for 100 s at 1
189 bar (5 capillary volumes).

190 For LNP plug-in-front experiment, the 60 cm long capillary was flushed with S_2 solution con-
191 taining diluted LNP (LNP/Tris-HCl, 1/20 v/v) for 7 min at 100 mbar (1 capillary volume). The
192 non-diluted LNP sample was next injected at 30 mbar for 12 s and mobilized with S_2 solution. Be-

193 tween each run, the eluent was flushed for 200 s at 1 bar (5 capillary volumes) with BGE without
194 LNP.

195

196 **2.4. Theoretical background.** In TDA, the diffusion coefficient D can be obtained using the entire
197 Taylor-Aris expression (Eq. (1) for $k=0$), as follows [2–4,16]

$$198 \quad D = \frac{1}{2} \left(D_{disp} - \sqrt{D_{disp}^2 - \frac{R_c^2 u^2}{12}} \right) \quad (3)$$

199 The dispersion coefficient can be experimentally determined as:

$$200 \quad D_{disp} = \frac{l^2 \sigma_t^2}{2t_0^3} \quad (4)$$

201 where σ_t is the temporal variance of the peak and l is the length of the capillary to the detector (in
202 m). Eq. 3 can be simplified when axial diffusion is negligible, leading to:

$$203 \quad D = \frac{R_c^2 t_0}{24 \sigma_t^2} \quad (5)$$

204 The validity of Eq. 5 depends on two conditions. First, the axial diffusion must be negligible com-
205 pared to the dispersion induced by the parabolic velocity profile. Within 3% relative error (ε) on
206 D , solute velocity must fulfill the following condition [23]:

$$207 \quad u \geq \frac{40D}{R_c} \quad (\text{or } Pe \geq 40) \quad (6)$$

208 where Pe is the Péclet number. If Pe is under 40, the entire Taylor-Aris expression Eq. 3 should be
209 used to determine D . The second condition is that t_0 must be longer than the characteristic diffu-
210 sion time of the solute in the cross section of the capillary [23]. Keeping the same relative error of
211 3%, t_0 must fulfill the following condition:

$$212 \quad t_0 \geq \frac{1.25 R_c^2}{D} \quad (7)$$

213 Finally, to determine the hydrodynamic radius R_h , the Stokes-Einstein is applied:

$$214 \quad R_h = \frac{k_B T}{6\pi\eta_s D} \quad (8)$$

215 where k_B is the Boltzmann constant, T is the temperature (in K), and η_s is the fluid viscosity.

216 The peak asymmetry (A_s) was determined at 10% of the peak apex, using Eq. (9):

$$217 \quad A_s = \frac{t_B - t_0}{t_0 - t_A} \quad (9)$$

218 where t_B and t_A represent the time at 10% of the maximum after and before peak apex, respective-
219 ly.

220

221 **2.5. Dynamic Light Scattering.** DLS experiments were carried out on an Anton Paar Litesizer 500
222 (Anton Paar, Graz, Austria). The measurement angle of the DLS was set at 90° and temperature of
223 the cuvettes was set at 25°C. LNP vaccine solution was diluted by adding 60 µL of vaccine solution
224 into 1 mL of eluent (20/17 mM Tris/HCl eluent, pH 7.4). Cumulant fit was used to estimate the
225 sample polydispersity. CONTIN-like fit provided by the Anton Paar software was used to obtain the
226 size distributions.

227

228 **3. Results and Discussion**

229 **3.1. Plug injection TDA of small molecules in the presence of adsorption.** In this first part of
230 this work, we focus on fast adsorption and desorption kinetics of small molecules on the capillary
231 wall, leading to equilibrium adsorption. As predicted by the general theory of chromatography²⁶
232 fast adsorption/desorption kinetics should lead to an increase of the solute retention time and to
233 higher peak broadening, keeping constant the symmetry of the taylorgram. Therefore, the diffu-
234 sion coefficient obtained from the working Eq. (5) - in which the observed analyte retention time,

235 t_r , replaces the carrier residence time in the capillary, t_0 - is an apparent diffusion coefficient, D^* ,
236 which differs from the true diffusion coefficient D_0 . From the Golay expression of the peak vari-
237 ance [26] and Eq. (1), it can be shown that:

$$238 \quad D^* = D_0 \times \frac{1+k}{1+6k+11k^2} \quad (10)$$

239 with $k=(t_r-t_0)/t_0$. In the case of low k values, applying a Taylor series expansion on Eq. (10) led to :

$$240 \quad \frac{D^*}{D_0} \approx 1 - 5k \quad (11)$$

241 As stated by Eq. (11), fast adsorption/desorption kinetics tends to increase the peak broadening
242 and thus to decrease the apparent diffusion coefficient by a factor of $-5k$.

243 To our knowledge, there is no experimental demonstration of the validity of Eqs. (10) and
244 (11) in modern implementation of TDA (i.e. using narrow bore capillaries of about 50 μm I.D.).
245 Plug injection TDA of three small apolar molecules (toluene, *p*-cymene and triphenylphosphine)
246 was performed using two commercial 50 μm . i.d. capillaries coated with apolar phases
247 (dimethylpolysiloxane (DB-1) and (50% phenyl)-methyl polysiloxane (DB-17)) in ACN/HEPES
248 (70/30 w/w, 0.39 cp) eluent. In these conditions, the solutes were all separated from the non-
249 retained polar molecule cyclo(Ala-Ala) used as the void-time marker (see top inserts of Figure 1).
250 Average k values were determined at $28.6 \pm 0.2 \times 10^{-3}$, $68.5 \pm 0.3 \times 10^{-3}$ and $95.4 \pm 0.4 \times 10^{-3}$ (on n
251 = 5 repetitions) for toluene (Figure 1A), *p*-cymene (Figure 1B) and triphenylphosphine (Figure
252 1C), respectively. The apparent diffusion coefficients D^* in presence of adsorption are gathered in
253 Table 1. Measuring diffusion coefficient D_0 in absence of adsorption was possible when using
254 ACN/HEPES (70/30 w/w) mobile phase on fused silica capillary (see bottom of Figure 1 for TDA
255 traces and Table 1 for D_0 values).

256 Table 1 demonstrates the validity of Eq. (11) for all small molecules, with experimental rati-
257 os of D^*/D_0 very close to $1-5k$. A R_h difference up to 0.40 nm (corresponding to 88% relative error)

258 was obtained for triphenylphosphine. As a conclusion, fast adsorption/desorption kinetics in TDA
259 is expected to overestimate the R_h result without changing the Gaussian shape of the Taylorgram. It
260 is however worth noting that fast adsorption kinetics with measurable retention is not very com-
261 mon in practical modern TDA using narrow bore fused silica capillary in aqueous solution.

262 For slow adsorption/desorption kinetics, non-Gaussian peak was generally observed, with
263 typical peak tailing due to non-equilibrium phenomena. This situation is much more common in
264 practice in TDA. However, Eq. (11) is not adequate to describe such a situation and practical solu-
265 tions are required to address this issue.

266

267 **3.2. Plug injection TDA of proteins in the presence and in the absence of adsorption.** When
268 performing simple plug TDA analysis of basic model proteins in acetic acid 2 M, pH 2.2 on a fused
269 silica capillary, no adsorption occurs due to the absence of electrostatic interaction (see Figure
270 2A). One way to generate solute adsorption is to increase the pH of the eluent from pH 2.2 (2M
271 acetic acid) to pH 5.8 using MES/NaOH (50/10 mM), to pH 7.0 using HEPES/NaOH (40/10 mM)
272 and finally to pH 9.1 using a 10 mM sodium tetraborate. The silica surface charge ($pK_a \approx 6$)
273 changes from neutral to negative when increasing the pH. Since the isoelectric point (pI) of Myo is
274 7 [52], adsorption is promoted at intermediate pH, as displayed in Figure 2A. At pH 5.8 and 7.0,
275 almost no signal was observed due to irreversible oppositely charged interactions. At pH 9.1, due
276 to electrostatic repulsion between negatively charged Myo and silica surface, no adsorption was
277 observed for Myo.

278 Since, in the case of slow adsorption/desorption kinetics, it is not possible to correct the im-
279 pact of adsorption onto the experimental determination of D (as Eq. (10) in the case of fast equi-
280 librium interactions), it is desirable to propose an experimental alternative to tackle adsorption
281 issue. Interestingly, strong interactions observed at pH 5.8 and 7.0 can be reduced using a

282 presaturation protocol of the capillary surface prior TDA analysis. Experimentally, this protocol
283 consisted in flushing the capillary with the solute solution in order to saturate the active sites of
284 adsorption. As shown in Figure 2B, Myo peak intensity increased owing to the presaturation of the
285 capillary. However, strong peak tailing was observed as calculated from peak asymmetry ($A_s = 4.0$
286 ± 1.2 ($n = 5$) at pH 7.02, see Eq. (9)). The improvement of the TDA signal after capillary
287 presaturation proves that desorption kinetics of the solute from the capillary wall is slow.

288 R_h of Myo with simple plug injection TDA at pH 2.2 was estimated around 3.43 ± 0.17 nm,
289 with A_s equal to 1.48 ± 0.26 ($n = 5$). A similar R_h value was found using presaturation protocol, but
290 with lower standard deviation and peak asymmetry values ($R_h = 3.54 \pm 0.09$ nm and $A_s = 1.17 \pm$
291 0.13 , $n = 5$). In that case, presaturation helped to reduce protein adsorption onto the capillary wall
292 by saturation of the adsorption sites, leading to more repeatable experiments. In contrast, no dif-
293 ference was observed between the two protocols at pH 9.1, i.e. R_h and A_s were similar ($R_h = 1.83 \pm$
294 0.09 nm and $A_s = 1.13 \pm 0.05$ with plug injection protocol, and $R_h = 1.89 \pm 0.06$ nm and $A_s = 1.33 \pm$
295 0.15 with presaturation protocol, $n = 5$), as shown in Table SI1. At pH 9.1, no adsorption occurs
296 and presaturating the capillary was thus not useful.

297 R_h values were in perfect agreement with the literature: R_h of Myo was found 1.83 ± 0.09 nm
298 ($n = 5$) at pH 9.1 (compared to 1.80-1.90 in literature [53,54]). In acidic conditions, R_h of Myo was
299 almost twice higher than the native protein R_h value. This can be easily explained by the fact that
300 Myo tends to aggregate in acidic conditions, leading to larger measured sizes [53].

301

302 **3.3. Plug-in-front injection TDA of proteins.** Based on the improved results obtained using
303 presaturation of the capillary inner surface, a new methodology is proposed to perform TDA using
304 what we name “plug-in-front” approach. In this new mode, TDA is performed by injecting a solute
305 plug (at 1 gL^{-1}) in a capillary prefilled with an eluent containing the solute at a lower concentra-

306 tion (C_{front} from 0.05 gL⁻¹ to 0.4 gL⁻¹, as indicated in Figure 3). Clearly, the peak symmetry im-
307 proved with increasing protein front concentration, as shown by the Gaussian fitting in Figure 3.
308 The influence of the solute concentration in the front on R_h and A_s was systematically investigated
309 and reported in Table 2. The A_s value decreased with increasing C_{front} and stabilized down to 1.0 –
310 1.2 above 0.4 gL⁻¹. It is also worth noting that the satellite peak observed after the main elution
311 peak disappeared above 0.4 gL⁻¹. Regarding the R_h value, an ANOVA test at 95% confidence level
312 proved that the results are not significantly different for C_{front} above or equal to 0.2 gL⁻¹. Average R_h
313 of Myo was 1.86 ± 0.04 nm at pH 5.8 and 1.84 ± 0.02 nm at pH 7.0 ($n = 5$), in good agreement with
314 the literature [53,54]. It is worth noting that the plug-in-front methodology allows analyzing pro-
315 teins on fused silica capillaries even at pH where electrostatic interactions occur. This results is
316 important in practice for TDA application and was not *a priori* expected. It can be explained by the
317 slow kinetics of desorption of the solute from the capillary surface.

318

319 **3.4. Optimizing the plug-in-front protocol to limit solute consumption.** One of the main ad-
320 vantages of using capillary electrophoresis apparatus to perform TDA experiments is its low sam-
321 ple consumption. With a 40 cm long fused silica (50 μ m i.d.), a 30 mbar injection for 3 s only rep-
322 resents 3.5 nL of analyte solution, while capillary conditioning and TDA run typically consume
323 respectively 5 and 2 μ L of eluent.

324 In the present study, the plug-in-front protocol was adapted to minimize the amount of sam-
325 ple used. In this optimized protocol, only 0.2 mg of Myo under solid form was required and dis-
326 solved in 100 μ L of eluent to prepare the mother solution S_0 (see Figure 4A). S_0 was further diluted
327 to prepare 100 μ L of S_1 and 200 μ L of S_2 solutions at 1 and 0.5 gL⁻¹, respectively. These solutions
328 allowed up to 27 consecutive plug-in-front TDA analyses.

329 As shown in Figure 4B and 4C, the signal obtained using this optimized protocol was similar
330 to that in Figure 3, with almost perfect Gaussian peaks and low A_s value (see Table 2). At pH 5.8,
331 almost symmetrical peaks were obtained ($A_s = 1.03 \pm 0.08$, $n = 5$), while at pH 7.0, no significant
332 change was observed between the experiments at 0.4 gL^{-1} and 0.5 gL^{-1} C_{front} plug. R_h values were in
333 very good agreement with the literature [53,54], with $1.89 \pm 0.09 \text{ nm}$ and $1.85 \pm 0.05 \text{ nm}$ at pH 5.8
334 and 7.0, respectively ($n = 5$). Low RSD values on both R_h and A_s proves the reliability of this proto-
335 col.

336

337 **3.5. Application to the analysis of mRNA lipid nanoparticles.** Finally, the previously optimized
338 protocol was applied to plug-in-front TDA of LNP. Indeed, since COVID19 pandemy, mRNA LNP
339 formulations have gained considerable interest for the development of mRNA vaccines and for
340 new cancer gene therapy. In a previous study [55], TDA of LNP was performed on a $\mu\text{SIL-FC}$ coated
341 capillaries. The analysis of LNP on fused silica capillary was not an option due to irreversible ad-
342 sorption onto capillary surface. This previous TDA method included a long rinsing protocol: 3-4
343 min rinsing with water and background electrolyte between each run, and a more intensive clean-
344 ing procedure every 10 runs composed of 20 min successive rinsing with Hellmanex / isopropanol
345 / water followed by 10 min LNP presaturation. In this work, we propose to apply the plug-in-front
346 protocol to the analysis of Pfizer Comirnaty™ vaccine against Covid 19. Compared to the “normal”
347 plug injection on bare fused silica capillary (Figure 5A) and to the plug injection after
348 presaturation of the capillary (see Figure 5B), the plug-in-front protocol leads to better signal
349 asymmetry without the resort to a coated capillary (Figure 5C).

350 The vaccine solution was injected as is without any dilution. The exact composition of the
351 vaccine solution being unknown, 20 mM /17 mM Tris-HCl buffer, pH 7.4 ($\eta = 0.94 \text{ cp}$) was used as
352 eluent. The plug-in-front optimized TDA protocol was implemented on a 60 cm long bare fused

353 silica capillary using as eluent a 20 times diluted LNP solution in Tris-HCl buffer. Figure 6A dis-
354 plays 5 plug-in-front TDA runs on Pfizer Comirnaty™ vaccine. The taylorgrams display a small thin
355 peak on the top of the elution profile, which can be attributed to small molecules and/or mismatch
356 buffer due to differences in composition between the sample and the front matrices. This small
357 molecule contribution corresponded to a hydrodynamic diameter D_h lower than 0.7 nm and was
358 not taken into account in the LNP size distribution. D_h distributions obtained by TDA using the
359 constrained regularized linear inversion algorithm (CRLI method as described in [56]) are dis-
360 played in Figures 6B and 6C, for five repetitions. Since hydrodynamic diameters are more general-
361 ly used than hydrodynamic radius in the pharmaceutical industry, LNP sizes are given in D_h . D_h of
362 56.60 ± 1.58 nm PDI of 0.0037 ± 0.0016 were determined ($n = 5$).

363 For a better comparison, the D_h distributions were represented both in linear and log scales
364 and were compared with those obtained by DLS. Figures 6B and 6C display the intensity weighted
365 D_h distributions centered at an average value of $D_h=91.65 \pm 2.64$ nm ($n = 5$) obtained by DLS using
366 CONTIN-like analysis and the polydispersity index obtained from the cumulant fit ($PDI=0.23$) in
367 good agreement with the literature (80 to 100 nm and PDI going from 0.2 to 0.25) [57–59].

368 TDA led to lower D_h values compared to DLS as already observed for LNP analysis [21] and
369 for any polydisperse sample [11,60,61]. This is due to intrinsic differences in the principle of anal-
370 ysis of both techniques. TDA basically leads to a mass-weighted size distribution for a mass con-
371 centration sensitive detector [11], while DLS is an intensity-weighted distribution which weighs
372 more the larger nanoparticles. This also explains why the DLS size distribution is much broader in
373 DLS compared to TDA.

374 We anticipate that plug-in-front protocol is a convenient, straightforward and simple way to
375 improve TDA repeatability and to limit the impact of slow adsorption /desorption kinetics on size
376 determination. Compared to the previously published article on TDA of LNP, we significantly im-

377 proved the TDA protocol by replacing expensive coated capillaries by fused silica capillaries and
378 by considerably reducing the rinsing times between runs down to 3.3 min rinse with the plug-in-
379 front protocol and no additional rinsing protocol between series of experiments.

380

381 **4. Conclusion**

382 In this work, we experimentally confirmed that the relative error on D (or R_h) is $5k$ in the
383 case of fast equilibrium adsorption/desorption, as predicted from the theory of chromatography.
384 More complex and larger solutes, such as proteins, can interact on the capillary wall with slow
385 desorption kinetics leading to peak tailing, a decrease of the signal intensity or even the absence of
386 detection for strong interactions. Capillary presaturation significantly improved the
387 shape/symmetry of the taylorgram. A new protocol, called plug-in-front TDA, consisting in adding
388 the solute in the eluent at a lower concentration compared to the injected sample, was successfully
389 tested and led to much better repeatability and shorter rinsing protocols. The interest of this new
390 approach was demonstrated on the analysis of protein and lipid nanoparticles (LNP) in mRNA
391 vaccines. Both peak asymmetry and standard deviation on R_h values were significantly reduced,
392 making this protocol of great interest for many applications.

393 It is interesting to note that the plug-in-front protocol presented here bears some similarity
394 with the step-and-pulse procedure used a long time ago in gas chromatography for the purpose of
395 determination of sorption isotherms [62]. In the latter method, the eluent was made of a given
396 concentration of solute in the carrier gas and the injected sample contained a slight concentration
397 excess of solute over that of the eluent, giving access to the slope of the sorption isotherm at the
398 solute concentration in the eluent. Repeating the process and various solute concentration in the
399 eluent allowed the determination of the whole isotherm. By contrast, in the present plug-in-front

400 case, for the purpose of preventing adsorption of the analyte on the TDA capillary wall, the solute
401 concentration in the carrier is significantly lower than that in the injected sample.

402

403 **Supporting Information**

404 **Table SI1.** Myoglobin R_h and A_s measurement of plug injection TDA at four different pH values.

405

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411

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570

571

LIST OF CAPTIONS

572 **Figure 1: TDA experiment for toluene (A), p-cymene (B) and triphenylphosphine (C) in presence of**
573 **adsorption on DB-1/DB-17 or in absence of adsorption on fused silica capillary ($n = 5$).** Experimental
574 conditions: capillary of 80 cm total length (71.5 cm to detector) \times 50 μm i.d. with or without DB-1/DB-17
575 coatings as indicated in the graph. Eluent: ACN/HEPES 70/30 (w/w). Hydrodynamic injection of solutes at
576 30 mbar for 4 s (0.16% capillary volume). 1: cyclo(Ala-Ala) used as the void-time marker, 2: toluene, 3: p-
577 cymene, 4: triphenylphosphine. Mobilization pressures as indicated in the figure. Temperature: 25°C. UV
578 detection: 214 nm. Dotted lines are Gaussian fits of the Taylorgrams.

579

580 **Figure 2: Plug injection TDA experiment on myoglobin, without (A) or with (B) capillary**
581 **presaturation, at four different pH values ($n = 5$).** Black lines correspond to experimental traces and red
582 dashed lines to Gaussian fits. Experimental conditions: fused silica capillary, 40 cm total length (30 cm to
583 detector) \times 50 μm i.d. Eluents: acetic acid (2 M, pH 2.2), MES/NaOH (50 mM/10 mM, pH 5.8), HEPES/NaOH
584 (40 mM/10 mM, pH 7.0) and sodium tetraborate (10 mM, pH 9.1). Capillary rinsing: BGE 1 bar, 5 min (fol-
585 lowed by solute solution injection for 10 min at 100 mbar in case of presaturation). Hydrodynamic injec-
586 tion of solutes: 30 mbar, 4 s (0.66% capillary volume). Mobilization pressure: 30 mbar. Myo concentration:
587 1 g.L⁻¹. Temperature: 25°C. UV detection: 214 nm.

588

589 **Figure 3: Influence of Myo front concentration (C_{front}) in plug-in-front TDA at pH 5.8 (A) and 7.0 (B),**
590 **$n = 5$.** Black lines correspond to experimental traces and red dashed lines to Gaussian fits. Experimental
591 conditions: fused silica capillary, 40 cm total length (31.5 cm to detector) \times 50 μm i.d. Eluents: MES/NaOH
592 (50 mM/10 mM, pH 5.8) or HEPES/NaOH (40 mM/10 mM, pH 7.0), both containing Myo as indicated on
593 the Figure. Capillary rinsing between runs: eluent at 1 bar, 5 min. Hydrodynamic injection: 30 mbar, 4 s
594 (0.66% capillary volume). Mobilization pressure: 30 mbar. Myo concentration in the plug: 1 g.L⁻¹. Tempera-
595 ture: 25°C. UV detection: 214 nm.

596

597 **Figure 4: Schematic representation of optimized plug-in-front TDA protocol (A) and five repetitions**
598 **of the corresponding taylorgrams for myoglobin at pH 5.8 (B) and pH 7.0 (C).** Experimental condi-
599 tions: fused silica capillary, 40 cm total length (31.5 cm to detector) × 50 μm i.d. Eluents: MES/NaOH (50
600 mM/10 mM, pH 5.8) and HEPES/NaOH (40 mM/10 mM, pH 7.0) both containing 0.5 gL⁻¹ Myo. Before the
601 first injection, the capillary was rinsed with the spiked eluent for 200 s at 100 mbar (1 capillary volume)
602 for saturation of the interacting sites. Between runs, the capillary was flushed with spiked eluent for 100 s
603 at 1 bar (5 capillary volumes). Hydrodynamic injection: 30 mbar, 4 s (0.66% of capillary volume). Myoglo-
604 bin plug concentration: 1 g.L⁻¹. Mobilization pressure: 30 mbar. Temperature: 25°C. UV detection: 214 nm.
605

606 **Figure 5: TDA experiments on Pfizer Comirnaty™ vaccine using plug injection without capillary**
607 **presaturation (A), with capillary presaturation (B) and using plug-in-front protocol (n =5) (C).** Ex-
608 perimental conditions: fused silica capillary, 60 cm total length (51.5 cm to detector) × 50 μm i.d. Eluent:
609 20 mM/17 mM Tris/HCl, pH 7.4 (A and B); 20 times diluted vaccine in Tris/HCl eluent (C). Hydrodynamic
610 injection: 30 mbar, 12 s (0.81% of capillary volume). Vaccine solution was injected as is. Mobilization pres-
611 sure: 45 mbar. Temperature: 25°C. UV detection: 214 nm. Rinsing between runs: 5 min eluent at 1 bar (A);
612 20 times diluted vaccine solution in Tris-HCl buffer for 200 s at 1 bar (5 capillary volumes) (B and C). Rins-
613 ing before the first run: 20 times diluted vaccine solution in Tris-HCl buffer for 420 s at 100 mbar (1 capil-
614 lary volume) to saturate all the interacting sites (B and C). Gaussian fits of the taylorgrams are presented in
615 red.

616

617 **Figure 6: Plug-in-front TDA of Pfizer Comirnaty™ vaccine solution with CRLI fit of 5 runs (A), over-**
618 **lay of the corresponding size distributions obtained by DLS and TDA in linear size scale (B) and log**
619 **scale (C).** CRLI fit of the taylorgram is represented in red in Figure 6A. Repetitions of $P(D_h)$ were normal-
620 ized within each method to the same peak area. Repetitions of $P(D_h)$ from TDA were then adjusted to the
621 maximum of $P(D_h)$ from DLS, to avoid too much spreading of the DLS distribution in the inter-method
622 comparison. Experimental conditions as in Figure 5C.

623

624 **Table 1: Values of retention factor (k), diffusion coefficient in presence (D^*) and in absence (D_0) of**
625 **adsorption and hydrodynamic radius in presence (R_h^*) and in absence (R_{h0}) of adsorption, obtained**
626 **for toluene, p-cymene and triphenylphosphine to verify the validity of Eq. (11). Experimental condi-**
627 **tions as in Figure 1 with DB-1/ DB-17 coatings.**

628

629 **Table 2: Impact of presaturated capillary and plug-in-front protocol on R_h and A_s values for myoglo-**
630 **bin TDA.**

631

632