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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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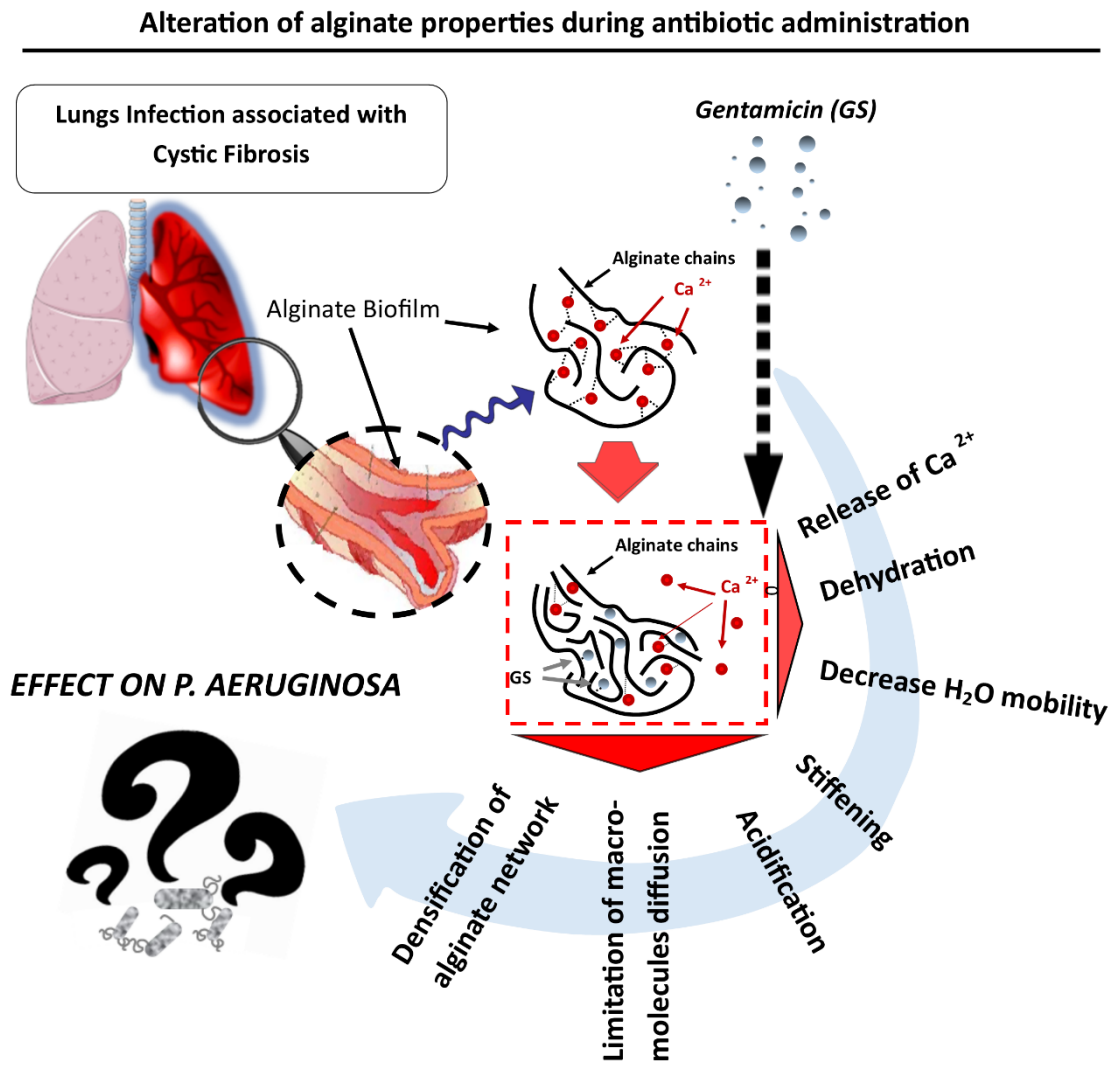
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12

13 **Graphical abstract**

14



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  $\text{Ca}^{2+}$  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,  $\text{Ca}^{2+}$  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 Algininate 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 Ca<sup>2+</sup>. Algininate 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 algininate) is one of the key  
52 factors to confer antibiotherapies resistance [2]. Such statements are not new, and in early works  
53 algininate 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 algininate 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 algininate, resulting in a limited antibiotics diffusion through algininate matrices [8-10]. Nevertheless,  
60 no study reports specifically on how antibiotics affect the physico-chemical characteristics of algininate,  
61 and how it alters locally the micro-environmental properties.

62 In this study, we deciphered how the nature and composition of algininate 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 algininate gels**

70           Acellular alginate gels were fabricated via an external ionic crosslinking procedure using  $\text{Ca}^{2+}$ .  
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  $\text{CaCl}_2$  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  $\text{CaCl}_2$  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 200 $\mu\text{L}$  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 (Viktor<sup>3</sup>, 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<sup>+</sup> and K<sup>+</sup> cations and PO<sub>4</sub><sup>3-</sup> and Cl<sup>-</sup> 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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  ions, and that  $\text{Ca}^{2+}$   
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  $\text{Ca}^{2+}$   
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 Ca<sup>2+</sup>) [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.

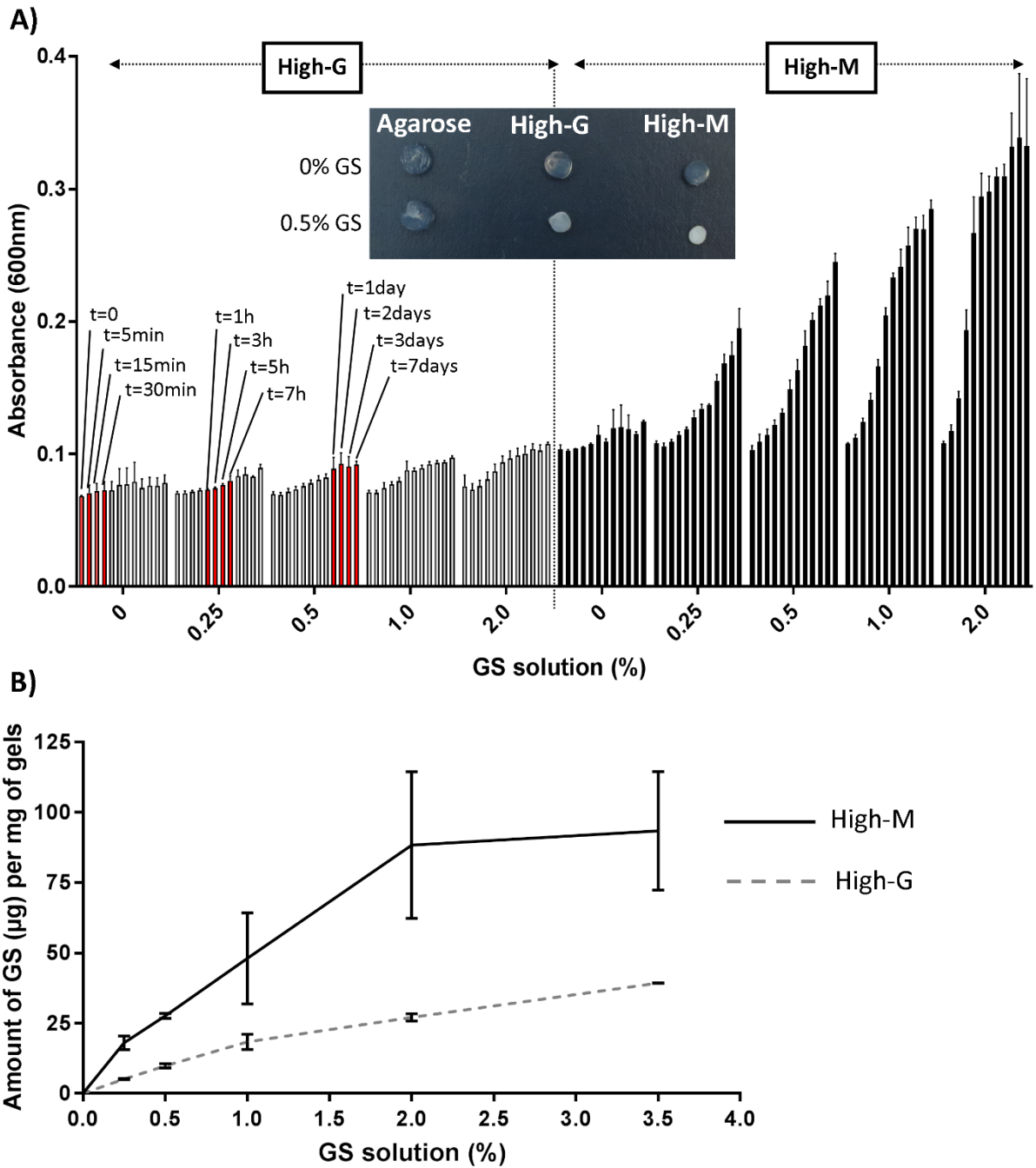
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## 284 **5. Acknowledgment.**

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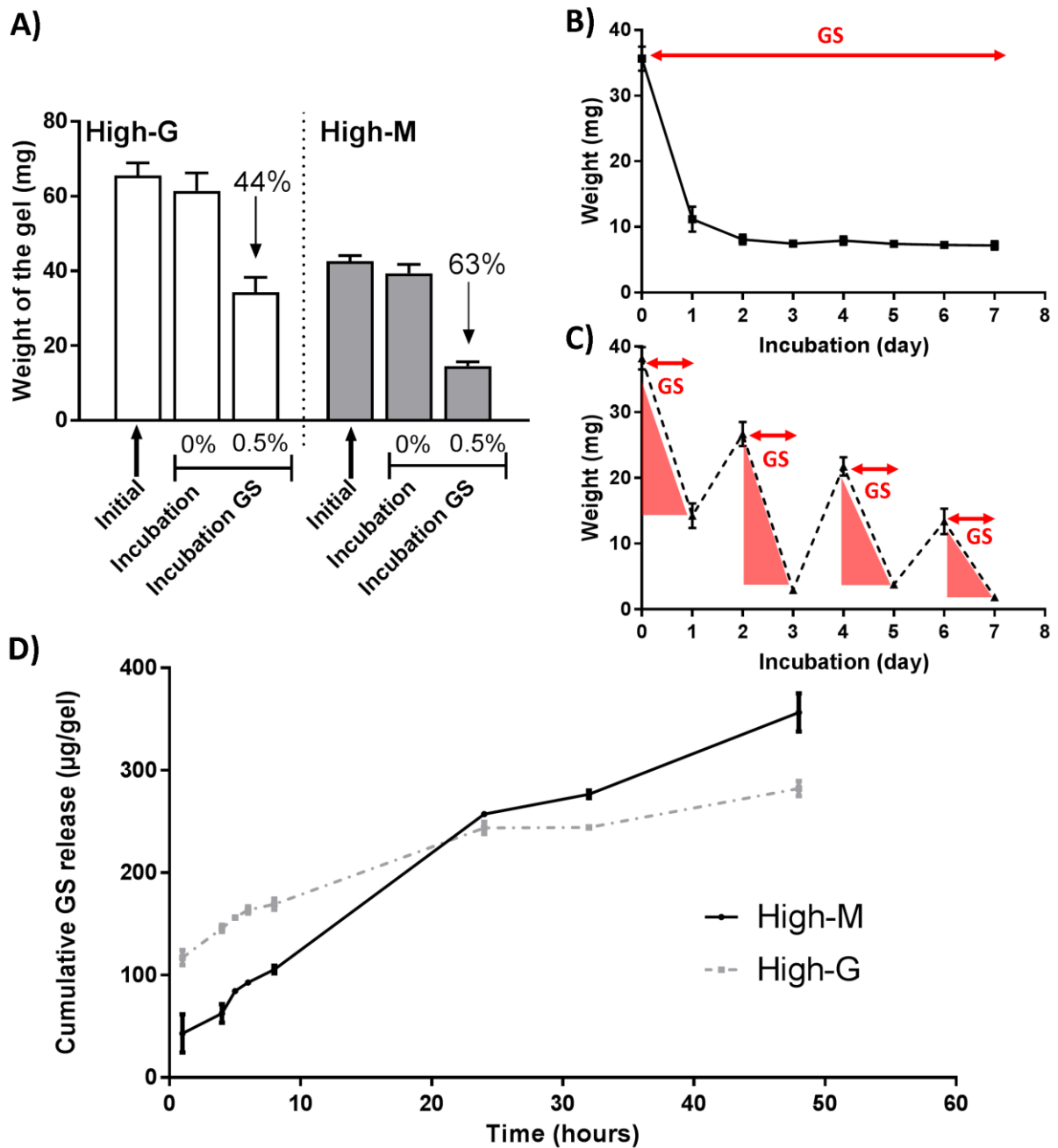
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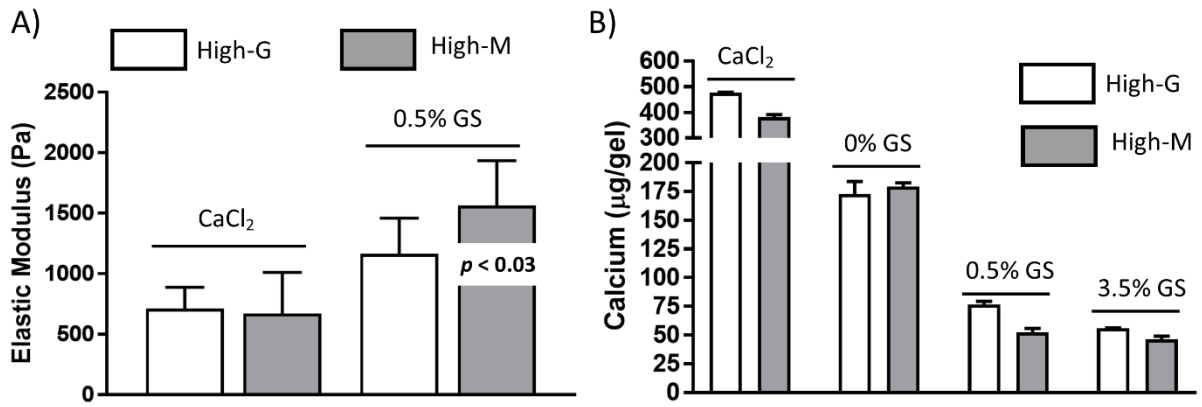
295 **Figure 1:** Evolution of the gels' opacity measured at 600nm over-time of incubation with GS solutions  
 296 (the photography shows the macroscopic difference observed between the different alginates before  
 297 and after incubation with GS (7 days), agarose is shown as control material, **A**).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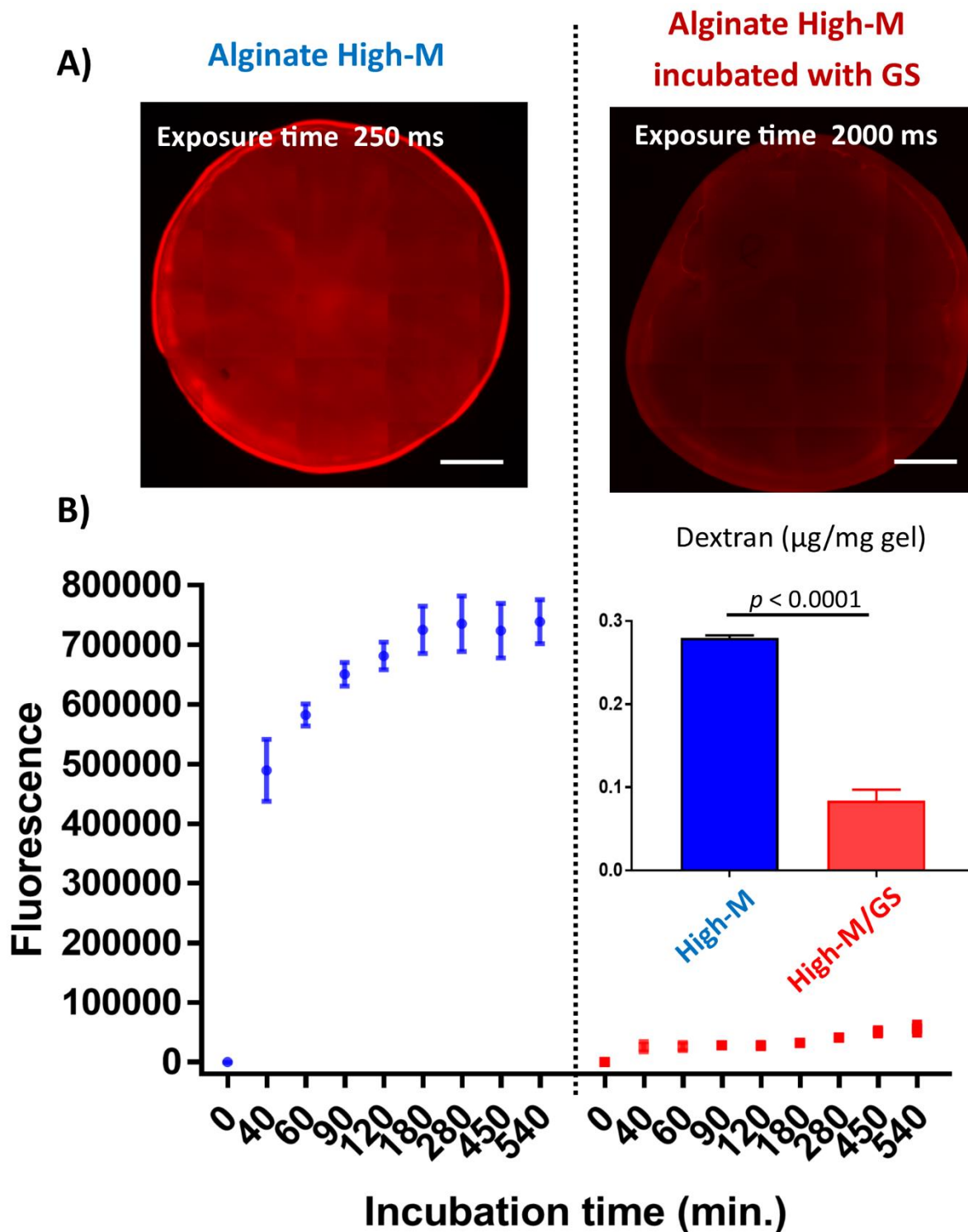


307

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**).

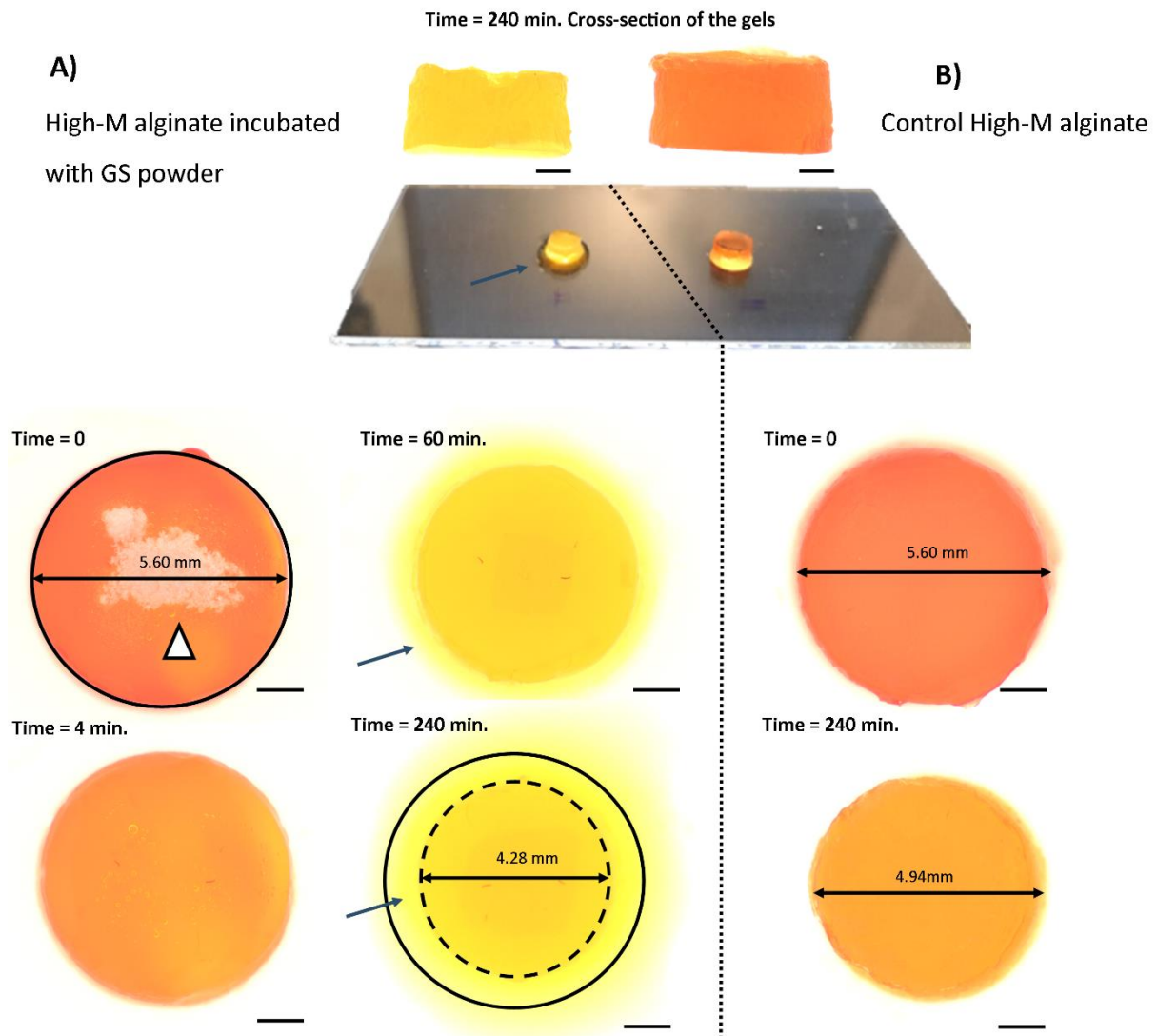
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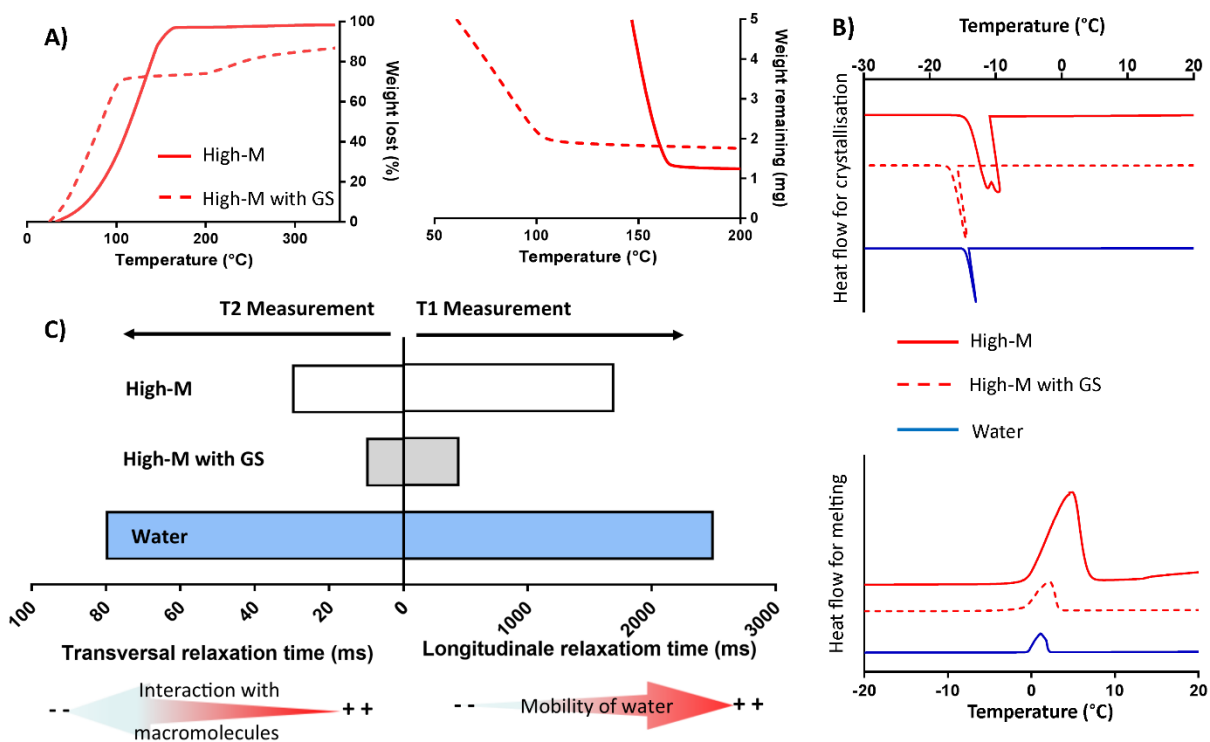
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313 **Figure 4:** Investigation of macromolecular permeability of alginate High-M without and with incubation  
 314 with GS, illustrated using fluorescent microscope (after 6hrs of incubation with Dextran-TRITC at 4.4  
 315 kDa, scale bars represent 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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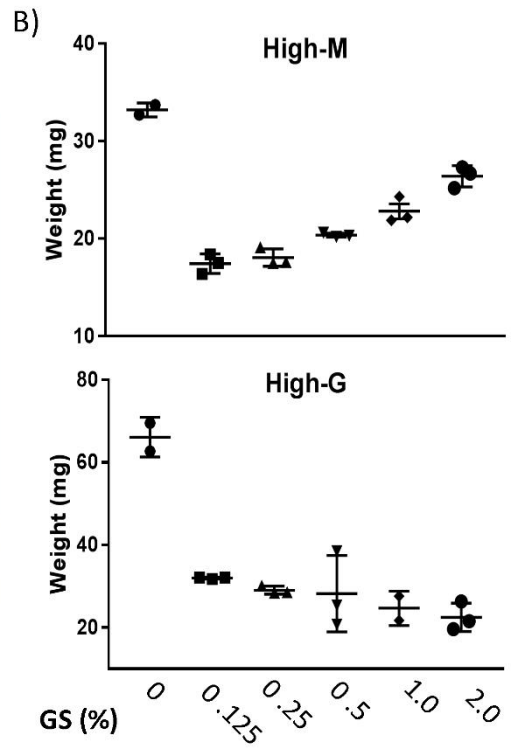
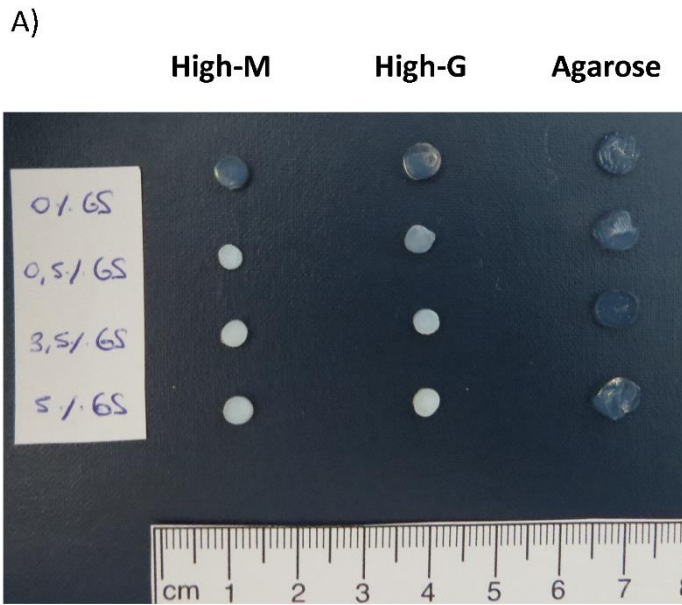
319 **Figure 5:** Acidification of alginate high-M alginate gels containing pH indicator red-phenol following  
 320 deposition of GS powder (shown with the white arrow, **A**) compared to drug-free group (**B**).  
 321 Subsequent dehydration and gel shrinkage are denoted by the blue arrows and dash-circle. Scale  
 322 bars represent 1mm.



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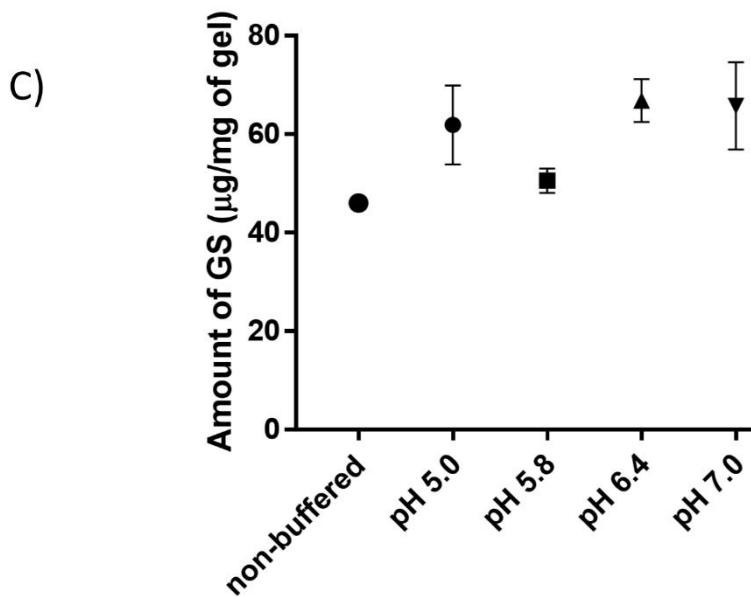
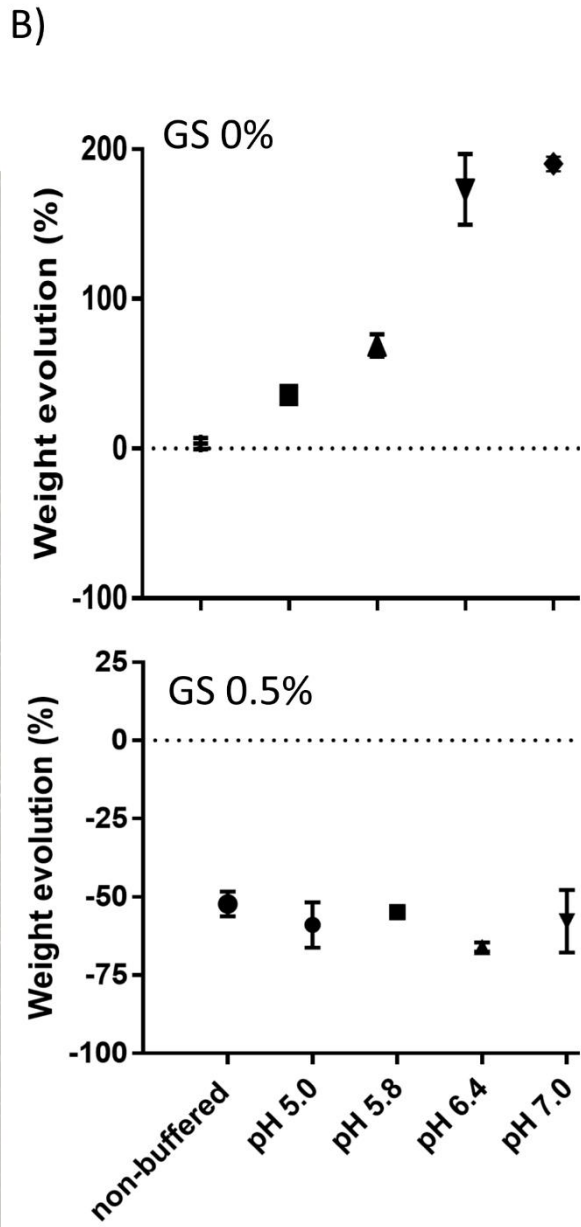
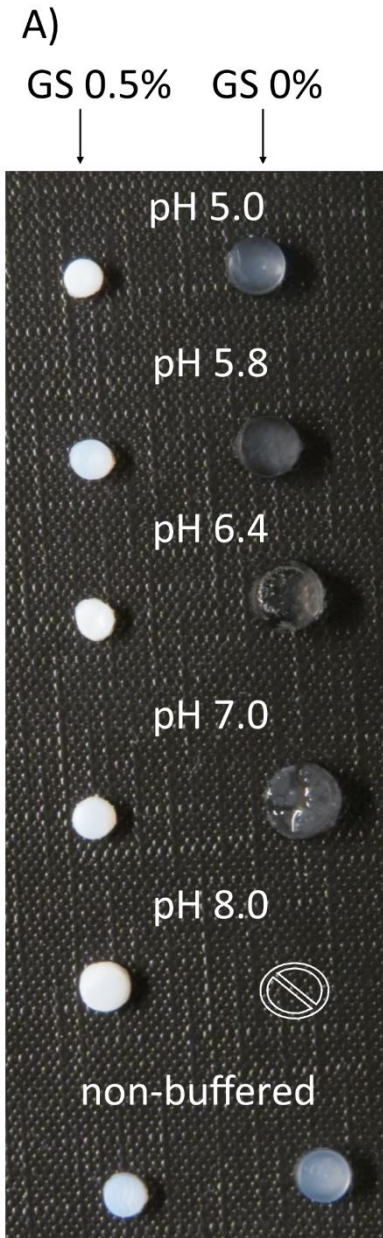
324 **Figure 6:** Thermogravimetric analyses of High-M alginate without and with GS, showing the two  
 325 different profiles of water evaporation and the difference in residual masses at the end of the  
 326 experiment (A). Differential scanning calorimetry analysing the different behaviours of the 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

340 **References:**

- 341 [1] W.W. Nichols, M.J. Evans, M.P. Slack, H.L. Walmsley, The penetration of antibiotics into  
342 aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*, *J Gen Microbiol* 135(5)  
343 (1989) 1291-303.
- 344 [2] T.B. May, D. Shinabarger, R. Maharaj, J. Kato, L. Chu, J.D. DeVault, S. Roychoudhury,  
345 N.A. Zielinski, A. Berry, R.K. Rothmel, et al., Alginate synthesis by *Pseudomonas*  
346 *aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis  
347 patients, *Clin Microbiol Rev* 4(2) (1991) 191-206.
- 348 [3] C.A. Demko, M.J. Thomassen, Effect of mucoid property on antibiotic susceptibility  
349 of *Pseudomonas aeruginosa*, *Current Microbiology* 4(2) (1980) 69.
- 350 [4] M.J. Thomassen, C.A. Demko, B. Boxerbaum, R.C. Stern, P.J. Kuchenbrod, Multiple of  
351 isolates of *Pseudomonas aeruginosa* with differing antimicrobial susceptibility patterns from  
352 patients with cystic fibrosis, *J Infect Dis* 140(6) (1979) 873-80.
- 353 [5] F. Eftekhar, D.P. Speert, Alginate treatment of mucoid *Pseudomonas aeruginosa*  
354 enhances phagocytosis by human monocyte-derived macrophages, *Infect Immun* 56(11)  
355 (1988) 2788-93.
- 356 [6] N. Hoiby, O. Ciofu, T. Bjarnsholt, *Pseudomonas aeruginosa* biofilms in cystic fibrosis,  
357 *Future Microbiol* 5(11) (2010) 1663-74.
- 358 [7] N. Bagge, M. Schuster, M. Hentzer, O. Ciofu, M. Givskov, E.P. Greenberg, N. Hoiby,  
359 *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene  
360 expression and beta-lactamase and alginate production, *Antimicrob Agents Chemother* 48(4)  
361 (2004) 1175-87.
- 362 [8] R.A. Hatch, N.L. Schiller, Alginate lyase promotes diffusion of aminoglycosides through  
363 the extracellular polysaccharide of mucoid *Pseudomonas aeruginosa*, *Antimicrob Agents*  
364 *Chemother* 42(4) (1998) 974-7.
- 365 [9] C.A. Gordon, N.A. Hodges, C. Marriott, Antibiotic interaction and diffusion through  
366 alginate and exopolysaccharide of cystic fibrosis-derived *Pseudomonas aeruginosa*, *J*  
367 *Antimicrob Chemother* 22(5) (1988) 667-74.
- 368 [10] D.G. Allison, M.J. Matthews, Effect of polysaccharide interactions on antibiotic  
369 susceptibility of *Pseudomonas aeruginosa*, *Journal of Applied Bacteriology* 73(6) (1992) 484-  
370 488.
- 371 [11] J. Gubernator, Z. Drulis-Kawa, A. Kozubek, A simply and sensitive fluorometric method  
372 for determination of gentamicin in liposomal suspensions, *Int J Pharm* 327(1-2) (2006) 104-9.
- 373 [12] M.M. Tunney, G. Ramage, S. Patrick, J.R. Nixon, P.G. Murphy, S.P. Gorman,  
374 Antimicrobial susceptibility of bacteria isolated from orthopedic implants following revision hip  
375 surgery, *Antimicrob Agents Chemother* 42(11) (1998) 3002-5.
- 376 [13] F. Song, D. Ren, Stiffness of cross-linked poly(dimethylsiloxane) affects bacterial  
377 adhesion and antibiotic susceptibility of attached cells, *Langmuir* 30(34) (2014) 10354-62.
- 378 [14] V. Iannuccelli, G. Coppi, R. Cameroni, Biodegradable intraoperative system for bone  
379 infection treatment. I. The drug/polymer interaction, *International Journal of Pharmaceutics*  
380 143(2) (1996) 195-201.
- 381 [15] M.H. Turakhia, W.G. Characklis, Activity of *Pseudomonas aeruginosa* in biofilms: effect  
382 of calcium, *Biotechnol Bioeng* 33(4) (1989) 406-14.
- 383 [16] X.X. Tang, L.S. Ostedgaard, M.J. Hoegger, T.O. Moninger, P.H. Karp, J.D. McMenimen,  
384 B. Choudhury, A. Varki, D.A. Stoltz, M.J. Welsh, Acidic pH increases airway surface liquid  
385 viscosity in cystic fibrosis, *J Clin Invest* 126(3) (2016) 879-91.
- 386 [17] D. Worlitzsch, R. Tarran, M. Ulrich, U. Schwab, A. Cekici, K.C. Meyer, P. Birrer, G.  
387 Bellon, J. Berger, T. Weiss, K. Botzenhart, J.R. Yankaskas, S. Randell, R.C. Boucher, G.  
388 Doring, Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of  
389 cystic fibrosis patients, *J Clin Invest* 109(3) (2002) 317-25.
- 390 [18] G. Borriello, E. Werner, F. Roe, A.M. Kim, G.D. Ehrlich, P.S. Stewart, Oxygen limitation  
391 contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms, *Antimicrob Agents*  
392 *Chemother* 48(7) (2004) 2659-64.

- 393 [19] T.F. Moriarty, J.S. Elborn, M.M. Tunney, Effect of pH on the antimicrobial susceptibility  
394 of planktonic and biofilm-grown clinical *Pseudomonas aeruginosa* isolates, *Br J Biomed Sci*  
395 64(3) (2007) 101-4.
- 396 [20] R.D. Coakley, B.R. Grubb, A.M. Paradiso, J.T. Gatzky, L.G. Johnson, S.M. Kreda, W.K.  
397 O'Neal, R.C. Boucher, Abnormal surface liquid pH regulation by cultured cystic fibrosis  
398 bronchial epithelium, *Proc Natl Acad Sci U S A* 100(26) (2003) 16083-8.
- 399 [21] J. Twiss, C. Byrnes, R. Johnson, D. Holland, Nebulised gentamicin-suitable for  
400 childhood bronchiectasis, *Int J Pharm* 295(1-2) (2005) 113-9.
- 401 [22] A. Gudmundsson, H. Erlendsdottir, M. Gottfredsson, S. Gudmundsson, Impact of pH  
402 and cationic supplementation on in vitro postantibiotic effect, *Antimicrob Agents Chemother*  
403 35(12) (1991) 2617-24.
- 404 [23] S. Klein, C. Lorenzo, S. Hoffmann, J.M. Walther, S. Storbeck, T. Piekarski, B.J. Tindall,  
405 V. Wray, M. Nimtz, J. Moser, Adaptation of *Pseudomonas aeruginosa* to various conditions  
406 includes tRNA-dependent formation of alanyl-phosphatidylglycerol, *Mol Microbiol* 71(3)  
407 (2009) 551-65.
- 408 [24] V. Gun'ko, I. Savina, S. Mikhalovsky, Properties of Water Bound in Hydrogels, *Gels* 3(4)  
409 (2017) 37.
- 410 [25] J.F. Chmiel, P.B. Davis, State of the art: why do the lungs of patients with cystic fibrosis  
411 become infected and why can't they clear the infection?, *Respir Res* 4 (2003) 8.
- 412 [26] D.J. Smith, G.J. Anderson, S.C. Bell, D.W. Reid, Elevated metal concentrations in the  
413 CF airway correlate with cellular injury and disease severity, *J Cyst Fibros* 13(3) (2014) 289-  
414 95.
- 415 [27] E.C. Dasenbrook, L. Lu, S. Donnola, D.E. Weaver, V. Gulani, P.M. Jakob, M.W.  
416 Konstan, C.A. Flask, Normalized T1 magnetic resonance imaging for assessment of regional  
417 lung function in adult cystic fibrosis patients--a cross-sectional study, *PLoS One* 8(9) (2013)  
418 e73286.
- 419 [28] M. Sonderholm, K.N. Kragh, K. Koren, T.H. Jakobsen, S.E. Darch, M. Alhede, P.O.  
420 Jensen, M. Whiteley, M. Kuhl, T. Bjarnsholt, *Pseudomonas aeruginosa* Aggregate Formation  
421 in an Alginate Bead Model System Exhibits In Vivo-Like Characteristics, *Appl Environ*  
422 *Microbiol* 83(9) (2017).
- 423 [29] B. Cao, L. Christophersen, M. Kolpen, P.O. Jensen, K. Sneppen, N. Hoiby, C. Moser, T.  
424 Sams, Diffusion Retardation by Binding of Tobramycin in an Alginate Biofilm Model, *PLoS*  
425 *One* 11(4) (2016) e0153616.