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1 **A mild and straightforward one-pot hyaluronic acid**
2 **functionalization through termination of poly-(2-alkyl-2-**
3 **oxazoline)**

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14 ABSTRACT

15 Hyaluronic acid (HA) of different molar masses from 38 000 to 990 000 g.mol⁻¹ were used
16 as terminating agents during the cationic ring-opening polymerisation of 2-methyl-2-
17 oxazoline. HA was first converted into a tetrabutylammonium salt to solubilize it in polar
18 aprotic organic solvents where the termination was conducted by varying the excess of HA
19 units to the growing poly(2-methyl-2-oxazoline) (PMeOx) chains (with various PMeOx
20 degree of polymerisation). This led to HA derivatives with various PMeOx degrees of
21 substitution from 4 % up to 13 % (determined by ¹H NMR). HA-*g*-PMeOx copolymers were
22 analysed by Size Exclusion Chromatography with on-line detectors (Multi-angle light
23 scattering, Viscometer, Refractive index) in 0.1 mol.L⁻¹ LiNO₃ to study their macromolecular
24 characteristics in dilute medium. The reaction was applied to functionalize HA with
25 thermo-responsive poly(2-isopropyl-2-oxazoline)-*co*-poly(2-*n*-butyl-2-oxazoline) moieties,
26 resulting in materials displaying thermally dependent hydrogel properties in aqueous
27 solutions which could be used for biomedical applications.

28
29 *Keywords: Hyaluronic acid; Poly(2-alkyl-2-oxazoline); Grafting to; One-pot reaction; Hydrogels*

30 1. Introduction

31 Among polysaccharides, hyaluronic acid (HA) is a material of choice for biomedical
32 applications as it is a component of the extracellular matrix and synovial fluid in vertebrates
33 (including humans) and some bacteria, hence its perfect biocompatibility. HA is the only non-
34 sulfated member of the glycosaminoglycan family [1][2]. It has a regular (1→4)-linked
35 disaccharide unit which consists of alternating β-D-glucuronic acid (1→3)-linked to *N*-acetyl-β-
36 D-glucosamine. It is involved in several physiological functions such as cell motility,
37 proliferation mechanisms, inflammatory response and articulation lubrication. In particular, HA
38 is used for treating osteoarthritis and as a wound healing promoter [1]. Its specific interaction
39 with cell receptor CD44, which is overexpressed in some cancerous cells, has also led to many
40 research articles probing its use in drug delivery systems [3]. In comparison to many other
41 polysaccharides, HA allows a wider range of chemical modifications thanks to both its alcohol
42 [3] and carboxylic acid groups [1] [2]. Carboxylic acid moieties also strongly influence the
43 solubility of HA depending on the counter ion accompanying the carboxylate anion. Sodium
44 hyaluronate (Na-HA, the sodium salt form of HA) is water-soluble and insoluble in any organic
45 solvents contrary to tetrabutylammonium hyaluronate (TBA-HA, the tetrabutylammonium salt
46 form of HA) which is soluble in polar aprotic solvents such as DMSO and DMF [4].

47 An ongoing challenge is to functionalize HA with suitable grafting moieties or with suitable
48 polymers to combine both the properties of HA and of grafted groups. “Grafting from” and
49 “grafting to” are the two main pathways to connect polymer chains on a backbone. In a
50 “grafting from” approach, the polysaccharide is used as a macro-initiator for polymerisation [5]
51 with the advantage that steric hindrance effect will be minimized compared to a “grafting to”
52 approach, **resulting in a generally higher reaction efficiency [6]**. The drawback is that the
53 resulting growth of the polymer chain might not be as well-controlled as with a “grafting to”
54 reaction scheme because of the use of a macro-initiator, which can be problematic when the
55 structure is closely related to the polymer properties [7]. Also, the characterisation of the
56 lateral chains will be more difficult. In the “grafting to” approach, the grown polymer chain is
57 directly grafted to the polysaccharide [3][8], allowing a better control of the resulting
58 copolymer structure. In this study, the “grafting to” approach was selected to finely design the
59 final material properties according to the grafted polymer structure. Nevertheless, this
60 functionalization pathway requires at least 2 steps (i.e. polymerisation of a monomer and its
61 subsequent grafting to the polysaccharide), sometimes even a supplementary step if an end-

62 chain functionalization is needed. Therefore, new approaches leading to a reduction of the
63 synthesis steps are of great interest.

64 Among grafted polymers, poly(ethylene glycol) (PEG) is one of the most used due to its
65 stealth properties making it untargeted by the immune system which is useful for designing
66 drug delivery materials. However, PEG is prone to oxidation [9] and to accumulate in organs
67 [10]. Furthermore, its manufacturing requires the use of highly toxic and hazardous ethylene
68 oxide monomer [9]. Polylactide-grafted polysaccharides copolymers are also good candidates
69 for biomedical or pharmaceutical applications because polylactide is biodegradable and can be
70 obtained from renewable sources [11][12]. For example, polylactide-grafted dextran has been
71 synthesized by the “grafting from” pathway in a three steps reaction involving a partial
72 reversible protection of the polysaccharide hydroxyl functions, the ring opening
73 polymerization (ROP) of D,L-lactide catalysed by stannous octoate and promoted by the
74 remaining OH groups of the partially protected dextran and finally a deprotection step [11].
75 Due to its toxicity, the presence of stannous salts remains a major problem for biomedical
76 applications. Poly(2-alkyl-2-oxazoline)s (POx) constitute alternative polymers for biomedical
77 applications due to their biocompatibility and their stealth properties [13]. They display other
78 interesting features such as modular physical properties (value of glass temperature,
79 amorphous or semi-crystalline nature, solubility) as well as thermo-responsiveness [7]
80 depending on the nature of the pendant alkyl group. The grafting of POx on polysaccharides
81 have already been undertaken using a “grafting to” approach by Aoi *et al.* on chitosan. The
82 amino groups of chitosan acted as terminating agents for the growing POx chains in DMSO [14]
83 [15]. They focused first on homopolymers: poly(2-methyl-2-oxazoline) and poly(2-ethyl-2-
84 oxazoline) [14] before extending their reaction to an amphiphilic poly(2-phenyl-2-oxazoline)-*b*-
85 poly(2-methyl-2-oxazoline) block copolymer [15]. However, this synthetic approach cannot be
86 extended to HA, which is lacking amine functionality. In another approach, Hruby and coll.
87 focused on the functionalization of glycogen and β -glucan with thermo-responsive poly(2-
88 isopropyl-2-oxazoline)-*co*-poly(2-*n*-butyl-2-oxazoline) [16] [17] [18]. The polysaccharides were
89 first activated by converting their alcohol moieties into alcoholate using a strong base, sodium
90 hydride, to be nucleophilic enough to induce the termination step. However, the drawback of
91 this approach is the requirement to use sodium hydride in DMSO which is preventing the
92 reaction scale-up, the combination of both being potentially hazardous on larger quantities
93 [19]. Also, extending this reaction to HA might be challenging, as this polysaccharide has been

94 proven to be quite easily degraded in the presence of bases [20], which questions its
95 compatibility with sodium hydride. As an alternative route for grafting of POx on HA without
96 using sodium hydride, we thought we could employ the carboxylic acid groups of HA as
97 terminating agents for the cationic ring-opening polymerisation (CROP) of oxazolines as these
98 functional groups have previously been used for such purpose with either small organic
99 molecules like methacrylates [21] [22] or synthetic polymers like poly(methyl methacrylate)s
100 [23].

101 In summary, our study focus on the use of HA as a terminating agent during the CROP of 2-
102 methyl-2-oxazoline (MeOx) to obtain HA grafted with poly-(2-methyl-2-oxazoline) (PMeOx)
103 named HA-*g*-PMeOx copolymers. We varied the HA/PMeOx ratios and the grafted PMeOx
104 chain length (with the DP_{PMeOx}) and checked their grafting amounts and the molar masses of
105 HA. HA-*g*-PMeOx copolymers have been characterised by FTIR and NMR spectroscopies.
106 PMeOx and HA being very hydrophilic, the successful grafting has been readily assessed using
107 both Size Exclusion Chromatography (SEC) with on-line Multi-Angle Light Scattering system
108 (MALS), Viscosity Detector (VD) and Differential Refractive Index detector (DRI) in aqueous
109 media and Diffusion-Ordered Spectroscopy (DOSY) NMR. The reaction was then extended to
110 the termination of HA with well-known thermo-responsive poly(2-isopropyl-2-oxazoline)-*co*-
111 poly(2-*n*-butyl-2-oxazoline) (PiPrOx-*co*-P*n*BuOx) [7] [16] in order to design physical hydrogels
112 whose formation would be triggered by temperature increase.

113

114 2. Experimental

115 2.1. Materials

116 Three different Na-HA batches were kindly provided by Givaudan France SAS (Pomacle,
117 France): CRISTALHYAL® $M_n = 990\ 000\ \text{g}\cdot\text{mol}^{-1}$ (HA_{990k}), BASHYAL® $M_n = 140\ 000\ \text{g}\cdot\text{mol}^{-1}$ (HA_{140k})
118 and RENOVHYAL® $M_n = 38\ 000\ \text{g}\cdot\text{mol}^{-1}$ (HA_{38k}) (absolute molar masses were determined
119 through SEC/MALS/VD/DRI measurement). 2-methyl-2-oxazoline, 2-*n*-isopropyl-2-oxazoline
120 (*i*PrOx), 2-*n*-butyl-2-oxazoline (*n*BuOx), methyl *p*-toluenesulfonate, anhydrous DMSO,
121 anhydrous acetonitrile, sodium chloride, tetrabutylammonium hydroxide (54.0-56.0 % in
122 water) (TBAOH), phosphate buffer solution (PBS) tablets, deuterium oxide (D₂O), sodium
123 deuterioxide solution (30 wt% in deuterium oxide), deuterated chloroform (CDCl₃) were
124 purchased from Sigma-Aldrich. Hydrochloric acid 36.5 %, sodium hydroxide pellets, amberlite

125 ion exchange resin (IRN77, cationic type, H⁺), acetone and ethanol 96% vol% were purchased
126 from VWR. Water was purified with the milli-Q water reagent system (Millipore, USA).

127

128 2.2. Conversion of Na-HA to TBA-HA

129 The principle of the conversion of Na-HA to TBA-HA is summed up in scheme 1 (a). The
130 three different HAs (HA_{990k}, HA_{140k} and HA_{38k}) were first transformed into their acidic form: 5 g
131 of Na-HA were first dissolved overnight in 830 mL of water at room temperature. Then the
132 cation exchange resin (H⁺) (45 g) was introduced in the solution, which was left to stir for one
133 day at room temperature. The resin was subsequently filtered off the solution through a
134 sintered glass filter. The resulting solution was then neutralized using TBAOH to obtain TBA-HA
135 as follow: TBAOH, diluted fivefold with water was added drop-wise to the previously prepared
136 hyaluronic acid solution until the pH reached 8. The solution was then freeze-dried and the
137 resulting TBA-HA was kept in a freezer until further use.

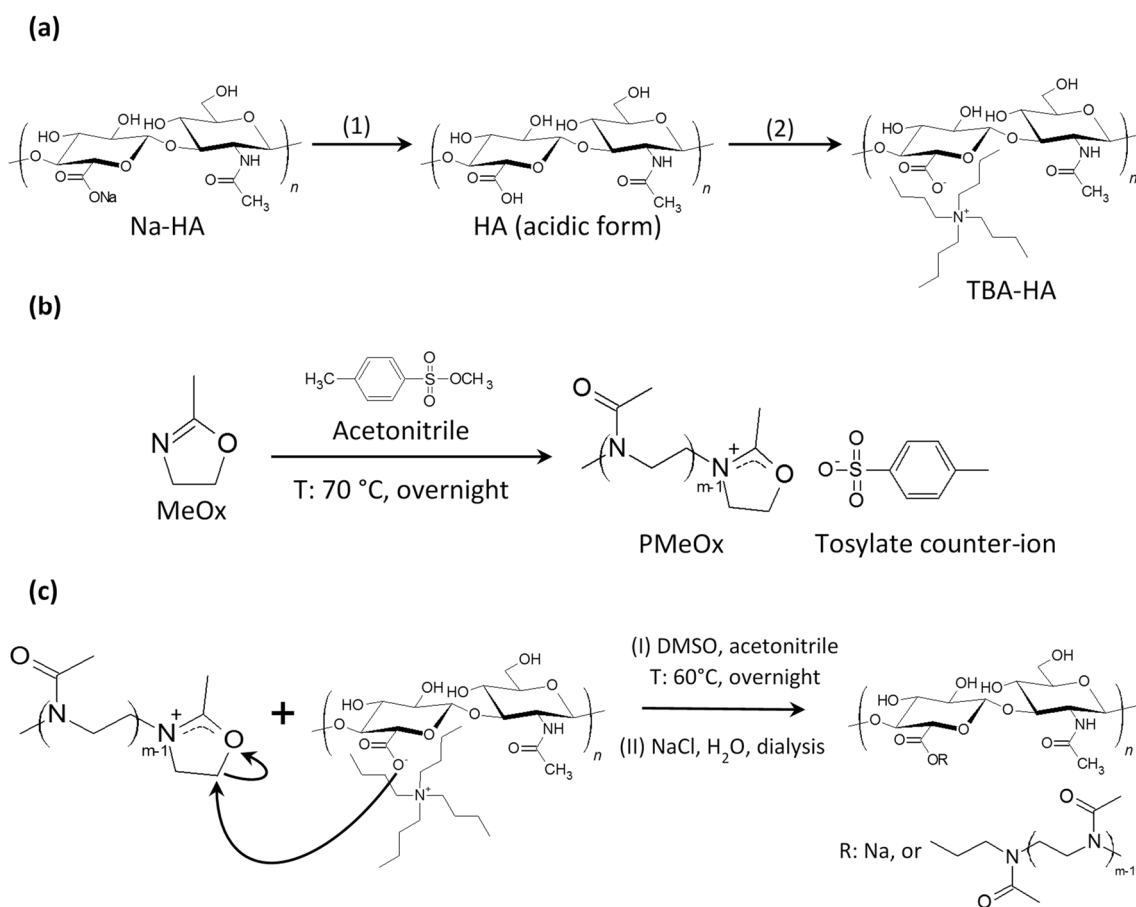
138 2.3. One-pot functionalization of TBA-HA with PMeOx through a “grafting to” approach

139 2.3.1. Polymerisation of MeOx (DP: 30 and 50)

140 The CROP protocol is presented in scheme 1 (b). MeOx (2.056 g, 24.16 mmol) was
141 introduced in an oven-dried round-bottom flask under nitrogen containing a solution of
142 methyl-*p*-toluenesulfonate (DP 30: 150 mg, 0.805 mmol; DP 50: 90 mg, 0.483 mmol) in 6 mL
143 dry acetonitrile. Nitrogen was bubbled within the mixture for 15 minutes at room temperature
144 after which the polymerisation mixture was stirred at 70 °C under nitrogen overnight. DPs
145 were checked by ¹H NMR (see Fig. S1) and calculated according to Eq. (S1). For the
146 polymerisation of *PiPrOx-co-PnBuOx* copolymers, the same protocol (target DP: 30) was
147 applied by substituting MeOx with both *iPrOx* (2.19 g, 19.4 mmol) and *nBuOx* (615 mg, 4.8
148 mmol) which were added simultaneously to the flask containing the initiator (target
149 *iPrOx/nBuOx* ratio: 80/20; experimental ratio: 75/25 as determined with NMR).

150 2.3.2. Termination of PMeOx with TBA-HA

151 The termination step is presented in scheme 1 (c) and described herein for PMeOx DP 30.
152 For PMeOx DP 50, the terminating agent TBA-HA molar quantity was adjusted to the PMeOx
153 polymer chain molar amount which was assumed to be equal to the initiator molar amount
154 introduced during the PMeOx polymerisation step (see section 2.3.1.).



155

156 **Scheme 1.** (a) Conversion of Na-HA to TBA-HA through two steps: (1) acidification with an
 157 amberlite ion exchange resin (cationic type, H⁺) and (2) neutralization of the resulting
 158 hyaluronic acid with TBA-OH, (b) CROP of MeOx, (c) direct termination of PMeOx chain with
 159 TBA-HA.

160 TBA-HA (500 mg, 0.805 mmol or 1 g, 1.6109 mmol, depending on the desired PMeOx
 161 (polymer chain): TBA-HA (disaccharide unit) molar ratio (respectively 1:1 or 1:2)) was dissolved
 162 in anhydrous DMSO in a three-neck flask under nitrogen. The solvent volume was adjusted
 163 depending on the starting polysaccharide: 50 g.L⁻¹ for TBA-HA_{38k}, 20 g.L⁻¹ for TBA-HA_{140k} and
 164 12.5 g.L⁻¹ for TBA-HA_{990k}. Furthermore, in the case of TBA-HA_{990k}, the polysaccharide had to be
 165 dissolved overnight at 60 °C while the dissolution was rather fast for the other two batches at
 166 room temperature. The PMeOx solution in acetonitrile was subsequently introduced in the
 167 flask containing TBA-HA and the following termination reaction was carried out at 60 °C under
 168 nitrogen overnight. Thereafter, the flask was left to cool down at room temperature and a 2.5
 169 mol.L⁻¹ NaCl solution (10 mL per gram of starting TBA-HA) was added drop-wise to the polymer

170 mixture to exchange the TBA counter-ions with sodium. The modified polysaccharide was
171 subsequently precipitated in acetone, then filtered and immediately placed in a 0.2 mol.L⁻¹
172 NaCl solution (100 mL per gram of starting TBA-HA) to substitute the remaining TBA counter-
173 ions with sodium. After one night, the resulting solution was dialyzed against distilled water
174 (dialysis advance was monitored with conductivity measurement, dialysis membrane cut off
175 (Repligen, MA, USA) was adjusted to starting polysaccharide: 6-8 kDa for TBA-HA_{38k} and 12-14
176 kDa for the two other batches). The material was recovered through freeze-drying. It was
177 subsequently washed by dispersing it in ethanol and recovering it through filtration before
178 drying it under vacuum, the process being performed three times to ensure removal of
179 remaining free PMeOx chains. Degrees of substitution (DS) were determined by ¹H NMR (see
180 section 2.4.2.). For the termination of HA with PiPrOx-co-PnBuOx, the same protocol was
181 applied with a PiPrOx-co-PnBuOx (polymer chain): TBA-HA (disaccharide unit) molar ratio of
182 1:1.

183 Finally, the samples will be coded as follow: HA_{M_n}-g-PMeOx_{DP}-DS (HA_{M_n}-g-(PiPrOx_{DP(PiPrOx)}-
184 co-PnBuOx_{DP(PnBuOx)})-DS for the thermo-responsive material), where M_n is the number average
185 molar mass of HA (e.g. 140k for 140 000 g.mol⁻¹), DP the degree of polymerisation of PMeOx
186 (DP(PiPrOx) and DP(PnBuOx) the DPs of PiPrOx and PnBuOx within the copolymer) and DS the
187 degree of substitution in grafted PMeOx.

188 2.4. Characterisation methods

189 2.4.1. Infrared spectroscopy

190 Infrared spectra were acquired on a spectrometer Nicolet IS50 FT-IR (Thermo Scientific,
191 USA). The samples were analysed with the OMNIC software by transmission from 500 to 4000
192 cm⁻¹ (32 scans with resolution 2 cm⁻¹).

193 2.4.2. NMR spectroscopy

194 ¹H NMR spectra were acquired on a Bruker Avance 300 MHz spectrometer (MA, USA). The
195 PMeOx homopolymer samples were prepared in CDCl₃ while HA derivatives were prepared at
196 a concentration of 5 g.L⁻¹ in D₂O containing 0.125 M sodium deuterioxide NaOD to increase HA
197 protons mobility, thus improving spectra resolution [24].

198 The experimental grafting degree of PMeOx to HA (DS) was estimated by ¹H NMR spectroscopy
199 using Eq. (1).

$$DS = \frac{2 \times (I_{Methyl} - 3)}{3 \times DP_{PMeOx} \times I_{Hanom}} \quad (1)$$

Where I_{Methyl} is the integration value of the overlapping peaks associated to methyl from HA and PMeOx respective *N-acetyl* moieties found at 1.8-2.1 ppm. DP_{PMeOx} stands for the experimental degree of polymerisation of MeOx (determined by 1H NMR spectroscopy before the termination step according to Eq. (S1)). I_{Hanom} accounts for the two anomeric protons of the disaccharide unit found near 4.5 ppm which were used as a reference peak as they were not overlapping with PMeOx or other HA proton peaks (Fig. 1).

DOSY NMR measurements were performed on a Bruker Avance III 600 MHz equipped with a CPTCI z-gradient probe (maximum gradient power of 50 G.cm⁻¹). The details on the experiment will be covered in the supplementary information.

2.4.3. SEC/MALS/VD/DRI

The SEC/MALS/VD/DRI measurements were conducted as follow. The carrier was a 0.1 mol.L⁻¹ LiNO₃ solution filtered through a 0.1 μm filter unit (Millipore, USA), degassed on-line (DGU-20A3, Shimadzu, Japan), and eluted at a 0.5 mL.min⁻¹ flow-rate (LC10Ai Shimadzu, Japan). The analysed solutions were prepared in 0.1 mol.L⁻¹ LiNO₃ at a concentration of respectively 0.5 g.L⁻¹ (HA_{990k} based materials) and 1 g.L⁻¹ (HA_{140k} and HA_{38k} based materials) and were filtered on a 0.45 μm filter prior to injection of 100 μL with an automatic injector (SIL-20A Shimadzu, Japan). The SEC line consisted of an OHPAK SB-G guard column for protection and two OHPAK SB 806 and 804 HQ columns (Shodex Showa Denko K.K., Japan) in series. The column packing was a poly(2-hydroxyethyl methacrylate) gel. The MALS detector was a DAWN Heleos-II (Wyatt Technology Inc., CA, USA) fitted with a 50 μL K5 cell and 18 photodiodes (normalized relative to the 90° detector using bovine serum albumin). The viscometer detector (VD) was a ViscoStar II (Wyatt Technology Inc., CA, USA). The collected data were analysed using the Astra® 6.1.7.16 software package. Molar masses were obtained with the Zimm order 1 method. The concentration of each eluted fraction was determined with DRI (RID 10A Shimadzu, Japan) according to the values of dn/dc (0.150 mL.g⁻¹ for HA and 0.158 mL.g⁻¹ for PMeOx), which were determined through DRI measurements using polymer solutions in 0.1 M LiNO₃ at different concentrations. The dn/dc of the HA-*g*-PMeOx samples were then calculated as the average of the dn/dc of HA and PMeOx according to the weight content of the respective polymers in the copolymer.

230

231 2.4.4. Rheology

232 Rheological measurements were performed on a Discovery HR-2 hybrid rheometer from
233 TA Instruments (UK). HA_{990k}-g-(PiPrOx₂₀-co-PnBuOx₇)-0.10 was dissolved in PBS (0.01 M
234 phosphate buffer, 0.15 M NaCl, pH: 7.4) at a concentration of 15 g.L⁻¹. The measurements were
235 performed with a double concentric cylinder measuring system and the rheometer was
236 operated using the TRIOS v4 software.

237

238 3. Results and discussion

239 3.1. Conversion of Na-HA to TBA-HA

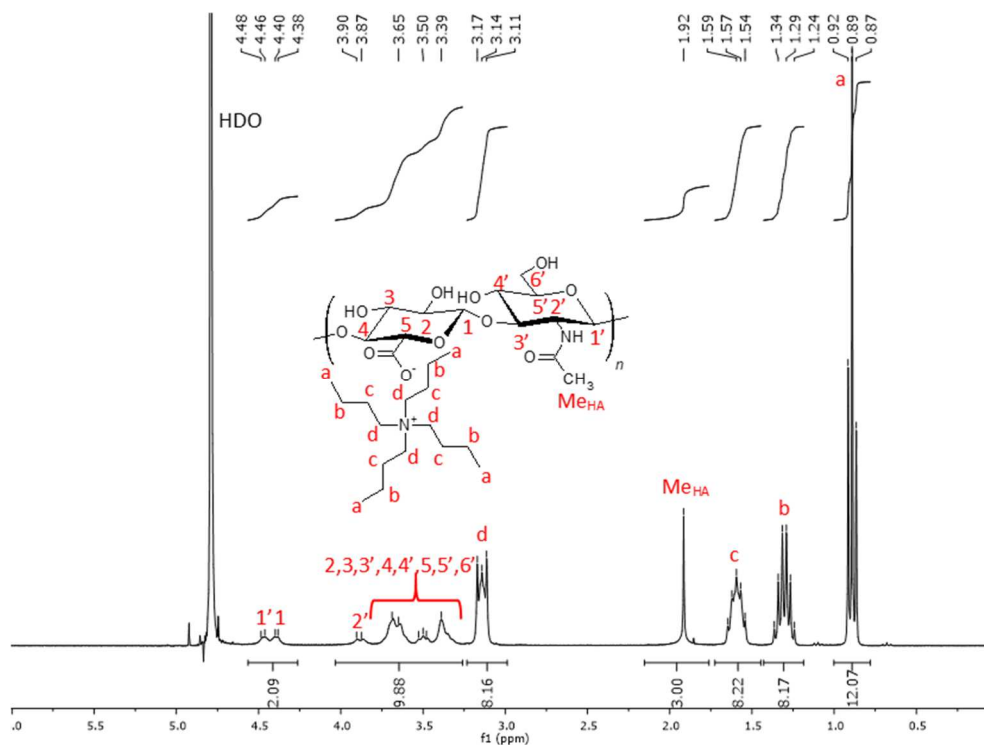
240 TBA-HA was obtained through a two-steps process (scheme 1a). Na-HA was first acidified
241 with an amberlite ion-exchange resin (cationic type) and then neutralized with TBA-OH. The
242 resulting TBA-HA was analysed by ¹H NMR spectroscopy to assess the full conversion of Na-HA
243 to TBA-HA (Fig. 1). Me_{HA} (δ =1.92 ppm), corresponding to the three protons of the methyl group
244 of HA, was taken as the reference peak. A complete conversion into TBA-HA was calculated
245 from either the H_b, H_c or H_d protons of TBA at respectively 3.14, 1.58 or 1.29 ppm (integration
246 value of 8 indicating full conversion) or the H_a protons of TBA at 0.89 ppm (integration value of
247 12 indicating full conversion).

248 SEC/MALS/VD/DRI analysis were conducted on Na-HA and TBA-HA salts in LiNO₃ 0.1 mol.L⁻¹
249 as summarised in Table 1. It clearly appeared that both Na-HA and subsequent TBA-HA salts
250 presented quite the same molar masses. For instance, Na-HA_{140k} had a M_n of 140 000 g.mol⁻¹
251 and a M_w of 210 000 g.mol⁻¹ which respectively shifted to 160 000 g.mol⁻¹ and 250 000 g.mol⁻¹
252 when converted into TBA-HA, which was within the margin of error of the measurement.

253 **Table 1.** Evolution of HA molar masses induced by the salt substitution reaction using
254 SEC/MALS/VD/DRI.

Sample	M _n (g.mol ⁻¹) (Na-HA)	M _w (g.mol ⁻¹) (Na-HA)	M _n (g.mol ⁻¹) (TBA-HA)	M _w (g.mol ⁻¹) (TBA-HA)
HA _{38k}	38 000	64 000	36 000	68 000
HA _{140k}	140 000	210 000	160 000	250 000
HA _{990k}	990 000	1 500 000	1 000 000	1 370 000

255 Accuracy of SEC/MALS/VD/DRI data: $M_n \pm 9\%$; $M_w \pm 6\%$.



256

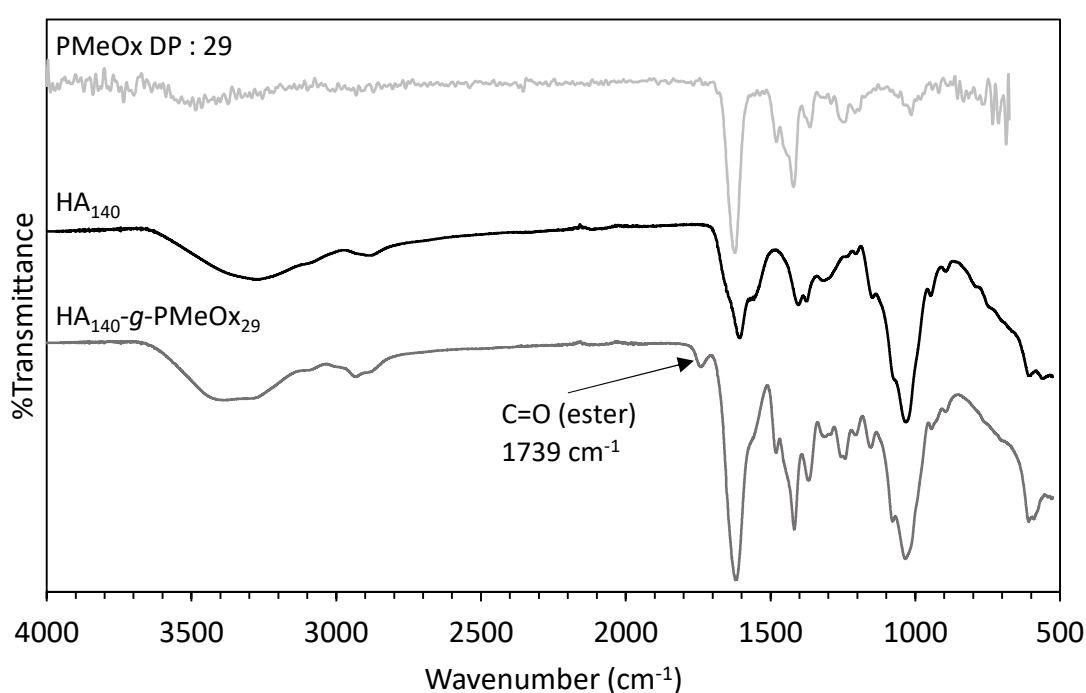
257 **Fig. 1.** ^1H NMR spectrum of TBA- $\text{HA}_{140\text{k}}$ in D_2O (with 0.125 M NaOD).

258

259 3.2. PMeOx grafting to HA: investigation of a one-step process

260 PMeOx was polymerised with an experimental DP of either 29 (Fig. S1) or 47 and
261 subsequently terminated with TBA- $\text{HA}_{38\text{k}}$, TBA- $\text{HA}_{140\text{k}}$ or TBA- $\text{HA}_{990\text{k}}$ by varying the PMeOx chain
262 to HA unit molar ratio (1:2 and 1:1) as summarized in Table 2. The nucleophilic termination of
263 CROP grown PMeOx was expected to be induced by the coupling of the cationic oxazolinium
264 end-group from the active propagating species on the anionic carboxylate moieties of HA, as
265 carboxylates have already been used as terminating agents during oxazoline polymerisation
266 [21]. Also, alcoholic moieties were not activated by turning them into alkoxide and were
267 therefore assumed not to be nucleophilic enough for terminating propagating oxazolinium.
268 The reaction was thus assumed to follow a similar pattern as the one described during
269 esterification of HA or alginate using alkyl halides (Scheme 1c) [4] [25]. The reaction was
270 stopped by adding an excess of sodium chloride solution to convert TBA- HA-g-PMeOx into Na-
271 HA-g-PMeOx , with the material being precipitated afterwards in acetone, recovered through
272 filtration and then purified by dialysis and several washings with ethanol.

273 FTIR analysis of resulting HA-g-PMeOx revealed characteristic bands of both starting HA at
 274 3610-2970 cm^{-1} (O-H stretching), 2888 cm^{-1} (C-H stretching), 1606 cm^{-1} (carboxylate C=O
 275 asymmetric stretching), 1558 cm^{-1} (N-H bending), 1404 cm^{-1} (carboxylate C=O symmetric
 276 stretching), 1318 cm^{-1} (carboxylate C-O stretching) and 1033 cm^{-1} (C-OH stretching), and of
 277 PMeOx at 1620 cm^{-1} (tertiary amide C=O stretching), 1420 cm^{-1} (amide C-N stretching), 1240
 278 cm^{-1} (backbone C-N stretching) [25] with an additional signal at 1739 cm^{-1} which corresponds
 279 to the ester bond between HA and PMeOx (Fig. 2). Qualitatively, the grafting appears
 280 successful.

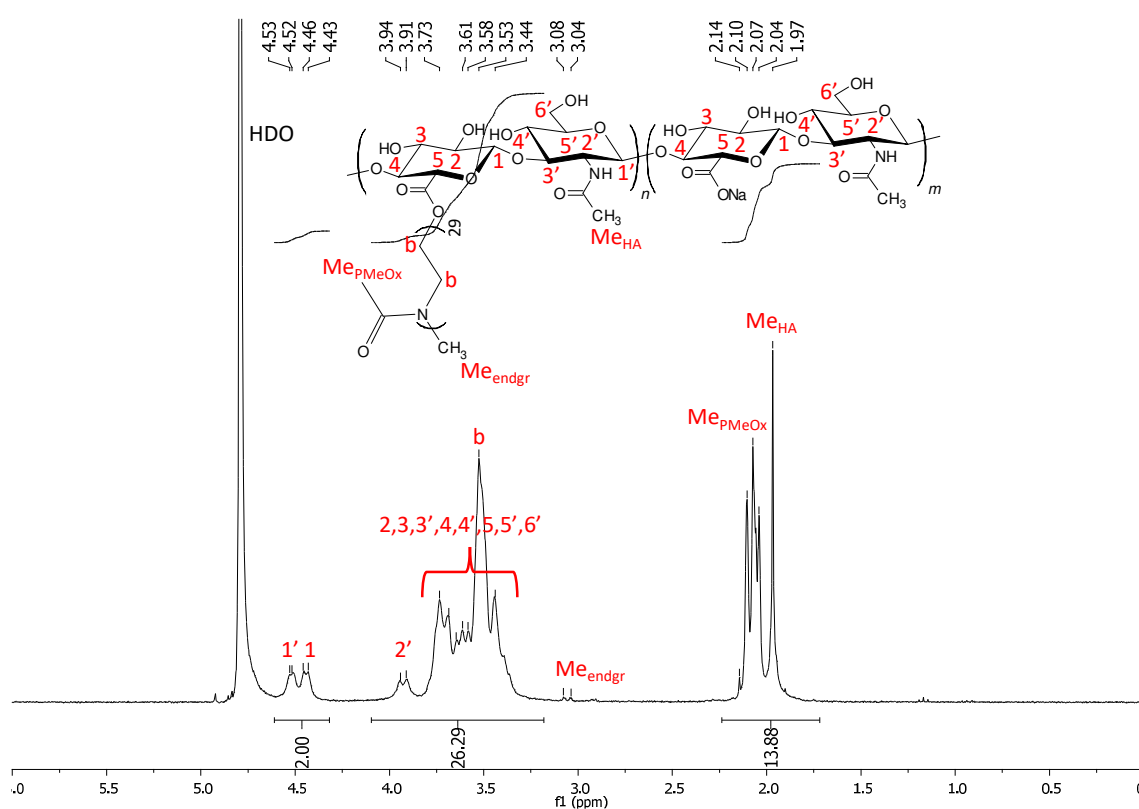


281
 282 **Fig. 2.** FTIR spectra of PMeOx DP: 29 terminated with KOH (1 M in MeOH) (top, light grey)
 283 HA_{140k} (middle, black) and HA_{140k}-g-PMeOx₂₉-0.13 (bottom, dark grey).

284 The NMR spectrum (Fig. 3) also displayed peaks of HA (anomeric protons (1 and 1') at 4.40-
 285 4.50 ppm, proton in α position of amide (2') around 3.9 ppm, the other protons from the sugar
 286 ring between 3.44 and 3.71 ppm, methyl Me_{HA} at 1.94 ppm), as well as peaks of PMeOx
 287 (methylene protons (b) at 3.44 ppm, methyl end group (Me_{endgr}) at 3.04-3.08 ppm and methyl
 288 groups (Me_{PMeOx}) around 2.1 ppm). The grafting degree (DS) was estimated according to Eq. (1),
 289 the results being summed up in Table 2. The highest obtained DS was 13% (for sample HA_{140k}-
 290 g-PMeOx₂₉-0.13 displayed in Fig. 3), which means 45 % of the weight of this material actually
 291 corresponds to PMeOx as calculated with Eq. (2).

$$Wt\%_{PMeOx} = \frac{Mn_{PMeOx} \times DS}{DS \times (Mn_{PMeOx} + Mu_{HA} - M_H) + (1 - DS) \times Mu_{NaHA}} \times 100 \quad (2)$$

Where $M_{n_{PMeOx}}$ is the number average molar mass of PMeOx chain calculated by 1H NMR spectroscopy using the following formula: $M_{n_{PMeOx}} = DP_{PMeOx} \times M_{MeOx} + M_{endgr}$ where $M_{MeOx} = 85 \text{ g.mol}^{-1}$ and $M_{endgr} = 15 \text{ g.mol}^{-1}$ (see Eq. (S1) and Fig. S1). DS is the grafting degree of PMeOx on HA, $M_{u_{HA}}$ is the molar mass of the acid form of HA disaccharide unit, M_H the molar mass of hydrogen and $M_{u_{NaHA}}$ the molar mass of the sodium form of HA disaccharide unit.



298

299 **Fig. 3.** 1H NMR spectrum of HA_{140k}-g-PMeOx₂₉-13% in D₂O (with 0.125 M NaOD).

300 The grafting efficiencies were ranging from 8 to 16 %, except with sample HA_{990k}-g-
 301 PMeOx₂₉-0.12 having a surprisingly higher grafting efficiency compared to the other samples
 302 (24%). Nevertheless, increasing the PMeOx/HA unit ratio led to a noticeable increase of the DS
 303 except for the highest HA molar masses (i.e. 990 000 g.mol⁻¹). This result suggests that the
 304 maximum achievable DS may not exceed the range of 11-13 %, which can be explained by the
 305 high steric hindrance induced by both the bulky polyoxazoline functionalities on neighbouring
 306 carboxylate moieties and by HA itself. **The starting HA molar mass did not significantly impact**
 307 **the outcome of the reaction. On the other hand**, increasing the DP_{PMeOx} of the grafted PMeOx
 308 chain from 29 to 47 led to lower grafting efficiencies when applying similar reaction conditions

309 as can be seen in Table 2 (e.g. from 16 % for sample HA_{140k}-g-PMeOx₂₉-0.08 to 8 % for sample
 310 HA_{140k}-g-PMeOx₄₇-0.04, which were both obtained using a PMeOx (chain) to HA (unit) ratio of
 311 0.5). Finally, the mass yield of the synthesis ranged from 40 to 72% which is satisfying
 312 considering the complete purification.

313 **Table 2.** Experimental conditions of PMeOx grafting to HA and experimental grafting rates.

Samples	$n_{\text{PMeOx (chain)}}/n_{\text{HA(unit)}}^{\text{a}}$	DP_{PMeOx}	DS^{b}	Grafting efficiency (%) ^c	Mass yield (%)
HA _{38k} -g-PMeOx ₄₇ -0.05	0.5	47	0.05	10	50
HA _{38k} -g-PMeOx ₂₉ -0.09	1.0	29	0.09	9	62
HA _{140k} -g-PMeOx ₄₇ -0.04	0.5	47	0.04	8	62
HA _{140k} -g-PMeOx ₂₉ -0.08	0.5	29	0.08	16	n.d.
HA _{140k} -g-PMeOx ₂₉ -0.13	1.0	29	0.13	13	40
HA _{990k} -g-PMeOx ₄₇ -0.05	0.5	47	0.05	10	82
HA _{990k} -g-PMeOx ₂₉ -0.12	0.5	29	0.12	24	72
HA _{990k} -g-PMeOx ₂₉ -0.11	1.0	29	0.11	11	58

314 ^a molar ratio

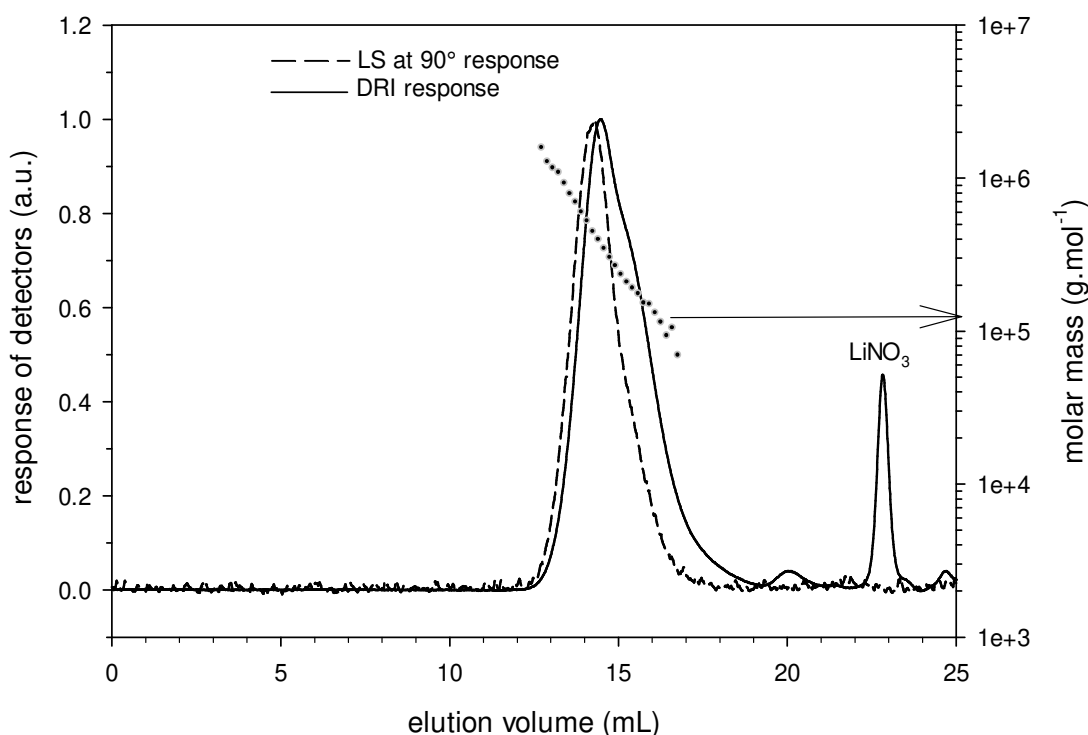
315 ^b determined by ¹H NMR spectroscopy according to Eq. 1

316 ^c defined as $\frac{\text{DS}}{n_{\text{PMeOx(chain)}}/n_{\text{HA(unit)}}}$

317 n.d.: not determined

318

319 The different HA-g-PMeOx were analysed with on-line coupled SEC/MALS/VD/DRI (Fig. 4).
 320 The resulting chromatograms display one distribution between 13 and 17 mL of elution
 321 corresponding to the grafted HA-g-PMeOx copolymer. SEC/MALS/VD/DRI was found out to be
 322 an effective method to check the HA derivative purity. Indeed, free PMeOx, with a much
 323 shorter chain than HA, would appear as a secondary distribution at around 20 mL (Fig. S2). For
 324 instance, HA-g-PMeOx was still displaying two distributions after the dialysis and subsequent
 325 freeze-drying steps and before the washing steps in ethanol (Fig. S3), indicating the dialysis
 326 step did not fully remove free PMeOx, despite the membranes cut off (6-8 kDa or 12-14 kDa)
 327 being higher than the PMeOx molecular weight (either 2.5 kDa or 4 kDa). This prompted us to
 328 additionally wash the material three times in ethanol, after which free PMeOx content was
 329 reduced to trace level (i.e < 2 % of the total sample weight) (Fig. 4).

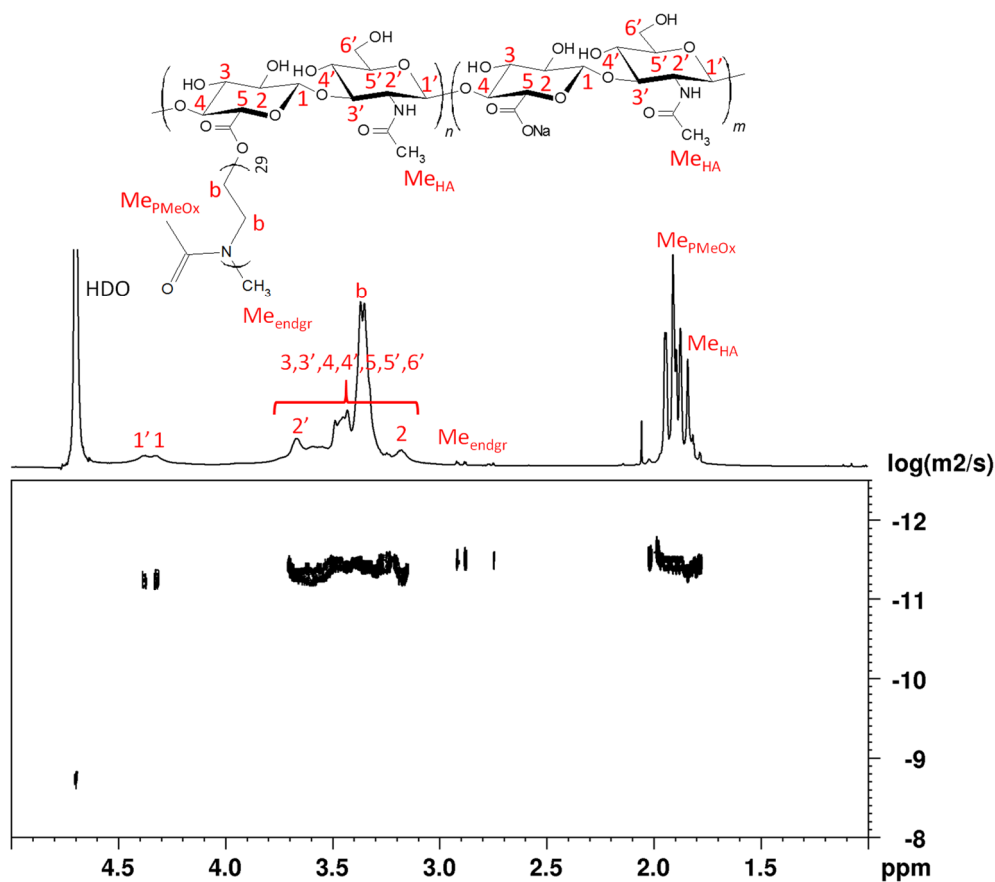


330

331 **Fig. 4.** Elution profiles and molar masses distribution of HA_{140k}-g-PMeOx₂₉-0.13 in 0.1 mol.L⁻¹
 332 LiNO₃ aqueous solution.

333 In order to further confirm the successful covalent grafting of PMeOx to HA and the
 334 absence of remaining free PMeOx after the washing steps with ethanol, diffusion-ordered
 335 NMR spectroscopy (DOSY) can be used for differentiating the species in solution according to
 336 their size *via* diffusion measurements. DOSY NMR measurements were then undertaken on
 337 PMeOx, HA, HA-g-PMeOx polymers (Fig. 5 and 6) and HA / PMeOx physical mixture (Fig. S4 and
 338 6). Unfortunately, in the range of 3-4 ppm, the proton signals of the two polymers were too
 339 close to give two different diffusion coefficient values. We decided to focus our attention on
 340 the isolated signals assigned to the HA anomeric protons at 4.38-4.32 ppm and PMeOx methyl
 341 end group at 2.92-2.88 ppm. For instance, the 2D ¹H DOSY spectra of HA_{140k}, PMeOx₂₉
 342 polymers and the physical mixture of these two polymers HA_{140k}/PMeOx₂₉, show that, as
 343 expected, HA_{140k} diffuses more slowly than PMeOx₂₉ and both polymers diffuse much more
 344 slowly than H₂O (Fig. 6 a-d-e).

345



346

347 **Fig. 5.** 2D ^1H DOSY NMR spectrum of $\text{HA}_{140\text{k}}\text{-g-PMeOx}_{29}\text{-}0.13$ in D_2O at 290 K.

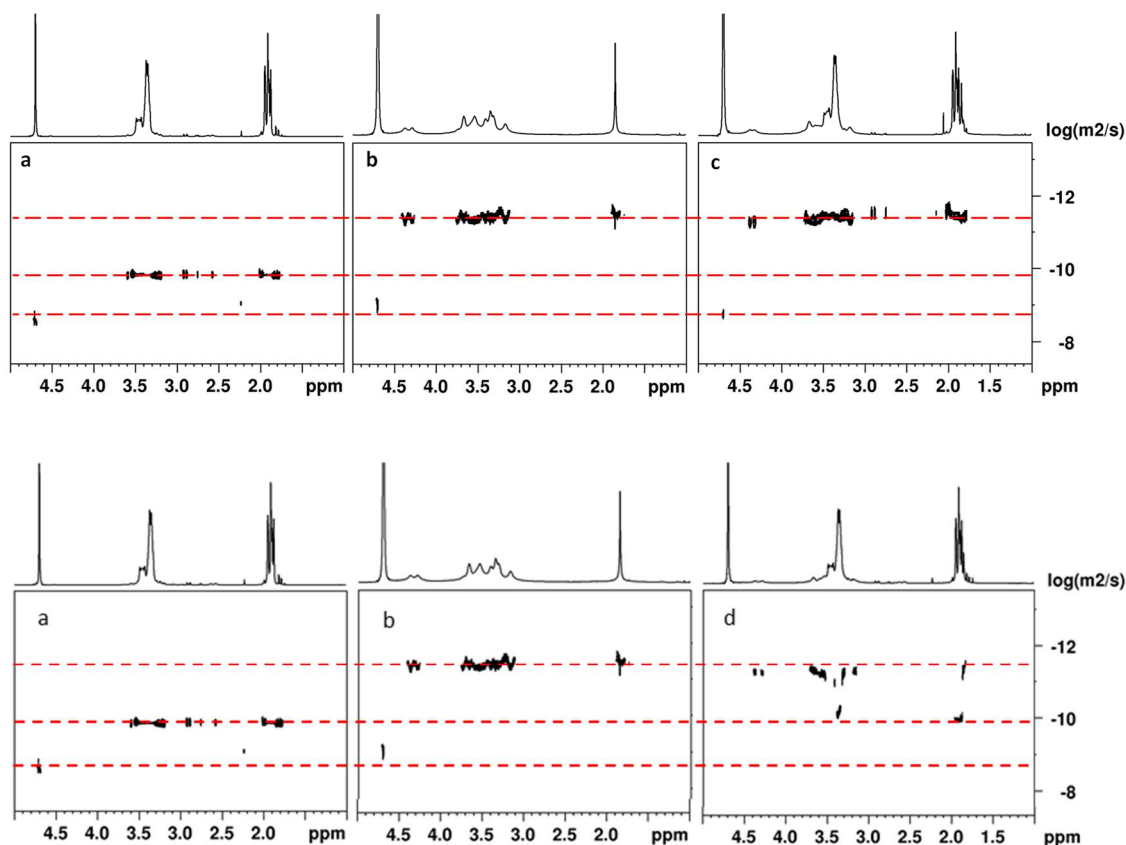
348 In the case of the HA-g-PMeOx copolymer, contrary to what was observed on the physical
 349 mixture, the 2D ^1H DOSY spectrum shows similar diffusion for the HA anomeric protons and
 350 the PMeOx methyl end chains (Fig. 6 a-b-c). This observation is consistent with the behaviour
 351 of the two polymers as a single unit and is a further proof of the successful grafting of PMeOx
 352 to HA through the formation of a covalent bond and that the sample was well purified.

353 3.3. Physico-chemical properties of HA-g-PMeOx

354 The physico-chemical properties of all the samples, obtained with SEC/MALS/VD/DRI
 355 analysis, are shown in Table 3.

356 For $\text{HA}_{38\text{k}}$ and $\text{HA}_{140\text{k}}$, the molar masses increased with the DS, which was expected as the
 357 average repetitive unit molar mass became higher when grafted with PMeOx chain [26].
 358 Furthermore, the DP_n (corresponding to the “degree of polymerisation” of HA-g-PMeOx) could
 359 be calculated according to both grafted PMeOx M_{nPMeOx} and DS (see Supplementary
 360 information for the calculation of DP_n). As a matter of fact, it appeared that for $\text{HA}_{38\text{k}}$ series the

361 DP_n was quite unchanged after the functionalization except a very slight decrease and
362 consequently a slight degradation for HA_{38k}-*g*-PMeOx₄₇-0.05, where the DP_n went from 95 to
363 77. For HA_{140k} series, a noticeable decrease of the DP_n was observed from 350 for the
364 precursor to 270, 300 and 330 for HA_{140k}-*g*-PMeOx₄₇-0.04, HA_{140k}-*g*-PMeOx₂₉-0.08 and HA_{140k}-
365 *g*-PMeOx₂₉-0.13 respectively. This tendency was fully confirmed and amplified with the highest
366 HA molar masses series (i.e. HA_{990k}-*g*-PMeOx). These results can be correlated to an important
367 HA chain degradation. The longer the starting HA chain, the higher the decrease of molar
368 masses. This appears in agreement with both the increase of flexibility of the chain [28] and
369 the amplified effect of one chain breaking when increasing the chain length (i.e. molar masses).
370 Such degradation could be the consequence of the reaction temperature (60 °C), HA being
371 sensitive to high temperatures [20].



372

373

374 **Fig. 6.** 2D ¹H DOSY NMR spectra (600 MHz, D₂O, 290 K) of PMeOx₂₉ (a); HA_{140k} (b); HA-*g*-PMeOx
375 (c); physical mixture of HA_{140k} and PMeOx₂₉ (d).

376

377 ~~It must also be noticed that HA₉₉₀ under its TBA-HA form was more difficult to solubilize in~~
 378 ~~DMSO than the HA of lower molar masses, and has been consequently placed at 60 °C for one~~
 379 ~~more day this treatment helping to homogenize the solution. This may also partly explain the~~
 380 ~~highest chain degradation observed for the HA₉₉₀-g-PMeOx samples.~~

381 **Table 3.** Macromolecular characteristics of the samples by SEC/MALS/VD/DRI (25 °C, LiNO₃ 0.1
 382 mol.L⁻¹).

Samples	M _n (g.mol ⁻¹)	M _w (g.mol ⁻¹)	DP _n	Đ	[η] _w (mL.g ⁻¹)	Rh _w (nm)	Rg _w (nm)	a (MHS)
HA _{38k}	38 000	64 000	95	1.68	145	11	^a	0.76
HA _{38k} -g-PMeOx ₄₇ -0.05	46 000	78 000	77	1.68	130	11	^a	0.78
HA _{38k} -g-PMeOx ₂₉ -0.09	60 000	103 000	96	1.70	130	12	^a	0.72
HA _{140k}	140 000	210 000	350	1.50	410	23	44	0.71 ^b
HA _{140k} -g-PMeOx ₄₇ -0.04	150 000	210 000	270	1.41	350	22	38	0.66 ^b
HA _{140k} -g-PMeOx ₂₉ -0.08	180 000	260 000	300	1.45	340	23	38	0.67 ^b
HA _{140k} -g-PMeOx ₂₉ -0.13	240 000	350 000	330	1.45	350	26	45	0.69 ^b
HA _{990k}	990 000	1 300 000	2 475	1.36	1 600	68	147	^{-c}
HA _{990k} -g-PMeOx ₄₇ -0.05	640 000	940 000	1065	1.47	890	49	94	^{-c}
HA _{990k} -g-PMeOx ₂₉ -0.12	470 000	650 000	675	1.37	570	38	66	^{-c}
HA _{990k} -g-PMeOx ₂₉ -0.11	650 000	910 000	967	1.40	750	46	80	^{-c}

383 MHS coefficient (a) has been determined by integrating the most linear part of bilogarithmic
 384 representation of intrinsic viscosity vs molar mass

385 SEC/MALS/VD/DRI data accuracy: M_n ± 9%; M_w ± 6%; Đ ± 10%; [η]_w ± 0.5%; Rh_w ± 2%; Rg_w ± 6%

386 ^a out of the range of the MALS detector (isotropic diffusion for lower masses fractions)

387 ^b See Fig. S5 for MHS plots

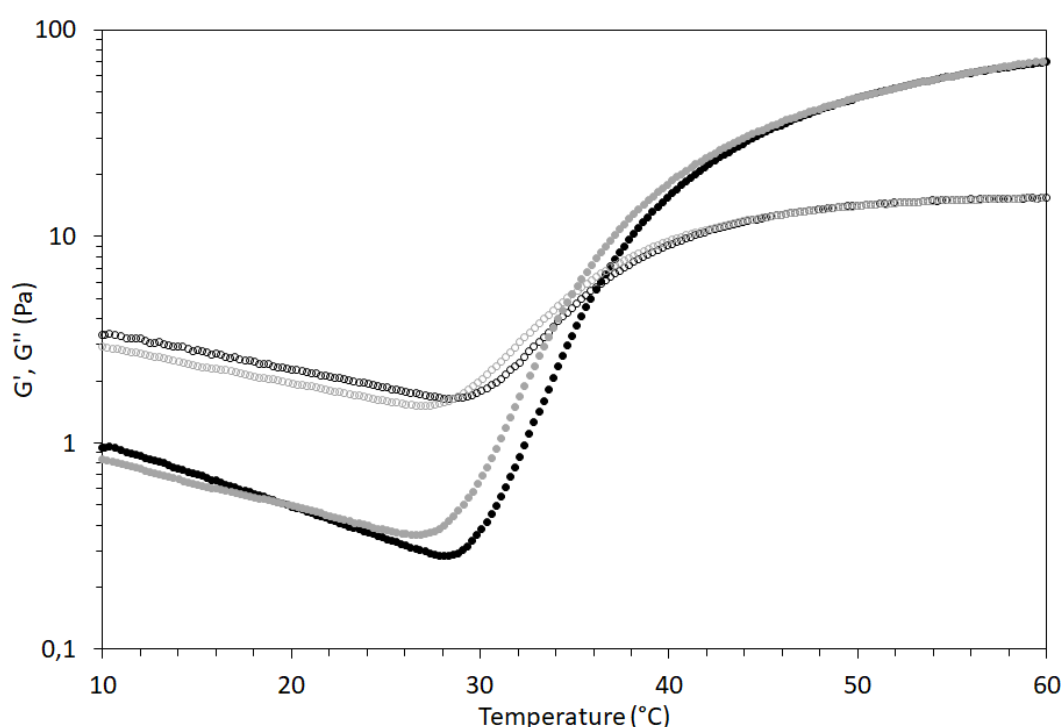
388 ^c undetermined values due to challenging analysis

389

390 Other physico-chemical parameters could also be determined by SEC/MALS/VD/DRI (Table
 391 3): gyration radii (Rg_w), intrinsic viscosities ([η]_w), hydrodynamic radii (Rh_w) and the Mark
 392 Houwink Sakurada (MHS) parameter 'a' (Mark-Houwink-Sakurada plots are given in Fig. S5).
 393 One can observe the decrease of size parameters (Rg_w, [η]_w and Rh_w) correlated with the
 394 above discussed degradation. For instance, Rg_w, [η]_w and Rh_w values of HA_{990k}-g-PMeOx₂₉-0.11
 395 (respectively 80 nm, 750 mL.g⁻¹ and 46 nm) were all lower than those of starting HA_{990k}

396 (respectively 147 nm, 1600 mL.g⁻¹ and 68 nm) which correlated well with the drop of the DP_n of
 397 the polysaccharide chain (from 2475 to 967 after modification). Concerning the MHS
 398 parameter, which gives conformational information, the results seem to indicate that the HA-
 399 g-PMeOx derivatives present quite the same conformation than the corresponding HA
 400 precursors. Meanwhile 'a' values were always around 0.7 which means all polymers present a
 401 classical random coil conformation in good solvent [28]. Logically, PMeOx moieties are clearly
 402 hydrophilic and do not affect the water-soluble character of HA derivatives.

403 3.4. Application of the reaction to devise HA-based physical hydrogels



404 **Fig. 7.** Rheological profile of HA_{990k}-g-(PiPrOx₂₀-co-PnBuOx₇)-0.10 (G': full circles; G'': empty
 405 circles; heating ramp: black circles; cooling ramp: grey circles) at 1.5 wt% in PBS during a
 406 temperature ramp (rate: 0.5 °C.min⁻¹; shear stress: 0.1 Pa; frequency: 1Hz).
 407

408

409 The poly(2-alkyl-2-oxazoline) polymerisation reaction was then performed on a mixture of
 410 *i*PrOx and *n*BuOx, the resulting copolymer, PiPrOx₂₀-co-PnBuOx₇ (DP: 27), being thermo-
 411 responsive [7] [16], with a cloud point at 26 °C for 10 g.L⁻¹ solutions) or at 32 °C for 2 g.L⁻¹
 412 solutions according to our measurements. It was subsequently terminated with HA_{990k} to result
 413 in HA_{990k}-g-(PiPrOx₂₀-co-PnBuOx₇)-0.10 (see Fig. S6 for ¹H-NMR spectrum), which was then
 414 dissolved in PBS as a 1.5 wt% solution to perform rheological measurements on it. Briefly, a

415 0.5 °C.min⁻¹ heating ramp was applied on the solution from 10 °C to 60 °C with a set stress of
416 0.1 Pa (linearity domain was checked beforehand) and a set frequency of 1 Hz, followed by a
417 corresponding cooling ramp. The evolution of the storage modulus G' and of the loss modulus
418 G'' with temperature were then plotted, the results being displayed on Fig. 7. A reversible
419 thermal transition was clearly seen, with G' being below G'' at the lower temperatures,
420 indicative of a predominantly viscous behaviour, before crossing it at 36.2 °C (34.9 °C during
421 the cooling ramp) and eventually becoming higher at the higher temperatures, indicative of a
422 predominantly elastic behaviour typical of hydrogels.

423

424 This result is quite promising for us and we will investigate the influence of the grafted
425 copolymer composition and length on the functionalized HA hydrogel transition in an
426 upcoming publication.

427 **4. Conclusion**

428 PMeOx was successfully grafted on HA through a direct termination route between the
429 oxazolinium terminal group of PMeOx chain and carboxylate moieties of HA without using any
430 activating agent. The successful grafting as well as the purity of the resulting HA-*g*-PMeOx have
431 been confirmed with SEC/MALS/VD/DRI and DOSY NMR experiments. The one-pot
432 functionalization has been conducted with various lengths (DP_{PMeOx}) of PMeOx chain and/or
433 target DS on various HA molar masses. The DS seems to cap up to about 13 % probably due to
434 the steric hindrance of both PMeOx and HA. Nevertheless, it should be noted such a DS is
435 already accounting for a PMeOx weight fraction in HA-*g*-PMeOx of 45 %. Characterisation of
436 the grafted HA derivatives indicated a HA chain degradation under high temperature (i.e.
437 60 °C) that was more pronounced when HA molar mass increases. From a conformational
438 aspect based on the MHS exponent analysis, HA derivatives (HA-*g*-PMeOx) and precursors (Na-
439 HA) seem to present the same random coil conformation in water (i.e. good solvent).

440 The reaction could then be extended to another poly(2-alkyl-2-oxazoline), namely
441 $PiPrOx_{20}\text{-}CO\text{-}PnBuOx_7$, which was terminated with HA_{990k} , the resulting copolymer showing a
442 hydrogel transition around 36 °C as a 1.5 wt% solution in PBS. Future work will further focus on
443 the HA grafting with thermo-responsive poly(2-alkyl-2-oxazoline). Both polymers are highly
444 biocompatible, HA being a component of the extracellular matrix and poly(2-alkyl-2-oxazoline)
445 being increasingly used for biomedical applications.

446 **Declaration of Competing Interest**

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

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454

455

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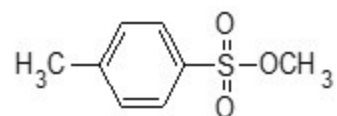
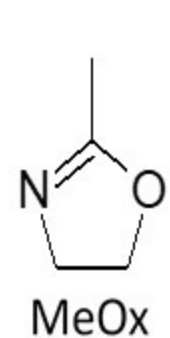
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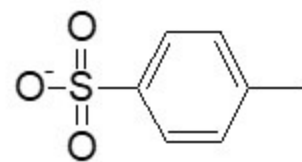
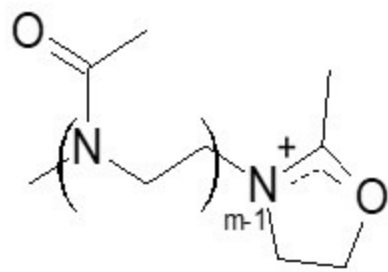
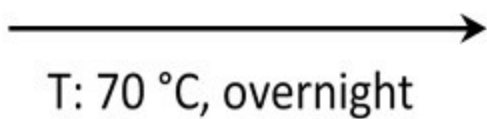
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556



Acetonitrile



Polymerization of MeOx



Termination step with TBA-HA



HA-g-PMeOx

One-pot functionalization of HA



HA



HA-g-PMeOx

Random coil conformation with no free PMeOx