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1 Interaction of gentamicin sulfate with alginate and consequences on the physico-
2 chemical properties of alginate-containing biofilms

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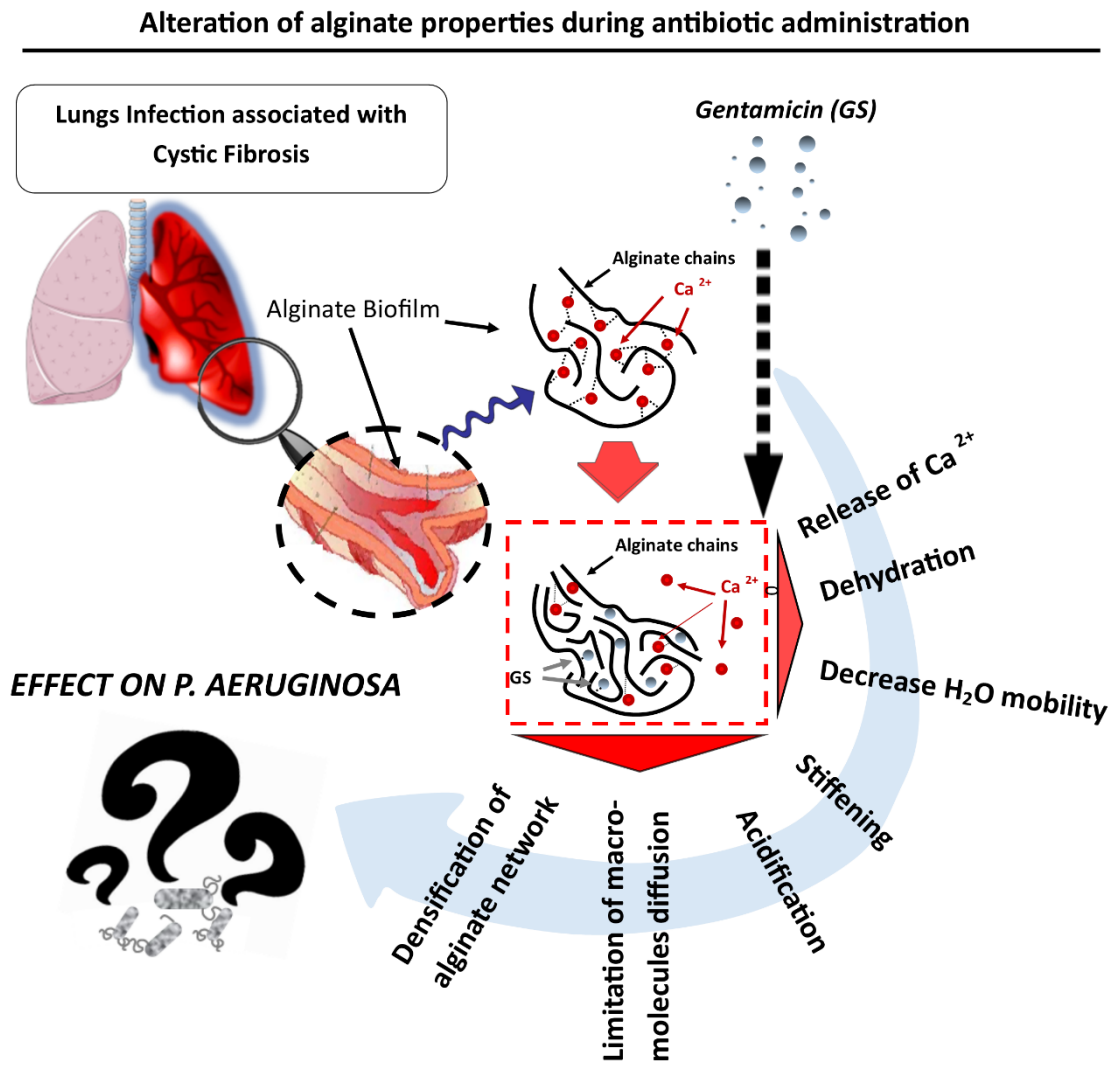
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12

13 **Graphical abstract**

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15

16

17 **Abstract**

18 Background: Alginate is one of the main extracellular polymeric substances (EPS) in biofilms secreted
19 by mucoid *Pseudomonas aeruginosa* in Cystic Fibrosis (CF) patients suffering from pulmonary
20 infections. Gentamicin, commonly used in CF patients, can strongly bind to alginate resulting in loss of
21 pharmacological activity; however neither the mechanisms of this affinity nor its repercussion are fully
22 understood. In this study, we investigated how the interaction with gentamicin sulfate (GS) modifies
23 alginate macromolecular network, with special emphasis on the physico-chemical features relevant for
24 CF-biofilm properties.

25 Methods: Alginate gels were crosslinked with Ca^{2+} and exposed to GS at varying times and
26 concentrations. Alginates with standard composition (enriched in guluronate units (G)) or enriched in
27 guluronate units (G) were used to study the influence of its composition on GS interaction. The
28 complexes were characterized via turbidimetry, mechanical tests, swelling, calorimetry techniques
29 nuclear magnetic resonance, Ca^{2+} displacement, diffusion of macromolecular probes and pH
30 alteration.

31 Results: In presence of GS, alginate network undergoes a tremendous reorganization. The gel turns
32 opaque, shrinks, becomes stiffer, decreases the diffusion of macromolecules and the state of the
33 molecules of water entrapped changes. Additionally, the environment of the gels is altered, with
34 release of calcium, expulsion of water and acidification.

35 Conclusions: We showed in this study that physico-chemical properties of alginate are dramatically
36 influenced by GS. These changes could potentially exacerbate the protective effect of the surrounding
37 EPS on the biofilm forming-microorganisms. Our results underline the importance of alginate as biofilm
38 component, its pernicious role during antibiotherapy, and could represent a potential molecular target
39 to improve existing therapies.

40 **Key-words:** Alginate, Gentamicin, Cystic Fibrosis, Aminoglycosides, Biofilm

41 **1. Introduction**

42 Alginate is a natural polysaccharide generally extracted from algae, containing repetition of M
43 and G units (being respectively mannuronate and guluronate). It was already reported that only G-
44 units are involved in the crosslinking mechanism with divalent ion such as Ca²⁺. Alginate is also one of
45 the main macromolecules of biofilms secreted by *Pseudomonas aeruginosa* in Cystic Fibrosis (CF)
46 patients, suffering from pulmonary infections. Once *P. aeruginosa* colonize the lungs, it can acquire a
47 mucoid phenotype, which renders infection extremely difficult to treat, despite intense antibiotherapies.
48 The degree of aminoglycoside-susceptibility of *P. aeruginosa* inside biofilm is up to 1000-fold less than
49 in planktonic form [1, 2], but it remains globally unchanged if we compared bacteria isolated from
50 mucoid-biofilm with non-mucoid phenotypes [3, 4]. This is in line with the fact that the surrounding
51 micro-environment of *P. aeruginosa* represented by the EPS (with M-rich alginate) is one of the key
52 factors to confer antibiotherapies resistance [2]. Such statements are not new, and in early works
53 alginate has already been described as acting as a protective niche for bacteria, by hampering
54 phagocytosis [5], by scavenging Reactive Oxygen Species [6], and by creating locally an anaerobic
55 environment favouring microorganisms low metabolic activity. Such responses can even be further
56 amplified as un-successful eradication of bacteria in initially non-mucoid biofilm can stimulate more
57 alginate production, compromising even more the chance of antibiotherapies success [7]. Many of the
58 early *in vitro* works have already stressed the fact there is a strong interaction between some drugs
59 and alginate, resulting in a limited antibiotics diffusion through alginate matrices [8-10]. Nevertheless,
60 no study reports specifically on how antibiotics affect the physico-chemical characteristics of alginate,
61 and how it alters locally the micro-environmental properties.

62 In this study, we deciphered how the nature and composition of alginate do interfere with the
63 gentamicin, from a molecular to a macroscopic scale. By understanding such phenomenon, this study
64 paves the road to further develop *in vitro* models of biofilm and alternative strategies to treat CF
65 patients affected by lung infection.

66

67 **2. Materials and Methods**

68 Except if stated differently, all the chemicals were purchased from Sigma-Aldrich.

69 **2.1 Preparation of alginate gels**

70 Acellular alginate gels were fabricated via an external ionic crosslinking procedure using Ca²⁺.
71 Two ultra-pure alginates from Pronova (UP-LVG enriched in guluronate motifs, G=67%) and UP-LVM
72 enriched in mannuronate motifs, M=59%) were used to form gels via incubation of alginate solution (at
73 1.5 % weight/volume) in wells of defined size created in agarose moulds. These moulds were
74 produced in presence of CaCl₂ 100mM to allow slow diffusion and gelling of the alginate. The obtained
75 gels, based on alginate LVG or alginate LVM (called High-G or High-M respectively), were kept
76 overnight in solution of 100mM CaCl₂ before any further experiment. To study of effect of gentamicin
77 sulfate (GS, purchased from Roth) on alginate gels characteristics, the gels were subsequently
78 incubated in solutions with defined GS concentrations. Unless otherwise stated, the GS concentration
79 was fixed at 0.5% in distilled water and the gels were incubated overnight (o/n) at room temperature
80 (RT).

81 **2.2 Investigation of alginate gel's alteration by GS**

82 After incubating each gel in a 96-well-plate covered with 200µL of GS solutions (n=6), the
83 opacity of the gels was monitored at 600nm using a spectrophotometer (Thermo Scientific, Multiskan
84 GO). Shrinking or swelling of the gels were determined by measuring weight and size with a balance
85 and with a digital caliper after blotting the excess of solution (n=3). The influence of GS on alginate
86 gels' stiffness was determined during a compression test (Instron, with static load cell of 10N, at
87 1mm/min). The samples were kept unconfined and immersed in phosphate buffer solution (PBS) at RT
88 for the duration of the experiment (n=3). The amount of GS adsorbed in the different gels was
89 determined after gel dissolution in 1mL of 150mM EDTA at pH 8 for 30min under rotation and
90 quantified using o-phthalaldehyde reaction (OPA) as described in this protocol [11] (n=2). The *in vitro*
91 release kinetic of GS from the different gels were performed after incubation of the gels in 1mL of NaCl
92 at 0.9% at 37°C under continuous agitation. The supernatants were removed and refreshed at every
93 time points and kept frozen until GS quantification using OPA as above indicated (n=3). Amount of
94 calcium ion present in the alginate gels before and after the incubation with GS solutions (at 0, 0.5 and
95 3.5%) was evaluated after mineralization of the samples in acidic condition and analysis
96 using inductively coupled plasma mass spectrometry (ICPMS 7700x Agilent) (n=2). The effect of a
97 continuous *versus* repeated incubation of alginate gels with GS solution was evaluated by incubating
98 gels with either only a solution of 0.5% GS in PBS (containing 10% bovine serum albumin (PBS and
99 BSA was added as source of ions and proteins to mimic *in vivo* conditions)) refreshed every 24hrs
100 (1mL per gel), or by alternating every day with either the GS or the drug-free PBS/BSA solution

101 (experiment performed at 37°C under agitation, n=3). At the end of every 24hrs incubation-slot period,
102 the gels were weighted and then re-incubated in the next solution, for a total of 7 days. To illustrate the
103 acidification triggered by GS on the gels, we formulated alginate gels containing pH-indicator (phenol
104 red, turning from red (when neutral), to yellow during acidification). The colour change was imaged
105 after adding 1mg of GS powder on top of the gel.

106 **2.3 Study of alginate permeability by macromolecules**

107 In order to assess how GS can alter the permeability of alginate gels, gels of either High-M
108 alginate or of alginate with GS (High-M/GS) were incubated in a solution of fluorescent Dextran
109 (Tetramethylrhodamine isothiocyanate, TRITC-Dextran, 4.4kDa) either dissolved in DI water (for High-
110 M group) or in DI water containing GS at 0.5% (for High-M/GS group). The penetration of Dextran-
111 TRITC was evaluated by continuous reading of the fluorescent intensity of the gels after a quick wash
112 with DI water between every reading to remove unbound dextran (Viktor³, 1420 Multilabel Count,
113 Perkin-Elmer). At the end of the experiment, the gels were washed, dissolved in EDTA solution, and
114 the amount of Dextran-TRITC was further quantified by fluorescence against a calibration curve (n=3).
115 In addition, the visualization of the Dextran-TRITC penetration in the gel was observed on gels' cross-
116 section using a fluorescent confocal microscope (Axiovert 200m microscope, Zeiss).

117 **2.4 Investigation of water mobility and of the state of water present in the different gels**

118 In order to investigate the amount of water in the gels and its degree of interaction with the
119 alginate/GS matrix, thermogravimetric analyses (TGA, TA Instrument Q50) were performed on gels
120 upon heating from 25 to 350°C at 10°C/min. The state of the molecules of water present in the gels
121 were investigated using differential scanning calorimetry (DSC, Perkin Elmer Instrument DSC 6000
122 Thermal Analyzer). Samples were submitted to a first cooling at -40°C, then to a heating ramp up to
123 +40°C (at 1°C/min) followed by a second cooling ramp to -40°C (at 2°C/min). The degree of water
124 mobility and its interaction with the alginate matrix was assessed on gels using magnetic resonance
125 imaging (MRI, 7T Bruker Biospec 70/20 USR Avance III), and measure of relaxation times of the
126 protons of water (longitudinal and transversal relaxations) were recorded using T1-weighted and T2-
127 weighted sequences respectively. T1-weighted imaging parameters were as follows: echo time (TE)
128 2.8ms, matrix 256*192, slice thickness 0.8mm, inversion time (T11 = 50ms; T1100 = 4980ms), recovery
129 time (TR) 7500ms. T2-weighted imaging parameters were as follows: TR 7500ms, TE from 8 to
130 200ms, matrix 256*192, slice thickness 0.8mm.

131

132

133 **3. Results and Discussion**

134 The incubation of Ca-crosslinked alginate gels with GS is responsible to a rapid increase of
135 the opacity for all alginates used in this study. Nevertheless, this effect was consistently more
136 pronounced for High-M, for every time point and all GS concentrations (Figure 1A). The agarose
137 control exposed to the same GS concentration did not display any opacity. Similarly to alginate,
138 agarose is a polysaccharide, however it consists of different repeating units and it is not polyanionic
139 like alginate. Therefore, the opacity observed is not due to unspecific GS precipitation in presence of
140 polysaccharides, but to specific chemical interactions between the Alginate and GS. The Figure 1B
141 demonstrated that GS can diffuse inside alginate gels, in a higher amount for high-M than for High-G.
142 Alginate does not hamper GS diffusion (in contradiction to previous reports [9, 10]); but the
143 polysaccharide network sequesters GS following internal diffusion. This first set of experiments
144 presented in Figure 1 illustrates that GS can generate specific interactions with alginate, resulting in
145 increased opacity during incorporation within the polymeric network. Both opacity and incorporation
146 occurred to a stronger extent with alginate enriched in M- rather than G- motifs. Besides the opacity,
147 the inset in Figure 1A is also indicative of network compaction and shrinking upon interaction. This
148 phenomenon was analysed with the experiments illustrated in Figure 2. The volume of the gels
149 decreased of 44% in case of High-G and 63% in case of High-M following o/n incubation with GS. It is
150 interesting to note that, in the case of alginate High-M, the shrinking of the structure is maximal when
151 low concentrations of GS are present in the solutions (SD1). Whereas, for High-G, the densification of
152 the gels followed a concentration-dependence pattern, with higher GS concentrations being
153 responsible to higher gels shrinkage (SD1). This shrinking process was not affected by the presence
154 of Na⁺ and K⁺ cations and PO₄³⁻ and Cl⁻ anions present in PBS and proteins (albumin present in BSA)
155 and reached a steady state rapidly after 1 day of continuous incubation with GS (Figure 2B). Serial
156 incubation of alginate gels in solutions with and then without GS resulted in repetitive shrinkage and
157 then re-swelling of the structures. The cycle was not totally reversible, as mass gained and lost was
158 slightly smaller at every cycle. This behaviour can be explained by GS adsorption from the GS-
159 containing medium, triggering shrinkage followed by GS diffusion as soon as the gels are soaked in
160 GS-free medium, with re-swelling of the structure. This specific behaviour is particularly meaningful
161 from a clinical perspective. CF-patients are regularly treated via repetitive inhalation of antibiotics, and

162 such a pulsatile administration could have major impact on biofilm hydration, volume and compaction,
163 with further impact on the embedded microorganisms.

164 Once the GS is in contact with alginate gel, a GS reservoir is formed in the gel, which is then released
165 for a prolonged period-of-time (Figure 2D). The GS release was shown to be more sustained in the
166 case of M-rich alginate than for G-rich alginate gels. This factor could result in the selection of resistant
167 bacteria if sub-inhibitory concentrations of antibiotics are released locally for extended duration [12].

168 The network compaction and dehydration in presence of GS was further investigated with
169 compression tests. GS-laden networks were consistently stiffer than GS-free controls, and for High-M
170 the Young modulus increase was 2.3-fold compared to pre-incubation controls, whereas it reached a
171 1.6-fold factor for High-G in the same conditions (Figure 3A). It was already reported that increasing
172 substrate stiffness influences *P. aeruginosa* attachment, growth and decreases susceptibility to
173 antibiotics [13]. The creation of a drug-dependent stiffening 3D microenvironment could have a
174 remarkable impact on the bacteria embedded, however the available literature only carries information
175 on bacteria cultivated on 2D surfaces [13].

176 Divalent ions such as Ca^{2+} act as alginate crosslinker, bridging monosaccharide units together
177 to form a macromolecular gel network. Previous reports suggested that GS does not compete with
178 Ca^{2+} ions in alginate gels [14]. Our results challenge such a statement as we did observe a
179 competitive mechanism between GS and calcium electrostatic interaction with the alginate chains
180 (Figure 3B). Indeed, once GS penetrates alginate gels, it does trigger a release of calcium ions initially
181 present as crosslinker of alginate chains, even when low GS concentrations are used. Those
182 experiments reveal that GS acts as external crosslinker on alginate, similar to Ca^{2+} ions, and that Ca^{2+}
183 is expelled from alginate gels in presence of GS in the environment. The differences observed
184 between high-M and High-G in Figure 3A and B corroborates previous report, showing that Ca^{2+}
185 exhibits preferential binding to G-units, and GS to M-units [14]. This molecular exchange could
186 potentially have a role in the tenacity of the CF-infection as increasing the concentration of calcium in
187 medium has been demonstrated to increase the rate and the extend of *P. aeruginosa* biofilm
188 production [15] and to elevate the airway surface liquid viscosity [16].

189 Network swelling or compaction can have a direct effect on the matrix diffusion properties.
190 Therefore, we employed fluorescent Dextran-TRITC as macromolecular penetration probe through

191 High-M gels in presence / absence of GS. The fluorescence microscopy images on Figure 4A showed
192 a clear difference with diffuse fluorescence for the gels not exposed to GS and a significantly lower
193 signal for the gels incubated with GS. The spectroscopic quantification of the fluorescent evolution
194 showed a similar trend, with a fast increase of the fluorescent intensity in the first 40min of incubation
195 with Dextran-TRITC and a plateau reached after 180min for the swollen gels in absence of GS in the
196 solution (Figure 4B). For the samples incubated with Dextran-TRITC in combination with GS, a lower
197 amount of Dextran diffused within the gels ($p < 0.0001$). The quantification of the Dextran-TRITC at the
198 end of the incubation period proved as well that the presence of GS in the gels environment was
199 directly responsible to a decrease of macromolecule diffusion of a factor 3.3. This proof-of-concept
200 using Dextran showed that treating alginate-rich biofilm with GS results in a significant reduction of the
201 capability of macromolecules to diffuse inside the structure. This result could have significant impact
202 on biofilm physiology and eradication, as following administration of GS in the lung, nutrient-
203 deprivation with metabolically-low active bacteria phenotypes could potentially emerge.
204 Macromolecular compaction within the biofilm could also hinder oxygen diffusion, contributing to
205 hypoxic domains formation. This hypothesis deserves particular attention, as *i*) the lung of CF-patients
206 with biofilm are already characterized by low oxygen diffusion [17], *ii*) *P. aeruginosa* production of EPS
207 increases under low oxygen concentration [17], and *iii*) antimicrobial activity of several antibiotics are
208 decreased in low oxygen concentration environment [18]. Further investigations are necessary to
209 determine oxygen diffusion in GS-laden alginate matrices and its clinical significance.

210 Another important characteristic of CF-lung is the local acidity ($\text{pH} < 6.5$ [19, 20]), therefore,
211 we investigated the influence of the pH on gel's swelling or shrinking. In absence of GS, the pH of the
212 incubating solutions impacted significantly on gels swelling. Such observation was completely inhibited
213 when the same buffers were supplemented with GS (similar shrinkage of $\pm 50\%$, SD2). Alginate gels
214 were able to adsorb similar amount of GS, independently of the pH of the medium (SD2). In addition,
215 we showed that due to its acidic nature [21], GS can modify the local pH. Indeed, when we deposited
216 a small amount of GS on top of the alginate gel, a rapid change of the pH-indicator was observed,
217 from red to yellow (Figure 5A), whereas no variation was seen on the control (Figure 5B). Low pH is
218 associated with a decrease of aminoglycosides bactericidal activity due to increase in ionisation
219 degree [22] and to a change in *P. aeruginosa* membrane composition (additional phospholipids)
220 affecting its sensitivity to antimicrobial therapies [23]. Along with the acidification, a decrease in
221 volume accompanied by gel syneresis (expulsion of water) were observed on the GS-supplemented

222 gel (denoted with blue arrows, Figure 5A). *P. aeruginosa* resists to dehydration by secreting more
223 alginate in biofilm due to upregulation of *algD* transcription [2]. Therefore, from our experimental
224 results, it is possible to expect that network dehydration in presence of GS incubation might have a
225 direct effect on alginate secretion by *P. aeruginosa*.

226 Thermal analyses were performed to investigate the nature of the water remaining in the gels. TGA
227 results, presented Figure 6A showed that the water evaporates at significantly lower temperatures for
228 High-M with GS compared to High-M (50% of the weight lost is reached at 84°C for High-M with GS,
229 against 115°C for High-M without GS). The difference of the remaining dry weight at the end of the
230 experiment between High-M and High-M with GS correspond to the amount of GS adsorbed in the last
231 group (equivalent to 0.4mg per mg of dry alginate). Further experiments conducted using DSC showed
232 a double peak in the crystallization and a shift in the melting temperature for the molecules of water
233 present inside the GS-free gels. In comparison, similar heat flows were registered between High-M
234 with GS and bulk water (containing only free molecule of water). This reveals that the water present in
235 High-M without GS is present under at least two states (most probably free and freezable-bound water
236 (being the portion of the water with thigh interaction with the alginate matrix) [24]), but that the
237 incorporation of GS inside the High-M network is responsible for water displacement, from bound to
238 free. This can explain the difference in water evaporation profiles recorded in TGA (Figure 6A), as free
239 water requires less energy to evaporate compared to bound water. To summarize Figure 6, we
240 demonstrated that the addition of GS did not only trigger a significant release of water in the
241 surrounding environment of the gels, but it alters the physico-chemical interactions of the molecules of
242 water remaining inside the gels with its surrounding alginate matrix.

243 Finally, MRI analyses validated the fact that the interaction of the molecules of water with the
244 alginate matrix depends on the presence or absence of GS (Figure 6C). In response to the drastic
245 network densification following GS incubation, the water mobility is highly limited in the alginate gels
246 (drop in T1) and its interaction with the chains increased (drop in T2). The effect of the state of the
247 molecules of waters on bacterial behaviour has not been reported to the best of our knowledge.
248 Nevertheless, it is well established, for example in the food industry, that environment with high activity
249 of water (corresponding to high degree of free water) are more prone to microorganisms proliferation
250 than products with low water activity.

251 Several *in vitro* observations presented in this manuscript correlate well with pathological signs
252 of CF-patient suffering chronic infection, such as accumulation of dehydrated mucus in the airways of
253 their lungs [25], extremely high and abnormal concentration of salts (including Ca²⁺) [26], abundance
254 of pulmonary oedema, acidity [16, 20] and decrease T1 signal in MRI [27]. The *in vitro* model of biofilm
255 presented here indicates that such symptoms could be exacerbated upon GS inhalation. The list of
256 phenomena described in this manuscript can be explained by ionic interaction between the positively
257 charged ammonium group in GS and the negatively charged carboxylic group present in alginate. This
258 strong ionic interaction is responsible to the gel's network reorganisation, resulting in the release of
259 calcium ion and of water, and in the rearrangement of the remaining water molecules. Our results
260 underline the importance of alginate as biofilm component, its pernicious role during antibiotherapy,
261 and could represent a potential molecular target to improve existing therapies. Outlook of this work will
262 be to investigate how bacteria embedded in biofilm-like structures react to such micro-environmental
263 dynamic evolutions. Using *P. aeruginosa* embedded in alginate beads (originating from seaweed) has
264 recently been suggested as suitable *in vitro* model to mimic CF-like condition and to screen antibiotics
265 activity [28]. Our study highlights the fact that alginate monosaccharide composition in M/G-units has
266 major impact on its behaviour and must be carefully considered. In addition, further strategies
267 originating from our results could focus on the prevention or the limitation of the alginate network
268 restructuring during incubation with GS (or other antibiotics which interact with alginate [9, 10, 28,
269 29]), using novel generation of adjuvants to antibiotherapies acting for examples as molecular "rigid
270 spacer".

271 **4. Conclusion**

272 In CF patient, once *Pseudomonas aeruginosa* bacteria have acquired mucoid phenotype,
273 infections become extremely difficult to eradicate. The presence of alginate in the biofilm acts as a
274 protective micro-environment for the bacteria from antibiotic therapies. In this study, we showed that
275 physico-chemical properties of alginate are influenced by gentamicin sulfate (GS). These changes
276 could potentially exacerbate the protective effect of the surrounding EPS on the microorganisms. In
277 addition, we demonstrated that the chemical structure of alginate (M- or G-rich alginate) impacts
278 strongly on its behaviour once in contact with solution of GS. Our results demonstrate that the
279 selection of an appropriate ratio M/G motif in alginate is of significance importance in the design of *in*
280 *vitro* biofilm models. By better understanding of how antibiotics interact with alginate and EPS present

281 in biofilm, the efficacy of existing antibiotics could be improved via alteration of EPS binding affinity to
282 antibiotics using novel therapeutic adjuvants.

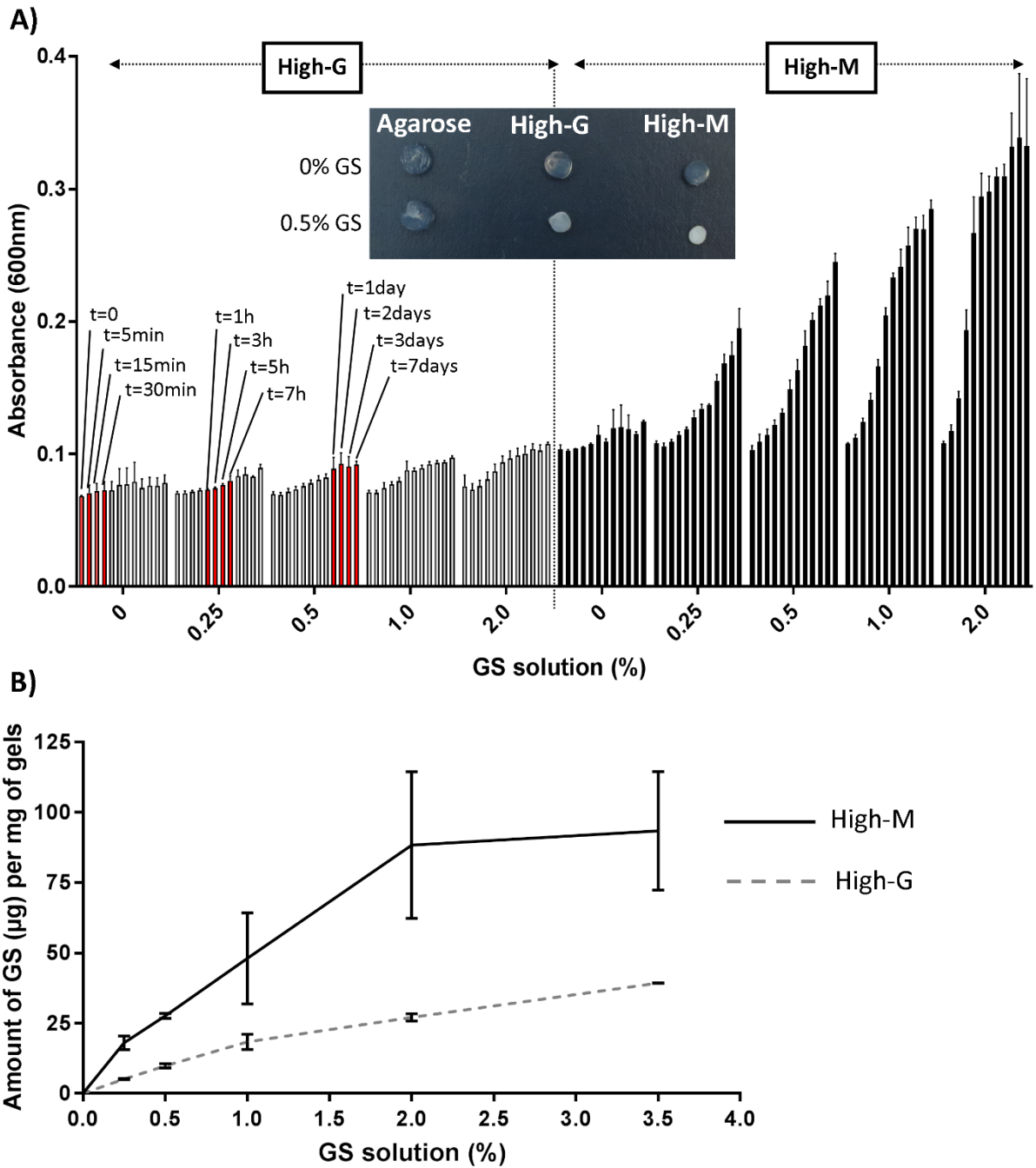
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284 **5. Acknowledgment.**

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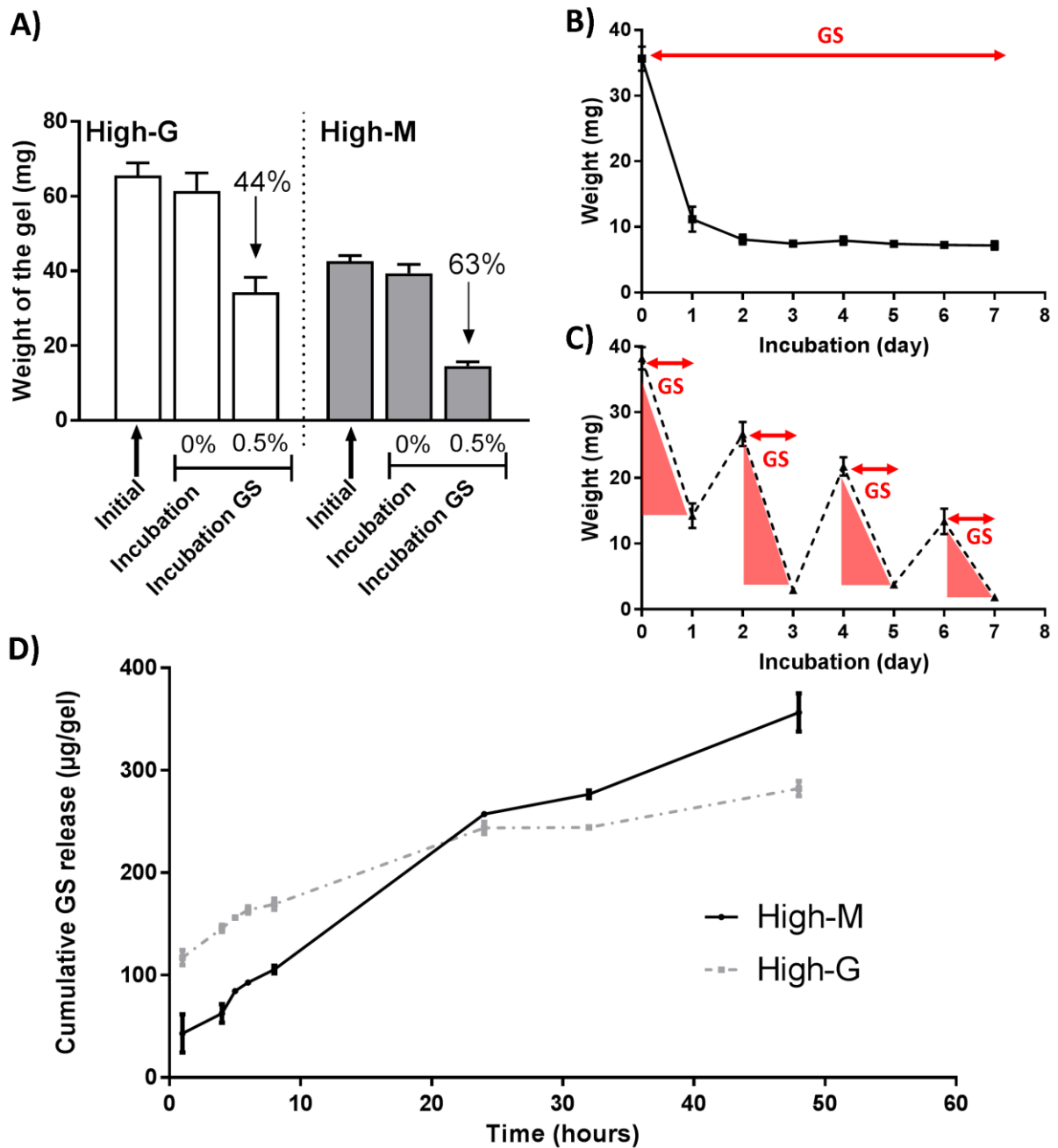
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294

295 **Figure 1:** Evolution of the gels' opacity measured at 600nm over-time of incubation with GS solutions
 296 (the photography shows the macroscopic difference observed between the different alginates before
 297 and after incubation with GS (7 days), agarose is shown as control material, **A**). Amount of GS
 298 adsorbed in the gels depending on the nature of the alginate and the concentration of GS in the
 299 solution (**B**).



300

301 **Figure 2:** Weight evolution of alginate gels in absence or presence of GS (after 18 hrs of incubation,

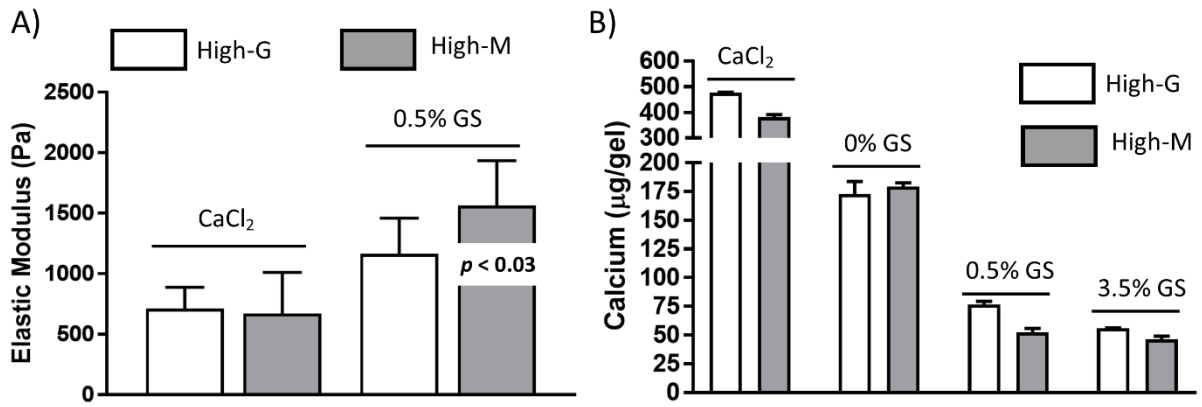
302 **A).** Difference in gels' weight of High-M alginate comparing a daily refreshing of GS solution (at 0.5%

303 dissolved in PBS with 10% BSA, **B)** to a discontinuous supply of GS (presence of GS in the incubating

304 solution is denoted in red, every other day, **C).** *In vitro* cumulative release of GS after incubation with

305 alginate High-M or High-G **(D).**

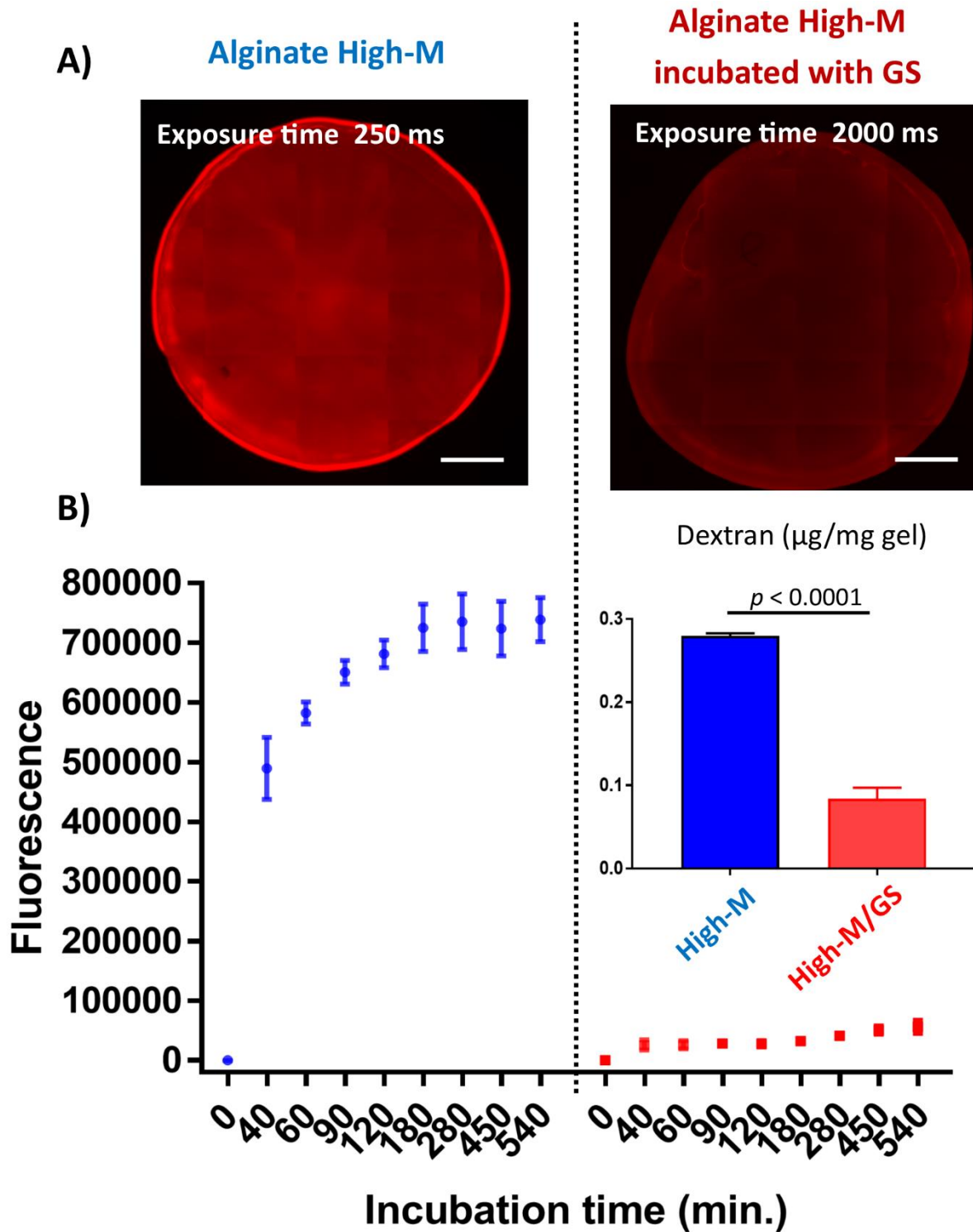
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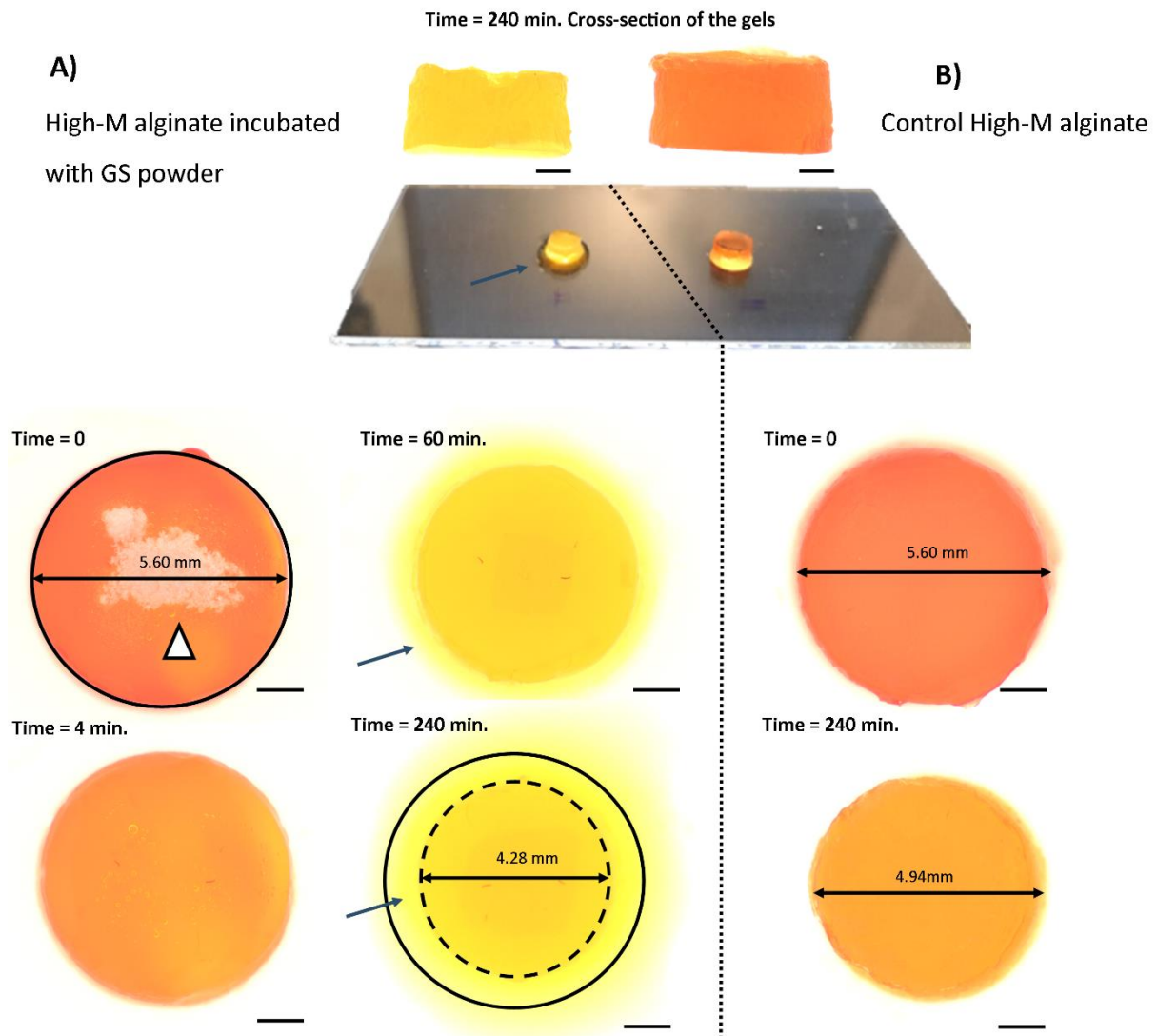
308 **Figure 3:** Influence of GS incubation on alginate gels stiffness (statistical difference was observed
 309 only between High-M groups, **A**) and on amount of calcium present inside the gels depending on the
 310 concentration of GS in the incubating solution (**B**).

311



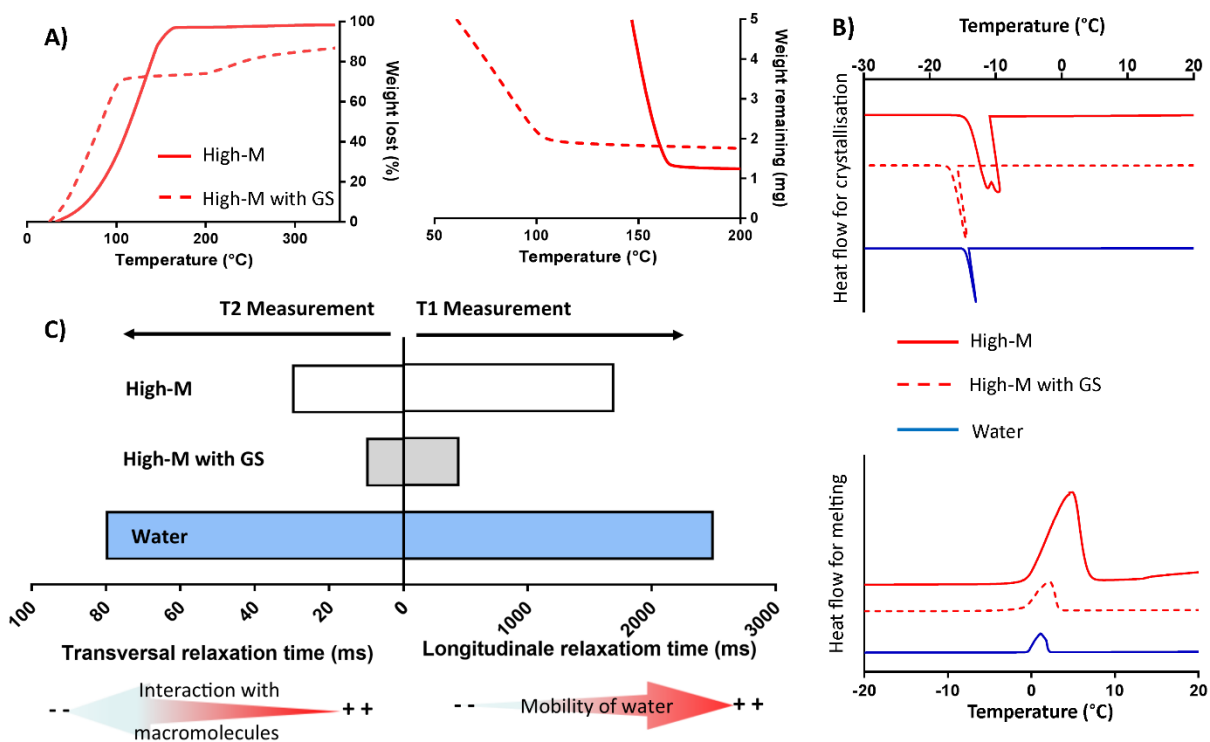
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313 **Figure 4:** Investigation of macromolecular permeability of alginate High-M without and with incubation
 314 with GS, illustrated using fluorescent microscope (after 6hrs of incubation with Dextran-TRITC at 4.4
 315 kDa, scale bars represent 1mm, **A**). Fluorescence intensity of the gels was evaluated by fluorimeter for
 316 540min, and the amount of Dextran-TRITC present inside the gels was subsequently quantified after
 317 gels dissolution (t-test was conducted to assess statistical differences, **B**).



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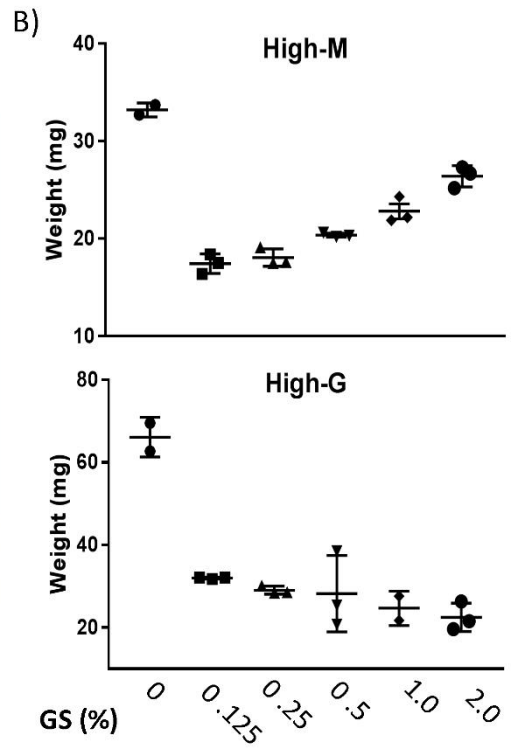
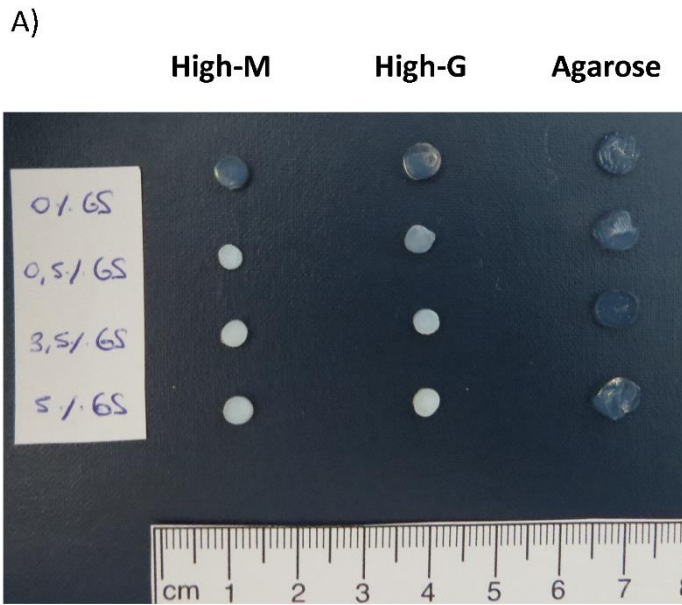
319 **Figure 5:** Acidification of alginate high-M alginate gels containing pH indicator red-phenol following
 320 deposition of GS powder (shown with the white arrow, **A**) compared to drug-free group (**B**).
 321 Subsequent dehydration and gel shrinkage are denoted by the blue arrows and dash-circle. Scale
 322 bars represent 1mm.



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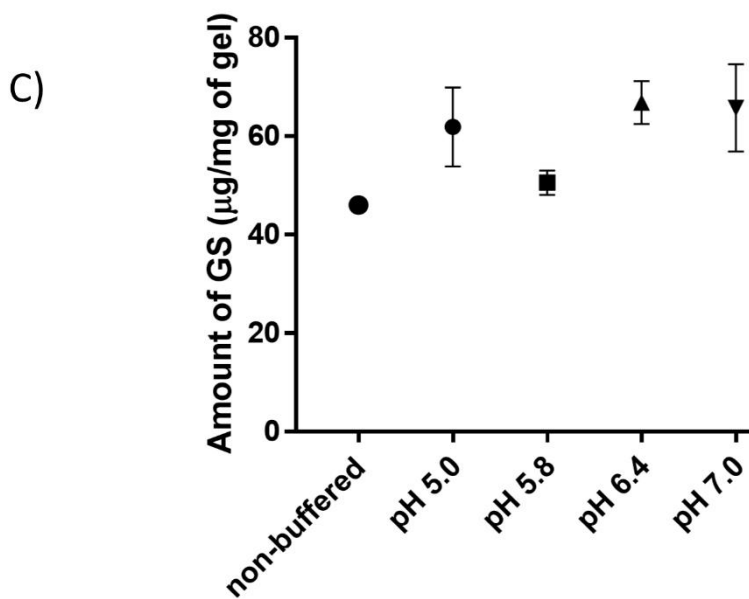
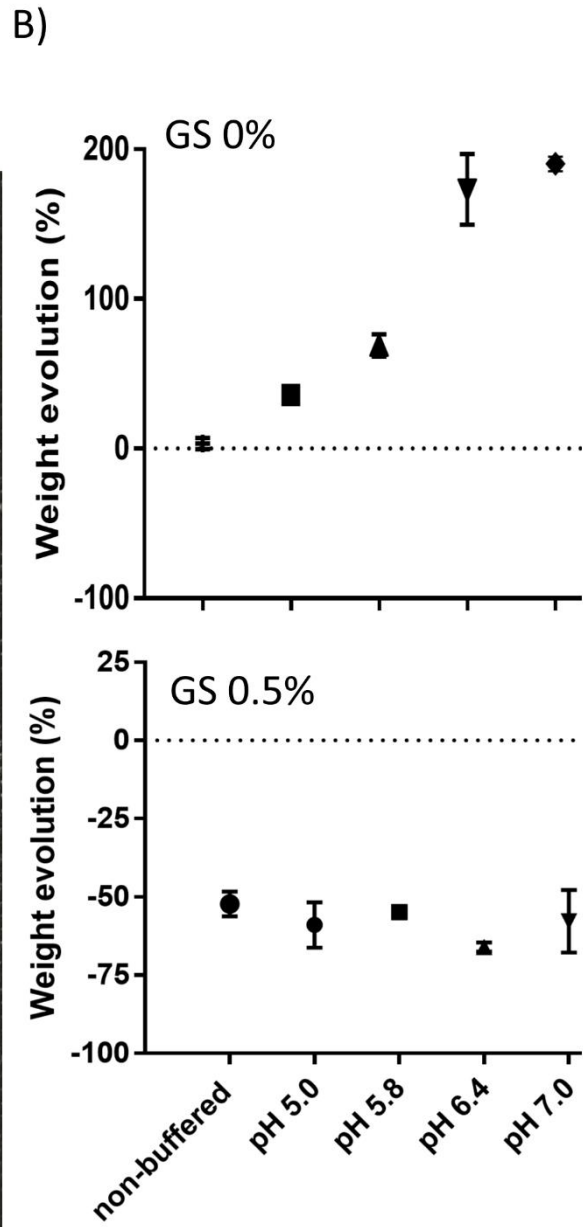
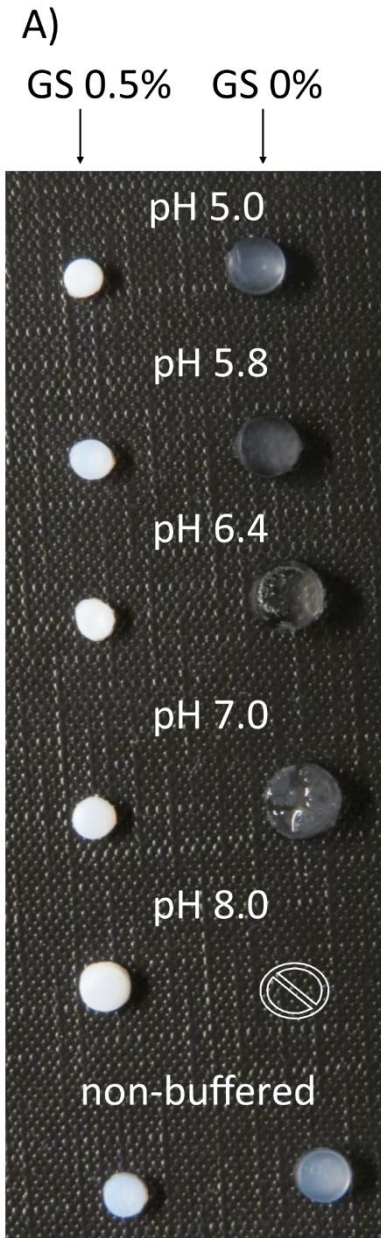
324 **Figure 6:** Thermogravimetric analyses of High-M alginate without and with GS, showing the two
 325 different profiles of water evaporation and the difference in residual masses at the end of the
 326 experiment (A). Differential scanning calorimetry analysing the different behaviours of the water
 327 molecules inside the gels containing or not GS in terms of water crystallisation (exothermic peak) and
 328 water melting (endothermic peak) (B). Measurement of transversal (T2) and longitudinal (T1)
 329 relaxation times of water protons inside the gels before and after incubation with GS (C).

330



331

332 **SD1:** Macroscopic observation of the opacity of the different gels (A) and determination of gels' weight
 333 depending on the concentration of GS in the incubation solution (B). Agarose was used as negative
 334 control.



336 **SD2:** Influence of pH microenvironment on the macroscopic aspect of the High-M gels (**A**), on their
337 swelling/shrinking behaviour depending on the absence or presence of GS in the solutions (**B**), and on
338 their ability to adsorb GS (**C**).

339

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