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# Thermoresponsive nanogels based on polyelectrolyte complexes between polycations and functionalized hyaluronic acid

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1 **Thermoresponsive nanogels based on polyelectrolyte complexes**  
2 **between polycations and functionalized hyaluronic acid**

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18 **ABSTRACT**

19 A novel kind of thermoresponsive polyelectrolyte complex-based nanogels (PEC-NGs) was  
20 elaborated by mixing hyaluronic acid (HA) functionalized with Jeffamine® M-2005 (M2005, a  
21 thermoresponsive amine-terminated polyether) and diethylaminoethyl dextran (DEAE-D) or  
22 poly-L-lysine (PLL) in water. The presence of M2005 grafts led to PEC-NGs with larger particle  
23 size, lower net surface charge and thermoresponsiveness, namely shrinkage with increasing  
24 hydrophobicity at higher temperature. Both M2005 grafts and replacing DEAE-D with PLL as  
25 polycation allowed PEC-NGs to have higher stability against salinity and better encapsulation of  
26 curcumin, most probably through intraparticle hydrophobic interactions, whereas interparticle  
27 hydrophobic interactions may facilitate particle aggregation over time. Curcumin encapsulation  
28 can be optimized by applying higher temperature during the complexation. Enzymatic  
29 degradability of PEC-NGs was also verified through particle size evolution in the presence of  
30 hyaluronidase. These results provide new insights into the physicochemical aspect of such  
31 systems as promising nanocarriers for drug delivery.

32

33 **Keywords:** nanogels; polyelectrolyte complexes; thermoresponsive; hyaluronic acid; Jeffamine®  
34 M-2005

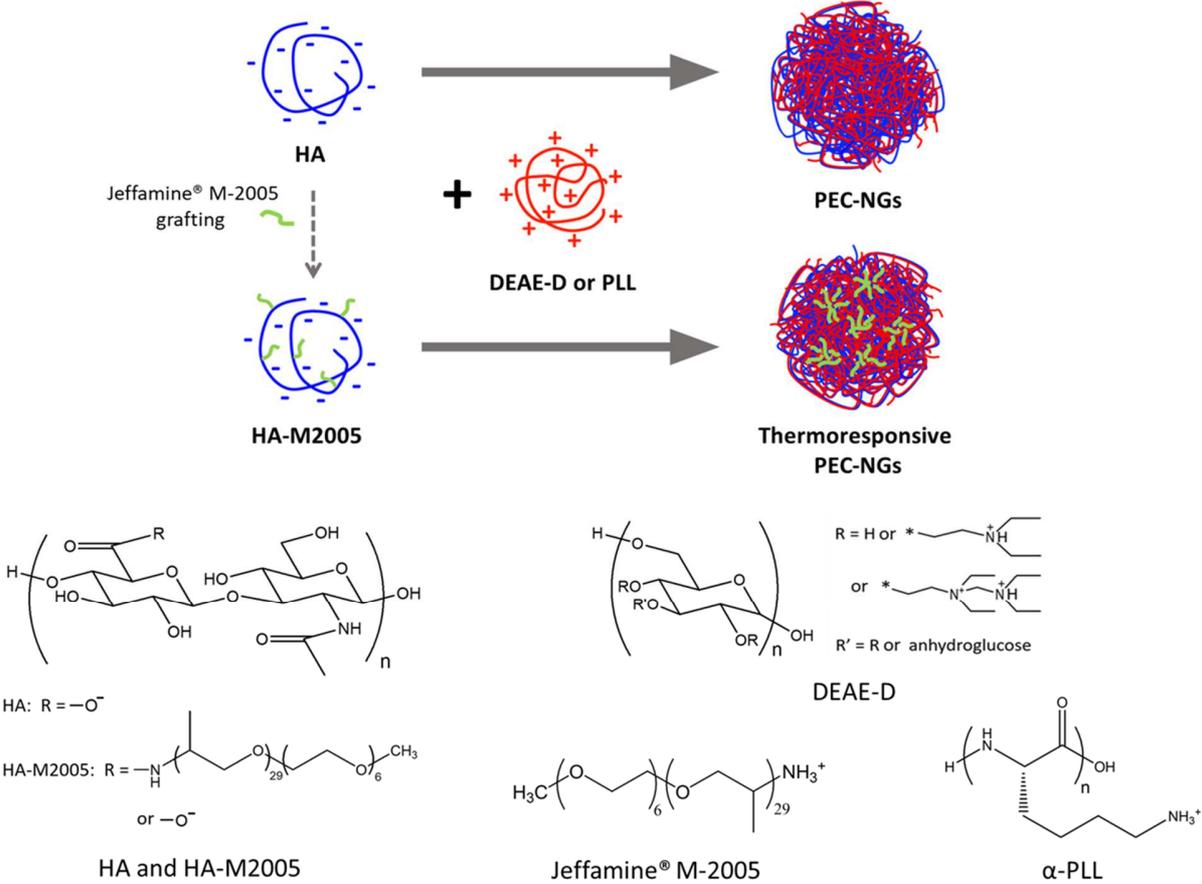
## 35 1. INTRODUCTION

36 Nanogels (NGs) based on polyelectrolyte complexes (PECs) can be formed spontaneously  
37 through ion pairing between polyanions and polycations upon simple mixing in aqueous media  
38 (Bonaccorso et al., 2021). Particularly, PEC-based NGs (PEC-NGs) from polysaccharides have  
39 attracted a great deal of interest as drug delivery systems (DDS) due to their biocompatibility and  
40 simple preparation requiring neither high energy nor organic solvents (Debele, Mekuria, & Tsai,  
41 2016).

42 Thermoresponsive nanocarriers have received increasing attention as robust DDS (Bordat,  
43 Boissenot, Nicolas, & Tsapis, 2019). They can be elaborated from thermoresponsive copolymers,  
44 which display a temperature-dependent hydrophilic-hydrophobic balance due to difference in  
45 solvation status between component monomers at different temperatures (Bordat et al., 2019;  
46 Gandhi, Paul, Sen, & Sen, 2015). Among them, polymers disposing lower critical solution  
47 temperature (LCST), which are water-soluble below a critical temperature but become insoluble  
48 at a higher temperature due to higher hydrophobicity, have been extensively studied and  
49 employed in DDS (Karimi et al., 2016). Nanoparticles from these polymers can show shrinkage  
50 along with higher hydrophobicity at higher temperature, leading to applications in either  
51 triggered-release or sustained-release of drugs (Karimi et al., 2016; Kim et al., 2019).  
52 Accordingly, employing thermoresponsive derivatives of polysaccharides (i.e. polysaccharides  
53 grafted with suitable LCST groups) in DDS has also become an emerging topic of research since  
54 they can improve the encapsulation and release of hydrophobic drugs while conserving the  
55 advantages of precursor biopolymers (Graham, Marina, & Blencowe, 2019).

56 From such perspectives, systems combining both the abovementioned aspects, i.e. PEC-NGs  
57 from thermoresponsive polysaccharide derivatives, would be of high interest as DDS, yet have

58 not been reported in the literature to the best of our knowledge. In this context, our work aimed  
 59 to elaborate and characterize such a novel kind of DDS from readily available materials, namely  
 60 HA-M2005 which is hyaluronic acid (HA) functionalized with Jeffamine® M-2005 (M2005)  
 61 and diethylaminoethyl dextran (DEAE-D) or poly-L-lysine (PLL) (**Figure 1**).



62  
 63 **Figure 1.** Scheme of PEC-NG formation from polyanions (blue) (HA: hyaluronic acid or HA-  
 64 M2005: hyaluronic acid functionalized with Jeffamine® M-2005 (green)) electrostatically  
 65 complexed with polycations (red) (DEAE-D: diethylaminoethyl dextran or PLL: poly-L-lysine)  
 66 and their chemical structures.

67 HA is an anionic polysaccharide extensively employed in DDS development due to its excellent  
68 biocompatibility and biodegradability (Necas, Bartosikova, Brauner, & Kolar, 2008). CD44  
69 receptors, which are natural receptors of HA, and hyaluronidase (HAase), which is a family of  
70 enzymes catalyzing HA degradation, are both overexpressed in many cancer cells, e.g. in breast  
71 and brain tumors (Stern, 2008; Xu, Niu, Yuan, Wu, & Liu, 2020). This renders HA-based nano-  
72 DDS particularly interesting for drug targeting since they can be specifically uptaken and  
73 degraded to release drugs inside these cells (Yang, Zhang, Chen, Chen, & Liu, 2016). M2005 is a  
74 commercial diblock copolyether monoamine made up of amine-terminated poly(propylene  
75 oxide) (PPO) and poly(ethylene oxide) (PEO), constituting a thermoresponsive copolymer with  
76 LCST temperature of 14-30 °C (Agut, Brûlet, Taton, & Lecommandoux, 2007; Dulong, Mocanu,  
77 Picton, & Le Cerf, 2012; Niang et al., 2016). In our previous work, M2005 was successfully  
78 grafted on HA to obtain HA-M2005 as a thermoresponsive HA derivative with a sol-gel  
79 transition depending on temperature (Madau, Le Cerf, Dulong, & Picton, 2021). DEAE-D is a  
80 polycation with cationic DEAE groups grafted on dextran, a natural polysaccharide extracted  
81 from plants or microbial fermentations (Díaz-Montes, 2021; Gulick, 1997). It is mainly used as  
82 means for nucleic acids transfection or proteins delivery with good biocompatibility and high  
83 solubility (Gulick, 1997; Huguet, Neufeld, & Dellacherie, 1996; Manosroi & Manosroi, 1997).  
84 PLL is a biocompatible and biodegradable cationic polyaminoacid, which is largely used as  
85 coating agent for cell adhesion promotion (Huguet et al., 1996). PLL coating can also increase  
86 cell uptake of nanoparticles, which would improve therapeutic efficacy in targeted nanomedicine  
87 (Siow et al., 2018).

88 Previously, our work has shown that mixing of HA and DEAE-D solutions can lead to formation  
89 of HA/DEAE-D PEC-NGs having mean size of 150-350 nm with relatively hydrophobic cores

90 surrounded by hydrophilic corona shells of polar uncomplexed polyelectrolyte segments (Le,  
91 Dulong, Picton, & Le Cerf, 2021). They can have good shelf stability in water but generally poor  
92 stability against salt and slight hydrophobicity (Le et al., 2021). Our hypothesis is that PEC-NGs  
93 prepared with HA-M2005 should present more interesting properties, such as dual-  
94 responsiveness (thermoreponsiveness beside enzymatic-induced degradation), than with non-  
95 modified HA. In this work, the effects of M2005 grafts and polycation structure on HA-based  
96 PEC-NGs were elucidated after their preparation and characterization in terms of morphology,  
97 particle size, zeta potential ( $\zeta$ ) and hydrophobicity with varying parameters, including constituent  
98 polymers (**Figure 1**), temperature, salinity and storage time. Encapsulation of curcumin as a  
99 model drug was realized to evaluate the potential of these systems as nanocarriers for delivering  
100 poorly water-soluble drugs. Enzymatic degradation of such PEC-NGs was also investigated and  
101 confirmed by observing particle size in the presence of HAase.

## 102 **2. MATERIALS AND METHODS**

### 103 **2.1. Materials**

104 HA and Jeffamine® M-2005 were kindly provided by Givaudan (France) and Huntsman (USA)  
105 respectively. DEAE-D hydrochloride and  $\alpha$ -PLL hydrobromide were purchased from Sigma-  
106 Aldrich (USA). HA-M2005 was obtained by grafting Jeffamine® M-2005 to HA (degree of  
107 substitution of 3.6 %) through EDC/NHS coupling reaction (Supplementary Information **SI-1**) as  
108 reported in our earlier study (Madau et al., 2021).

109 **Table 1** summarizes macromolecular characteristics of the polyelectrolytes (Supplementary  
110 Information **SI-2**), with those of HA and DEAE-D retrieved from our previous work (Le et al.,  
111 2021). Pyrene was purchased from Acros Organics (USA). Curcumin, phosphate-buffered saline  
112 (PBS) tablets and HAase from sheep testes (400 IU.mg<sup>-1</sup>) were purchased from Sigma-Aldrich  
113 (USA). Water purified by Milli-Q system (Millipore, USA) was used for all experiments.

114 **Table 1.** Characteristics of polyelectrolytes

Polyelectrolyte	M <sub>n</sub> (g.mol <sup>-1</sup> )	M <sub>w</sub> (g.mol <sup>-1</sup> )	Đ
HA	210,000	270,000	1.3
DEAE-D	270,000	700,000	2.6
PLL	200,000	490,000	2.5
HA-M2005	180,000	240,000	1.3

M<sub>n</sub>: number average molecular weight; M<sub>w</sub>: weight average molecular weight; Đ: dispersity

## 115 **2.2. Preparation of PEC-NGs**

116 PEC-NGs were prepared following the same method as our previous work (Le et al., 2021).  
117 Polyelectrolyte solutions at defined total polymer concentrations (C<sub>p</sub>, excluding counterions)  
118 were prepared beforehand by dissolving polyelectrolytes in Milli-Q water under stirring  
119 overnight at room temperature and afterwards filtered through regenerated cellulose 0.45 μm  
120 membrane filter units (Sartorius, Germany). Each PEC-NG suspension at a definite C<sub>p</sub> and n-/n+  
121 (molar ratio of negative to positive charges on component polyelectrolytes) was obtained by one-  
122 shot addition of polyanion to polycation solutions of the same C<sub>p</sub> with the volume ratio deduced  
123 from n-/n+ (Supplementary Information **SI-3**), followed by 30 min of stirring at room  
124 temperature. At least three batches were prepared for each formulation.

125 For preparing curcumin-loaded NGs, before polycation addition, there was an additional step of  
126 dropping an amount of curcumin 5 g.L<sup>-1</sup> stock solution in acetone to the polyanion solution. The

127 ratio of curcumin to polyelectrolytes was fixed at 2/5 m/m. After 30 min of stirring at studied  
128 temperatures (25 °C, 50 °C or 70 °C), all samples were left at room temperature over 12 h for  
129 acetone evaporation. The precipitated non-encapsulated curcumin was then removed by  
130 centrifugation at  $3,000\times g$  for 15 min and the supernatant was filtered through polyvinylidene  
131 difluoride (PVDF) 0.45  $\mu\text{m}$  membrane filter units (Millipore, Germany) to obtain the filtrate as  
132 PEC-NG suspension. Samples without polymers were also prepared as reference. All samples  
133 were protected from light to avoid curcumin degradation.

### 134 **2.3. Particle size, zeta potential ( $\zeta$ ) and morphology analyses**

135 Particle size and  $\zeta$  were characterized only for visually homogeneous NG suspensions without  
136 further dilution using Zetasizer Ultra (Malvern Panalytical, UK). Mean hydrodynamic diameter  
137 ( $D_h$ ) and polydispersity index (PDI) were determined by dynamic light scattering (DLS) with  
138 back-scatter mode.  $\zeta$  was measured by electrophoretic light scattering. Every sample was  
139 measured at least in triplicate after stabilization for 120 s at 25 °C or 300 s at 37 °C or 50 °C.

140 The morphologies of PEC-NGs were examined using a transmission electron microscope (TEM)  
141 FEI Tecnai 12 BioTwin (Philips, The Netherlands) operating at 80 kV. For specimen  
142 preparation, 10  $\mu\text{L}$  of a PEC-NG suspension was placed on a formvar and carbon coated copper  
143 grid for 15 min for adhesion of PEC-NGs on the grid and the excess suspension was then  
144 removed by filter paper. 10  $\mu\text{L}$  of phosphotungstic acid 1% solution was thereafter deposited on  
145 the grid for contrast enhancement and wicked away after 30 s, followed by air-drying of the grid  
146 before observation.

147 **2.4. Pyrene fluorescence**

148 Samples for pyrene fluorescence analyses were prepared as previously described (Le et al.,  
149 2021). 1 mL of pyrene  $8 \times 10^{-7}$  mol.L<sup>-1</sup> aqueous solution was mixed with an equal volume of  
150 PEC-NG suspensions ( $C_P = 0.5$  g.L<sup>-1</sup>) under stirring overnight. Fluorescence emission spectra of  
151 samples upon excitation at 335 nm wavelength were recorded at certain temperatures (25 °C, 37  
152 °C or 50 °C) using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon, France). The ratio  
153  $I_I/I_{III}$  was calculated as intensity ratio of the first to the third vibronic bands recorded respectively  
154 at 373 and 383 nm. All measurements were done at least in triplicate.

155 **2.5. Stability studies**

156 Stability of PEC-NGs in Milli-Q water and physiological salines (NaCl 0.9% eq. 0.154 mol.L<sup>-1</sup>  
157 and PBS) was evaluated (Dioury et al., 2021). To reach NG suspensions having final salinity of  
158 NaCl 0.9% or PBS 1X, 80  $\mu$ L NaCl 4 mol.L<sup>-1</sup> or 52  $\mu$ L PBS 40X was added to 2 mL PEC-NG  
159 suspension ( $C_P$  variation assumed to be inconsiderable). All samples were then vortexed and left  
160 for 30 min before DLS analysis at 25 °C, then stored at 4 or 25 °C for revaluation by DLS after  
161 1, 3, 7 and 30 days. pH measurements for certain samples were done using a pH meter (Mettler  
162 Toledo, Switzerland).

163 **2.6. Enzymatic degradation studies**

164 NG degradation was studied through particle size evolution in the presence of HAase (Yang et  
165 al., 2016). Firstly, HAase 30 and 3 mg.mL<sup>-1</sup> solutions were prepared by dissolving HAase in  
166 Milli-Q water. 10  $\mu$ L of these solutions was then vortexed with 1 mL of PEC-NG suspensions of  
167 interest to reach final HAase concentrations of 0.3 or 0.03 mg.mL<sup>-1</sup>. All samples, including  
168 samples without HAase as reference, were then analyzed by DLS at predetermined time points  
169 during their incubation at 37 °C.

## 170 **2.7. Curcumin quantification**

171 Curcumin quantification method was adapted from other studies with some modifications  
172 (Gómez-Mascaraque, Sipoli, de La Torre, & López-Rubio, 2017; Sarika & James, 2016).  
173 Briefly, 200 mL PEC-NG suspension was vortexed with 20 mL of HCl 1 N to protonate HA and  
174 facilitate PEC disintegration (Kayitmazer, Koksall, & Iyilik, 2015). The mixture was then  
175 vortexed with 1.1 mL (880 mg) of ethanol for curcumin solubilization. The precipitated polymer  
176 was then separated by centrifugation at  $3,000 \times g$  for 15 min. The curcumin concentration  $C$  in  
177 the supernatants ( $\mu\text{g}\cdot\text{mL}^{-1}$ ) was determined by UV-visible spectrophotometer Cary 100 (Agilent  
178 Technology, USA) with a standard curve of absorbance at 420 nm ( $A_{420}$ ) in function of curcumin  
179 concentration between 0.05-10  $\mu\text{g}\cdot\text{mL}^{-1}$  in water/HCl 1N/EtOH 10/1/55 v/v ( $A_{420} = 0.1407 \times C -$   
180  $0.0022$  with  $R^2 = 0.9999$ ). Curcumin concentrations in the starting PEC-NG suspensions  
181 ( $C_{\text{CUR/SUS}}$ ) were accordingly deducted, with densities of PEC-NG suspensions and the  
182 supernatant (EtOH/aqueous phase 5/1 v/v or 4/1 m/m) at room temperature being 1 and 0.84  
183  $\text{g}\cdot\text{mL}^{-1}$  respectively (Rumble, 2021). The loading capacity (LC) and encapsulation efficiency  
184 (EE) were calculated as followed:

$$185 \quad \text{LC} = (C_{\text{CUR/SUS}} - C_{\text{CUR/WATER}})/C_{\text{P}} \times 100 (\%) \quad (\text{Equation 1})$$

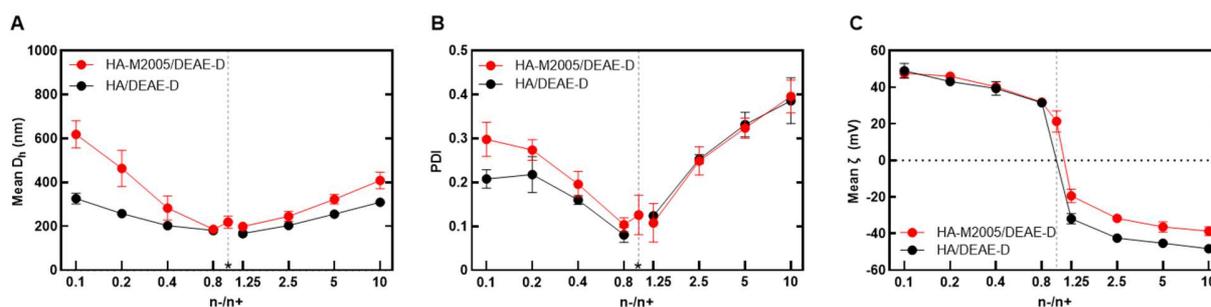
$$186 \quad \text{EE} = (C_{\text{CUR/SUS}} - C_{\text{CUR/WATER}})/C_{\text{CUR0}} \times 100 (\%) \quad (\text{Equation 2})$$

187 with  $C_{\text{CUR/WATER}}$  as curcumin concentration of reference sample, which contained solely water  
188 saturated in curcumin,  $C_{\text{P}}$  as polymer concentration and  $C_{\text{CUR0}}$  as the concentration of added  
189 curcumin with respect to the aqueous phase during the preparation.

190 **3. RESULTS AND DISCUSSION**

191 **3.1. PEC-NG initial characterization**

192 PEC-NGs were generated by simply mixing polyanion with polycation solutions, which was  
193 spontaneous *via* electrostatic interactions since no chemical reaction was observed from their  
194 FT-IR spectra (**Figure S3**). Such preparation process is thus simple and rapid, requiring no  
195 organic solvent, chemical agent or high energy. For clarifying the effect of M2005 grafting,  
196 **Figure 2** shows particle properties of HA-M2005/DEAE-D PEC-NGs at  $C_P = 0.5 \text{ g.L}^{-1}$  after 30  
197 min of complexation, compared to those of HA/DEAE-D PEC-NGs from our previous study (Le  
198 et al., 2021).

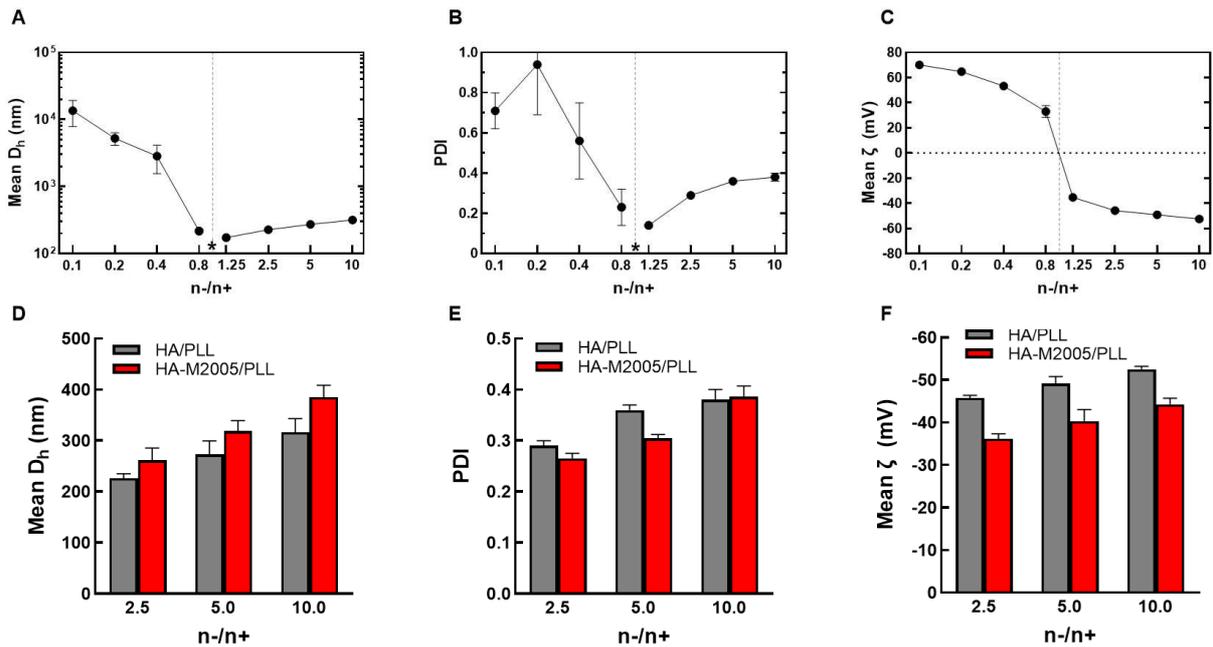


199 **Figure 2.** Mean  $D_h$  (A), PDI (B) and mean  $\zeta$  (C) measured at 25 °C of HA/DEAE-D and HA-  
200 M2005/DEAE-D PEC-NGs ( $C_P = 0.5 \text{ g.L}^{-1}$  in Milli-Q water) after 30 min of complexation.  
201 Asterisk represents  $n-/n+ = 1$ , at which precipitation was observed for HA/DEAE-D PEC-NGs.  
202 Data are presented as mean  $\pm$  standard deviation ( $n \geq 3$ ).  
203

204 As seen from **Figure 2A-B**, homogeneous HA-M2005/DEAE-D PEC-NGs samples have mean  
205  $D_h$  of 200-600 nm and PDI of 0.1-0.4. However, such  $D_h$  is always larger than that of  
206 HA/DEAE-D PEC-NGs at the same n-/n+ (**Figure 2A**). This may stem from steric effects of the  
207 bulky M2005 grafts inside PEC-NGs. It should also be noticed that for the same n-/n+, the mass

208 ratio of polyanion to polycation in HA-M2005/DEAE-D PECs was higher than in HA/DEAE-D  
209 PECs to compensate the loss of negative charges after M2005 grafting, which may also  
210 contribute to the difference in their particle sizes. Furthermore, the difference in  $D_h$  and PDI  
211 between the two kinds of PEC-NGs is more obvious at  $n-/n+ < 0.8$ , where DEAE-D exceeds HA  
212 (**Figure 2A-B**). As discussed in our previous work, at  $n-/n+ < 0.8$ , PECs from intermediate  
213 molecular weight HA and high molecular weight DEAE-D showed rapid aggregation continuing  
214 after 30 min of complexation, being ascribed to hydrophobic segregation of unstable short  
215 double-stranded PEC segments (Le et al., 2021). With M2005 grafts, this effect may be enhanced  
216 due to higher hydrophobicity of such segments and hence generate much larger secondary PECs.  
217 Regarding net surface charge, HA-M2005/DEAE-D and HA/DEAE-D PEC-NGs show quite a  
218 similar curve of mean  $\zeta$  in function of  $n-/n+$  (**Figure 2C**), except the less negative  $\zeta$  observed at  
219  $n-/n+ \geq 1$  for PECs possessing M2005 grafts. This could be attributed to shrinkage of HA  
220 backbone towards particle interior due to hydrophobic interactions of M2005 on HA and  
221 therefore its negative charges are less accessible on NG surface. Mean  $\zeta$  of PECs of  $n-/n+ = 1$  is  
222 hence deviated away from zero when HA-M2005 replaces HA (**Figure 2C**), rendering  
223 precipitation absent at stoichiometric  $n-/n+$  as observed but assumedly present between  $n-/n+$  of  
224 1 and 1.25.

225 For preparing HA/PLL PEC-NGs, we also used intermediate molecular weight HA with PLL of  
226 higher molecular weight. Such PEC-NGs also present physicochemical parameters (**Figure**  
227 **3A-C**) following the same trends in function of  $n-/n+$  as HA/DEAE-D PEC-NGs (**Figure 2A-C**).



228

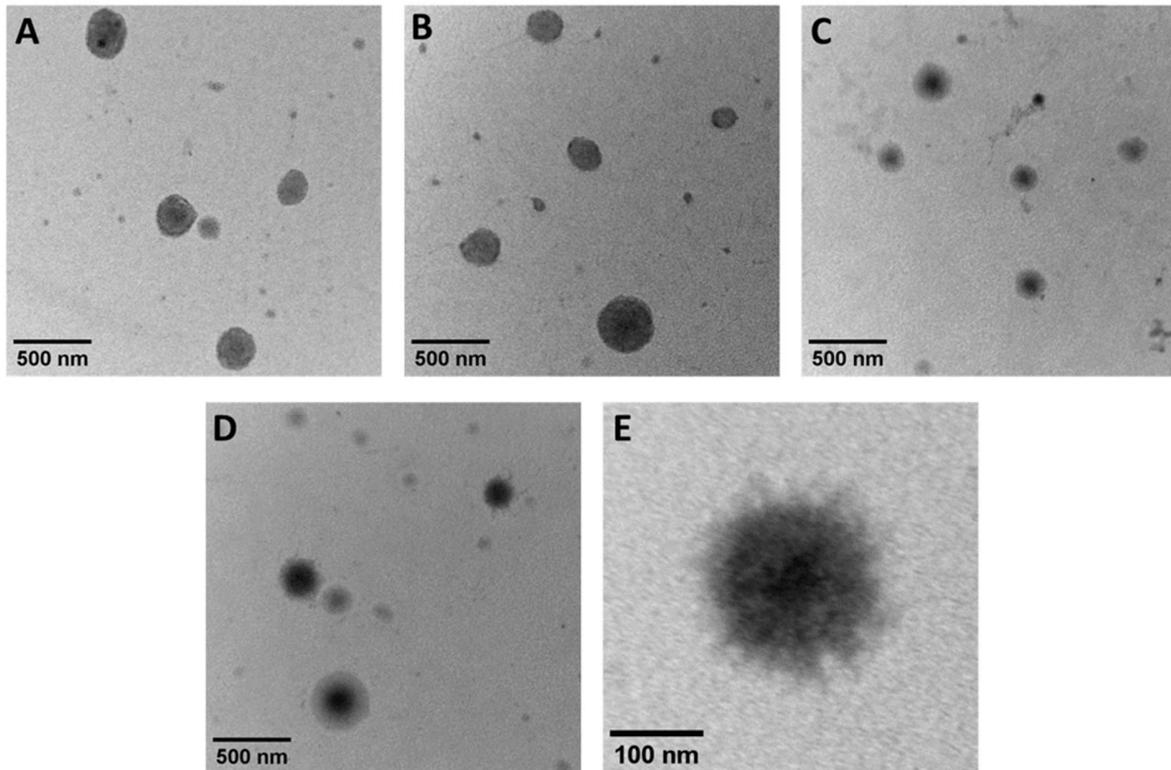
229 **Figure 3.** Mean  $D_h$ , PDI and mean  $\zeta$  of HA/PLL PEC-NGs (A-C respectively) and of HA-  
 230 M2005/PLL PEC-NGs in comparison with HA/PLL PEC-NGs (D-F respectively) at  $C_p = 0.5$   
 231  $g \cdot L^{-1}$  in Milli-Q water after 30 min of complexation. Asterisk represents precipitation at  
 232  $n-/n+ = 1$  (DLS not performed). Data are presented as mean  $\pm$  standard deviation ( $n \geq 3$ ).

233 HA/PLL PEC-NGs at  $n-/n+ > 1$  exhibited consistent characteristics with mean  $D_h$  between  
 234 100-300 nm and PDI  $< 0.4$ . In contrast, at  $n-/n+ < 0.8$ , unusually large  $D_h$  (1000-10,000 nm) and  
 235 PDI (higher than 0.5) were remarked (**Figure 3A-B**). These results approach the upper limit of  
 236 reliability for DLS and should not be considered as accurate characteristics but rather a relative  
 237 indication of large aggregates uncontrolled in size. PEC-NGs at  $n-/n+$  of 0.8, despite their more  
 238 relevant  $D_h$  (around 200 nm), still showed low batch-to-batch consistency in PDI (**Figure 3B**).  
 239 Large aggregates were also reported for HA/PLL PECs of  $n-/n+ < 1$  with increasing particle size  
 240 over time, from 400 to 800-1000 nm after 8 h (Pan et al., 2019). Such aggregation is probably  
 241 caused by stronger intercomplex hydrophobic interactions between PECs containing mainly

242 PLL, which has been known to be considerably hydrophobic upon charge neutralization due to  
243 the four succeeding methylene groups in lysine units (Dyson, Wright, & Scheraga, 2006; Pan et  
244 al., 2019).

245 Considering the results of HA/PLL PEC-NGs, for HA-M2005/PLL PEC-NGs, we focused only  
246 on  $n-/n+ > 1$  due to their more consistent and measurable properties. However, for  $n-/n+$  of 1.25,  
247 precipitation was observed and DLS characterization was not performed. For the other  $n-/n+$ ,  
248 M2005 grafts seem to display the same impact as in HA/DEAE-D PECs (**Figure 3D-F** vs.  
249 **Figure 2A-C**), i.e. increasing  $D_h$  and reducing  $\zeta$ . Accordingly, this may explain the observed  
250 precipitation at  $n-/n+$  of 1.25 since the readily low net surface charge at such  $n-/n+$  can be further  
251 reduced and thus facilitate aggregation.

252 For further characterization of PEC-NGs, the morphologies of all the four types of PEC-NGs at  
253  $n-/n+ = 2.5$  and  $C_p = 0.5 \text{ g.L}^{-1}$  were also examined by TEM (**Figure 4**), which verified the  
254 formation of PEC-NGs as particles having generally spherical shape and particle size in  
255 coherence with DLS results. Particle morphologies seem to be distinct depending on the  
256 constituent polycation, while M2005 grafts led to no visible difference in this aspect. At higher  
257 magnification, some particles can clearly show their structure comprising a dense core  
258 surrounded by a corona shell (**Figure 4E**), which confirms the suggested core-shell structure of  
259 PEC-NGs.

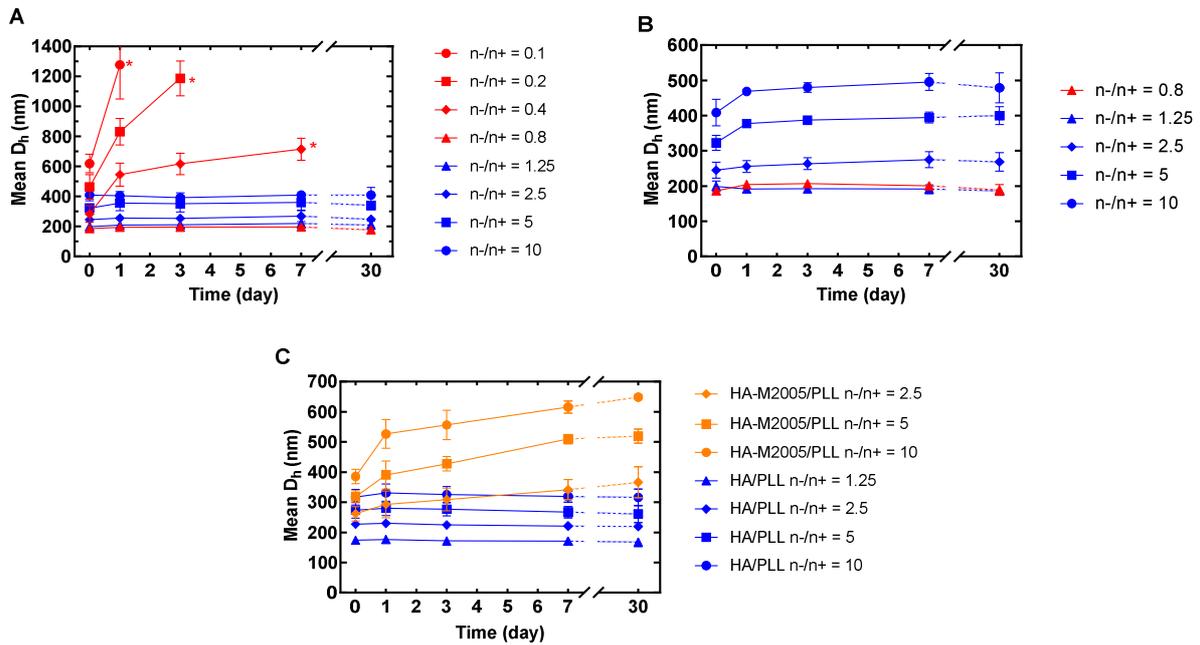


260

261 **Figure 4.** TEM images of PEC-NGs from HA/DEAE-D (A), HA-M2005/DEAE-D (B), HA/PLL  
 262 (C and E) and HA-M2005/PLL (D) at  $n^-/n^+ = 2.5$  and  $C_p = 0.5 \text{ g.L}^{-1}$ .

263 **3.2. Stability studies**

264 Particle sizes of different PEC-NGs at  $C_p = 0.5 \text{ g.L}^{-1}$  were monitored during one month in  
 265 different conditions (i.e. storage temperature, saline and buffered media) to evaluate the  
 266 influence of M2005 grafts and polycation nature on their stability. Stability of mean  $D_h$  is shown  
 267 in **Figure 5** and their PDIs with the same trend can be consulted from **Figure S4**.



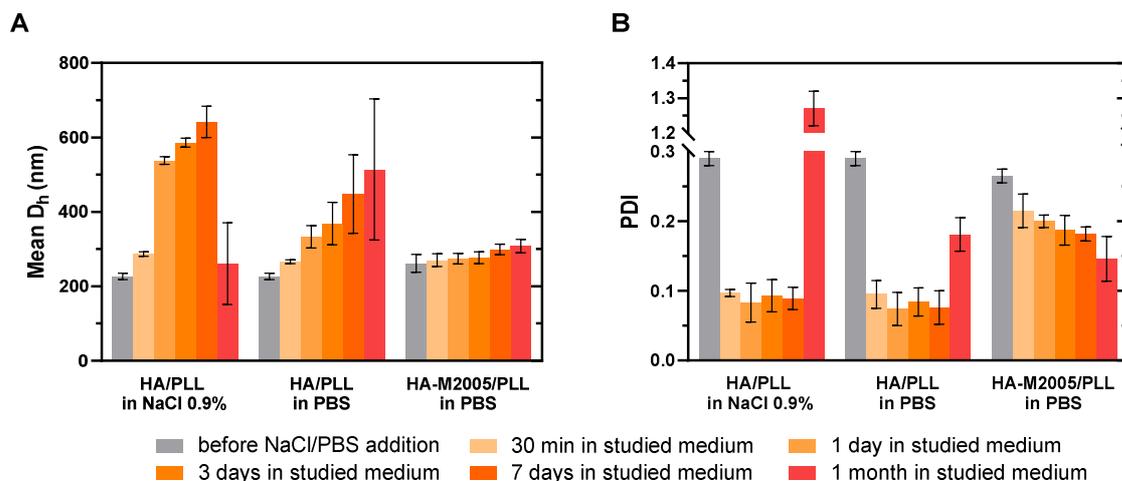
268

269 **Figure 5.** Mean D<sub>h</sub> of PEC-NGs (C<sub>P</sub> = 0.5 g.L<sup>-1</sup> in Milli-Q water) as a function of time: (A) HA-  
 270 M2005/DEAE-D PEC-NGs stored at 4 °C, (B) HA-M2005/DEAE-D PEC-NGs stored at 25 °C,  
 271 (C) HA-M2005/PLL and HA/PLL PEC-NGs stored at 4 °C. Data are presented as mean ±  
 272 standard deviation (n ≥ 3). Asterisks represent samples having large particle sizes (mean D<sub>h</sub> >  
 273 700 nm) with inconsistently large distribution.

274 As shown in **Figure 5A**, when stored at 4 °C, HA-M2005/DEAE-D PEC-NGs of n-/n+ ≥ 0.8  
 275 were stable but those of n-/n+ < 0.8 showed particle size increased after one day. Stability  
 276 evaluations were ceased when their mean particle sizes became too high (over 700 nm) with  
 277 largely varying PDIs. This is similar to HA/DEAE-D PEC-NGs in our previous work and could  
 278 be explained by the same hypothetical mechanism, that neutral double-stranded segments in  
 279 these PECs would be very short and hence unstable, favoring their segregation over time to reach  
 280 a more stable state (Le et al., 2021).

281 In a second time, HA-M2005/DEAE-D PEC-NGs having good stability at 4 °C were subjected to  
282 stability evaluation at 25 °C. Interestingly, higher storage temperature seems to render such  
283 PEC-NGs less stable, evidenced by increasing  $D_h$  at  $n-/n+ \geq 2.5$  (**Figure 5B**). This was not  
284 observed with PEC-NGs without M2005 (Le et al., 2021) and therefore can be interpreted as  
285 particle aggregation due to enhanced hydrophobic interactions between M2005 grafts on PEC-  
286 NG surfaces at higher temperature. Meanwhile, HA-M2005/PLL PEC-NGs at the same  $n-/n+$   
287 showed  $D_h$  increasing over time despite being stored at low temperature (4 °C), in contrast to  
288 HA/PLL PEC-NGs which were stable (**Figure 5C**). This suggests that replacing DEAE-D with  
289 PLL renders PEC-NGs containing M2005 more susceptible to agglomeration, probably because  
290 of stronger interparticle hydrophobic interactions contributed by PLL as mentioned in **Section**  
291 **3.1** beside that of M2005.

292 In order to study the stability of PEC-NGs against physiological saline conditions, different PEC-  
293 NG formulations were subjected to saline solutions (NaCl 0.9 % and PBS) and stored at 4 °C for  
294 stability monitoring. However, the suspensions of HA/DEAE and HA-M2005/DEAE-D  
295 PEC-NGs ( $C_p = 0.5 \text{ g.L}^{-1}$ ) showed precipitation after 30 min in NaCl 0.9 % or PBS (results not  
296 shown) and were thus not suitable for DLS analysis. This is in accordance with the low stability  
297 of HA/DEAE-D PECs upon increasing ionic strength in our previous work (Le et al., 2021) and  
298 confirms that such PECs are still highly sensitive to ionic strength even with M20005 grafts.  
299 Interestingly, replacing DEAE-D with PLL led to very different behaviors of PECs, which  
300 displayed no precipitation and therefore satisfactory for DLS characterization (**Figure 6**).



301  
 302 **Figure 6.** (A) Mean  $D_h$  and (B) PDI of different PEC-NGs ( $C_p = 0.5 \text{ g.L}^{-1}$ ) stored at  $4 \text{ }^\circ\text{C}$  in  
 303 different media. Data are presented as mean  $\pm$  standard deviation ( $n \geq 3$ ).

304 Following NaCl or PBS addition, HA/PLL PEC-NGs showed significantly increased  $D_h$  with an  
 305 abrupt fall in PDI. This is most probably due to particle aggregation when electrostatic repulsion  
 306 between particles is weakened by counterions through charge screening. As PDI represents the  
 307 relation between distribution width and mean value of particle size, the fall of PDI during the  
 308 aggregation can be interpreted as the absolute distribution width remaining the same or changing  
 309 to a lesser extent than the increase of the mean particle size.

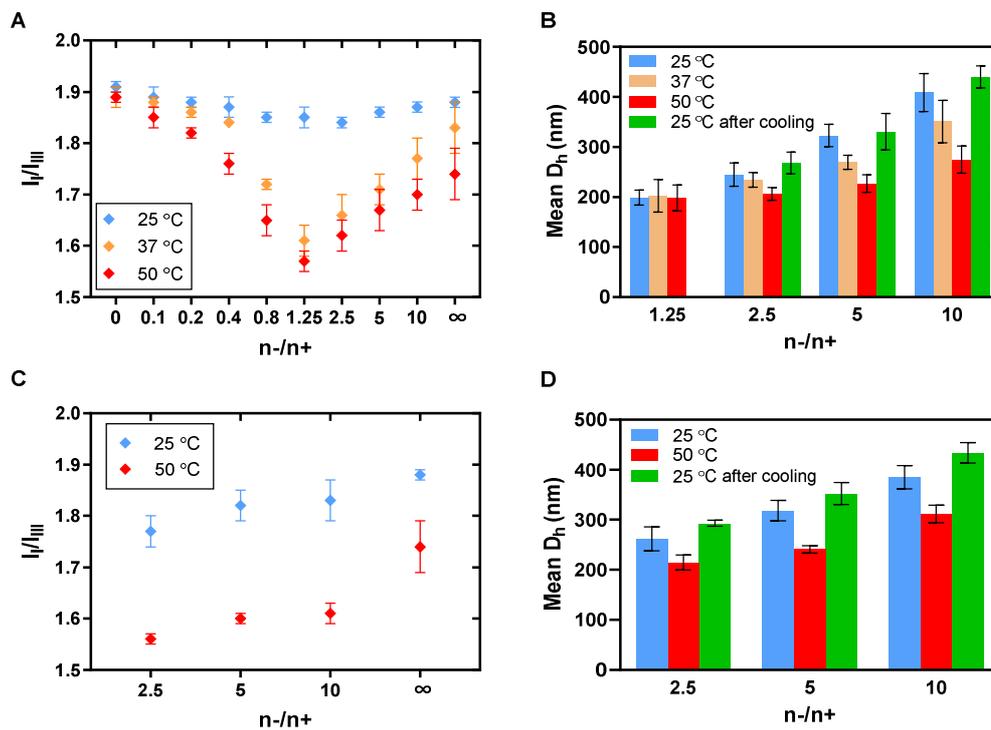
310 Nevertheless, in NaCl 0.9 %, the  $D_h$  continued to increase after seven days but decreased after  
 311 one month, coinciding with a significant increase in PDI to more than 1. This can be explained  
 312 by the appearance of another particle population having lower size of around 10-100 nm (**Figure**  
 313 **S5**). As discussed in our previous work (Le et al., 2021), charge screening by salt ions can take  
 314 place not only at particle level to cause particle aggregation but also at macromolecular level,  
 315 which may cause PEC decomplexation into smaller fragments. In the current case, the latter  
 316 effect seems to take place much later than the former. This could be explained by a longer time  
 317 required for infiltration of salt ions into the interior of PEC-NGs to interfere with ion pairing.

318 This was also the case in PBS, as shown by the increase in PDI after one month, but seemingly  
319 to a lesser extent than in NaCl 0.9 % since the mean particle size did not obviously decrease.  
320 Such difference could hardly be explained by the difference between their initial pH, which were  
321 measured as  $6.28 \pm 0.18$  and  $7.40 \pm 0.04$  in NaCl 0.9 % and PBS respectively and thus cause no  
322 considerable difference in ionization rate and actual n-/n+ of PECs (Supplementary Information  
323 **SI-3**). Such a better stability of PEC-NGs in PBS may be due to the long-term pH stability in this  
324 buffer. More interestingly, HA-M2005/PLL PEC-NGs showed relatively stable particle size after  
325 PBS addition (**Figure 6A**). However, a minor increase in particle size over one month was still  
326 observed but to a smaller extent than in Milli-Q water at the same condition (**Figure 5C**) and  
327 their PDI was gradually decreased over time. Such results evidence that the presence of salt does  
328 not deteriorate but indeed improve the stability of PEC-NGs having M2005 grafts. This finding  
329 is especially interesting since PECs from polyelectrolytes disposing weak charges are generally  
330 known to be highly unstable under physiological ionic strength (Wu & Delair, 2015). The exact  
331 mechanism of such stabilizing effect remains unclear but may be related to salting-out effect of  
332 salt, which can reinforce intraparticle hydrophobic interactions between PPO blocks of M2005  
333 grafts (Dulong et al., 2012; Gupta et al., 2015; Niang et al., 2016) and therefore stabilize PEC  
334 structure.

335 Collectively, such results prove that PEC-NGs with PLL as polycation are more resistant against  
336 ionic strength than with DEAE-D. As an explanation, regular charge distribution on both PLL  
337 and HA (**Figure 1**) may contribute to more close-knit ion pairing and hence their stronger  
338 association. On the contrary, arbitrary distribution of positive charges on DEAE-D may lead to  
339 nonoptimal charge compensation with HA and thus more loosely and unstable structure of PECs.

### 340 3.3. Thermoresponsiveness studies

341 Thermoresponsive properties of nanocarriers possessing LCST pendant groups, i.e. shrinkage  
342 and increase in hydrophobicity at higher temperature, have been known to be the mechanism and  
343 hence the prerequisite for their interesting drug delivery applications, namely triggering and/or  
344 controlling the drug release spatiotemporally *in vivo* by applying local hyperthermia (Molina et  
345 al., 2015). To verify the thermoresponsiveness of PEC-NGs from HA-M2005, we evaluated at  
346 different temperatures their hydrophobicity by fluorescence spectroscopy with pyrene probe and  
347 their particle sizes by DLS. The principle of the former technique is that a decrease in intensity  
348 ratio of the first to the third peaks ( $I_I/I_{III}$ ) in the emission spectra upon excitation at 335 nm of  
349 pyrene incorporated in the systems indicates the presence of more hydrophobic nanodomains  
350 (Burdíková, Mravec, & Pekař, 2016). Compared to previous  $I_I/I_{III}$  results of HA/DEAE-D PEC-  
351 NGs incorporating pyrene (HA/DEAE-D/Py) (Le et al., 2021), those of HA-M2005/DEAE-D/Py  
352 PEC-NGs suggest that M2005 grafts do not significantly enhance the hydrophobicity of PEC-  
353 NGs at 25 °C (**Figure S6A**). However, whereas the former system showed unchanged  $I_I/I_{III}$  upon  
354 heating (**Figure S6B**), HA-M2005/DEAE-D/Py PEC-NGs exhibited lower  $I_I/I_{III}$  when the  
355 temperature was increased from 25 °C to 37 °C and 50 °C, more significantly at  $n-/n+ > 1$  than at  
356  $n-/n+ < 1$  (**Figure 7A**). These results prove an increase in hydrophobicity upon heating, probably  
357 due to stronger hydrophobic association of the thermosensitive M2005 grafts. This tendency was  
358 the same for HA-M2005/PLL/Py PEC-NGs (**Figure 7C**). Furthermore, this effect seems to be  
359 more important when  $n-/n+$  is nearer to 1 regardless of component polycation (**Figure 7A** and  
360 **C**). This may be attributed to PEC structure being readily more compact at such  $n-/n+$  (Le et al.,  
361 2021), rendering M2005 chains already located in close proximity and hence favoring their  
362 approximation to form more hydrophobic clusters.



363  
 364 **Figure 7.**  $I_p/I_{III}$  in pyrene fluorescence and particle sizes at different temperatures of HA-  
 365 M2005/DEAE-D PEC-NGs (A-B respectively) and HA-M2005/PLL PEC-NGs (C-D  
 366 respectively) in Milli-Q water.  $C_P = 0.5 \text{ g.L}^{-1}$  for particle size analyses and  $0.25 \text{ g.L}^{-1}$  for pyrene  
 367 fluorescence studies due to dilution. The values 0 and  $\infty$  represent solutions containing solely  
 368 polycations and polyanions respectively. Data are presented as mean  $\pm$  standard deviation ( $n \geq$   
 369 3).

370 For characterizing the thermal dependence of particle size, we also focused on PEC-NGs with  
 371  $n/n+ > 1$  since these systems showed more consistent results of initial particle size (**Section 3.1**).  
 372 Upon heating, HA-M2005/DEAE-D PEC-NGs showed a decrease in  $D_h$  (**Figure 7B**) and the  
 373 same for their PDI (**Figure S7A**). For HA-M2005/PLL PEC-NGs, except for those at  $n/n+ =$   
 374 1.25 which could not be characterized by DLS because of precipitation (**Section 3.1**), PEC-NGs  
 375 at  $n/n+ \geq 2.5$  also displayed lower  $D_h$  (**Figure 7D**) and PDI (**Figure S7B**) at higher temperature.

376 Such decrease in particle size takes place within only 200-300 s of heating before reaching an  
377 equilibrium (**Figure S7D**). In contrast, PECs without M2005 grafts did not show such change  
378 upon heating (**Figure S7C**). Together with the earlier results on hydrophobicity, these results  
379 suggest that LCST property of M2005 grafts on HA contributes to a quasi-instantaneous series of  
380 thermoresponsive behaviors of our PEC-NGs, namely compaction of intraparticle hydrophobic  
381 clusters which leads to rapid particle shrinkage. However, different from the increase in  
382 hydrophobicity, the shrinkage is less obvious when  $n-/n+$  is nearer to 1 and even unobservable at  
383  $n-/n+ = 1.25$  for HA-M2005/DEAE-D PEC-NGs, presumably because the readily compact  
384 structure of PEC-NGs at  $n-/n+$  nearer to 1 is less susceptible to shrinkage than the highly porous  
385 structure at other  $n-/n+$ . Furthermore, after one hour of cooling at room temperature, their  $D_h$   
386 resurged but slightly surpassed the initial values (**Figure 7B and D**) and less clearly for PDI  
387 (**Figure S7A-B**). This suggests a completely reversible thermoresponsive behavior but which  
388 may accompany a slight rearrangement within PEC-NG structure. This experiment was repeated  
389 up to three cycles of heating-cooling and such reversibility was still conserved during the whole  
390 process (**Figure S7E**).

### 391 **3.4. Curcumin encapsulation**

392 Curcumin was used as a model compound to verify the potential of our system in encapsulating  
393 poorly water-soluble drugs. Our previous study reported that pyrene is encapsulated more  
394 efficiently in PECs if it is added before the complexation (Le et al., 2021). Thus, the preparation  
395 of PEC-NGs encapsulating curcumin in the current work was performed by adding polycation to  
396 polyanion solutions readily mixed with curcumin. From pyrene fluorescence studies (**Figure 7**),  
397 PEC-NGs having  $n-/n+$  of 1.25-2.5 seem to be the most potential for encapsulating hydrophobic  
398 molecules. However, our preliminary curcumin encapsulation trials with PEC-NGs of  $n-/n+ =$

399 1.25 showed inconsistent results, namely large batch-to-batch variation in visual aspect and  
 400 curcumin concentration of the samples (results not shown). This possibly stems from the weak  
 401 net surface charge of PEC-NGs at this n-/n+, which facilitate their precipitation and  
 402 sedimentation along with the insoluble curcumin in excess. PEC-NGs having n-/n+ of 2.5,  
 403 displaying more consistent results (**Table 2**), were thus chosen as the main subjects for these  
 404 studies. As shown in **Table 2**, curcumin concentration in HA/DEAE-D/Cur PEC-NGs is higher  
 405 than in curcumin-saturated water, proving the encapsulation of curcumin inside such PEC-NGs.  
 406 Furthermore, the encapsulation was improved with PEC-NGs having M2005 grafts, most  
 407 probably due to M2005 hydrophobic clusters in the NGs.

408 **Table 2.** Curcumin quantification results in different formulations

Formulation parameters		Curcumin quantification results <sup>c</sup>		
System	T <sub>prep</sub>	C <sub>CUR</sub> (µg.mL <sup>-1</sup> )	LC (%)	EE (%)
Milli-Q/Cur <sup>a</sup>	25 °C	0.30 ± 0.05	-	-
HA/DEAE-D/Cur <sup>b</sup>	25 °C	1.11 ± 0.05	0.16 ± 0.01	0.40 ± 0.03
HA-M2005/DEAE-D/Cur <sup>b</sup>	25 °C	1.70 ± 0.13	0.28 ± 0.03	0.70 ± 0.06
HA/DEAE-D/Cur <sup>b</sup>	50 °C	1.15 ± 0.13	0.17 ± 0.03	0.43 ± 0.06
HA-M2005/DEAE-D/Cur <sup>b</sup>	50 °C	3.55 ± 0.41	0.65 ± 0.08	1.63 ± 0.20
HA-M2005/DEAE-D/Cur <sup>b</sup>	70 °C	3.61 ± 0.18	0.66 ± 0.04	1.65 ± 0.09
HA-M2005/PLL/Cur <sup>b</sup>	50 °C	5.06 ± 0.25	0.94 ± 0.05	2.38 ± 0.12

T<sub>prep</sub>: preparation temperature, C<sub>CUR</sub>: curcumin concentration, LC: loading capacity, EE: encapsulation efficiency  
<sup>a</sup> Milli-Q water saturated in curcumin, <sup>b</sup> C<sub>P</sub>= 0.5 g.L<sup>-1</sup> and n-/n+ = 2.5, <sup>c</sup> mean ± standard deviation (n ≥ 3)

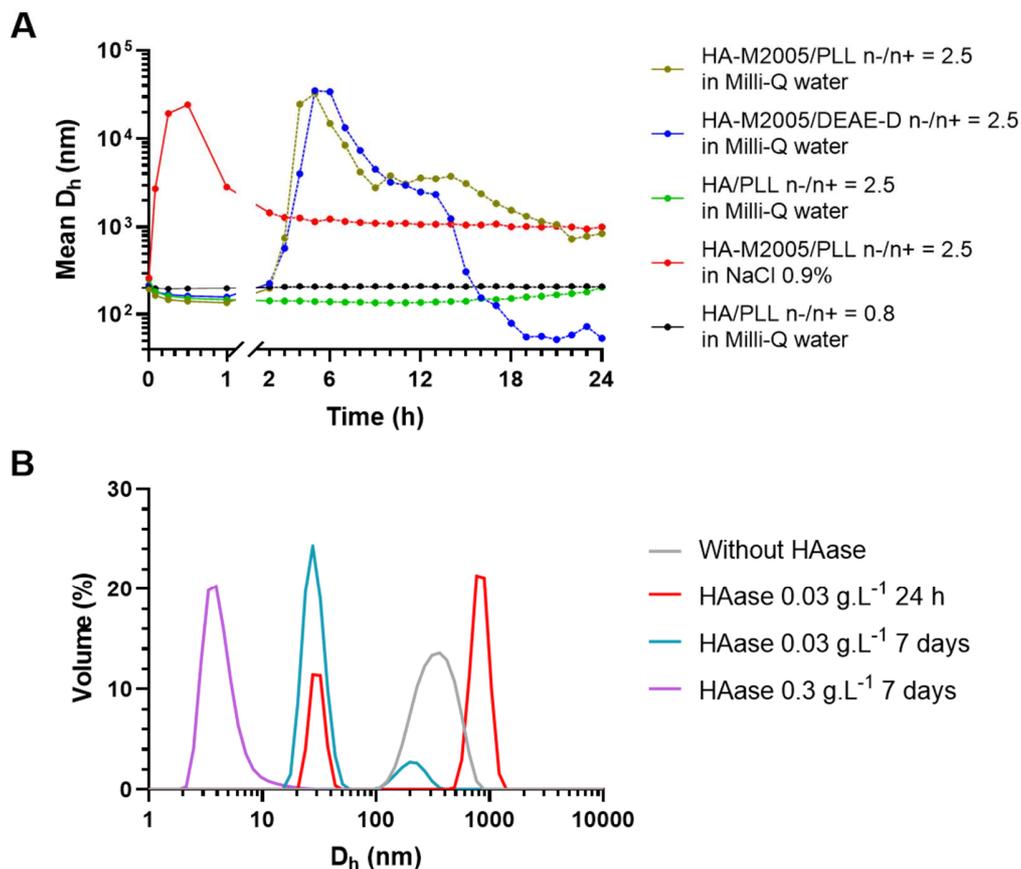
409 To optimize the encapsulation, we carried out the complexation at a higher temperature (50 °C or  
 410 70 °C), hypothesizing that this approach would lead to more hydrophobic clusters of M2005 and  
 411 incorporate more curcumin, which is then protected from leakage upon cooling by the  
 412 hydrophilic shell of PEC-NGs. As expected, HA-M2005/DEAE-D/Cur PEC-NGs prepared at

413 50 °C displayed remarkably higher curcumin content than those prepared at 25 °C, which was  
414 not observed for PEC-NGs without M2005 grafts (**Table 2**). This is in coherence with pyrene  
415 fluorescence studies and confirms the role of the temperature-dependent hydrophobic  
416 interactions of M2005 grafts in encapsulating hydrophobic molecules. However, increasing the  
417 complexation temperature from 50 °C to 70 °C did not further improve the encapsulation rate  
418 (**Table 2**), suggesting that the hydrophobic interactions of M2005 grafts are already optimal at  
419 50 °C. In addition, replacing DEAE-D with PLL also significantly improve the encapsulation  
420 (**Table 2**), confirming the more hydrophobic nature of PLL compared with DEAE-D as  
421 suggested in **Section 3.1**. Furthermore, PEC-NGs encapsulating curcumin prepared at 50 °C  
422 presented comparable mean  $D_h$  and  $\zeta$  (**Table S1**) to those of PEC-NGs without curcumin  
423 prepared at room temperature (**Section 3.1**), suggesting that curcumin and the preparation  
424 temperature have no considerable effect on ultimate state of PEC-NGs. The modest EE (**Table 2**)  
425 may be ascribed to the excessively large amount of starting curcumin during the preparation and  
426 the highly hydrophobic nature of this compound with respect to our PEC-NGs which are still  
427 globally hydrophilic. Formulation and process optimization is thus still in need before further  
428 characterizations like drug release studies or bioassays. Anyway, these results evidence that such  
429 PEC-NGs can increase the aqueous solubility of curcumin with possibility of encapsulation  
430 tuning and optimization through temperature control, showing therefore the potential for  
431 encapsulating other hydrophobic drugs.

### 432 **3.5. Enzymatic degradation**

433 The current PEC-NGs, as other HA-based nanocarriers, are potential for target drug delivery  
434 towards cancer cells since they are supposed to be degraded by HAase overexpressed  
435 specifically inside these cells and exhibit therefore more efficient intracellular drug release than

436 in normal cells (Yang et al., 2016). From this perspective, it is of high interest to verify and  
 437 understand HAase-induced degradation of our PEC-NGs. To this aim, we investigated the  
 438 evolution of their particle size in the presence of HAase 0.03 g.L<sup>-1</sup> (12 IU.mL<sup>-1</sup>) during 24 h at 37  
 439 °C. Such evolution of D<sub>h</sub> is reported in **Figure 8A**, with PDI following relatively similar trends  
 440 in **Figure S8**.



441  
 442 **Figure 8.** (A) Temporal evolution of mean D<sub>h</sub> of different PEC-NGs in the presence of HAase  
 443 0.03 g.L<sup>-1</sup> (12 IU.mL<sup>-1</sup>) during 24 h at 37 °C. (B) Volume-weighted particle size distribution of  
 444 HA-M2005/PLL PEC-NGs (n-/n+ = 2.5, C<sub>P</sub> = 0.5 g.L<sup>-1</sup>) in NaCl 0.9% before and after being  
 445 treated with HAase at different enzyme concentrations and time points. Data were obtained from  
 446 one experiment, results of replicated experiments showed similar trends.

447 Whereas PEC-NGs displayed no significant change in  $D_h$  over time in the absence of HAase  
448 (results not shown), HA-M2005/PLL and HA-M2005/DEAE-D PEC-NGs of  $n-/n+ = 2.5$  in  
449 Milli-Q water showed  $D_h$  changed after HAase addition, including a decrease in the first one  
450 hour followed by a sudden increase to reach a maximum after 5-6 h. For HA/PLL PEC-NGs of  
451 the same  $n-/n+$ , such phenomenon evolved much more slowly as their  $D_h$  only started to  
452 gradually increase after 12 h of slight diminution since HAase addition (**Figure 8A**) and then  
453 showed mean  $D_h$  of nearly 2000 nm with PDI of 0.88 after 3 days (results not shown). Such  
454 change in particle size may be because HAase preferentially degrades uncomplexed HA on the  
455 corona shell of PEC-NGs, leading to reduction of particle size at the beginning but also loss of  
456 surface charge and steric effect, which would in turn reduce interparticle electrostatic repulsion  
457 and cause hydrophobic aggregation. The speediness of such evolution for PEC-NGs containing  
458 M2005 probably stems from their weaker net surface charge and more hydrophobic structure  
459 brought about by M2005 grafts (see **Section 3.1**). Meanwhile, HA-M2005/PLL PEC-NGs in  
460 NaCl 0.9 % showed an immediate increase in particle size after HAase addition, probably  
461 because of two synergistic effects of salt: (i) charge screening to restrict non-specific  
462 complexation between HA and HAase, thus enhancing enzyme activity (Astériou, Vincent,  
463 Tranchepain, & Deschrevel, 2006) and (ii) reducing electrostatic repulsion between PEC-NGs  
464 while reinforcing hydrophobic interactions of M2005 to enhance particle aggregation (see  
465 **Section 3.2**). However, after reaching the maxima, the particle sizes started to decrease (**Figure**  
466 **8A**), which can be interpreted by PEC deterioration following HA degradation. This was more  
467 considerable with DEAE-D than with PLL as polycation, presumably due to the more fragile  
468 PEC structure between HA and DEAE-D as described in **Section 3.2**. Meanwhile, HA/PLL PEC-

469 NGs at  $n-/n+ = 0.8$  presented no change in  $D_h$  in the presence of HAase (**Figure 8A**), suggesting  
470 a protective effect of PLL shell on HA inside PEC-NGs against the enzyme.

471 Regarding  $D_h$  distribution (**Figure 8B**), HA-M2005/PLL PEC-NGs of  $n-/n+ = 2.5$  treated with  
472 HAase  $0.03 \text{ g.L}^{-1}$  in NaCl 0.9 % over 24 h had a population of low  $D_h$  (20-50 nm) beside few  
473 large aggregates (500-1000 nm). The former population was further accentuated with  
474 disappearance of the aggregates after 7 days, confirming the degradation of PEC-NGs by HAase.  
475 For the same treatment time, a higher enzyme concentration of  $0.3 \text{ g.L}^{-1}$  led to PEC-NGs being  
476 broken down into much smaller fragments (2-20 nm). These results further confirm that PEC-NG  
477 degradation by HAase is dependent on treatment time and enzyme concentration.

#### 478 **4. CONCLUSION**

479 In this work, mixing the solutions of HA or HA-M2005 with DEAE-D or PLL allowed  
480 spontaneous formation of PEC-NGs through electrostatic interactions, which is a rapid and  
481 simple elaboration process. M2005 grafts on HA can prompt inter- and intraparticle hydrophobic  
482 interactions while also rendering PEC-NGs thermoresponsive, which is applicable in optimizing  
483 drug encapsulation. Compared with DEAE-D, PLL can offer additional hydrophobicity and  
484 better stabilization of PEC-NGs. A synergistic combination between HA-M2005 and PLL can  
485 therefore generate PEC-NGs showing good stability under physiological ionic strength and the  
486 highest encapsulation capacity. With HA-M2005 as the main component, these PEC-NGs are not  
487 only thermosensitive but also degradable in the presence of HAase, thus promising as a dual-  
488 responsive system for targeted delivery of poorly water-soluble drugs. This should be especially  
489 advantageous for targeting tumors, where the overexpression of CD44 receptors and HAase  
490 should lead to enhanced cellular uptake and degradation of our NGs for drug release, while the  
491 thermoresponsiveness of the system may allow controlling not only encapsulation of anticancer

492 drugs, which usually have low aqueous solubility, but also their spatiotemporal delivery *in vivo*  
493 by applying local hyperthermia. However, further studies, namely formulation and conservation  
494 optimization by freeze-drying and drug release studies, remain to be done before biological *in*  
495 *vitro* and *in vivo* evaluations.

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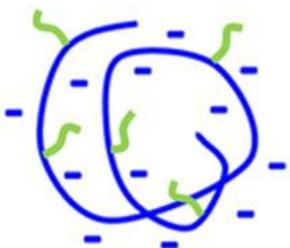
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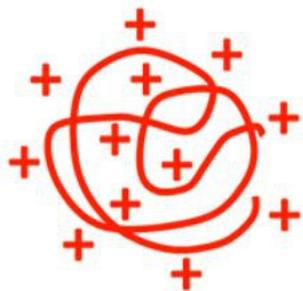


HA-M2005

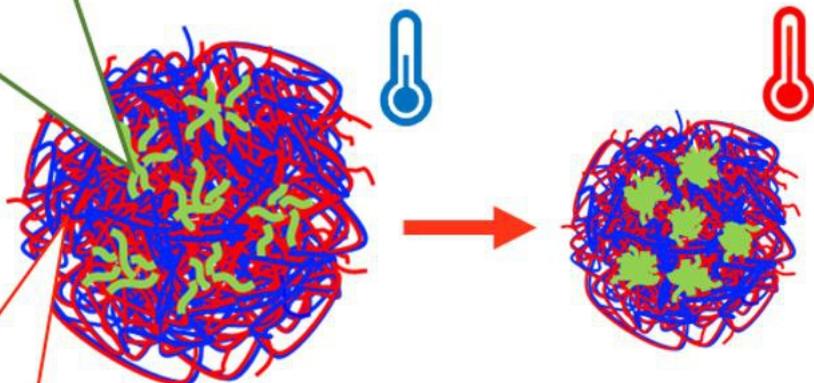
**M2005 grafts**  
[Poly(ethylene oxide)-co-poly(propylene oxide)]  
✓ Thermoresponsiveness  
↑ Hydrophobicity + NG stability



Simple mixing



DEAE-D or PLL



Thermoresponsive  
PEC-NGs

**Polycation = PLL**  
↑ Hydrophobicity + NG stability

