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► **To cite this version:**

Ahmed Zeeshan, Hicheme Hadji, Hiba Khelifa, Mickael Bourge, Kawthar Bouchemal. Understanding the interplay between surface properties and the aspect ratio of ellipsoidal nanomaterials. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 2024, 680, pp.132680. 10.1016/j.colsurfa.2023.132680 . hal-04281942

HAL Id: hal-04281942

<https://hal.science/hal-04281942v1>

Submitted on 13 Nov 2023

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1 *Understanding the Interplay Between Surface Properties and*
2 *the Aspect Ratio of Ellipsoidal Nanomaterials*

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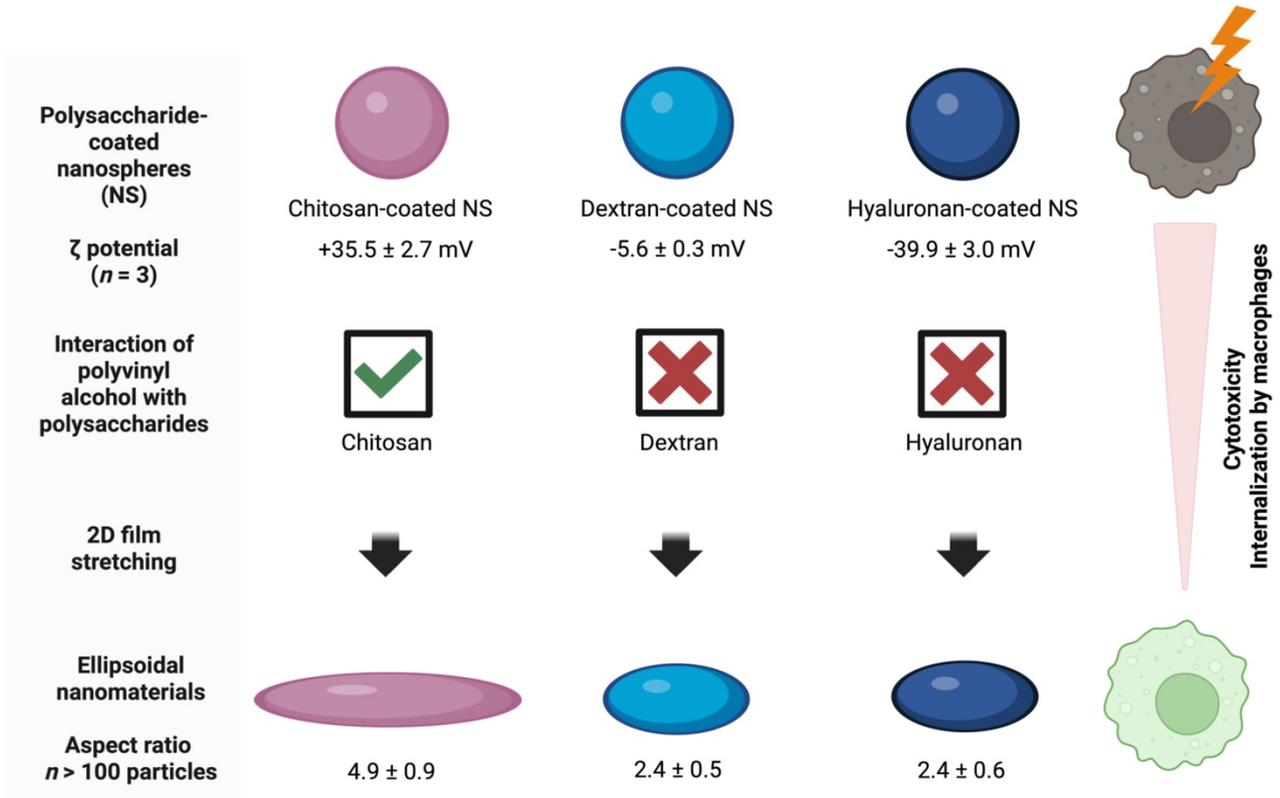
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Graphical abstract

15 **ABSTRACT**

16 Polymeric nanomaterials (NM) with nonspherical morphologies have increasingly captured the
17 spotlight in biomedical applications. Physical deformation of nanospheres (NS) represents a versatile
18 technique for obtaining ellipsoidal NM (E-NM) that exhibit unique properties regarding their
19 interactions with biological systems. Despite this technique's versatility and simplicity, the film's
20 detailed composition and the parameters controlling the efficiency of the NS's physical deformation
21 still need to be clarified. Herein, we detailed a step-by-step formulation of a film composed of
22 polyvinyl alcohol (PVA), and we investigated the impact of NS surface properties on the efficiency of
23 the physical stretching. To reach those objectives, we designed two morphologies (NS and E-NM),
24 and for each morphology, the surface composition was modified by using three different
25 polysaccharides (chitosan, dextran, and hyaluronan). Physicochemical analysis revealed a connection
26 between NS surface potential, their interaction with PVA, and the aspect ratio of ellipsoidal NM.
27 Chitosan-coated E-NM had the highest aspect ratio ($\Gamma = 4.9$), which decreased to 2.4 with dextran and
28 hyaluronan coatings. Isothermal titration calorimetry demonstrated strong interaction between PVA
29 and chitosan but little interaction with dextran and hyaluronan. Finally, the impact of NM morphology
30 and surface properties on phagocytosis showed that E-NM exhibited a reduced internalization by
31 macrophages and reduced cytotoxicity compared to NS. Overall, in this study, we highlighted that the
32 aspect ratio of E-NM is controlled by the strength of the interaction with PVA with the polysaccharide
33 on the NS surface and revealed the role of NM morphology in reducing cytotoxicity and escaping
34 internalization by macrophages.

35 **KEYWORDS:** Nanomaterials; Morphology; Surface properties; Cytotoxicity.

Abbreviations

- 36 DDA, Degree of deacetylation
- 37 DMEM, Dulbecco-modified eagle médium
- 38 DMSO, Dimethyl sulfoxide
- 39 E-NM, Ellipsoidal nanomaterial
- 40 EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
- 41 FBS, Fetal bovine serum
- 42 FITC, Fluorescein isothiocyanate Isomer I
- 43 HPLC, High-performance liquid chromatography
- 44 IC₅₀, The concentration of polysaccharide preparations to reduce cell viability by 50% relative to untreated
- 45 control cells
- 46 ITC, Isothermal titration calorimetry
- 47 J774A.1, Murine monocyte macrophages
- 48 MFI, Mean fluorescence intensity
- 49 NHS, *N*-hydroxysuccinimide
- 50 NM, Nanomaterials
- 51 NS, Nanospheres
- 52 NS_{PVA}, Nanospheres incubated with PVA and purified by dialysis
- 53 PBS, Phosphate buffer saline
- 54 PVA, Poly(vinyl alcohol)
- 55 QELS, Quasi-Elastic Light Scattering
- 56 TEM, Transmission Electron Microscopy

1. INTRODUCTION

Since the 1970s, polymeric nanomaterials (NM) have emerged as promising systems with numerous advantages over conventional formulations.[1, 2] They have shown ground-breaking achievements in improving drug efficacy/toxicity ratio, controlled drug release, and targeted drug biodistribution by efficiently bypassing biological barriers.[3] Among criteria defining synthetic identity, NM morphology has been considered during the last decade as a novel parameter that plays a pivotal role in controlling biological processes.[4, 5] Nevertheless, a close inspection of the literature reveals that most experimental works conducted on nonspherical NM have been devoted to metal[6, 7] or carbon nanotubes,[8] while NM composed of polysaccharides and polymers were less investigated. Among the various techniques used to produce nonspherical NM, the film stretching technique offers the advantage of simultaneously modifying the morphology and surface properties of NM composed of polymers and polysaccharides.[9-12] This technique involves dispersing nanospheres (NS) in a polyvinyl alcohol (PVA) solution, followed by drying and stretching of the resulting PVA film containing the NS. Ellipsoidal particles are then obtained by dissolving the film in water at ambient temperature. PVA is considered a class of water-soluble synthetic polymers, but water solubility primarily depends upon the degree of hydrolysis of the acetate groups in the polyvinyl acetate.

Previous works utilized PVA with a minimum degree of hydrolysis of 87% and a molecular weight of approximately $\approx 115 \times 10^3 \text{ g mol}^{-1}$. [9] In that study, the films were immersed in an oil bath maintained at 200 °C before stretching. In another publication, the stretching of PVA was performed at a lower temperature (50 °C), using a commercial film known as STBT[®]. [11, 12] However, the STBT[®] film contains insoluble debris.[12] The PVA concentration in the STBT[®] film, the degree of hydrolysis, and the molecular weight are unknown. In this context, herein we report the first step-by-step formulation of the PVA film in which the NS are dispersed before stretching. The PVA molecular

81 weight, its degree of hydrolysis, the concentration in water, and the concentration of glycerol used as
82 a plasticizer were varied in a rational way.

83 On the other hand, the parameters leading to efficient film stretching and, consequently, high
84 aspect ratio, still need to be clarified. Several works hypothesized that parent NS's glass transition
85 temperature (T_g) was a crucial parameter for the stretching efficiency.[9, 13] Within this framework,
86 the second objective of this investigation is to understand the effect of NM surface properties and the
87 T_g on the efficiency of the physical deformation of the NS characterized by the aspect ratio of
88 ellipsoidal NM.

89 To reach those objectives, our strategy is to consider two NM morphologies: spherical particles
90 (nanospheres, NS) and ellipsoidal NM (E-NM). After optimizing the PVA formulation, we
91 investigated three types of polysaccharides for each morphology: chitosan, dextran, and hyaluronan.
92 We selected three preparation types with comparable T_g but varying surface compositions. Spherical
93 particles composed of poly(isobutyl cyanoacrylate) (PIBCA) were obtained by polymerizing isobutyl
94 cyanoacrylate monomer and polysaccharides.[14-17] Shape-persistent E-NM with a high aspect ratio
95 ($T = 3.4$) were achieved through the physical deformation of NS.[12] The E-NM shape was stable for
96 6 months at different temperatures (4 °C, 20 °C, and 40 °C).[12] Subsequently, we will characterize
97 the NM's morphology, dimensions, and surface potential. Finally, we will explore the effects of NM
98 morphology and surface composition on cell cytotoxicity and endocytosis.

99 **2. MATERIALS**

100 **2.1. Reagents for polysaccharide chemical modification and NM design**

101 Chitosan ($\approx 20 \times 10^3 \text{ g mol}^{-1}$ and degree of deacetylation DDA > 85% according to the
102 manufacturer) was purchased from Amicogen (Seoul, Korea). Hyaluronan ($\approx 61 \times 10^3 \text{ g mol}^{-1}$) was
103 purchased from Lifecore Biomedical (Minnesota, USA). Dextran ($\approx 64 \times 10^3 \text{ g mol}^{-1}$), glycerol,
104 polyvinyl alcohol (PVA, fully hydrolyzed, 99% hydrolyzed ($\approx 89 \times 10^3 - 98 \times 10^3 \text{ g mol}^{-1}$), 80%

105 hydrolyzed ($\approx 9 \times 10^3 - 10 \times 10^3 \text{ g mol}^{-1}$), and 87 - 90% hydrolyzed ($\approx 30 \times 10^3 - 70 \times 10^3 \text{ g mol}^{-1}$),
106 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), fluorescein
107 isothiocyanate isomer I (FITC), FITC-dextran ($\approx 70 \times 10^3 \text{ g mol}^{-1}$), dimethylsulfoxide (DMSO), nitric
108 acid (HNO_3), NaOH (1 N), and acetic acid were purchased from Sigma Aldrich (St. Quentin Fallavier,
109 France). Methanol (HPLC grade) and acetonitrile were purchased from Carlo Erba® (Val de Reuil,
110 France). Isobutyl cyanoacrylate monomer was purchased from Afinitica Technologies (Barcelona,
111 Spain). Polyfluor 570 (methacryloxyether thiocarbamyl rhodamine B) was purchased from Biovalley
112 Polyscience Inc (Warrington, USA). A-float-layer dialysis device ($\approx 3 \times 10^5 \text{ g mol}^{-1}$) was purchased
113 from Spectrum Labs (Rancho Dominguez, CA, USA). Dialysis membranes with molecular weight
114 cutoffs $\approx 1 \times 10^5 \text{ g mol}^{-1}$, $\approx 12 \times 10^3 \text{ g mol}^{-1}$, and $\approx 3 \times 10^3 \text{ g mol}^{-1}$ were purchased from Spectra/Por
115 (Biovalley, Marne la Vallée, France). A dialysis membrane with a molecular weight cutoff \approx
116 $1 \times 10^3 \text{ g mol}^{-1}$ was purchased from Spectrum-Labs (New Jersey, USA). Syringe filters (30 mm, pore
117 size 1 μm PTFE membrane, non-sterile) were purchased from Sigma Aldrich (St. Quentin Fallavier,
118 France). Milli-Q® water was used for all the experiments (Resistivity 18.2 M Ω .cm at 21 °C, Millipore
119 purification system, Millex, SLAP 0225, Millipore, France).

120 **2.2. Reagents for cell culture**

121 Murine hematopoiesis-derived macrophages (J774A.1) were from CLS (Eppelheim, Germany).
122 Dulbecco-modified eagle medium (DMEM), streptomycin, and penicillin were purchased from
123 ThermoFisher Scientific (Illkirch, France). Phosphate buffer saline (PBS, pH 7.4), fetal bovine serum
124 (FBS), trypan blue, and 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were
125 from Sigma Aldrich (St. Quentin Fallavier, France). Penicillin/streptomycin solution contains 10,000
126 units of penicillin and streptomycin dissolved in a citrate buffer at a concentration of 10 mg mL⁻¹.

127 **3. METHODS**

128 **3.1. Polysaccharide chemical coupling of FITC**

129 ***3.1.1. Chitosan labeling***

130 The reaction between the isothiocyanate group of FITC Isomer I and the primary amine groups of
131 the D-glucosamine units of chitosan was performed according to the method developed elsewhere.[18,
132 19] Powder of chitosan (200 mg) was dispersed in 20 mL of acetic acid (0.1 mol L⁻¹) in an amber flask
133 and under magnetic stirring (400 rpm). Then, methanol (20 mL) was added, followed by a slow
134 addition of 10 mL of a solution of FITC in methanol at a concentration of 2 mg mL⁻¹. The reaction
135 was left to proceed for 3 h, protected from light. The reaction was stopped, and the labeled chitosan
136 was precipitated by raising the pH to ~ 9 - 10 by adding drop-by-drop NaOH solution (1 N). FITC
137 grafted chitosan was centrifuged at 4500 × g for 30 min at 25 °C and washed 3 times with acetic acid
138 to eliminate unreacted components. The products were re-dispersed in 20 mL of acetic acid
139 (0.1 mol L⁻¹) and dialyzed against water for 3 days (molecular weight cutoff ≈ 3 × 10³ g mol⁻¹). The
140 obtained FITC-chitosan was freeze-dried for 24 h (Alpha 1-2 freeze-dryer, Fisher Scientific Bioblock,
141 Illkirch, France).

142 ***3.1.2. Hyaluronan labeling***

143 Hyaluronan (400 mg) was dissolved in water (60 mL) in an amber flask. Then, a solution of NHS
144 (8.63 mg) and EDC (14.37 mg) in water (2.5 mL) was added to the hyaluronan solution and
145 magnetically stirred for 30 min at 25 °C to activate the carboxylic groups on hyaluronan. Then, a
146 solution containing FITC (50 mg) was dissolved in DMSO (70 mL) (equivalent to 2.5% moles of the
147 disaccharides units) was added dropwise to the previously prepared mixture. The reaction occurs at
148 25 °C under magnetic stirring (300 rpm) for 12 h. At the end of the reaction, the FITC-labeled
149 hyaluronan was dialyzed against water for 32 h (Dialysis membrane with a molecular weight cutoff
150 ≈ 1 × 10³ g mol⁻¹). This dialysis step will eliminate DMSO, and other impurities produced by NHS

151 and EDC. In this dialysis step, water was changed after 1 h, 3 h, 5 h, and overnight. To eliminate any
152 residual FITC that did not react with hyaluronan, PD-10 desalting columns (Sephadex G-25, GE
153 Healthcare Life Sciences, Amersham, UK) were used to separate the eluted product from the unreacted
154 molecules, which stack in the stationary phase. Water was used as the mobile phase. The obtained
155 products were freeze-dried for 24 h.

156 **3.2. Preparation of NS and E-NM**

157 **3.2.1. NS preparation**

158 NS composed of PIBCA core and polysaccharide shell were prepared using an anionic emulsion
159 polymerization of isobutyl cyanoacrylate monomer and the polysaccharides in an acidic medium. This
160 method was adapted from previous reports using other polysaccharides, including chitosan [14, 15],
161 dextran [16], and hyaluronan [17]. Non-labeled NS and FITC-labeled NS were prepared by dissolving
162 69 mg of each polysaccharide (FITC-labeled or not) in 5 mL of dilute nitric acid at a concentration of
163 0.2 M under magnetic stirring (300 rpm) in a glass tube at 40 °C for 30 min. After polysaccharide
164 dissolution, 250 µL of isobutyl cyanoacrylate monomer was added under vigorous magnetic stirring
165 (adjusted to 750 rpm). After firmly closing the vials, the reaction continued at 40 °C under stirring for
166 24 h. The sample was cooled to room temperature. Then, nanospheres were purified first by filtration
167 through a 1 µm filter to remove polymer aggregates and then by dialysis using a Spectra/Por membrane
168 with a molecular weight cutoff $\approx 100 \times 10^3 \text{ g mol}^{-1}$ twice for 30 min, twice for 60 min, and once
169 overnight against 1 L of water. The pH of the final suspension was adjusted to 6.3 - 6.5 with NaOH
170 (0.02 N). The concentration of the NS in the suspensions was 18 mg mL^{-1} , as evaluated by freeze-
171 drying.

172 NS labeled with rhodamine (denoted as Rh-labeled) were prepared similarly, except that 5 min
173 after adding isobutyl cyanoacrylate, a solution of PolyFluor 570 (1 mL) (a fluorescent competitive
174 monomer at a 2 mg mL^{-1} concentration in acetonitrile) was added. The reaction continued at 40 °C

175 under magnetic stirring for 24 h. Then, the NS were purified by filtration followed by dialysis
176 according to the process described above for non-labeled and FITC-labeled NS.

177 **3.2.2. E-NM preparation**

178 **3.2.2.1. Optimization of the preparation of a stretchable film containing the NS**

179 Ellipsoidal particles were obtained by the physical deformation of NS according to protocols
180 published elsewhere.[11, 12] PVA film formulation was first optimized without adding NS.
181 Homogeneity and sufficient stretchability of the films were the two target properties for optimizing
182 the formulation and process parameters, as shown in Table S1. Optimized formulation parameters were
183 the concentration and hydrolysis degree of PVA and the percentage of glycerol (Table S1). Briefly,
184 solutions of PVA were prepared by dissolving PVA powders in water at different temperatures (20 °C,
185 40 °C, 50 °C, and 75 °C). Mixing duration was also optimized (from 4 to 12 h). The plasticizer was
186 then added to the PVA solution under magnetic stirring. The formulation was observed visually for
187 each parameter, and only homogenous solutions were selected for film casting and stretching
188 experiments.

189 **3.2.2.2. Preparation of PVA film containing the NS**

190 First, a PVA solution was prepared by adapting protocols reported in the publications of Champion
191 et al.,[20] and Ahmed et al.[12] Briefly, 20 mL of water was added to 2 g of PVA powder (87 - 90%
192 hydrolyzed) under magnetic stirring for 1 h at room temperature (20 °C). Then, 440 mg of glycerol
193 was added to the mixture. Glycerol was used as a plasticizer to decrease the T_g of the PVA film.[20]
194 The final mixture was magnetically stirred at room temperature for 16 h to obtain a homogenous
195 solution. The concentrations of PVA and glycerol were 10 wt% and 2 wt%, respectively. Then, a
196 suspension of NS (720 μ L) was dispersed in 1 mL of this solution to reach a final concentration of
197 13 wt% of NS per PVA solution.[11, 12] The resulting mixture of NS and PVA/glycerol solution was
198 gently stirred for 30 min, spread on a glass slide (25 \times 75 mm), and dried for 12 h at 37 °C to evaporate
199 water. After drying, the films were immediately recovered from the glass slide and placed in a

200 thermoregulated stretching machine. Film stretching was operated at 50 °C, and the stretching speed
201 was 0.5 mm min⁻¹. Ellipsoidal NM were obtained by stretching the films in one axis (x, x') to a final
202 length of 130 mm. At the end of the stretching, the films were immediately detached from the machine
203 and rapidly cooled to 5 °C using a flux of cold air using dry ice (temperature -16.5 °C) while the films
204 were maintained at the maximum stretched position. After cooling, the film was recovered in 1 mL of
205 water. The recovered film was poured into a folate A-lyser dialysis device (molecular weight cutoff
206 $\approx 300 \times 10^3$ g mol⁻¹) after its initial preparation according to the manufacturer's instructions. The
207 dialysis was maintained for 24 h. The pH of the obtained suspensions was then adjusted to 6.5 with
208 NaOH 0.1 N. To investigate the effect of PVA on NS surface modification, cytotoxicity, and
209 endocytosis, NS were immersed in PVA and purified using the above-described protocol.

210 **3.3. Physicochemical characterizations**

211 ***3.3.1. Quasi-elastic light scattering***

212 NS mean hydrodynamic diameters were measured in a Zetasizer Nanoseries (Nano-ZS, Malvern
213 Instruments, France). Samples (100 µL) were diluted in 1 mL of water before measurement at 25 °C.
214 The scattered angle was 173°.

215 ***3.3.2. Surface potential***

216 Zeta (ζ) potential measurements characterize the surface potentials of NS and E-NM were
217 characterized by electrophoretic mobility. This method was already used for surface potential
218 characterization of spherical and nonspherical NM [21-24]. The ζ potentials were calculated at 25 °C
219 by measuring the electrophoretic mobility using an electrophoretic light scattering technique (Zetasizer
220 Nanoseries Nano-ZS, Malvern Instruments, France). For sample preparation, 60 µL of each suspension
221 was diluted with 2 mL NaCl (1 mM). Then, 1 mL of each suspension was placed in a disposable folded
222 capillary cell. The scattered angle was 173°. Each experiment was replicated three times on three
223 independent formulations.

224 **3.3.3. Transmission electron microscopy**

225 TEM was used in this investigation to characterize NS and E-NM's shape, size, and aspect ratio of
226 nonspherical NM. TEM characterizations were performed at 80 kV transmission using a transmission
227 electron microscope (JEOL 1400) coupled to TEM Domain Centre software. The suspensions were
228 diluted in water (1/10 for NS and E-NM). The dilution was optimized to observe particles with good
229 contrast. Then, 25 μL NS and E-NM suspensions were placed on a carbon grid and stained with
230 phosphotungstic acid 1% (pH 7.4) before observation. The grid was then placed on a slide and inserted
231 into the microscope for observation. Different frames were randomly selected for each TEM
232 microphotograph set to analyze the geometrical dimensions of NS or E-NM ($n > 100$ particles). Size
233 distribution analysis was performed on TEM images using ImageJ and fitted to a normal Gaussian law.
234 Statistical analyses were performed using GraphPad Prism software.

235 **3.3.4. ITC**

236 An isothermal calorimeter (VP-ITC, MicroCal Inc., USA) has been used for characterizing the
237 interaction of polysaccharides with PVA. A typical experiment used a syringe filled with 250 μL of
238 chitosan, dextran, or hyaluronan (0.5 mM) aqueous solution to titrate an aqueous solution of PVA
239 (1 mM) into the calorimetric cell at 293.15 K (20 °C) or 323.15 °K (50 °C). Aliquots of 10 μL of titrant
240 (polysaccharide solution) were delivered over 20 s, intervals between injections were 180 s, and
241 agitation speed was 310 rpm. A background titration was obtained by injecting polysaccharide solution
242 in water in the sample cell.

243 **3.4. Cell culture**

244 **3.4.1. Cell growth conditions**

245 Cells were incubated at 37 °C in a humidified atmosphere (5% CO_2). Cells were maintained in cell
246 culture flasks (75 cm^2 , Sigma Aldrich, St. Quentin Fallavier, France) and passaged every three days,
247 reaching 70 - 80% confluency. Cells were cultured on DMEM supplemented with 10% FBS,
248 100 $\mu\text{g mL}^{-1}$ streptomycin, and 100 IU mL^{-1} penicillin.

249 **3.4.2. Cytotoxicity assay**

250 In vitro cytotoxicity was investigated using the MTT assay. Macrophages were first cultured in 96-
251 well at 10^3 cells/well concentration. The plates were preincubated for 48 h at 37 °C. Then, NS before
252 and after their soaking in PVA and E-NM, as well as their negative controls (native polysaccharides),
253 were diluted in the culture medium to reach the following concentrations (0.1, 0.5, 1, 10, 25, 50, 100,
254 and 250 $\mu\text{g mL}^{-1}$). After 24 h of incubation of each preparation with the cells, 20 μL of an MTT
255 solution in PBS (5 mg mL^{-1}) was added to each well. After two hours of incubation, the content of the
256 wells was removed, and 200 μL DMSO was added to solubilize the formazan crystals that formed after
257 adding the MTT reagent. Then, 96-well plates were stirred at 450 rpm for 30 min. After that, the
258 absorbances of solubilized formazan crystals were measured with a microplate reader at 570 nm. IC_{50}
259 values (concentration of each preparation to reduce cell viability by 50% relative to untreated control
260 cells, respectively) were calculated from the dose-response curve. All experiments were repeated six
261 times.

262 **3.4.3. Particle endocytosis**

263 Cells were analyzed in a CytoFlex cytometer (Beckman-Coulter) driven by Cytexpert 2.4 software
264 for endocytosis studies. Cells were excited by a 488 nm laser for fluorescence measurements, and
265 fluorescence emission was collected through a 525/40-nm bandpass filter. FITC-labeled preparations
266 were incubated at 37 °C with cells to measure endocytosis efficiency. After a washing step, cells were
267 resuspended in PBS, and 0.01% trypan blue was added to quench FITC fluorescence outside the cells.
268 The threshold of positive cells was determined by comparison with cells without preparations.
269 Frequency (F) and Mean Fluorescence Intensity (MFI) were used to calculate an internalization index
270 (I) ($I = F \times \text{MFI}$). For each condition, a total of 15,000 - 20,000 cells were analyzed. The effect of the
271 particle morphology on internalization in macrophages was studied on hyaluronan NS pre-incubated
272 in PVA and purified hyaluronan E-NM. The polysaccharide composition (hyaluronan) was kept

273 constant while the concentrations varied as 0.1, 1, or 10 $\mu\text{g mL}^{-1}$. The incubation duration was kept
274 constant at two hours.

275 **3.5. Data analysis**

276 Graphics of cellular viability and aspect ratio were analyzed by using Graphpad prism[®]. Graphics
277 that compare the cellular viability of the two cell lines after treatment with the different types of
278 particles and negative controls were performed with R programming language using “dplyr” package
279 to manipulate data, “ggplot2” package to plot data, and “gridExtra” package to pool data in
280 comparative graphics.

281 **4. RESULTS AND DISCUSSION**

282 In this investigation, two NM morphologies were designed: spherical and ellipsoidal. Surface
283 properties were tailored using different polysaccharides, namely chitosan, dextran, and hyaluronan.
284 Each type of particle was conjugated to FITC to monitor NM cell internalization by flow cytometry.
285 The fluorescence-quenching method was employed to distinguish between NM attached to the cell
286 surface and those ingested by the cell. This method involves adding a component that quenches the
287 fluorescence of attached FITC-conjugated NM while the ingested particles remain fluorescent.[25]
288 Trypan blue, a non-permeant dye, quenched membrane-bound fluorescein-labeled compounds for flow
289 cytometry analyses. Trypan blue is known to quench the green fluorescence of cell-surface attached
290 particles.[26] Dextran and hyaluronan were conjugated with FITC, while dextran-FITC was
291 commercially available. The three FITC-labeled polysaccharides were then used for the preparation of
292 FITC-labeled NM.

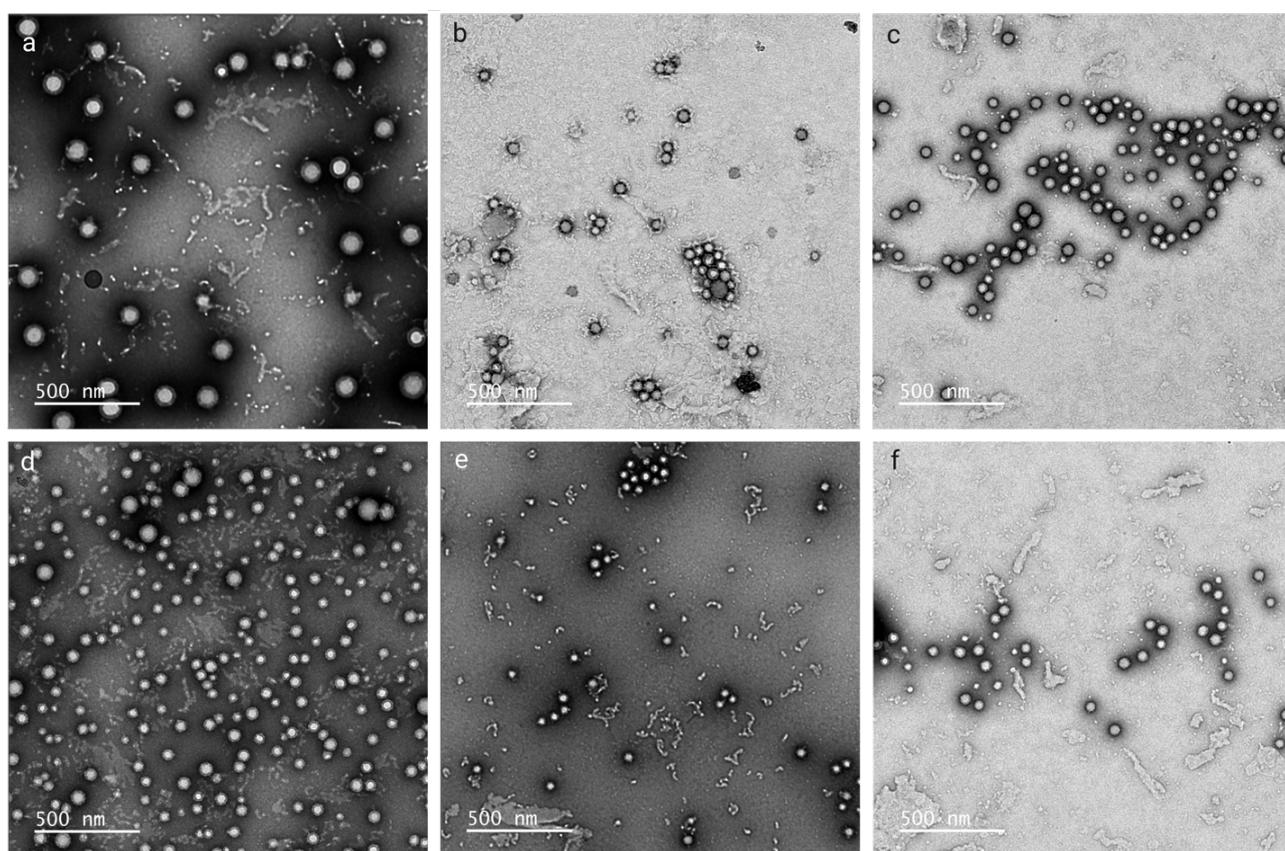
293 **4.1. NS and E-NM preparation and physicochemical characterizations**

294 **4.1.1. NS**

295 **4.1.1.1. Mean hydrodynamic diameter**

296 Spherical particles were designed by emulsion polymerization of isobutylcyanoacrylate.
297 Representative TEM images of non-labeled NS and FITC-labeled NS are given in Figure 1. FITC
298 labeling slightly affected the mean hydrodynamic diameter of NS (Table 1). The labeling used in this

299 investigation differed from the one reported earlier with PIBCA NS.[27] In most reports on
300 fluorescently labeled PIBCA NS, a fluorescent monomer is added to the polymerization medium,
301 which competes with PIBCA during polymerization. However, this strategy significantly increased the
302 mean hydrodynamic diameter of NS, as observed from the size measurement in Table 1. The mean
303 hydrodynamic diameter of rhodamine-labeled NS rose from 197 to 388 nm for chitosan and 126 to
304 326 nm for dextran. Hyaluronan NS showed an increase in mean hydrodynamic diameter from 355 to
305 984 nm (Table 1).



306 **Figure 1:** Representative TEM images of non-labeled NS (a,b,c), and FITC-labeled NS (d,e,f),
307 composed of PIBCA and chitosan (a,d), dextran (b,e), and hyaluronan (c,f).

308 **4.1.1.2. ζ potential**

309 Before labeling, chitosan NS exhibited a ζ potential of +35.5 mV due to the protonated amine
310 groups at the pH of 6.3 - 6.5.[28] NS coated with dextran displayed a negative ζ potential (-5.6 mV),
311 which agrees with previous findings.[29, 30] Hyaluronan NS exhibited a strong negative charge
312 (-39.9 mV) (Table 1) due to carboxylic groups of hyaluronan.[31] Chemical structures of chitosan,
313 dextran, and hyaluronan are given in Figure S2.a,b, and c, respectively. NS labeling with FITC
314 decreased the ζ potential of chitosan NS from +35.5 mV to +27.2 mV. FITC grafting on the primary
315 amine groups of chitosan could explain this decrease in the ζ potential. Similarly, FITC resulted in a
316 reduction of the ζ potential of NS composed of dextran and hyaluronan. This decrease in the ζ potential
317 could be due to the presence of carboxylic groups of the FITC isomer I grafted on the polysaccharides.

318 **Table 1:** Effect of labeling method on mean hydrodynamic diameter (d_h) and zeta potential (ζ) of NS prepared by polymerizing IBCA and chitosan,
 319 dextran, or hyaluronan. The polysaccharides were labeled or not with FITC (FITC-labeled) or rhodamine (Rh-labeled). $n = 3$ different preparations.

Physicochemical parameter	Chitosan			Dextran			Hyaluronan		
	Non-labeled	FITC-labeled	Rh-labeled	Non-labeled	FITC-labeled	Rh-labeled	Non-labeled	FITC-labeled	Rh-labeled
d_h (nm)	197 ± 8	153 ± 2	388 ± 37	126 ± 25	116 ± 2	326 ± 21	355 ± 5	358 ± 11	984 ± 22
ζ (mV)	+35.5 ± 2.7	+27.9 ± 0.5	+40.5 ± 0.2	-5.6 ± 0.2	-12.4 ± 0.7	-2.1 ± 0.8	-39.9 ± 3.0	-42.6 ± 2.1	-41.0 ± 0.5

320 **Table 2:** Physicochemical properties of NS and E-NM prepared by polymerizing IBCA and chitosan, dextran, or hyaluronan. The polysaccharides were
 321 labeled or not with FITC. $n = 3$ different preparations.

Particle morphology	Physicochemical parameter	Chitosan		Dextran		Hyaluronan	
		Non-labeled	FITC-labeled	Non-labeled	FITC-labeled	Non-labeled	FITC-labeled
NS	d_h^a (nm)	197 ± 8	153 ± 2	126 ± 25	116 ± 2	355 ± 5	358 ± 11
	d_{TEM} (nm)	85 ± 25	80 ± 17	59 ± 7	51 ± 6	62 ± 8 ^a	59 ± 13
E-NM	w_{TEM} (nm)	310 ± 97	127 ± 50	95 ± 31	96 ± 38	106 ± 42 ^b	67 ± 28
	h_{TEM} (nm)	64 ± 15	51 ± 18	40 ± 10	50 ± 15	44 ± 13 ^b	28 ± 9
	Γ	4.9 ± 0.9	2.5 ± 0.4	2.4 ± 0.5	1.9 ± 0.4	2.4 ± 0.6	2.4 ± 0.7
	$\Gamma_{min} - \Gamma_{max}$	3.5 - 7.6	1.6 - 4.1	1.6 - 4.0	1.1 - 3.3	1.6 - 4.0	1.3 - 3.9
NS	ζ potential (mV)	+35.5 ± 2.7 ^a	+27.9 ± 0.5 ^a	-5.6 ± 0.3 ^a	-12.4 ± 0.7 ^a	-39.9 ± 3.0 ^a	-42.6 ± 2.1 ^a
		+5.8 ± 0.2 ^b	+1.7 ± 0.3 ^b	-1.2 ± 0.2 ^b	-1.4 ± 0.1 ^b	-28.3 ± 3.1 ^b	-17.9 ± 3.6 ^b
		+2.7 ± 0.6 ^c	+5.4 ± 0.1 ^c	-2.0 ± 0.9 ^c	-7.1 ± 0.6 ^c	-32.1 ± 0.8 ^c	-34.3 ± 1.4 ^c
E-NM		+1.5 ± 0.2 ^d	+1.4 ± 2.1 ^d	-1.9 ± 1.0 ^d	-5.6 ± 1.0 ^d	-32.4 ± 1.1 ^d	-36.4 ± 2.1 ^d

322 d_h : mean hydrodynamic diameter.

323 d_{TEM} : particle diameters determined by analyzing TEM images of NS. $n > 100$ particles analyzed.

324 Γ : aspect ratio calculated by dividing the mean width of E-NM (w_{TEM}) by the mean height of E-NM (h_{TEM}).

325 ^a: as prepared, without purification.

326 ^b: NS after incubation with PVA/glycerol solution (10/2 wt%). PVA hydrolysis degree was 87 - 90%.

327 ^c: NS after incubation with PVA/glycerol solution and purification by dialysis (cutoff $\approx 3 \times 10^5$ g mol⁻¹).

328 ^d: E-NM obtained after incubation of NS with PVA/glycerol solution (10/2 wt%) followed by drying and film stretching at 50 °C.

329 **4.1.1.3. TEM**

330 TEM analysis allowed the characterization of NS morphology and the diameter of individual
331 particles. The NS size obtained from TEM images is denoted d_{TEM} . The results in Table 2 revealed
332 that particle diameters obtained from TEM images were lower than the mean hydrodynamic diameters
333 determined by QELS. Indeed, the QELS technique does not distinguish between an individual particle
334 and an aggregate of several particles.

335 This difference is particularly marked for hyaluronan NS, where the d_h was equal to 355 nm for
336 non-labeled particles while the d_{TEM} was 62 nm. Similarly, for FITC-labeled NS composed of
337 hyaluronan, d_h was equal to 358 nm while d_{TEM} was 59 nm. This discrepancy could be attributed to
338 the swelling behaviors of hyaluronan present on the NS surface during QELS measurements, leading
339 to the formation of a gel-like layer around the NS during QELS measurements. It has been
340 demonstrated that low hyaluronan molecular weight ($\sim 50 - 60 \times 10^3 \text{ g mol}^{-1}$), similar to the molecular
341 weight of hyaluronan used in this investigation, has a higher volumetric swelling ratio than high
342 molecular weight ($\sim 10^6 \text{ g mol}^{-1}$).[32, 33] According to phantom network models, mesh sizes for
343 hyaluronan $6 \times 10^4 \text{ g mol}^{-1}$, $5 \times 10^5 \text{ g mol}^{-1}$, and 10^6 g mol^{-1} were 35 ± 2 , 24 ± 3 , and 13 ± 1 nm,
344 respectively.[33]

345 **4.1.2. E-NM**

346 **4.1.2.1. Optimization of the formulation of homogenous and stretchable PVA film**

347 In this section, we aimed to optimize PVA molecular weight and its degree of hydrolysis to obtain
348 films with optimized characteristics suitable for producing ellipsoidal particles by stretching parent
349 NS. An ideal film should dissolve at room temperature (*e.g.*, 20 °C), be homogeneous after drying, and
350 resist stretching. The chemical structure of PVA is given in Figure S2.d, and the equation to calculate
351 the degree of hydrolysis is in Eq.S1 (Supporting data). Different grades of PVA with variable degrees
352 of hydrolysis were tested to optimize film formulation. The concentrations of PVA and glycerol, used
353 as a plasticizer, were optimized.

354 Furthermore, the temperature and duration of mixing were modified to identify the best parameters
355 for film formation. For each parameter, the homogeneity of the resulting solutions was observed
356 visually. Then, the impact of each parameter on the film's stretchability was observed.

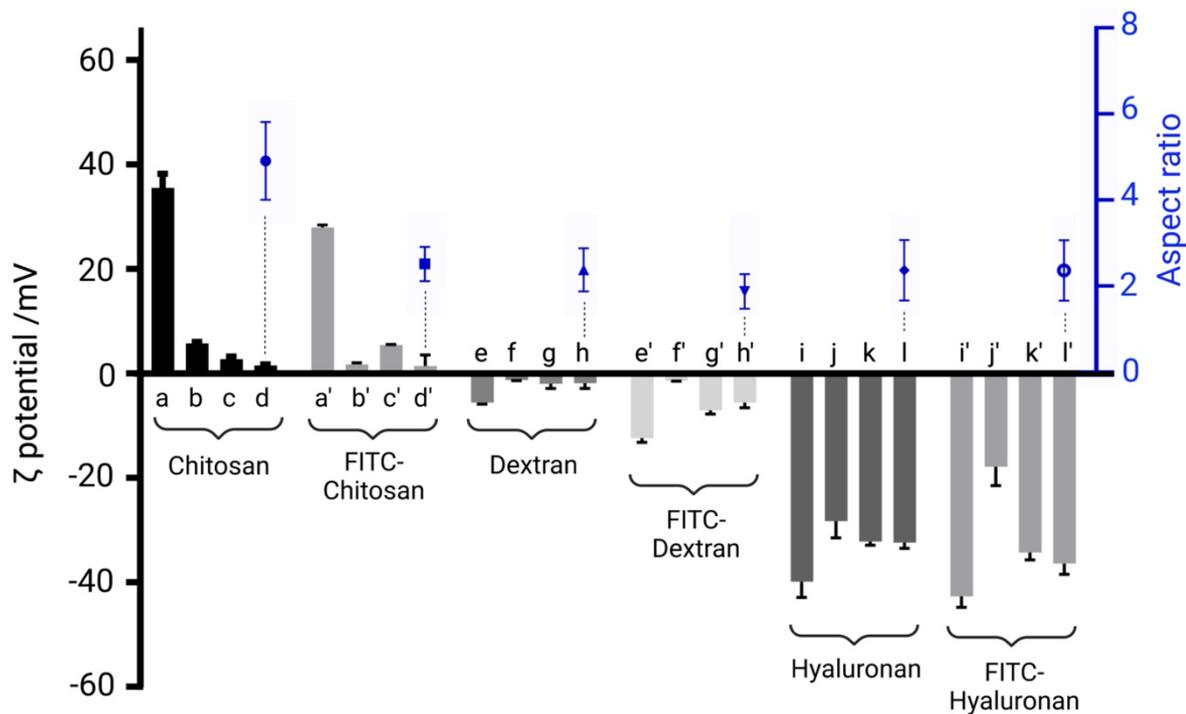
357 Varying the degree of hydrolysis from 80% to 100% revealed that the water solubility of PVA
358 decreased when the hydrolysis degree increased (Table S1). Those results are explained by highly
359 hydrolyzed PVA's high degree of crystallinity. Partially hydrolyzed PVA (80% and 87 - 90% of
360 hydrolysis degrees) were soluble in water under magnetic stirring at 20 °C (Table S1 in the Supporting
361 Information). Higher hydrolysis grades of PVA (99% or 100%) were insoluble in water at 20 °C,
362 40 °C, and 50 °C (Table S1). They are soluble at temperatures above 75 °C after 6 - 12 h of magnetic
363 stirring. A higher crystallinity is due to the formation of strong inter and intra-chain hydrogen bonding.
364 Thus, a high degree of hydrolysis requires higher temperatures for dissolution by disrupting inter and
365 intra-hydrogen bonding.[34] The films obtained with partially hydrolyzed PVA (87 - 90%) were
366 homogeneous, non-sticky, and smooth. In particular, the film obtained with partially hydrolyzed PVA
367 at a concentration of 10 wt% was easily stretchable. In contrast, the films obtained with a lower PVA
368 concentration (5 wt%) were thin and brittle when stretched. At a concentration of 5 wt%, fully
369 hydrolyzed PVA and 99 wt% hydrolyzed PVA formed homogeneous and easily stretchable films. This
370 difference could be explained by the higher molecular weight of fully hydrolyzed PVA and 99 wt%
371 hydrolyzed PVA ($89 - 98 \times 10^3 \text{ g mol}^{-1}$) compared with partially hydrolyzed PVA (87 - 90%), where
372 the molecular weight was ($30 - 70 \times 10^3 \text{ g mol}^{-1}$). This difference also explains why the 80%
373 hydrolyzed PVA concentration required to form a stretchable film was 20 wt%. Probably because its
374 molecular weight was ($9 - 10 \times 10^3 \text{ g mol}^{-1}$), which is ten-fold lower than fully hydrolyzed and 99%
375 hydrolyzed PVA.

376 Glycerol was added to the PVA solutions at different concentrations ranging from 0.5 to 2 wt%.
377 Without glycerol, the films were brittle and fragile upon stretching. Notably, the films prepared with
378 fully hydrolyzed and 99 wt% hydrolyzed PVA and supplemented with 2 wt% of glycerol were easily

379 stretchable. However, they dissolved at 75 °C after 12 h of magnetic stirring. They were not selected
380 for the following experiments. The final PVA solution formulation used for the subsequent
381 experiments was composed of PVA (87 - 90%) at 10 wt% in water and glycerol (2 wt%).

382 **4.1.2.2. ζ potential characterization after NS incubation with PVA solution**

383 In the article of Ahmed et al.,[12] we suggested that the PVA in the STBT[®] film adsorbed on the
384 particle surface of chitosan NS. Herein, we measured the surface potentials of NS composed of
385 chitosan, dextran, and hyaluronan before and after incubation with PVA solution. PVA solution was
386 prepared according to the previous section. Once soaked into the PVA solution, we noticed a net
387 decrease in the values of ζ potentials of chitosan NS from +35.5 mV to +5.8 mV for non-labeled
388 chitosan NS and from +27.9 mV to +1.7 mV for FITC-labeled chitosan NS. The results are shown in
389 Table 2 and Figure 2. Then, NS suspensions were purified by dialysis, and the efficacy of NS
390 purification from PVA was estimated from the change in the NS ζ potential measurement before and
391 after dialysis. The results in Table 2 and Figure 2 revealed that dialysis did not allow a complete
392 purification of chitosan NS suspension from PVA since the ζ potentials were +2.7 mV and +5.4 mV
393 for non-labeled and FITC-labeled chitosan NS, respectively.



394

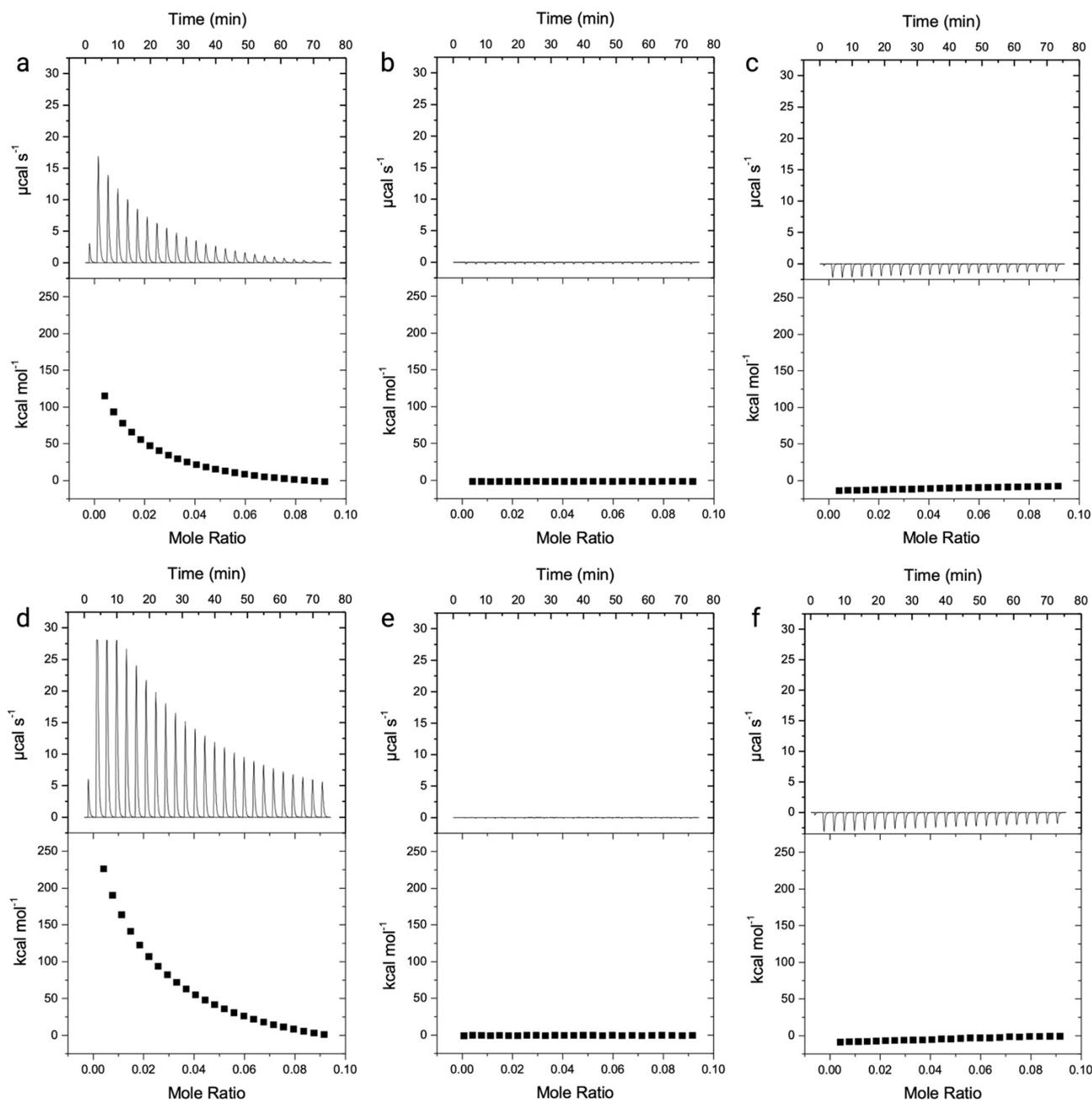
395 **Figure 2:** Representation of the effect of incubation of nanospheres with PVA on ζ potential (left y
396 axis) and impact of polysaccharide type on the aspect ratio (right y axis). The preparations are
397 composed of chitosan (a,b,c,d), FITC-chitosan (a',b',c',d'), dextran (e,f,g,h), FITC-dextran
398 (e',f',g',h'), hyaluronan (i,j,k,l), and FITC-hyaluronan (i',j',k',l'). The preparations were composed as
399 prepared nanospheres (NS) without PVA (chitosan NS in (a), FITC-chitosan NS in (a'), dextran NS in
400 (e), FITC-dextran NS in (e'), hyaluronan NS in (i), FITC-hyaluronan NS in (i')), NS after incubation
401 with PVA denoted as NS+PVA (chitosan NS+PVA in (b), FITC-chitosan NS+PVA in (b'), dextran
402 NS+PVA in (f), FITC-dextran NS+PVA in (f'), hyaluronan NS+PVA in (j), FITC-hyaluronan
403 NS+PVA in (j')). NS after incubation with PVA and purification by dialysis denoted as NS_{PVA}
404 (chitosan NS_{PVA} in (c), FITC-chitosan NS_{PVA} in (c'), dextran NS_{PVA} in (g), FITC-dextran NS_{PVA} in
405 (g'), hyaluronan NS_{PVA} in (k), FITC-hyaluronan NS_{PVA} in (k')). Ellipsoidal nanomaterials denoted as
406 E-NM (chitosan E-NM in (d), FITC-chitosan E-NM in (d'), dextran E-NM in (h), FITC-dextran E-
407 NM in (h'), hyaluronan E-NM in (l), FITC-hyaluronan E-NM in (l')). ζ potential was the mean of $n =$
408 3 different preparations. Aspect ratio (I) was the mean of $n > 100$ particles analyzed.

409 Concerning NS composed of dextran, after incubation with PVA, the ζ potential varied from
410 -5.6 mV to -1.2 mV for non-labeled dextran NS and from -12.4 mV to -1.4 mV for FITC-labeled
411 dextran NS. Interestingly, purification of NS suspension by dialysis decreased the ζ potential from
412 -1.2 mV to -2.0 mV for non-labeled dextran NS and from -1.4 mV to -7.1 mV for FITC-labeled dextran
413 NS. In the case of dextran, purification was most effective in recovering the initial surface potential
414 compared to chitosan. Similar observations were reported with hyaluronan NS. Indeed, the ζ potential

415 varied from -39.9 mV to -28.3 mV after incubation with PVA. Purification of NS suspension decreased
416 the ζ potential from -28.3 mV to -32.1 mV for non-labeled hyaluronan NS and from -17.9 mV to
417 -34.3 mV for FITC-labeled hyaluronan NS. For dextran and hyaluronan, purification by dialysis brings
418 the ζ potential to values close to the ones reported with NS before incubation. The difference in the ζ
419 potential before incubation and after purification is less marked than with chitosan. Those results could
420 be attributed to the stronger interaction of PVA with chitosan than with dextran and hyaluronan on the
421 NS surface. Consequently, the purification of chitosan NS after PVA adsorption is less efficient than
422 dextran and hyaluronan. To explore this hypothesis, the interaction of PVA with the three
423 polysaccharides was studied by ITC, as detailed in the following section.

424 **4.1.2.3. ITC**

425 ITC titration curves and enthalpies of the interactions of PVA with chitosan, dextran, and
426 hyaluronan solutions are shown in Figure 3. The interaction was studied at 20 °C to mimic the
427 temperature of NS incubation with PVA and at 50 °C to mimic the stretching temperature. The results
428 revealed that the interaction of PVA was higher with chitosan than with dextran and hyaluronan at
429 both temperatures, 20 °C and 50 °C (Figure 3). Strong interaction of PVA with chitosan could be due
430 to hydrogen-bound formation between hydroxyl groups of PVA with amine groups, hydroxymethyl
431 groups, hydroxyl groups, or carboxylic acid groups on chitosan. Those findings agree with data from
432 the literature demonstrating that -CH₂-OH groups on PVA interacted with hydroxymethyl groups[35]
433 and amine groups on chitosan.[36] Figure 3 revealed that the interaction of chitosan with PVA was
434 higher at 50 °C (Figure 3.d) than at 20 °C (Figure 3.a). This behavior could be due to the decreased
435 viscosity of PVA[34] and chitosan solutions[37] upon temperature increase. The interaction of PVA
436 with dextran and hyaluronan solutions was lower than with chitosan at 20 °C (Figure 3.b,c) and 50 °C
437 (Figure 3.e,f). The heat flows fall in the same range as dextran and hyaluronan dilution curves in water
438 (Figure S3).



439

440 **Figure 3:** ITC titration curves and enthalpies of the interaction of PVA with chitosan (a,d), dextran
 441 (b,e), and hyaluronan (c,f). The concentration of chitosan, dextran, and hyaluronan in the syringe was
 442 0.5 mM, while the concentration of PVA in the titration cell was 1 mM. The temperature of the
 443 experiment was maintained constant at 20 °C (a,b,c) or 50 °C (d,e,f).

444

4.1.2.4. Physicochemical characterizations of E-NM

445

In this section, E-NM were characterized by determining ζ potential, morphology, and dimensions

446

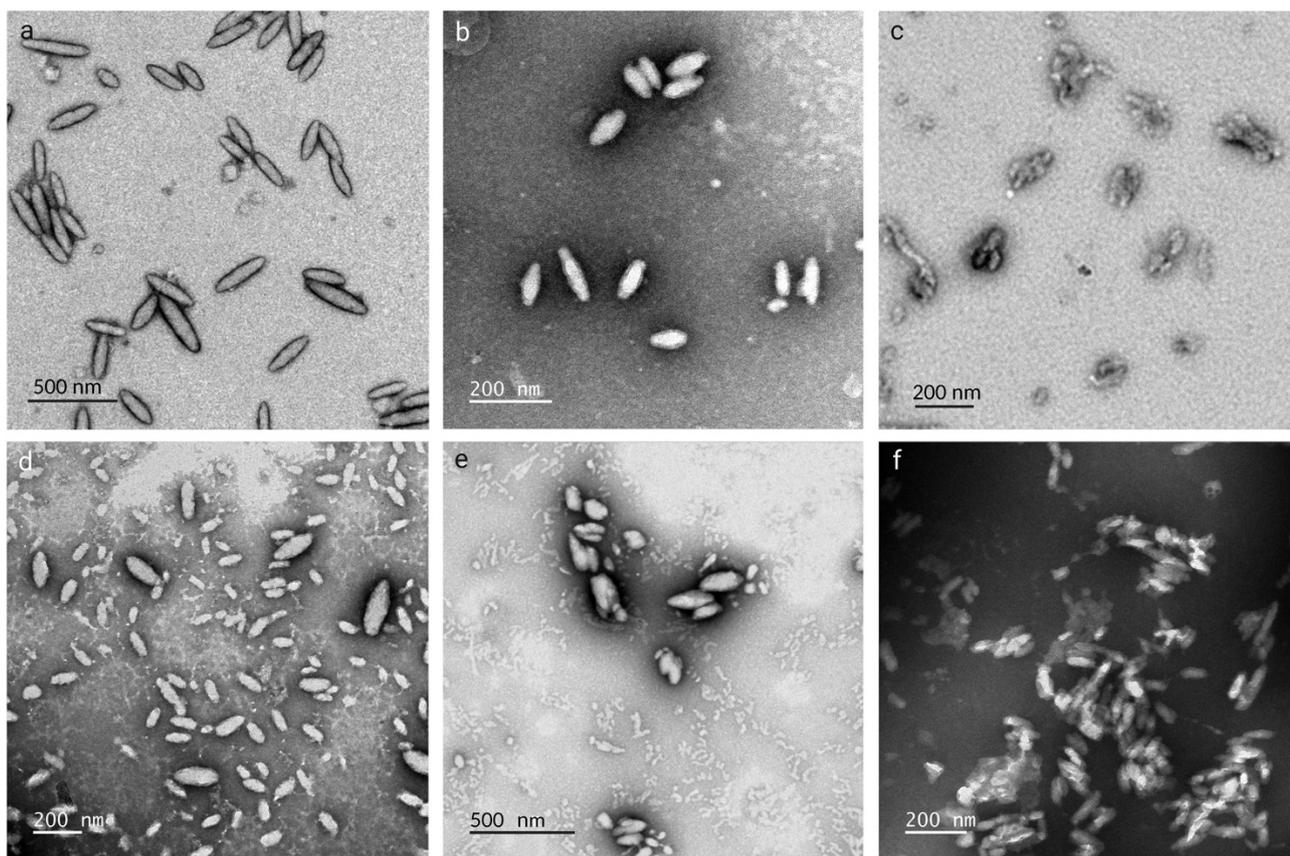
from TEM images. The ζ potentials of the E-NM reported in Table 2 and Figure 2 were in the same

447

range as spherical particles after incubation with a PVA solution and purification by dialysis.

448 Electrophoretic mobility was already used to characterize spherical and nonspherical NM surface
449 potential.[21, 22]

450 The size of nonspherical particles was analyzed from TEM images. Indeed, the non-sphericity of
451 NM complexifies their physicochemical characterizations compared with spherical particles. In many
452 analytical techniques, the particles were assimilated to spheres. For example, in the quasi-elastic light
453 scattering technique, the hydrodynamic size (d_h) is calculated from the diffusion coefficient (D_t) of the
454 NM in suspension using the Stokes-Einstein equation $d_h = \frac{K_B T}{3\pi\eta D_t}$, where K_B is the Boltzmann constant,
455 T the temperature and η the dynamic viscosity of the suspension. When dealing with the
456 characterization of nonspherical particles, more relevant methods are required for analyzing shape,
457 morphology, and size distribution. Transmission electron microscopy is a rapid and straightforward
458 technique for characterizing the size of NM with a spherical or nonspherical morphology. TEM
459 analysis of preparations composed of chitosan, dextran, hyaluronan, and FITC-labeled counterparts
460 revealed that ellipsoidal particles were obtained by the physical deformation of parent NS regardless
461 of the polysaccharide used (Figure 4). However, we observed that chitosan E-NM in Figure 4.a were
462 well stretched compared with dextran and hyaluronan E-NM in Figure 4.b and 4.c, respectively. In the
463 case of dextran and hyaluronan, the ellipsoids appeared less stretched than with chitosan. In Figure
464 4.b, dextran ellipsoids appeared more round-shaped than with chitosan. Calculation of the mean long
465 axis dimension (width, w_{TEM}) and the mean small axis dimension (height, h_{TEM}) of more than 100 E-
466 NM revealed that chitosan ellipsoids had the highest width (310 ± 97 nm) compared with the width of
467 dextran (95 ± 31 nm) and hyaluronan (106 ± 42 nm). The aspect ratios (Γ) were calculated by dividing
468 w_{TEM} by h_{TEM} for each preparation. The mean aspect ratios of more than 100 particles observed from
469 TEM images are reported in Table 2.[12] The results indicated that the mean aspect ratio of non-labeled
470 chitosan E-NM (4.9 ± 0.9) was higher than the ones obtained with dextran (2.4 ± 0.5) and hyaluronan
471 (2.4 ± 0.6). This difference was visible by plotting the mean aspect ratios in Figure 2 (right y axis).



472
 473 **Figure 4:** Representative TEM images of non-labeled E-NM (a,b,c) and FITC-labeled E-NM (d,e,f),
 474 composed of PIBCA and chitosan (a,d), dextran (b,e), and hyaluronan (c,f).

475 The results also revealed that the FITC grafting of chitosan decreased the mean aspect ratio from
 476 (4.9 ± 0.9) to (2.5 ± 0.4) . We suspected that the decrease in the mean aspect ratio is correlated to the
 477 change in the ζ potential. Consequently, we plotted the mean aspect ratio on the same figure as the ζ
 478 potential. In Figure 2 and Table 2, we noticed a decrease in the mean aspect ratio of chitosan ellipsoids
 479 due to FITC grafting. Indeed, FITC Isomer I was grafted on the NH_2 groups of chitosan, which could
 480 reduce the interacting sites with PVA. Lower interaction with PVA results in a lower aspect ratio.
 481 Those findings suggest that high ζ potential could result from high interaction with PVA. The reduced
 482 aspect ratio in the case of dextran and hyaluronan could be due to a lower interaction of those
 483 polysaccharides with PVA, as characterized by ITC in the previous section. In the case of chitosan,
 484 FITC grafting on amine groups reduces the ζ potential and, in turn, the interaction with PVA.

485 We also noticed that the change in the aspect ratio was slightly influenced by the T_g . Indeed, the
486 T_g of PIBCA was 46 °C.[38] The T_g of chitosan was in the range of 140 ~ 150 °C,[39] while the T_g of
487 dextran was 149 °C.[40] Concerning hyaluronan, the T_g was observed at -70 °C to -100 °C.[41]

488 **4.2. Cytotoxicity**

489 Herein, the cytotoxicity of NS and E-NM was investigated in macrophages. Cytotoxicity was also
490 investigated for native polysaccharides used as controls. As expected, native polysaccharides were
491 non-toxic toward macrophages in the tested concentration range (Figure 5) since the IC_{50} values were
492 higher than 250 $\mu\text{g mL}^{-1}$ (Table 3). As the PVA modifies the particle's surface potential, the effect of
493 the particle incubation in PVA solution on the cell viability was investigated. Figure 5 represents the
494 relative cell viability curves of NS and E-NM composed of PIBCA. Analysis of IC_{50} values revealed
495 that regardless of the polysaccharide type, the as-prepared PIBCA NS, without incubation with PVA
496 solution, caused significant cell death in a concentration-dependent fashion (Figure 5). We noticed the
497 presence of a threshold concentration around 1 $\mu\text{g mL}^{-1}$, beyond which the percentage of cell viability
498 starts to decrease drastically for both cell lines (Figure 5). The cytotoxicity mechanism of
499 cyanoacrylate polymers was attributed to their degradation, suggesting that the degradation products
500 are responsible for the toxicity.[42] In particular, the cytotoxicity of poly(isobutyl 2-cyanoacrylate)
501 polymers was attributed to formaldehyde release upon polymer degradation.[42] Other investigations
502 suggested that the cytotoxicity observed with cyanoacrylate polymers may be due to cellular damage
503 leading to oxidative conversion of membranal lipids.[43]

504 Interestingly, this investigation revealed that NS cytotoxicity was reduced after incubating with
505 PVA solution, followed by purification. The IC_{50} values were reported in Table 3 and Figure 6.
506 Analysis of the results in Table 3 and Figure 6 revealed that regardless of the polysaccharide type, the
507 particles denoted as NS_{PVA} , had a higher IC_{50} than prepared NS (Table 3). Reduced cytotoxicity could
508 be due to the adsorption of the PVA on the NS surface, as discussed in the previous section. Those
509 findings also suggested that PVA was not entirely removed from the preparation after purification by

510 dialysis. The dose-response cytotoxicity curves in Figure 5 revealed that the ellipsoidal shape of the
 511 E-NM did not significantly modify their cytotoxicity in both cell lines compared with NS_{PVA}. The
 512 values of the IC₅₀ were in the same range for NS_{PVA} and E-NM (Table 3 and Figure 6). This result
 513 could be due to comparable surface properties of NS incubated with PVA and E-NM due to the PVA
 514 adsorption, which partially masked the polysaccharide on the particle surface. Considering those
 515 results, we can conclude that cytotoxicity is linked to the particle's surface properties rather than
 516 morphology.

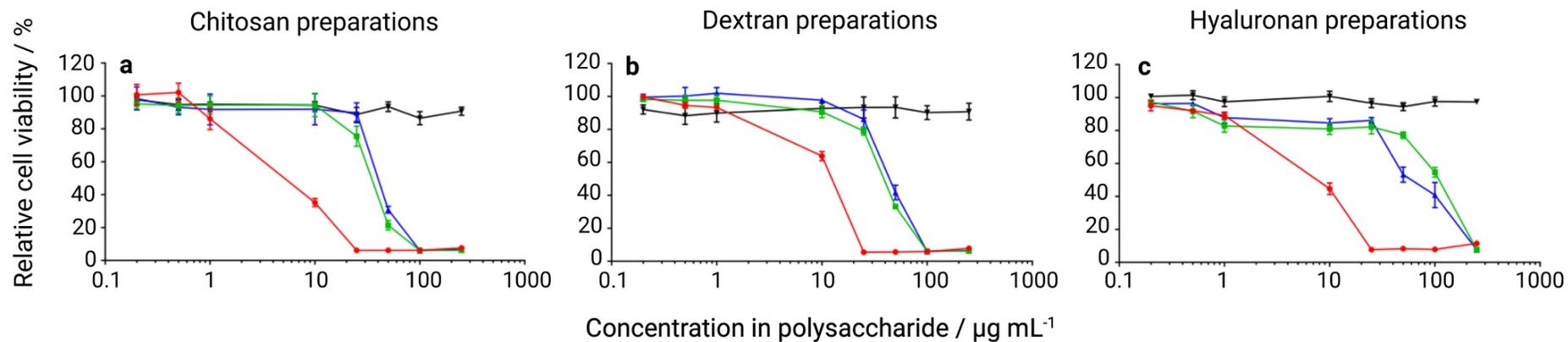
517 **Table 3:** IC₅₀ values (μg mL⁻¹) after 24-h-incubation of macrophages J774A.1 with polysaccharide
 518 preparations composed of chitosan, dextran, and hyaluronan. For each polysaccharide, the preparations
 519 were composed of solutions, nanospheres as prepared (NS), and nanospheres after incubation with
 520 PVA and purification (denoted as NS_{PVA}). The IC₅₀ values were obtained from relative cell viability
 521 curves in Figure 5.

Preparations		IC ₅₀ (μg mL ⁻¹)*
Chitosan preparations	Solution	> 250
	NS ^a	5.0
	NS _{PVA} ^b	34.2
	E-NM	39.5
Dextran	Solution	> 250
	NS ^a	12.3
	NS _{PVA} ^b	38.4
	E-NM	43.2
Hyaluronan	Solution	> 250
	NS ^a	7.5
	NS _{PVA} ^b	107.2
	E-NM	109.4

522 *: Macrophages RAW264.7

523 ^a: as prepared

524 ^b: NS were previously incubated with PVA/glycerol (10/2 wt%) solution and dialyzed. PVA hydrolysis
 525 degree was 87 - 90%.



526

527 **Figure 5:** Macrophage viability testing of polysaccharide preparations composed of chitosan (a), dextran (b), and hyaluronan (c). The preparations
 528 were composed of native polysaccharides (black q, corresponding to chitosan solution in (a), dextran solution in (b) and hyaluronan solution in
 529 (c)), NS without PVA (red l, corresponding to chitosan NS in (a), dextran NS in (b) and hyaluronan NS in (c)), NS after incubation with PVA,
 530 denoted as NS_{PVA} (green n, corresponding to chitosan NS_{PVA} in (a), dextran NS_{PVA} in (b) and hyaluronan NS_{PVA} in (c)), and elongated nanomaterials
 531 (blue p, corresponding to chitosan E-NM in (a), dextran E-NM in (b) and hyaluronan E-NM in (c)). The incubation duration was kept constant at
 532 24 h. Data are presented as the mean ± s.d. (*n* = 6 independent experiments).

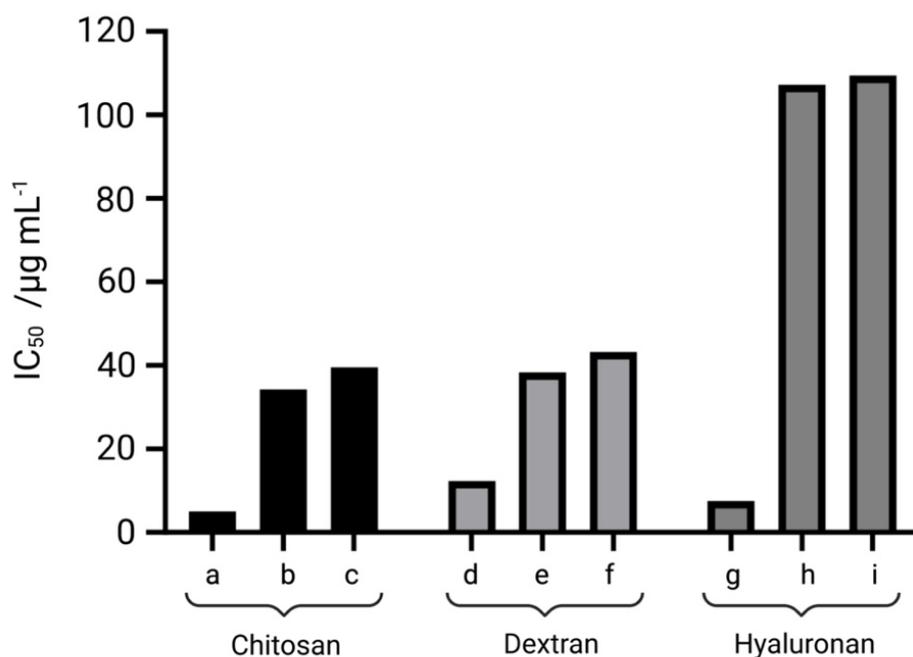


Figure 6: IC₅₀ values (µg mL⁻¹) after incubation of macrophages J774A.1 with polysaccharide preparations composed of chitosan (a,b,c), dextran (d,e,f), and hyaluronan (g,h,i). The preparations were composed of nanospheres as prepared (NS) without PVA (chitosan NS in (a), dextran NS in (d) and hyaluronan NS in (g)), NS after incubation with PVA, denoted as NS_{PVA} (chitosan NS_{PVA} in (b), dextran NS_{PVA} in (e) and hyaluronan NS_{PVA} in (h)). The incubation duration was kept constant at 24 h. Data are presented as the mean of $n = 6$ independent experiments). The IC₅₀ values were obtained from relative cell viability curves in Figure 5.

4.3. Cell internalization

The polysaccharide composition was kept constant, and NM morphology was modified for the following experiments. Hyaluronan was selected because its interaction with cells involves several types of receptors called hyaladherins. Hyaladherins include CD44,[44] CD38, RHAMM (receptor for hyaluronan-mediated motility, designated as CD168), stabilin-2,[45] lymphatic vessel endothelial hyaluronan receptor (LYVE-1), inter- α -inhibitor (I α I), and TSG-6 (tumor necrosis factor-stimulated gene-6). Internalization of hyaluronan NS_{PVA} and E-NM by macrophages was studied at three particle concentrations (0.1, 1, and 10 µg mL⁻¹). The results in Figure 7, presenting the internalization index before and after trypan blue quenching, revealed that NS_{PVA} and E-NM were internalized by macrophages for the tested concentrations. At concentrations of 0.1 µg mL⁻¹ and 1 µg mL⁻¹, before trypan blue quenching, internalization indexes of E-NM were in the same order of magnitude as NS_{PVA}. However, after adding trypan blue, internalization indexes of E-NM were reduced for both

concentrations, while internalization indexes of NS_{PVA} increased (Figure 7). At a high concentration of 10 $\mu\text{g mL}^{-1}$, E-NM adsorption to macrophages was higher than NS_{PVA}, while the internalization index drastically reduced for E-NM after trypan blue quenching. Those results could be due to the difference in the actin structures formed upon particle interaction with the macrophage surface. Indeed, among the four endocytosis mechanisms of NM (micropinocytosis, clathrin-mediated, caveolae-mediated, translocation), micropinocytosis is the most reported mechanism for NM, with a size ranging from 200 nm to 5 μm . [4, 46] Macropinocytosis is mostly initiated by actin-driven membrane ruffles (Figure 8.a).

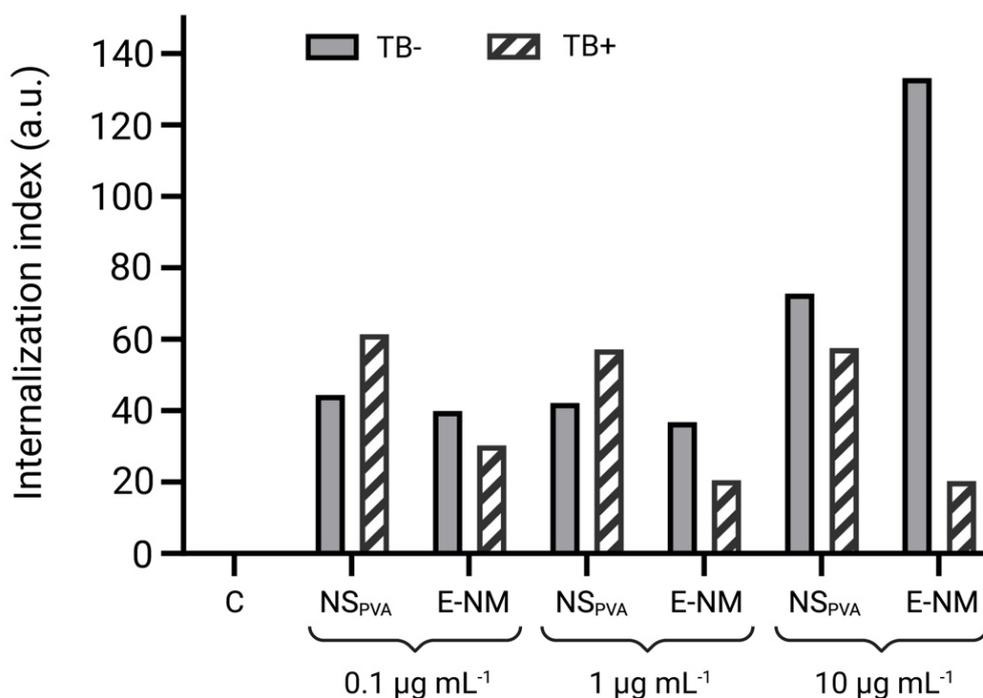


Figure 7: Effect of particle concentration and morphology on internalization index in macrophages without adding trypan blue (TB-) and after adding trypan blue (TB+). Macrophages were incubated 2 h with preparations composed of FITC-hyaluronan nanospheres after incubation with PVA and purification by dialysis (NS_{PVA}) and ellipsoidal nanomaterials (E-NM). The concentration of each preparation was varied as 0.1, 1, and 10 $\mu\text{g mL}^{-1}$. The internalization index was calculated by multiplying the percent of fluorescent cells with fluorescence intensity and normalized by taking the internalization index of cells without particles at 100%. Control experiment (C) comprised macrophage suspension without adding hyaluronan preparations. At least 50 cells were counted for each sample.

We hypothesize that E-NM could spread on the macrophage surface by particle long axis (Figure 8). Consequently, actin polymerized at points of contact failed to create the adequate actin structure necessary to engulf the particle (Figure 8). Another hypothesis to explain those findings is the surface

property of the E-NM. According to previous investigations,[47] the hypothesis of the organization of hyaluronan at the surface of the NS is given in the article of Coty and coworkers.[48] Film stretching technique could result from a stretching of hyaluronan located at the surface of the E-NM. Consequently, the interaction of hyaluronan with hyaladherins located on the macrophage surface could be lowered. Regardless of the phagocytosis mechanism of hyaluronan E-NM was reduced, limiting capture by macrophages could be advantageous since phagocytosis can create a substantial barrier to successful drug delivery. In some applications, macrophage internalization of particles is necessary.[49, 50] However, phagocytosis could prevent particles from releasing the encapsulated active drug near or within the desired cell population if macrophages are not the targeted cell type. Consequently, those findings could be exploited to reduce phagocytosis compared with conventional NS.

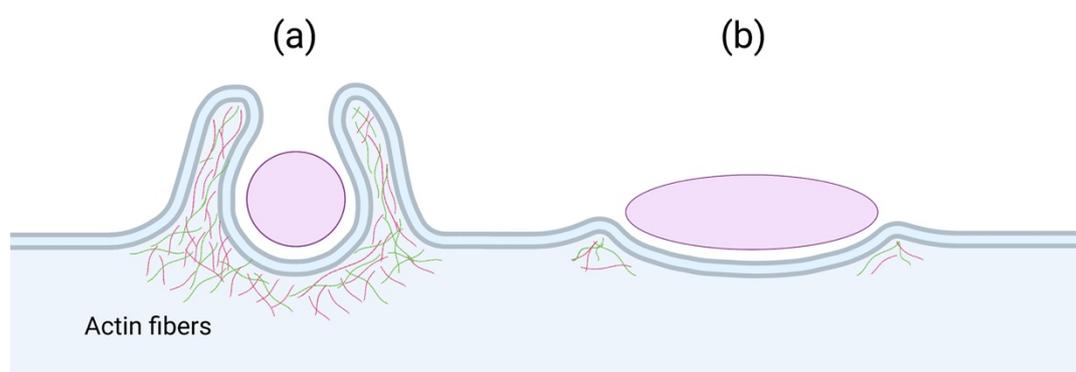


Figure 8: Schematic representation of hypothesis of the interaction of a nanosphere (a) and an ellipsoidal nanomaterial (b) with macrophage surface and involvement of actin fibers.

5. CONCLUSION

The investigation unveiled a correlation between NS surface potential, aspect ratio, and the interaction of PVA and the polysaccharides. Spherical and ellipsoidal NM were designed with surface properties adjusted using different polysaccharides (chitosan, dextran, and hyaluronan). The NM were labeled with FITC to track their internalization by cells. The FITC grafting on the NS surface resulted in modifications to the surface potential, while the size was relatively unchanged. Transmission electron microscopy analysis provided insights into the size and morphology, particularly for

ellipsoidal particles, revealing aspect ratio variations influenced by polysaccharide type and FITC grafting. Indeed, high PVA interaction with chitosan led to a high aspect ratio (4.9), while lower interaction resulted in a lower aspect ratio (2.4). Future investigations could predict the aspect ratio of ellipsoidal NM by studying the interaction of NS surfaces with the dispersing polymer.

Cytotoxicity studies demonstrated reduced toxicity after incubating NS with a PVA solution and subsequent purification, indicating the potential of PVA adsorption on NM surfaces to mitigate their toxic effects. Cell internalization experiments revealed that NS, after PVA incubation, were internalized by macrophages. However, ellipsoidal NM exhibited lower internalization than NS, despite having comparable surface potentials. This reduced phagocytosis of ellipsoidal NM may be attributed to their nonspherical morphology. This finding suggests a potential advantage for applications where macrophage internalization is undesirable. Future perspectives include visualizing the kinetics of NM interactions with cells, particularly NM with varying morphologies and surface properties. Developing stretchable polymers capable of interacting with NS surfaces would be interesting, allowing for their physical deformation during stretching. These polymers should be easily removable through conventional purification techniques to limit surface modification of stretched NM.

6. Funding

This work was supported by the Institut Universitaire de France, the ANR-17-CE09-0038 and ANR-21-CE09-0015.

7. Acknowledgements

The present work has benefited from the Imagerie-Gif core facility supported by l'Agence Nationale de la Recherche (ANR-11-EQPX-0029/Morphoscope, ANR-10-INBS-04/FranceBioImaging; ANR-11-IDEX-0003-02/Saclay Plant Sciences). The present work has benefited from the platform PIM of I2BC supported by French Infrastructure for Integrated Structural Biology (FRISBI) ANR-10-INBS-05. The authors thank Dr. Josh Mc Graw and Pr. Gilles Ponchel for scientific discussion and helpful advice.

8. Credit Author Statement

The manuscript was written through the contributions of all authors. All authors have given approval for the final version of the manuscript. ZA: Chemical modifications, formulation, characterization, data curation, formal and statistical analysis, writing original experimental section. HH: Chemical modifications, formulation, characterization, cell culture experiments, data curation, formal and statistical analysis, writing original experimental section. HK: Involvement in the data analysis and discussion. MB: Monitoring and data analysis of flow cytometry. K.B: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing - original draft; Writing - review & editing.

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Supporting data

1. Optimization of the PVA film formulation

Table S1: Optimization of PVA film formulation. Formulation parameters were modified by adjusting the degree of hydrolysis, molecular weight, and concentration of PVA, and glycerol concentration. Process parameters were modified by adjusting the dissolution temperature and duration of stirring. Stirring speed was maintained at 400 rpm. Film stretchability was ranked as follows: brittle if the film cannot be manipulated and stretchable if the film can be deformed at least 2 folds without breaking up to this pre-set deformation.

PVA hydrolysis degree	Molecular weight $\times 10^3 \text{ g mol}^{-1}$	[PVA] (wt%)	Glycerol (wt%)	Solubility of the preparation	T and stirring duration	Macroscopic aspect after drying	Handling	Detachment	Stretchability
80%	9 – 10	5	0	Soluble	20 °C, 3 h	Sticky, homogenous	+	Easy detachment	Brittle
			2	Soluble	20 °C, 3 h	Sticky, homogenous		Too sticky	Brittle
		10	0	Soluble	20 °C, 3 h	Sticky, homogenous		Easy detachment	Brittle
			2	Soluble	20 °C, 3 h	Sticky, homogenous		Too sticky	Brittle
		15	0.5	Soluble	20 °C, 3 h	Non sticky, homogenous		Easy detachment,	Rigid
			1	Soluble	20 °C, 3 h	Non sticky, homogenous		Easy detachment	Rigid
			2	Soluble	20 °C, 3 h	Non sticky, homogenous		Easy detachment	Slightly rigid
			0	Soluble	20 °C, 3 h	Non sticky, homogenous		Easy detachment	Tough
		20	0.5	Soluble	20 °C, 3 h	Non sticky, homogenous		Easy detachment	Good to stretching
			1	Soluble	20 °C, 3 h	Non sticky, homogenous		Easy detachment	Good stretching
			2	Soluble	20 °C, 3 h	Non sticky, homogenous		Easy detachment	Stretchable but rigid
		87 – 90%	30 – 70	5	2	Soluble		20 °C, 6 h	Homogenous
10	2			Soluble	20 °C, 6 h	Homogenous	Easy detachment	Good stretching	
99%	89 – 98	5	2	Insoluble	40 °C, 6 h	Heterogenous, rough	-	Difficult detachment	Breaks during stretching
		5	2	Insoluble	50 °C, 6 h				
		5	2	Soluble	75 °C, 6 h	Homogenous smooth	+	Easy detachment	Good stretching
		10	2	Soluble	75 °C, 6 h				
100%	89 – 98	5	0	Insoluble	40 °C, 12 h	Heterogenous, rough	+	Difficult detachment	Breaks during stretching
		5	2	Insoluble	40 °C, 12 h		-		
		10	0	Insoluble	40 °C, 12 h		+		
		10	2	Insoluble	40 °C, 12 h		-		
		5	2	Soluble	75 °C, 12 h	Homogenous, smooth	+	Easy detachment	Good stretching
		10	2	Soluble	75 °C, 12 h				

(+): Recovered in one piece; (-): Cracks on film

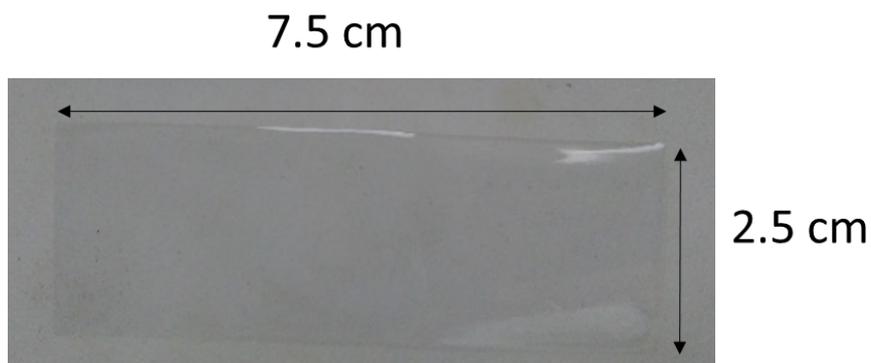


Figure S1: Photo of 87 – 90% hydrolysed PVA film before stretching. Film forming solution: 10 wt% PVA powder and 2 wt% glycerol.

2. Chemical structures of chitosan, dextran, hyaluronan and polyvinyl alcohol

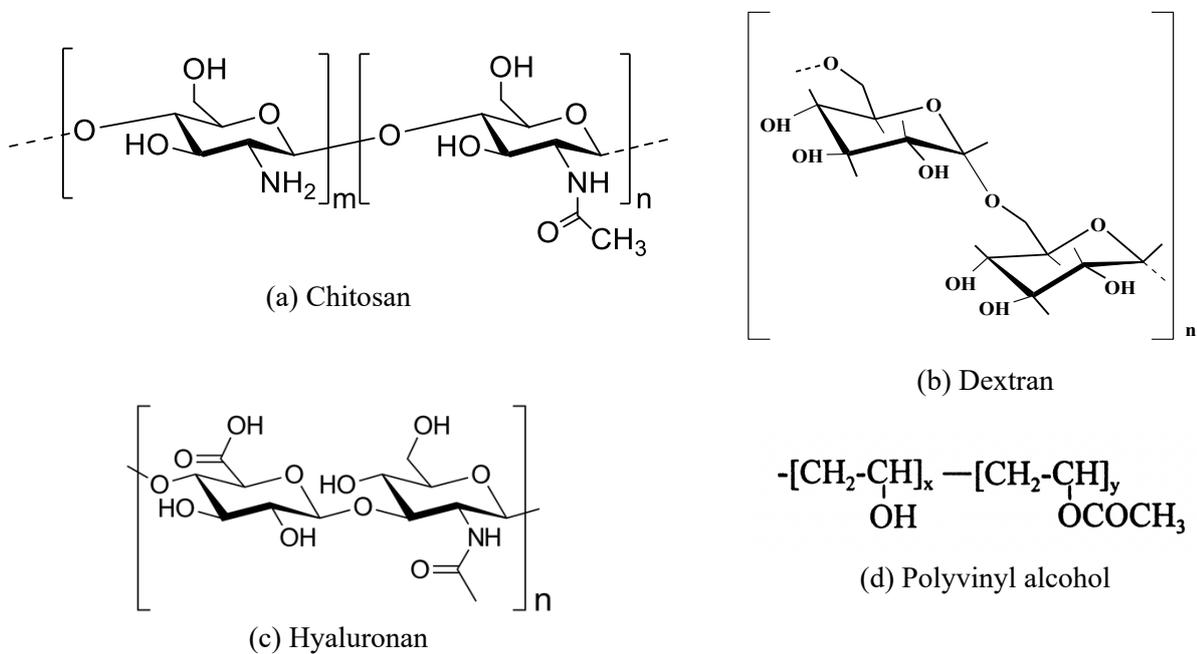


Figure S2: Chemical structures of chitosan (a), dextran (b), hyaluronan (c), and polyvinyl alcohol (d). In (d), the degree of hydrolysis was calculated according to the equation Eq.S1 where x and y are the molar fractions of the hydroxyl and the acetate groups.

$$\text{PVA degree of hydrolysis} = \frac{x}{x+y} \quad \text{Eq.S1}$$

3. ITC

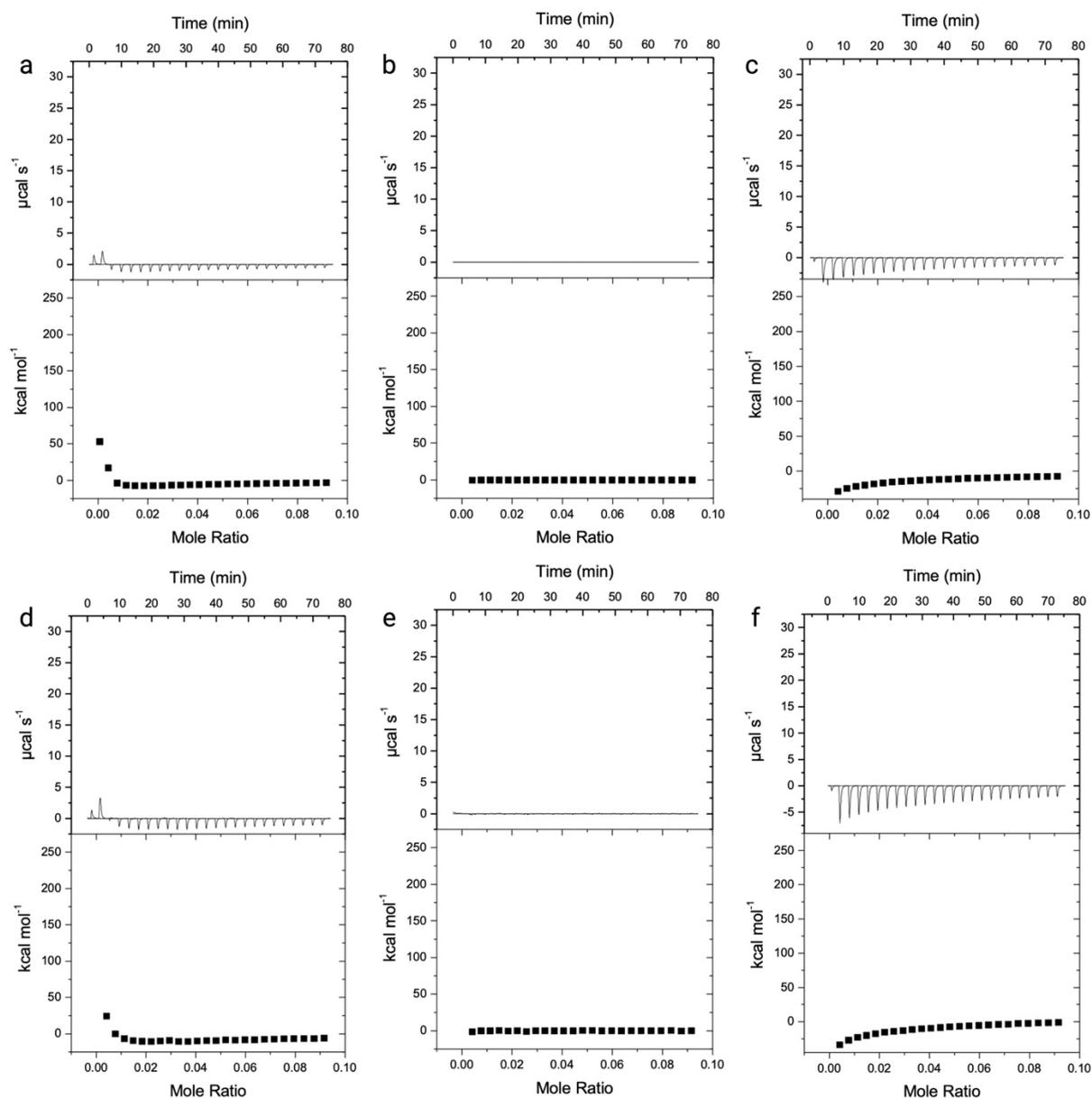


Figure S3: ITC titration curves and enthalpies of dilution of CS (a,d), DEX (b,e), and HA (c,f) in water. The concentration of CS, DEX, and HA in the syringe was 0.5 mM. Water was placed in the titration cell. The temperature of the experiment was maintained constant at 20 °C (a,b,c) or 50 °C (d,e,f).