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## Characterization of dextran particle size: How frit-inlet asymmetrical flow field-flow fractionation (FI-AF4) coupled online with dynamic light scattering (DLS) leads to enhanced size distribution

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1 **Characterization of dextran particle size: how Frit-Inlet**  
2 **Asymmetrical Flow Field-Flow Fractionation (FI-AF4)**  
3 **coupled online with dynamic light scattering (DLS) leads**  
4 **to enhanced size distribution.**

5

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## 24 **HIGHLIGHTS**

- 25 - An FI-AF4 separation method for dextran nanoparticles was developed.
- 26 - FI-AF4 coupled to the DLS detector showed the whole distribution of nanoparticles.
- 27 - The enhanced resolution of the FI-AF4 technique was proven.
- 28 - Batch-mode DLS was not suitable and provided misleading information.
- 29 - The good reproducibility of the particle preparation method was demonstrated.

30

## 31 **ABSTRACT**

32

33 Accurate determinations of particle size and particle size distribution (PSD) are essential to  
34 achieve the clinical translation of medical nanoparticles (NPs). Herein, dextran-based NPs  
35 produced via a water-in-oil emulsification/crosslinking process and developed as  
36 nanomedicines were studied. NPs were first characterized using traditional batch-mode  
37 techniques as dynamic light scattering (DLS) and laser diffraction. In a second step, their  
38 analysis by frit-inlet asymmetrical flow field-flow fractionation (FI-AF4) was explored. The  
39 major parameters of the AF4 procedure, namely, crossflow, detector flow, crossflow decay  
40 programming and relaxation time were set up. The sizes of the particle fractions eluted under  
41 optimized conditions were measured using DLS as an online detector. We demonstrate that  
42 FI-AF4 is a powerful method to characterize dextran-NPs in the 200 nm -1  $\mu$ m range. It  
43 provided a more realistic and comprehensive picture of PSD, revealing its heterogenous  
44 character and clearly showing the ratio of different populations in the sample, while batch-  
45 mode light scattering techniques only detected the biggest particle sizes.

## 46 **Keywords**

47 Frit-Inlet Asymmetrical Flow Field-Flow Fractionation (FI-AF4)

48 Dextran

- 49 Nanoparticles
- 50 Dynamic Light Scattering (DLS)
- 51 Size-based separation

## 52        **1. Introduction**

53        Nanoparticle (NP) based technologies have found numerous applications in the biomedical  
54        field, ranging from controlled and targeted delivery of therapeutic compounds to imaging,  
55        diagnostics, and monitoring of disease processes [1,2]. Once systematically administered, the  
56        fate and biological performance of NPs rely on their physicochemical properties: shape,  
57        surface chemistry, charge and, in particular, their size [3]. Particle size and particle size  
58        distribution (PSD) appear to dictate NP blood circulation half-life [3,4], biodistribution  
59        throughout the body and within specific organs [4,5], tumour penetration [6,7], cellular uptake  
60        mechanism [8,9], targeting [10], toxicity [11], therapeutic index [12] and even contrast  
61        properties [13]. The development of NPs with narrow and controlled PSD as well as reliable  
62        routine methods for their quality control and characterization are therefore of key importance  
63        for clinical translation.

64        Several methods are available for size and PSD analysis. Among them, scanning and  
65        transmission electron microscopy (SEM and TEM) approaches are commonly used for direct  
66        visualization of NP shape, surface, and geometric size [14,15]. Nevertheless, size assessment  
67        is often laborious and time consuming, as a very large number of particles must be observed  
68        to obtain statistically significant PSD information [16,17]. Moreover, under sample  
69        preparation conditions (dried state and high-energy electron source), the observed size and  
70        morphology may not be representative of those in biological aqueous environments [18]. In  
71        this regard, dynamic light scattering (DLS), also called quasi-elastic light scattering (QELS),  
72        has become the standard approach to determine NP size in suspension. DLS measures the  
73        translational diffusion coefficient of NPs in dispersing media by quantifying dynamic  
74        fluctuations in scattered light [19,20]. This information is then converted into a particle size  
75        by applying the Stokes-Einstein equation. When used in batch-mode, DLS allows accurate,  
76        simple, and fast characterization of monomodal NPs in different dispersing media while

77 requiring limited sample quantities. However, size resolution can be significantly  
78 compromised in the case of complex mixtures or highly dispersed samples.

79 One way to overcome this drawback is to introduce a fractionation step to separate the  
80 particles, according to their size, prior to DLS detection. In asymmetrical flow field flow  
81 fractionation (AF4) method, this separation can be achieved into a narrow, opened, and  
82 unpacked channel [21–25]. Briefly, a single carrier flow is pumped from the channel inlet and  
83 split into both the channel flow and the crossflow (Figure 1). The channel flow displays a  
84 parabolic velocity profile and carries NPs to the channel outlet where they are detected. The  
85 crossflow, which moves from the top to the bottom of the channel, forces NPs down to the  
86 accumulation wall (an ultrafiltration membrane overlaying a porous frit). Finally, natural NP  
87 diffusion counteracts the crossflow field, allowing size fractionation. Due to their higher  
88 translational diffusion coefficients, smaller particles tend to reach an equilibrium position  
89 higher up in the channel. They thus experience a faster channel flow velocity and are eluted  
90 earlier than larger particles.

91 Coupled with downstream detectors such as refractive index (RI), multiangle light scattering  
92 (MALS), ultraviolet-visible spectrophotometry (UV-Vis) and DLS, AF4 has been effectively  
93 used for the analysis and characterization of different NPs including liposomes [26], lipid-  
94 based NPs [27,28], polymeric NPs [14,23,29–31], metallic NPs [32,33], and metal oxide NPs  
95 [34–36]. To the best of our knowledge, very few examples have dealt with pure  
96 polysaccharide NPs, and almost all of them were made of chitosan [37–39]. It is well known  
97 that a new and specific AF4 method needs to be established for each kind of NP depending on  
98 its surface properties, composition, average size, and size distribution. Herein, we present the  
99 development and optimization of a separation method for dextran-based NPs produced via a  
100 simple water-in-oil emulsification/crosslinking process. The potential of such NPs as MRI  
101 contrast agent carriers for atherothrombosis detection was recently reported [40]. AF4 could

102 provide deeper characterizations of their average size, PSD and preparation method  
103 reproducibility, which are essential to complete their preclinical development. The main  
104 parameters influencing AF4 separation, including spacer height, crossflow rate and relaxation  
105 time, were studied. The optimized procedure was then applied to assess particle size and PSD.  
106 AF4 results were finally compared against batch-mode DLS data to draw conclusions about  
107 the suitability of the latter as a routine quality control technique. Orthogonal methods, such as  
108 TEM, SEM, and confocal microscopy, were performed to support the results.

109

## 110 **2. Materials and methods**

### 111 *2.1 Chemicals*

112 Dextran T70 ( $\bar{M}_n = 55\ 000$  g/mol,  $\bar{D} = 1.12$ ) was purchased from Pharmacosmos (Holbæk,  
113 Denmark). Trisodium trimetaphosphate (STMP) was obtained from Sigma Aldrich (Saint-  
114 Quentin-Fallavier, France). Fluorescein isothiocyanate-dextran (FITC-dextran,  $\bar{M}_w = 70\ 000$   
115 g/mol) was purchased from TdB Consultancy (Uppsala, Sweden). Polyglycerol  
116 polyricinoleate (PGPR 4150) was a gift from Palsgaard SAS (Lyon, France). Sunflower oil  
117 (Lesieur, France) is a mixture of saturated fatty acids (palmitic acid ~5 wt%- and stearic acid  
118 ~6 wt%) and unsaturated fatty acids (mainly oleic acid ~37 wt% and linoleic acid ~51 wt%).  
119 All materials were of analytical grade and were used without further purification. Ultrapure  
120 water was used throughout this work.

### 121 *2.2 Dextran-NPs synthesis*

122 Dextran T70 particles were obtained via a water-in-oil emulsification-crosslinking method as  
123 previously described [40]. The typical experimental conditions were as follows: 1.2 g of  
124 dextran and 1.4 g of NaCl were solubilized in 4 mL of water. Then, 1.2 g of this solution was

125 mixed with 120  $\mu$ L of NaOH (10 M) and 240  $\mu$ L of the crosslinking agent trisodium  
126 trimetaphosphate (STMP) (30% (w/v) in water). A total of 600  $\mu$ L of the mixture was slowly  
127 injected into 30 mL of sunflower oil containing 6.0% (w/v) polyglycerol polyricinoleate  
128 (PGPR) as an emulsion stabilizer and dispersed with a homogenizer at 24,000 rpm for 4 min  
129 (IKA T18 Basic equipped with a dispersing element IKA S 18 N-19 G, Germany). The  
130 resulting emulsion was then put in an oil bath (50 °C) wherein the crosslinking step took place  
131 for 20 min. Polysaccharide particles were recovered by ultracentrifugation (15,000 x g,  
132 45 min), washed once with 1X PBS twice with SDS (0.04% (w/v)) and four times with water.  
133 The resulting pellets were resuspended in water and stored at 4 °C until use.

### 134 *2.3 Dynamic light scattering (DLS) and $\zeta$ -potential*

135 Batch DLS measurements were performed using a Malvern Zetasizer Ultra instrument  
136 (Worcestershire, UK) equipped with a 633 nm He-Ne laser. Particles were analysed as  
137 synthesized without prior filtration to avoid material losses, e.g., aggregates and large  
138 populations (previous filtration with a 1.2  $\mu$ m filter did not affect the size distribution results).  
139 The particle concentration was fixed to 0.5 mg/mL to obtain an attenuator factor of 6 or 7.  
140 Experimental measurements were performed with a fixed 173° scattering angle at 25°C after  
141 an equilibration step of 2 min. Each sample was recorded in triplicate using at least 20 data  
142 sets acquired for 10 s each. The correlation function was analysed-by the general-purpose  
143 method (using a nonnegative least square algorithm) for derivation of the intensity-weighted  
144 particle size distribution, intensity-weighted mean diameter ( $D_{h-DLS}$ ) and polydispersity index  
145 (PDI). Average values of replicates (n=3) are reported.

146 The  $\zeta$ -potential was determined in 1 mM KCl at 25°C with the same instrument. The  
147 measurements of the electrophoretic mobility were converted to  $\zeta$ -potential (mV) using the



148 Smoluchowski approximation. Average values of three consecutive measurements (n=3) are  
149 reported.

150

#### 151 *2.4 Laser diffraction measurements*

152 Batch laser diffraction measurements were performed using a Mastersizer 3000 (Malvern  
153 Instruments, UK). The refractive indexes were 1.20 for the dispersed phase (dextran-NPs) and  
154 1.330 for the dispersant (water). An absorption index of 0.01 was applied. Volume and  
155 number-based PSDs obtained by Mie theory are reported together with the corresponding  
156 median diameters (D50v and D50n). The width of the PSD is commonly described by the  
157 sizes of the finest (D10) and coarsest (D90) fractions. Using the same convention as D50,  
158 10% of the total particles have a diameter below D10, while 90% have a diameter below D90.  
159 Average values of three consecutive measurements (n=3) are given.

#### 160 *2.5 Frit Inlet Asymmetrical Flow-Field Flow Fractionation (FI-AF4)*

161 Flow-field flow fractionation of polysaccharide NPs was carried out in a frit-inlet  
162 asymmetrical channel (270 mm length, Wyatt technology, CA, USA) linked to: i) an Eclipse  
163 AF4 flow control module (Wyatt technology, CA, USA), ii) a differential refractive index  
164 detector (RID-10A, Shimadzu, Japan), iii) a multiangle light scattering detector (MALS,  
165 Dawn-EOS, Wyatt technology, CA, USA), and iv) a quasi-elastic light scattering detector  
166 (Wyatt-QELS (or DLS), Wyatt technology, CA, USA) connected to the MALS photodiode at  
167 an angle of 111°. The accumulation wall was an ultrafiltration membrane of regenerated  
168 cellulose with a 10-kDa cut-off. Two spacers were used: 350 µm and 490 µm. An isocratic  
169 pump (LC-10A, Shimadzu, Japan) equipped with an in-line vacuum degasser (DGU 20A3,  
170 Shimadzu, Japan) and an autosampler (SIL-20A, Shimadzu, Japan) provided the carrier flow  
171 and handled the sample injection into the channel, respectively. The carrier liquid was

172 composed of Milli-Q water containing 0.02% NaN<sub>3</sub> prefiltered at 0.1 μm. The particle  
173 concentration was set to 0.5 mg/mL and the injection volume was 10 μL. Analyses were  
174 performed at room temperature. The collected data were analyzed using the Astra 6.1.7.  
175 software package. The weight-based size distribution was calculated by using the  
176 hydrodynamic diameter acquired by DLS and sample concentration measured by the RI  
177 detector.

## 178 *2.6 SEM, TEM and Confocal Microscopy*

179 Particles were visualized by transmission electron microscopy (TEM, Tecnai 12, 80 kV; FEI;  
180 Hillsboro, OR, USA), scanning electron microscopy (SEM, Philips XL30 ESEM-FEG,  
181 Netherlands) and confocal microscopy (Zeiss LSM 780 with 63× oil objective, Carl Zeiss  
182 Microscopy, Germany). For TEM observation, 3 μL of particle suspension was dropped onto a  
183 400-mesh copper grid and positively stained with uranyl acetate (1% (w/v)) and allowed to air-  
184 dry at room temperature. For SEM images, one drop of particle suspension was dropped onto  
185 a silicon wafer, air-dried and coated with a thin layer of gold before observation.

## 186 **3. Results and discussion**

### 187 **3.1 Particles preparation and batch mode characterization**

188 Dextran-NPs were prepared by a water-in-oil emulsion/crosslinking method using STMP as a  
189 crosslinking agent. The optimization of this method is described elsewhere [40]. Due to the  
190 anionic charges brought by STMP, dextran-NPs exhibited a negative zeta potential (-28±3  
191 mV) and behaved as three-dimensional and hydrophilic polymeric networks able to swell and  
192 hold a great amount of water (Figure 2A). The water content of dextran-NPs dispersed in  
193 Milli-Q water was estimated to be approximately 98% of the wet weight.

194 Dextran-NP size was first characterized in batch mode by DLS. As shown in Figure 2B,  
195 dextran-NPs exhibited a monomodal profile, with particle sizes ranging from 350 nm to 1.6

196  $\mu\text{m}$ , an intensity-weighted mean diameter ( $D_{h\text{-DLS}}$ ) of  $760\pm 13$  nm and a polydispersity index  
197 (PDI) of  $0.17\pm 0.02$ . These results were consistent with a PSD of moderate width. As stated in  
198 the international standard (ISO 22412:2017), intensity-based results are the most reliable  
199 parameters provided by DLS to describe particle size and PSD. Nevertheless, for comparison  
200 purposes, the intensity distribution was converted into volume and number distributions using  
201 Mie theory. This theory assumes that all the particles are spherical and that the optical  
202 properties of both the particles, and the dispersing medium, are known. As expected, the  
203 number PSD shifted towards smaller sizes with a maximum peak size of approximately 750  
204 nm (Figure 2B). In contrast, the volume PSD favoured larger particles with a peak maximum  
205 of approximately  $1 \mu\text{m}$ .

206 Laser diffraction was used as complementary batch sizing technique. In this case, particles  
207 cause light diffraction with a scattering angle that depends on particle size under the  
208 assumption that the particles are spherical. In principle, small particles will scatter light at  
209 large angles while large particles will scatter light at small angles [41]. The scattering pattern  
210 produced by the sample could once again be directly related to PSD by applying Mie theory.  
211 Laser diffraction provided both volume and number size distributions of dextran-NPs (Figure  
212 2C). As for DLS analysis, only one population was detected. The volume and number-median  
213 diameters ( $D_{50v}$  and  $D_{50n}$ ) of dextran-NPs were evaluated to be  $910\pm 10$  nm and  $720\pm 12$   
214 nm, respectively, which closely corresponds to the previous observations by DLS. On a  
215 volume basis,  $D_{10v} = 630$  nm and  $D_{90v} = 1.2 \mu\text{m}$  whereas in number one,  $D_{10n} = 530$  nm  
216 and  $D_{90n} = 1.0 \mu\text{m}$ .

217 Both DLS and laser diffraction suggested a quite homogeneous batch with most of the  
218 particles in the submicronic size range. To confirm these results and validate the use of batch-  
219 mode light scattering methods as fast and simple quality control techniques, dextran-NPs were

220 analysed by AF4. The use of a fractionation step, which separates the particles according to  
221 their size, and continuous online detection by DLS were expected to provide a PSD with  
222 higher resolution.

## 223 **3.2 AF4 method development**

### 224 **3.2.1 Channel characteristics**

225 When NPs are injected into an AF4 system, a relaxation process occurs prior to separation.  
226 This process allows NPs to reach their diffusion-dependent equilibrium positions and thus  
227 different velocity streamlines of the parabolic flow profile. In a conventional AF4 channel,  
228 relaxation takes place during a focusing step in which the sample is concentrated in a  
229 relatively narrow band near to the injection point by two counterdirected flows [42].  
230 However, the focusing process may cause sample aggregation, mass losses due to adsorption  
231 into the accumulation wall, incomplete relaxation due to the high local concentration or  
232 baseline fluctuations because of the rapid flow rate changes [43–45]. In this study, a long AF4  
233 channel (270 mm) equipped with a frit inlet (FI-AF4) was used (Figure 1). The focusing step  
234 was completely avoided, and the sample was relaxed hydrodynamically as it entered the  
235 channel by the compressing effect of the frit inlet flow [45,46]. The FI-AF4 channel has been  
236 shown to allow for a higher injection mass than the conventional channel while limiting  
237 overloading effects and normally leading to higher recoveries [42].

238 In an AF4 system, particles can be separated following two different modes of retention. We  
239 have already described the so-called normal mode where fractionation is governed by  
240 Brownian motion and smaller particles elute earlier than larger particles. Nevertheless,  
241 beyond a certain size limit, the diffusive transport opposing the crossflow field is negligible,  
242 and the elution becomes dependent on the position of the particles centre of mass in the  
243 parabolic flow profile [47]. In this case, larger particles protruding farther into the channel

244 experience higher velocities and are eluted before the smaller particles [48,49]. This is  
245 referred to as the steric mode. Between the two modes, in the so-called steric transition  
246 region, two groups of particles having different sizes are eluted together, one by the normal  
247 mode and the other by the steric mode [50,51]. This makes it difficult to accurately interpret  
248 size data, especially when the sample has a broad size distribution. Thus, AF4 separation  
249 conditions need to be adjusted to ensure that the entire population of the sample is governed  
250 by only one kind of elution mechanism.

251 The crossover between normal and steric modes, also called the steric inversion diameter,  
252 depends on the channel dimensions, field strength and flow rates. Steric inversion diameters  
253 of 0.2-0.5  $\mu\text{m}$  were reported by Dou et al. [52] for polystyrene particles eluted in a  
254 conventional AF4 channel using a constant-crossflow method. Kim et al. [53] investigated the  
255 steric transition of polystyrene particles in an FI-AF4 channel. They demonstrated that the  
256 steric inversion diameter can be increased by programming the crossflow field and by  
257 increasing the channel thickness. Values of 0.7, 1.1 and 1.8  $\mu\text{m}$  were determined for channel  
258 spacers of 190, 350 and 490  $\mu\text{m}$ , respectively. Based on these studies and because of the  
259 expected dextran-NPs size ( $\sim 760$  nm), spacers heights equal to or above 350  $\mu\text{m}$  appeared  
260 suitable to elute all NP batches in the normal mode. However, initial screening performed  
261 with a spacer of 350  $\mu\text{m}$  did not lead to convenient separation. The spacer thickness was thus  
262 increased to 490  $\mu\text{m}$  for the optimization approach described below.

### 263 **3.2.2 AF4 flow settings**

264 The typically applied AF4 sequence is summarized in Figure 3. Step 1 allowed system  
265 equilibration at the desired flow rates. Injection and relaxation of the sample took place  
266 simultaneously in step 2 under a constant initial crossflow rate. The actual sample separation  
267 and elution occurred in steps 3 and 4 after a gradual decrease in the crossflow field. In step 5,

268 the crossflow was set to zero to investigate whether a fraction of the sample experienced  
269 strong retention in the channel. All separations were performed at room temperature using  
270 Milli-Q water as liquid carrier. A regenerated cellulose membrane with a cut-off of 10 kDa  
271 was chosen as the accumulation wall. Electrostatic repulsions between the negative surfaces  
272 of both the membrane and dextran-NPs were expected to prevent potential attachment or  
273 adsorption of NPs on the membrane. A dextran-NP mass of 5  $\mu\text{g}$  was injected to obtain an  
274 exploitable signal in all detectors.

275 The frit inlet flow (FF) and the crossflow (CF) are the key parameters determining the  
276 resolution and separation quality of an FI-AF4 system. Previous studies have shown that  
277 efficient hydrodynamic relaxation can be obtained using a high ratio of the FF rate to the  
278 sample injection flow rate (IF). FF/IF values greater than or equal to 20 are normally  
279 recommended [53–55]. Since there is no incoming CF in an asymmetrical channel, the sum of  
280 the channel flow and the outgoing CF corresponds to the FF (Figure 1). Following the  
281 recommendations of the instrument supplier, the injection and channel flow rates were set to  
282 0.1 and 0.6 mL/min, respectively. Both the field strength and the FF rate were varied  
283 simultaneously by using different initial CFs. For these experiments, the sample was injected  
284 and relaxed for 10 min (step 2) at the set CF rate. A linear decay of the CF in 1 min (step 3)  
285 was found to be the better choice for field strength programming.

286 As shown in light scattering (LS) fractograms (Figure 4), a too low initial CF (0.1 mL/min,  
287 FF/IF= 7) led to a high number of unfractionated particles eluting prematurely in the so-called  
288 void peak. This peak disappeared for CF rates equal to or above 0.2 mL/min (FF/IF= 8).  
289 Nevertheless, when using too high CFs, the baseline could not be reached within 60 min of  
290 the sample run. At these crossflow fields (1.0 mL/min and 2.2 mL/min) and the resulting  
291 FF/IF ratios (16 and 28, respectively), particles were probably compressed too close to the

292 accumulation wall and were subsequently highly retained by increased particle-membrane  
293 interactions. A CF rate of 0.3 mL/min, corresponding to an FF rate of 0.9 mL/min and an  
294 FF/IF ratio of 9, was ultimately chosen because it was high enough to obtain suitable particle  
295 separation but low enough to provide a proper shape peak. Under these conditions, a retention  
296 time of 18 min and a peak width of 20 min were obtained.

297 To examine the effect of the relaxation time, CF was fixed to 0.3 mL/min, and the duration of  
298 step 2 was varied from 5 to 15 minutes. In all cases, particle elution started in step 4 after the  
299 end of the relaxation phase and CF linear decay. Ten minutes was found to be the optimal  
300 time. A further rise in relaxation time did not result in better separation quality. The effect of  
301 the CF decay pattern (step 3) was also explored. Linear, stepwise, exponential and power  
302 crossflow decays with decay times ranging from 1 to 20 minutes were tested. Nevertheless, a  
303 1-minute linear decay allowed for the best compromise between size resolution and overall  
304 run duration.

305 A screening of the channel flow rate between 0.5 and 0.8 mL/min was also performed, and all  
306 other run conditions were the same. It was observed that the axial flow velocity should be as  
307 low as possible to provide enough residence time for suitable sample relaxation. While the  
308 overall dextran-NPs were eluted in normal mode at 0.5 and 0.6 mL/min, a combination of  
309 both steric and normal retention mechanisms was observed at higher channel flow rates (see  
310 supporting information, Figure S1). A 0.6 mL/min volumetric flow rate was ultimately  
311 selected, which resulted in better resolution and narrower elution peaks. Table 2 summarizes  
312 the parameters of the final AF4 separation method.

313

### 314 **3.2.3 Dextran-NPs size distribution by FI-AF4**

315 The hydrodynamic size of fractionated dextran-NPs was measured with an in-line DLS  
316 detector at an angle of 110°. The resulting fractogram and size distribution profile are  
317 depicted in Figure 5A. The optimized FI-AF4 method allowed the detection of NPs with sizes  
318 ranging from 260 nm to 1 µm while maintaining normal separation mode. An intensity-  
319 weighted mean diameter of 340 nm was estimated. The AF4 method showed good  
320 reproducibility, as the relative standard deviation of the mean diameter was below 3.0% (n =  
321 3). DLS signal showed a single monomodal distribution, although a small shoulder is  
322 apparent at higher particle sizes. Weight-based PSD, deduced from both QELS and RI data,  
323 confirmed the presence of a major population of approximately 320 nm and a long tail  
324 between 400 nm and 1 µm (Figure 5B). The relative proportion of both main peak and tail  
325 was assessed from the cumulative weight-based distribution. Seventy percent of dextran-NPs  
326 were found to have a diameter equal to or below 400 nm, whereas particles with a diameter  
327 between 400 nm and 1 µm represented only 30% of the sample. The intensity correlation  
328 functions of particle fractions obtained at different elution times are depicted in Figure 5C. In  
329 all cases, cumulant analysis provided a good fit of the single exponential decay, attesting to  
330 the quality of the data acquired from the DLS detector.

### 331 **3.3 FI-AF4 vs. batch-mode DLS**

332 Batch-mode DLS and FI-AF4 analysis provided quite different pictures of dextran-NP size  
333 distribution. On the one hand, DLS measurements of unfractionated particles resulted in an  
334 average diameter of 760 nm in combination with a monomodal PSD. On the other hand, FI-  
335 AF4 delivered the whole size distribution of the sample between 260 nm and 1 µm with a  
336 mean size that was almost two times lower (340nm) and a long-tailed. To discriminate  
337 between the two techniques, dextran-NPs were directly visualized by TEM, SEM and  
338 confocal microscopy. The resulting micrographs, depicted in Figure 6, clearly supported FI-  
339 AF4 analysis. The heterogeneity of the dextran-NP batch became evident together with the



340 coexistence of particles of very different sizes. In addition, all techniques confirmed the  
341 obtention of spherical shaped NPs, and diameters between approximately 300 nm and 1  $\mu$ m  
342 were observed by confocal microscopy, where particles were imaged under hydrated  
343 conditions.

344 Batch-mode DLS failed to differentiate the dextran-NPs mixed population and delivered a  
345 size distribution skewed towards larger particles visibly present to a lower extent (Figure 6).  
346 These findings were consistent with comparative studies performed on multimodal  
347 dispersions prepared intentionally by mixing at least three particle sizes. Mixtures of gold NPs  
348 (5-60 nm) [56], pegylated gold NPs (30-90 nm) [57] and silver NPs (30-100 nm) [58,59] were  
349 examined, and similar conclusions were drawn in all cases. While AF4 was able to  
350 discriminate and quantify the different populations due to sufficient resolution, batch-DLS  
351 gave one broad peak weighted towards the biggest particles in the sample. In another work,  
352 Varenne et al. [23] reported a complete evaluation of the PSD of dextran-covered  
353 poly(isobutylcyanoacrylate) NPs. Nine different sizing techniques were used, including DLS,  
354 SEM, TEM and AF4. DLS did not reveal the complexity of the PSD, showing only one  
355 population of NPs of approximately 200 nm with narrow polydispersity. In contrast, AF4  
356 identified a multimodal dispersion with four peaks of significantly different sizes (97, 168,  
357 417 and 446 nm), in agreement with observations made on TEM and SEM micrographs.  
358 These examples highlighted the limits of batch-mode DLS in regard to resolving complex  
359 samples. This lack of sensitivity, leading in most cases to the overestimation of particle size,  
360 appear to be an inherent artifact of DLS and is directly related to its measurement principle.  
361 The Rayleigh approximation states that the intensity of the scattered light is proportional to  
362 the particle diameter to a power of six [20]. This means that NPs of 50 nm scatter one million  
363 times as much light as NPs of 5 nm. Consequently, in batch-mode some larger particles or  
364 aggregates can mask the presence of smaller particles, ultimately biasing the DLS response. In

365 addition, DLS requires particles different in size by at least a factor of 3 to offer enough peak  
366 resolution. This makes difficult to analyse polydisperse samples with particles of closer sizes  
367 as is the case of Dextran-NPs [60]. In those situations, particle fractionation prior to DLS  
368 detection appeared essential to obtain reliable PSD information.

### 369 **3.4 Batch to batch reproducibility**

370 Reproducible manufacturing of nanomedicines is one of the key parameters determining their  
371 successful translation into commercial products. Batch-to batch consistency should thus be  
372 checked from the early stages of development, since significant variation of the PSD between  
373 batches could result in distinct biological properties [61]. In this work, four batches of  
374 dextran-NPs were prepared using the same protocol and their PSD was determined by FI-AF4  
375 and batch-mode DLS. As shown in Figure 7, the manufacturing process of dextran-NPs  
376 appeared to be highly reproducible. No significant differences were detected in the weight-  
377 based size distribution given by FI-AF4, except for batch B where particles below 400 nm  
378 were present to a lesser extent. As expected, batch-mode DLS gave similar monomodal  
379 distributions systematically biased towards larger particles, with intensity-weighted mean  
380 diameters between 700 and 760 nm and PDI values below or equal to 0.18 (Table 2).

381 With all this evidence, we can conclude that batch-mode DLS is not suitable for dextran-NPs  
382 characterization. Conversely, FI-AF4 not only was able to measure the PSD of dextran-NPs  
383 with high resolution but also allowed us to resolve small changes in size between different  
384 batches. FI-AF4 can thus be considered a promising technique for the synthesis optimization  
385 and quality control of this nanosystem.

## 386 **4. Conclusion**

387

388 Polysaccharide-based nanoparticles are being developed as promising imaging and  
389 therapeutic tools for multiple diseases. Their translation to commercial products involves deep  
390 characterization of their physicochemical properties, especially their size and size distribution.  
391 In this study we developed and validated an FI-AF4 protocol allowing accurate and  
392 reproducible characterization of dextran-NPs in the 200 nm -1  $\mu$ m range. The results  
393 presented here once again highlight the inherent limitations of batch-mode DLS. Although  
394 widely used in laboratories for an initial quick and easy check of new samples, DLS data  
395 should be interpreted carefully and systematically contrasted with other sizing techniques, to  
396 avoid drawing misleading conclusions. In this respect, FI-AF4 was found to be a valuable  
397 technique that is able to provide a realistic and complete knowledge of dextran-NPs. Even if  
398 FI-AF4 method development is more laborious and time consuming, once established, it  
399 becomes a robust, accurate and sensitive tool for the routine characterization of complex  
400 nanosystems.

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412 **CRedit author statement**

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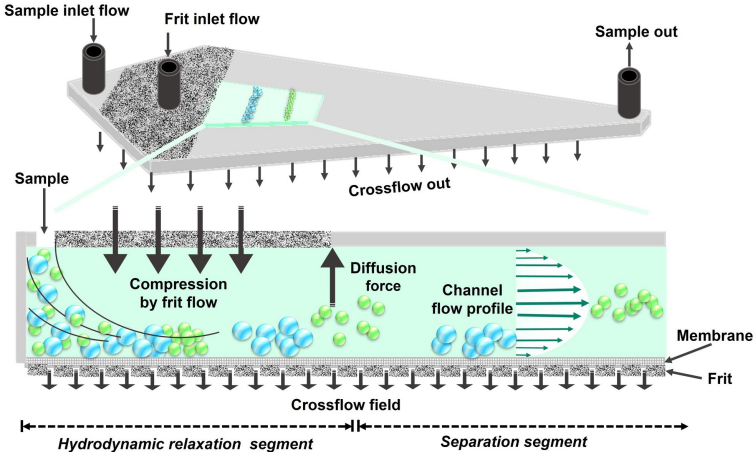
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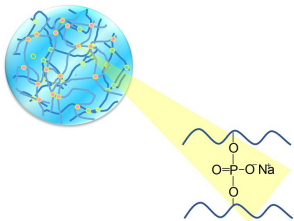
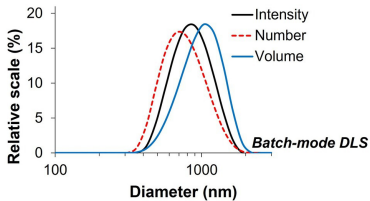
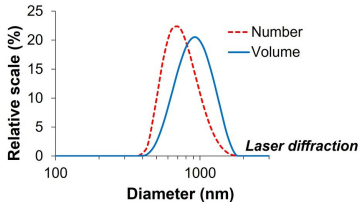
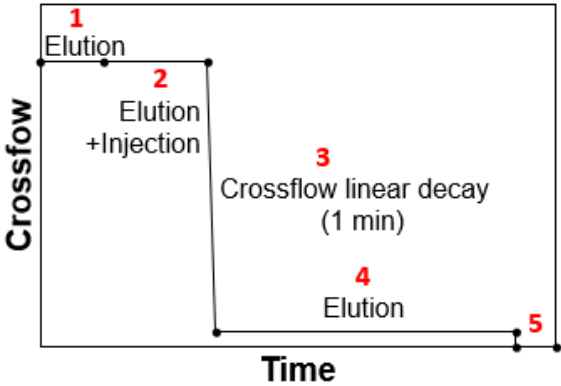
**A****Dextran-NPs****B****C**

Figure 3 (Forero Ramirez et al) – revised



**Figure 3.** Schematic representation of FI-AF4 flow method. Samples were injected at a NPs concentration of 0.5 mg/mL (10  $\mu$ L injection volume). Detector flow was held constant at 0.6 mL/min and the NPs were injected at 0.1 mL/min. A spacer thickness of 490  $\mu$ m was used.

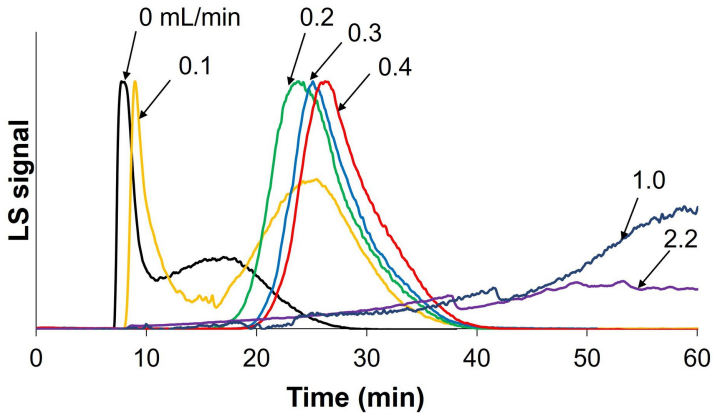
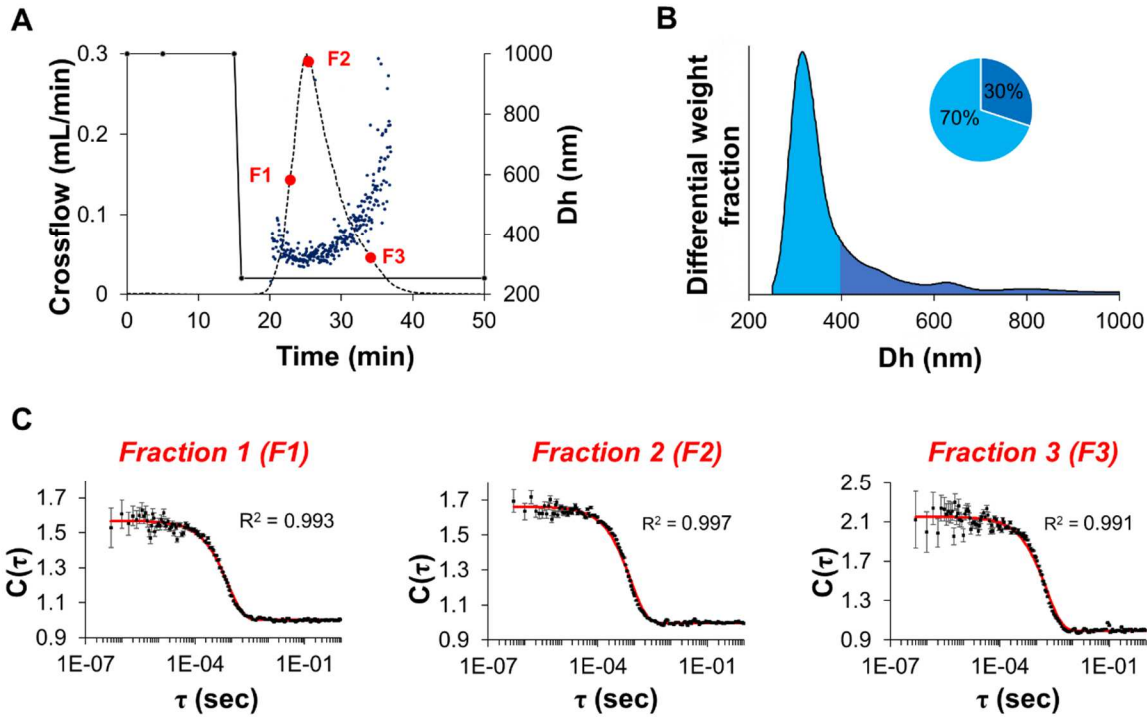


Figure 5 (Forero Ramirez et al) revised



**Figure 5.** Size characterization of dextran-NPs by FI-AF4 coupled with DLS detector. (A) DLS fractogram and size distribution profile, (B) ~~WeightNumber~~-based distribution and (C) DLS correlation function plot of particles fractions recovered at different times as indicated on (A).

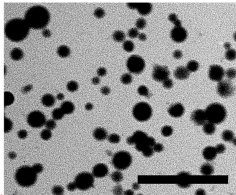
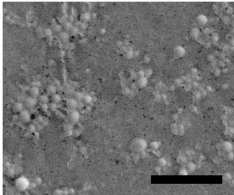
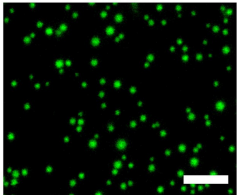
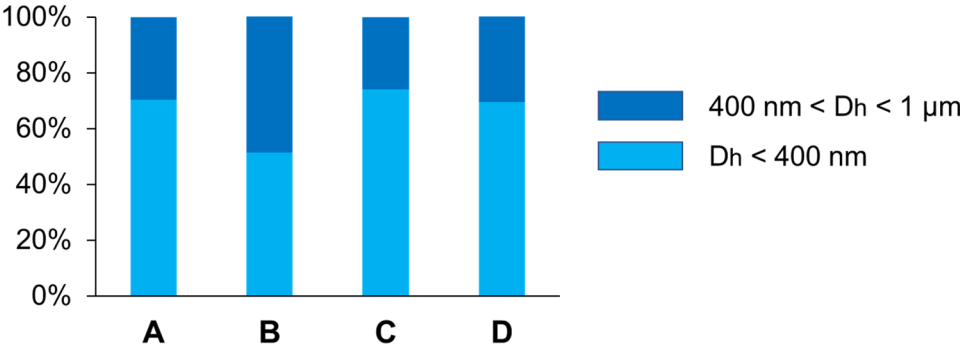
**A****B****C**

Figure 7 (Forero Ramirez et al) revised



**Figure 7.** ~~Weight~~Number-based PSD obtained by FI-AF4 for four independent batches of dextran-NPs prepared under the same experimental conditions.