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Heriot Marine[1,2], Nottelet Benjamin[1], Garric Xavier[1], D'Este Matteo[2], Richards G. Geoff[2], Moriarty T. Fintan[2], Eglin David[2] and Guillaume Olivier[2*]

1- IBMM (UMR5247), University of Montpellier, CNRS, ENSCM, Montpellier, France

2- AO Research Institute Davos, Clavadelerstrasse 8, CH 7270 Davos, Switzerland

* corresponding author at: AO Research Institute Davos, Clavadelerstrasse 8, CH 7270 Davos, Switzerland

E-mail address: olivier.guillaume@aofoundation.org

13 **Graphical abstract**

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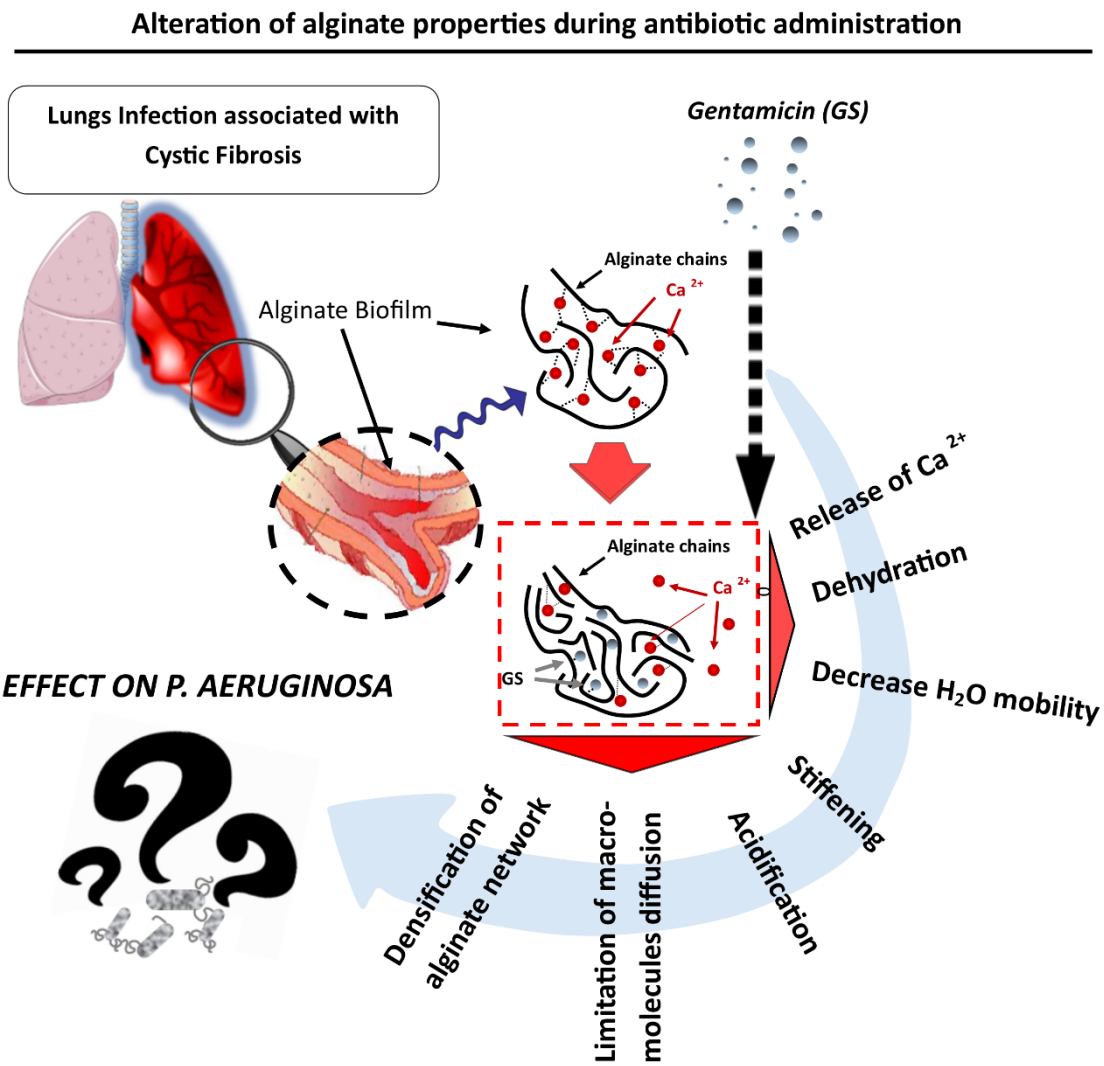

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Abstract

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

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

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

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

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

1. Introduction

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

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

2. Materials and Methods

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

2.1 Preparation of alginate gels

Acellular alginate gels were fabricated via an external ionic crosslinking procedure using Ca^{2+}. Two ultra-pure alginates from Pronova (UP-LVG enriched in guluronate motifs, G=67%) and UP-LVM enriched in mannuronate motifs, M=59%) were used to form gels via incubation of alginate solution (at 1.5 % weight/volume) in wells of defined size created in agarose moulds. These moulds were produced in presence of $CaCl_2$ 100mM to allow slow diffusion and gelling of the alginate. The obtained gels, based on alginate LVG or alginate LVM (called High-G or High-M respectively), were kept overnight in solution of 100mM $CaCl_2$ before any further experiment. To study of effect of gentamicin sulfate (GS, purchased from Roth) on alginate gels characteristics, the gels were subsequently incubated in solutions with defined GS concentrations. Unless otherwise stated, the GS concentration was fixed at 0.5% in distilled water and the gels were incubated overnight (o/n) at room temperature (RT).

2.2 Investigation of alginate gel's alteration by GS

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

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

2.3 Study of alginate permeability by macromolecules

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

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

In order to investigate the amount of water in the gels and its degree of interaction with the alginate/GS matrix, thermogravimetric analyses (TGA, TA Instrument Q50) were performed on gels upon heating from 25 to 350°C at 10°C/min. The state of the molecules of water present in the gels were investigated using differential scanning calorimetry (DSC, Perkin Elmer Instrument DSC 6000 Thermal Analyzer). Samples were submitted to a first cooling at -40°C, then to a heating ramp up to +40°C (at 1°C/min) followed by a second cooling ramp to −40°C (at 2°C/min). The degree of water mobility and its interaction with the alginate matrix was assessed on gels using magnetic resonance imaging (MRI, 7T Bruker Biospec 70/20 USR Avance III), and measure of relaxation times of the protons of water (longitudinal and transversal relaxations) were recorded using T1-weighted and T2-weighted sequences respectively. T1-weighted imaging parameters were as follows: echo time (TE) 2.8ms, matrix 256*192, slice thickness 0.8mm, inversion time (TI1 = 50ms; TI100 = 4980ms), recovery time (TR) 7500ms. T2-weighted imaging parameters were as follows: TR 7500ms, TE from 8 to 200ms, matrix 256*192, slice thickness 0.8mm.

3. Results and Discussion

The incubation of Ca-crosslinked alginate gels with GS is responsible to a rapid increase of the opacity for all alginates used in this study. Nevertheless, this effect was consistently more pronounced for High-M, for every time point and all GS concentrations (Figure 1A). The agarose control exposed to the same GS concentration did not display any opacity. Similarly to alginate, agarose is a polysaccharide, however it consists of different repeating units and it is not polyanionic like alginate. Therefore, the opacity observed is not due to unspecific GS precipitation in presence of polysaccharides, but to specific chemical interactions between the Alginate and GS. The Figure 1B demonstrated that GS can diffuse inside alginate gels, in a higher amount for high-M than for High-G. Alginate does not hamper GS diffusion (in contradiction to previous reports [9, 10]); but the polysaccharide network sequesters GS following internal diffusion. This first set of experiments presented in Figure 1 illustrates that GS can generate specific interactions with alginate, resulting in increased opacity during incorporation within the polymeric network. Both opacity and incorporation occured to a stronger extent with alginate enriched in M- rather than G- motifs. Besides the opacity, the inset in Figure 1A is also indicative of network compaction and shrinking upon interaction. This phenomenon was analysed with the experiments illustrated in Figure 2. The volume of the gels decreased of 44% in case of High-G and 63% in case of High-M following o/n incubation with GS. It is interesting to note that, in the case of alginate High-M, the shrinking of the structure is maximal when low concentrations of GS are present in the solutions (SD1). Whereas, for High-G, the densification of the gels followed a concentration-dependence pattern, with higher GS concentrations being responsible to higher gels shrinkage (SD1). This shrinking process was not affected by the presence of Na^+ and K^+ cations and PO_4^{3-} and Cl^- anions present in PBS and proteins (albumin present in BSA) and reached a steady state rapidly after 1 day of continuous incubation with GS (Figure 2B). Serial incubation of alginate gels in solutions with and then without GS resulted in repetitive shrinkage and then re-swelling of the structures. The cycle was not totally reversible, as mass gained and lost was slightly smaller at every cycle. This behaviour can be explained by GS adsorption from the GS-containing medium, triggering shrinkage followed by GS diffusion as soon as the gels are soaked in GS-free medium, with re-swelling of the structure. This specific behaviour is particularly meaningful from a clinical perspective. CF-patients are regularly treated via repetitive inhalation of antibiotics, and

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

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

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

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

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

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

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

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

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

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

Several *in vitro* observations presented in this manuscript correlate well with pathological signs of CF-patient suffering chronic infection, such as accumulation of dehydrated mucus in the airways of their lungs [25], extremely high and abnormal concentration of salts (including Ca^{2+}) [26], abundance of pulmonary oedema, acidity [16, 20] and decrease T1 signal in MRI [27]. The *in vitro* model of biofilm presented here indicates that such symptoms could be exacerbated upon GS inhalation. The list of phenomena described in this manuscript can be explained by ionic interaction between the positively charged ammonium group in GS and the negatively charged carboxylic group present in alginate. This strong ionic interaction is responsible to the gel's network reorganisation, resulting in the release of calcium ion and of water, and in the rearrangement of the remaining water molecules. Our results underline the importance of alginate as biofilm component, its pernicious role during antibiotherapy, and could represent a potential molecular target to improve existing therapies. Outlook of this work will be to investigate how bacteria embedded in biofilm-like structures react to such micro-environmental dynamic evolutions. Using *P. aeruginosa* embedded in alginate beads (originating from seaweed) has recently been suggested as suitable *in vitro* model to mimic CF-like condition and to screen antibiotics activity [28]. Our study highlights the fact that alginate monosaccharide composition in M/G-units has major impact on its behaviour and must be carefully considered. In addition, further strategies originating from our results could focus on the prevention or the limitation of the alginate network restructuration during incubation with GS (or other antibiotics which interact with alginate [9, 10, 28, 29]), using novel generation of adjuvants to antibiotherapies acting for examples as molecular "rigid spacer".

4. Conclusion

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

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

5. Acknowledgment.

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Figure caption

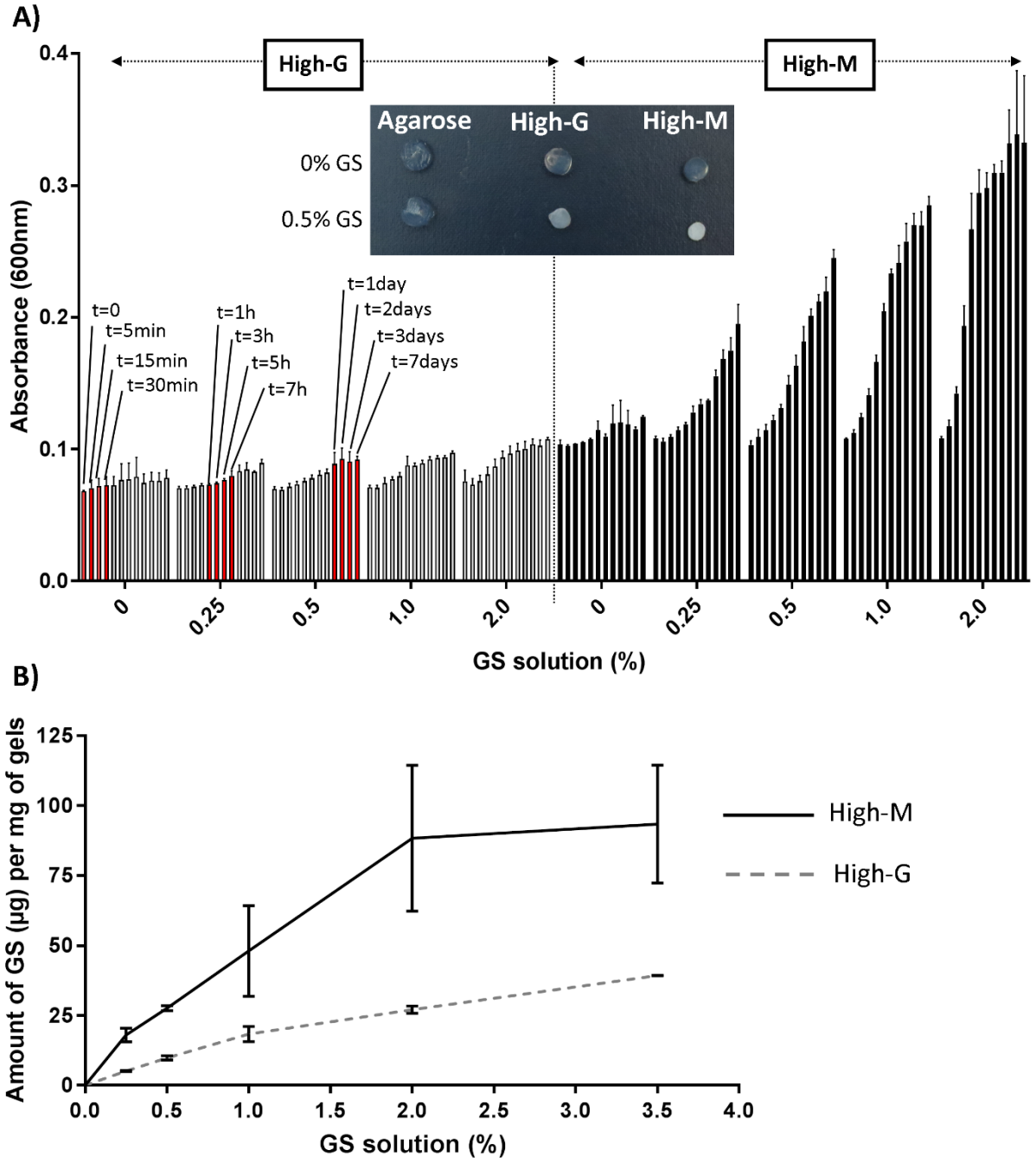


Figure 1: Evolution of the gels' opacity measured at 600nm over-time of incubation with GS solutions (the photography shows the macroscopic difference observed between the different alginates before and after incubation with GS (7 days), agarose is shown as control material, **A**). Amount of GS adsorbed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

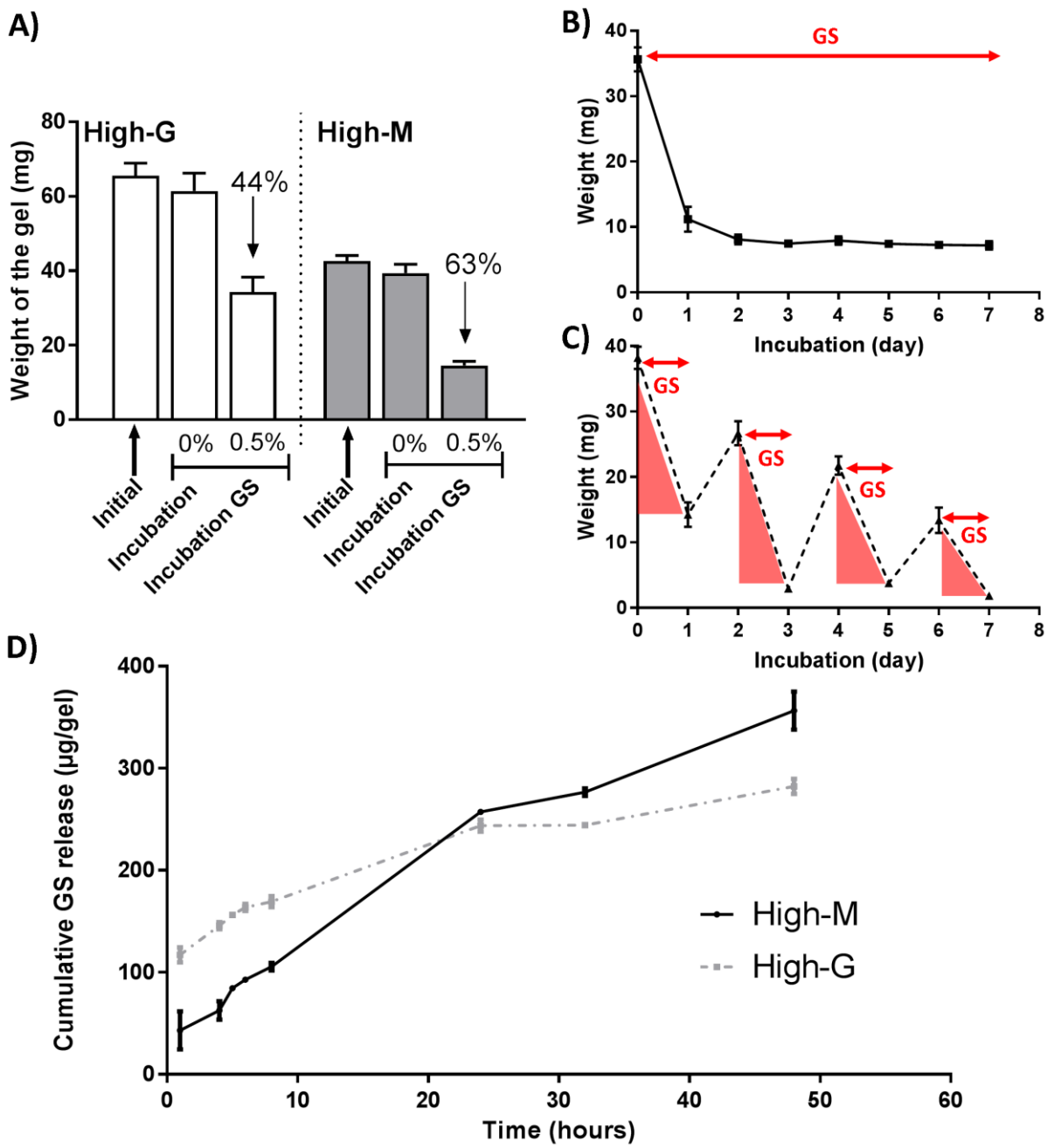


Figure 2: Weight evolution of alginate gels in absence or presence of GS (after 18 hrs of incubation, **A**). Difference in gels' weight of High-M alginate comparing a daily refreshing of GS solution (at 0.5% dissolved in PBS with 10% BSA, **B**) to a discontinuous supply of GS (presence of GS in the incubating solution is denoted in red, every other day, **C**). *In vitro* cumulative release of GS after incubation with alginate High-M or High-G (**D**).

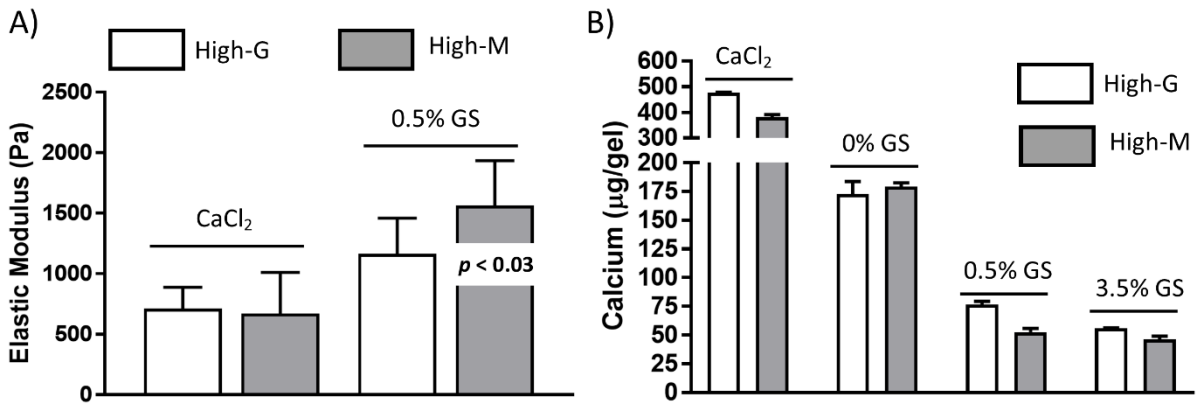
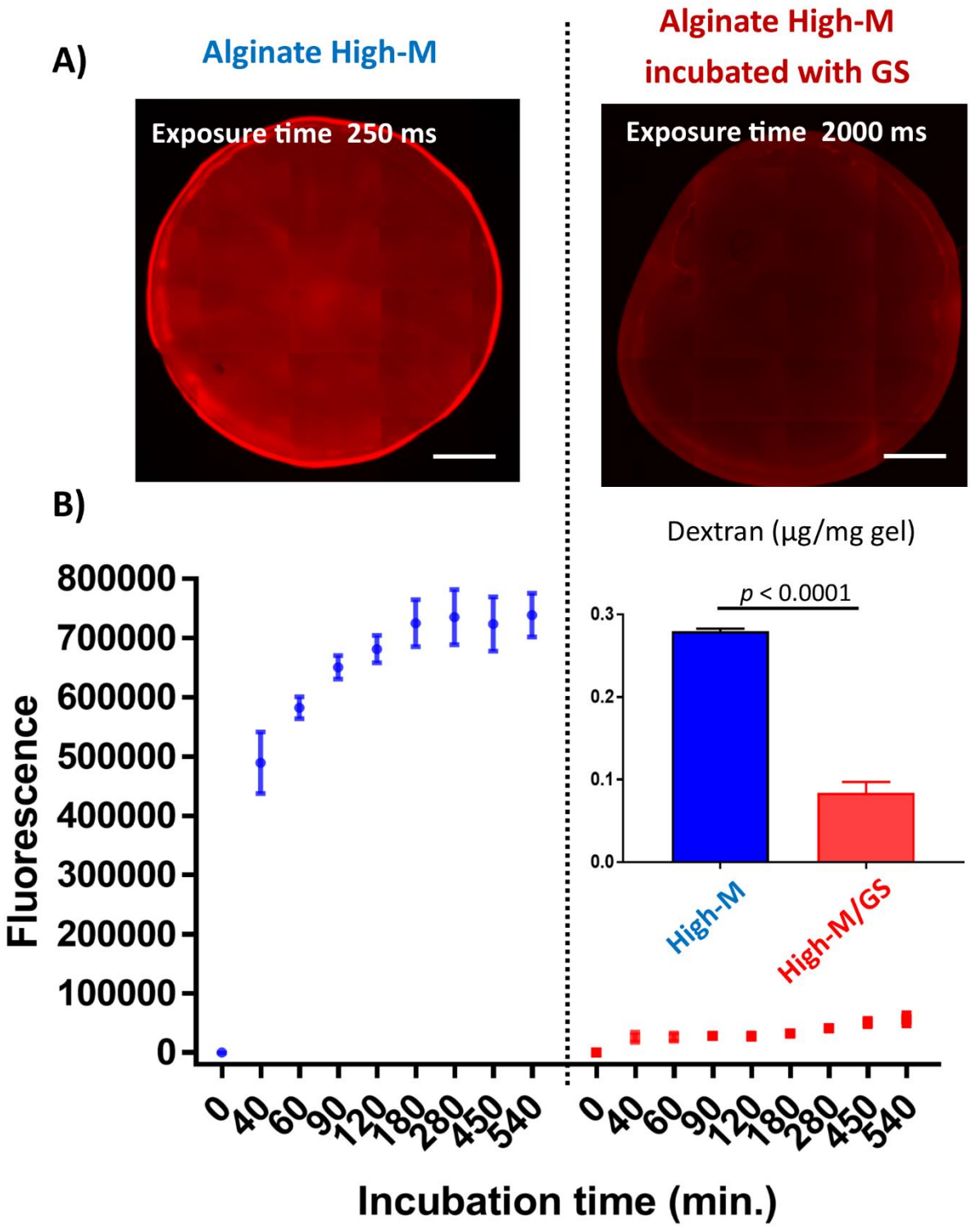


Figure 3: Influence of GS incubation on alginate gels stiffness (statistical difference was observed only between High-M groups, **A**) and on amount of calcium present inside the gels depending on the concentration of GS in the incubating solution (**B**).



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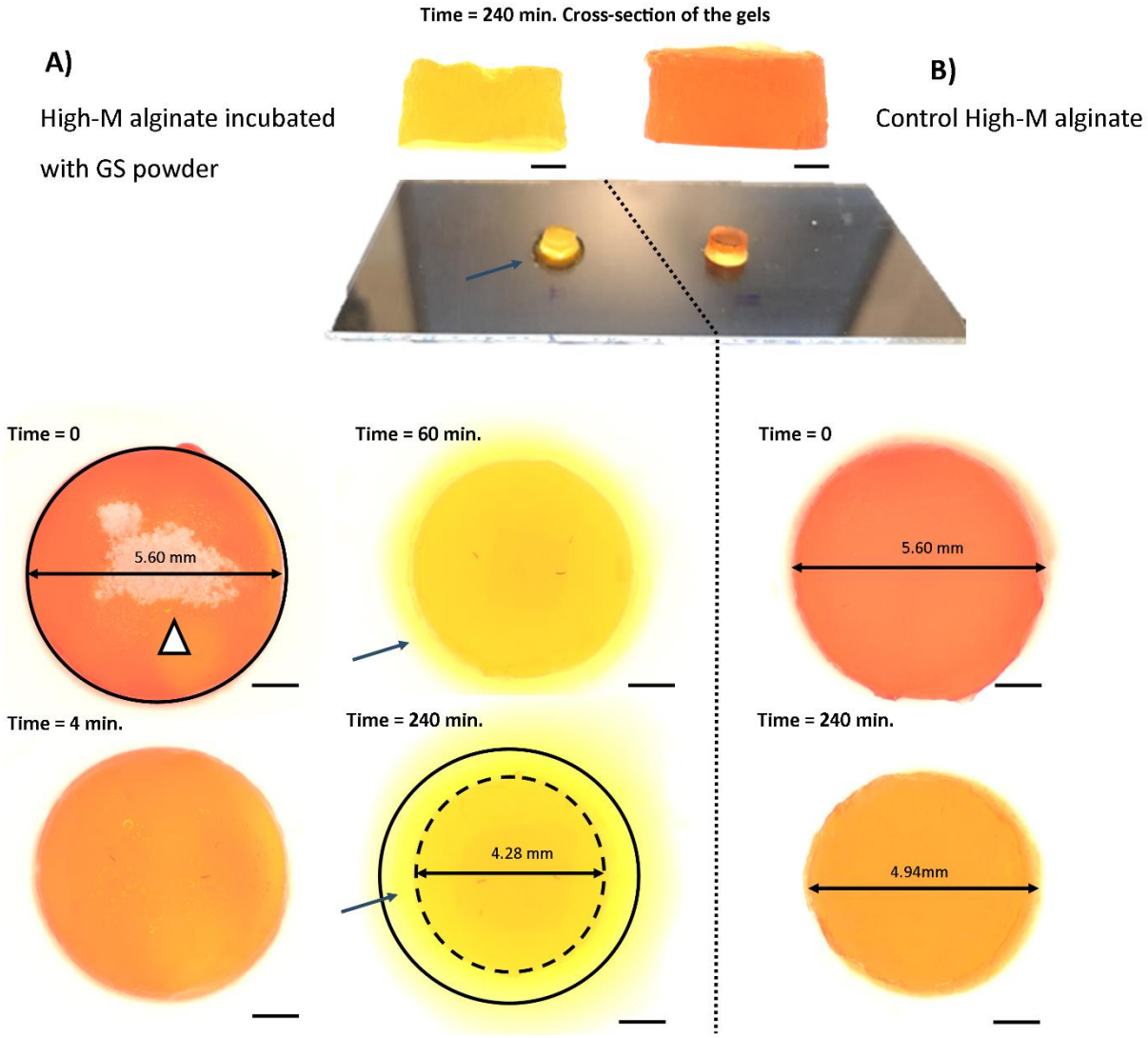
313 **Figure 4:** Investigation of macromolecular permeability of alginate High-M without and with incubation
314 with GS, illustrated using fluorescent microscope (after 6hrs of incubation with Dextran-TRITC at 4.4
315 kDa, scale bars represent 1mm, **A**). Fluorescence intensity of the gels was evaluated 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.

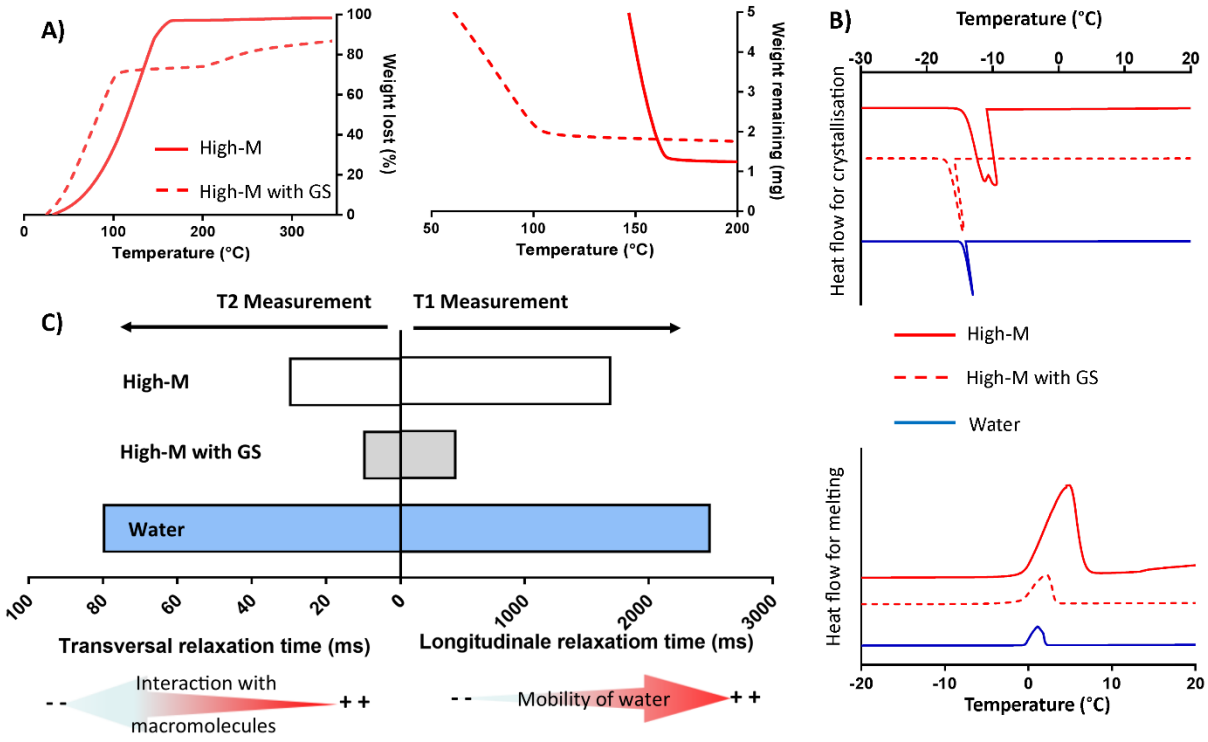
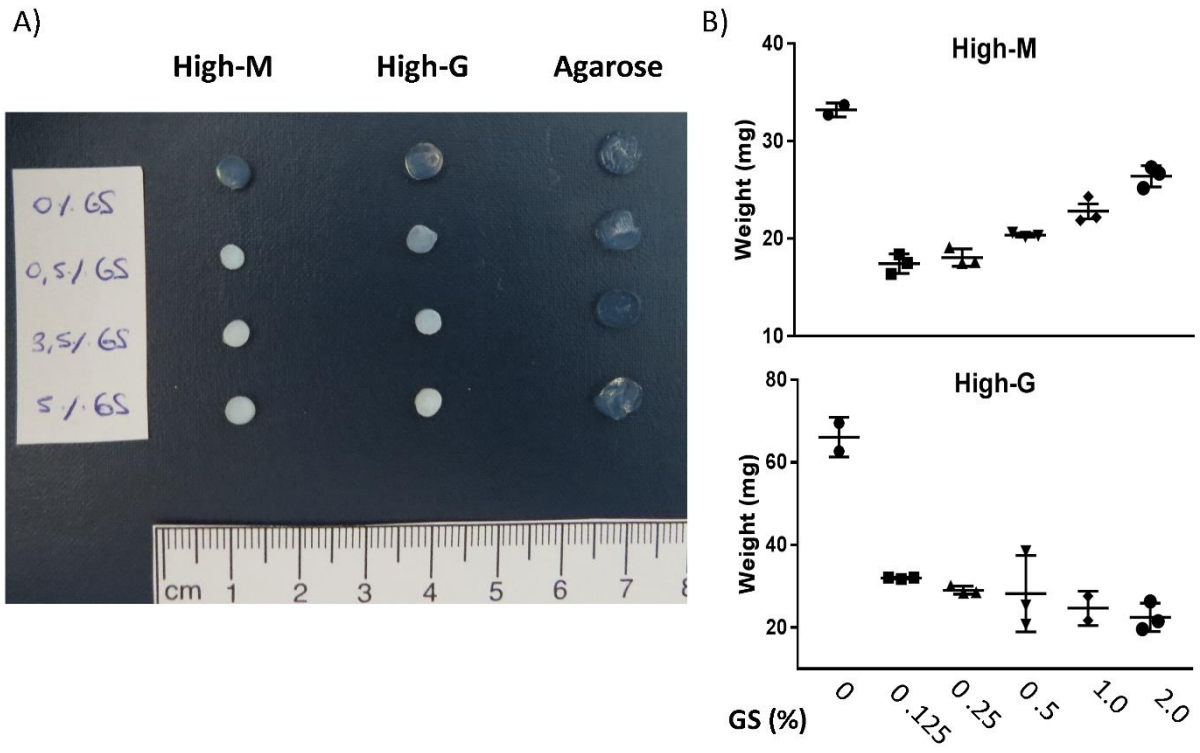


Figure 6: Thermogravimetric analyses of High-M alginate without and with GS, showing the two different profiles of water evaporation and the difference in residual masses at the end of the experiment (**A**). Differential scanning calorimetry analysing the different behaviours of the water molecules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



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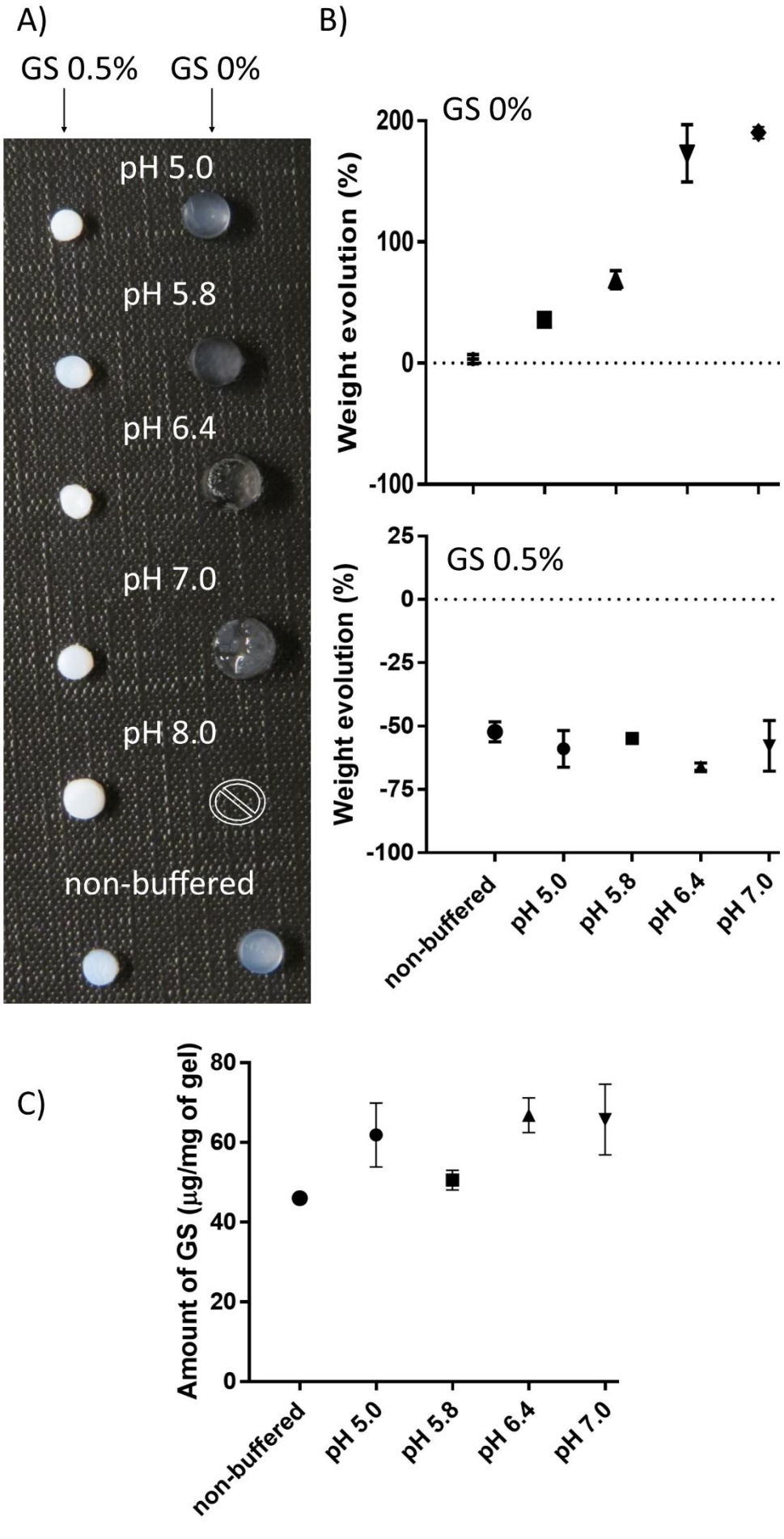
332 **SD1:** Macroscopic observation of the opacity of the different gels (**A**) and determination of gels' weight
333 depending on the concentration of GS in the incubation solution (**B**). Agarose was used as negative
334 control.


A)
GS 0.5%
GS 0%
pH 5.0
pH 5.8
pH 6.4
pH 7.0
pH 8.0
non-buffered
B)
GS 0%
Weight evolution (%)
200
100
0
-100
GS 0.5%
Weight evolution (%)
25
0
-25
-50
-75
-100
non-buffered
pH 5.0
pH 5.8
pH 6.4
pH 7.0
C)
Amount of GS (µg/mg of gel)
80
60
40
20
0
non-buffered
pH 5.0
pH 5.8
pH 6.4
pH 7.0

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

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