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

Marine Heriot [a,b], Benjamin Nottelet [a], Xavier Garric [a], Matteo D'Este [b], Geoff R. Richards [b], Fintan T. Moriarty [b], David Eglin [b], Olivier Guillaume [b,*]

[a] IBMM (UMR5247), University of Montpellier, CNRS, ENSCM, Montpellier, France
[b] AO Research Institute Davos, Clavadelerstrasse 8, CH 7270 Davos, Switzerland

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ABSTRACT

Background: Alginate is one of the main extracellular polymeric substances (EPS) in biofilms of Cystic Fibrosis (CF) patients suffering from pulmonary infections. Gentamicin sulfate (GS) can strongly bind to alginate resulting in loss of pharmacological activity; however neither the mechanism nor its repercussion is fully understood. In this study, we investigated how GS modifies the alginate macromolecular network and its microenvironment.
Material and methods: Alginate gels of two different compositions (either enriched in guluronate units (G) or enriched in mannuronate units (M)) were crosslinked with Ca^{2+} and exposed to GS at varying times and concentrations. The complexes formed were characterized via turbidimetry, mechanical tests, swelling assay, calorimetry techniques, nuclear magnetic resonance, Ca^{2+} displacement, macromolecular probe diffusion and pH alteration.
Results: In presence of GS, the alginate network and its environment undergo a tremendous reorganization in terms of gel density, stiffness, diffusion property, presence and state of the water molecules. We noted that the intensity of those alterations is directly dependent on the polysaccharide motif composition (ratio M/G).
Conclusion: Our results underline the importance of alginate as biofilm component, its pernicious role during antibiotherapy and could represent a potential macromolecular target to improve anti-infectious therapies.

1. Introduction

Alginate is a natural polysaccharide composed of repeating mannuronate (M) and guluronate (G) units, with the G-units being responsible for crosslinking with divalent ions such as Ca^{2+}. [1] Alginate is most commonly found in, and extracted from, algae, but it is also one of the main macromolecules secreted by *Pseudomonas aeruginosa* and key component in biofilm formation, for example in the lungs of people with cystic fibrosis (CF) disease. Once *P. aeruginosa* colonize the lungs, they can acquire a mucoid phenotype, which is characterized by excessive alginate production, which renders infection extremely difficult to treat. In fact, when growing as a biofilm, *P. aeruginosa* become tolerant to antibiotic concentrations up to 1000-fold higher than when cultivated under the planktonic form [2,3]. In contrast, the antibiotic resistance of *P. aeruginosa* remains unchanged whether the isolate is mucoid or not. [4,5] This is consistent with the fact that alginate is one of the key factors that confers tolerance to antibiotics. [3] Alginate has previously been shown to protect bacteria by hampering phagocytosis, [6] by scavenging reactive oxygen species, [7] and by creating a local anaerobic environment favouring microorganisms low metabolic activity. [8] This protective effect can 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 success of any antibiotic treatments. [9] Early studies also identified a strong interaction between some drugs (i.e. gentamycin, tobramycin and ciprofloxacin) and alginate, resulting in limited antibiotic diffusion through alginate matrices. [10–12] When *P. aeruginosa* are embedded within alginate gels as in vitro CF model, gentamicin concentrations up to 30 times of the MIC were unable to eradicate the microorganisms. [13] However, to date, no study has provided any specific measurements or has elucidating the mechanisms of how antibiotics affect the physico-chemical characteristics of alginate gels, which could help in elucidating the problem of antibiotic tolerance encountered in this field.

In this study, we investigated how the nature and composition of alginate interferes with gentamicin, from a molecular to a macroscopic scale. By understanding this phenomenon, this study enables the development of more clinically-relevant in vitro models of *P. aeruginosa*

* Corresponding author.
E-mail address: olivier.guillaume@aofoundation.org (O. Guillaume).

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biofilm and will pave the way to discover alternative strategies aiming to treat CF patients affected by lung infection.

2. Materials and methods

Unless stated otherwise, all 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 1.5% weight/volume alginate aqueous solutions in wells of defined size (6 mm Ø × 3 mm H) created in agarose moulds at room temperature. These moulds were produced in presence of $CaCl_2$ 100 mM for in-situ gelling of the alginate solutions (through external cross-linking mechanism with Ca^{2+}). The obtained gels, based on alginate LVG and alginate LVM, called High-G or High-M respectively, were kept overnight (o/n) in a solution of 100 mM $CaCl_2$ before use.

2.2. Investigation of the alteration of alginate gels by gentamicin sulfate

To study the effect of gentamicin sulfate (GS, 661 I.U./mg, Roth) on alginate, the gels were placed in a 96-well-plate and incubated with 200 μL of solution containing 0.5 GS w:v % (corresponding to 5 mg/mL) in distilled water, unless stated differently. The range of GS concentrations tested in this study is based on the numerical estimation of antibiotic repartition in the airway surface liquid (ASL) after aerosol inhalation proposed by Hasan et al. [14] They calculated that by inhaling aerosol (nebulized aminoglycoside at 60 mg/mL), local concentrations ranging between 1 and 5 mg/mL of antibiotic could be reached in the ASL. Unless stated otherwise, the gels were incubated in GS solutions o/n at room temperature (RT). The opacity of the gels (n = 6) was monitored at 600 nm using a spectrophotometer (Thermo Scientific, Multiskan GO). Shrinking or swelling of the gels were determined by measuring the weight and the size with a balance and with a digital caliper respectively, after blotting the excess of solution (n = 3). The stiffness of the gels was determined in an unconfined compression test (Instron 5866), with a static load cell of 10 N at a displacement of 1 mm/min. During this experiment, the gels were immersed in phosphate buffer solution (PBS) and kept at RT (n = 3).

The amounts of GS adsorbed in the different gels were determined after material dissolution in 1 mL of 150 mM EDTA at pH 8 for 30 min under rotation and GS was quantified using *o*-phthalaldehyde reaction (OPA) as described previously (n = 2). [15] The in vitro release kinetic of GS from the gels was performed after incubation of the gels in 1 mL of NaCl at 0.9% at 37 °C under continuous agitation. The supernatants were removed and refreshed at every time point and kept frozen until GS quantification using OPA as indicated above (n = 3). The amount of calcium ions present in the alginate gels before and after the incubation with GS solutions (at 0, 0.5 and 3.5 w:v %) 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 the alginate gels with GS solutions was evaluated by incubating gels with either a solution of 0.5 w:v % GS in PBS containing 10 w:v % bovine serum albumin (PBS and BSA was added as source of ions and proteins to mimic in vivo conditions) refreshed every 24 h (1 mL per gel), or by alternating every day the incubating solution with either the GS or the drug-free PBS/BSA solution (experiment performed at 37 °C under agitation, n = 3). Every day, 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, alginate gels containing phenol red, turning from red when neutral, to yellow during acidification were formulated. The colour change was imaged after adding 1 mg of GS powder on top of the gel.

2.3. Study of alginate permeability by macromolecules

In order to assess how GS alters the permeability of alginate gels, gels without GS (High-M) and with GS (High-M/GS) were incubated in solutions of fluorescent dextran (tetramethyl rhodamine isothiocyanate, TRITC-Dextran, 4.4 kDa) either dissolved in DI water for High-M gel or in DI water containing GS at 0.5 w:v % for High-M/GS gel. The transport of the dextran-TRITC inside the gels was evaluated by a 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 extensively washed, dissolved in EDTA solution, and the total amount of dextran-TRITC was quantified by fluorescence reading against a calibration curve (n = 3). In addition, the visualization of the dextran-TRITC penetration in the different gels was observed on gels' cross-sections using a fluorescent confocal microscope (Axiovert 200 m microscope, Zeiss).

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

To investigate the amount of water present 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 molecular state of the water present in the gels was 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, 7 T Bruker Biospec 70/20 USR Avance III), and the 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 following: echo time (TE) 2.8 ms, matrix 256*192, slice thickness 0.8 mm, inversion time (TI1 = 50 ms; TI100 = 4980 ms), recovery time (TR) 7500 ms. T2-weighted imaging parameters were as following: TR 7500 ms, TE from 8 to 200 ms, matrix 256*192, slice thickness 0.8 mm.

2.5. Statistical analyses

Statistical analysis of data was performed using Prism software (GraphPad Software, La Jolla, CA, USA). We assumed normal distribution of data. Either one-way ANOVA with Tukey's multiple comparison or *t*-test were applied to detect significant differences between experimental groups (with $p < 0.05$). Data presented are means ± standard deviation (SD) unless stated otherwise. Data presented are means ± standard deviation (SD) unless stated otherwise.

3. Results and discussion

The incubation of Ca^{2+}-crosslinked alginate gels with GS results in a rapid increase of the opacity of both High-M and High-G alginates. Nevertheless, the rate and the degree of the opacity reached were higher for High-M at every time point and was observed for all the tested GS concentrations (Fig. 1a). The agarose control exposed to the same GS concentrations did not display any opacity. Similar to alginate, agarose is a polysaccharide, however it consists of different repeating units and it is not polyanionic like alginate. As the GS in solution contains positively charged ammonium groups and sulfate ions, we conducted similar experiment as the one presented Fig. 1a by replacing GS with ammonium sulfate (at 5 w:v %) in DI water. The incubation of alginate gels with

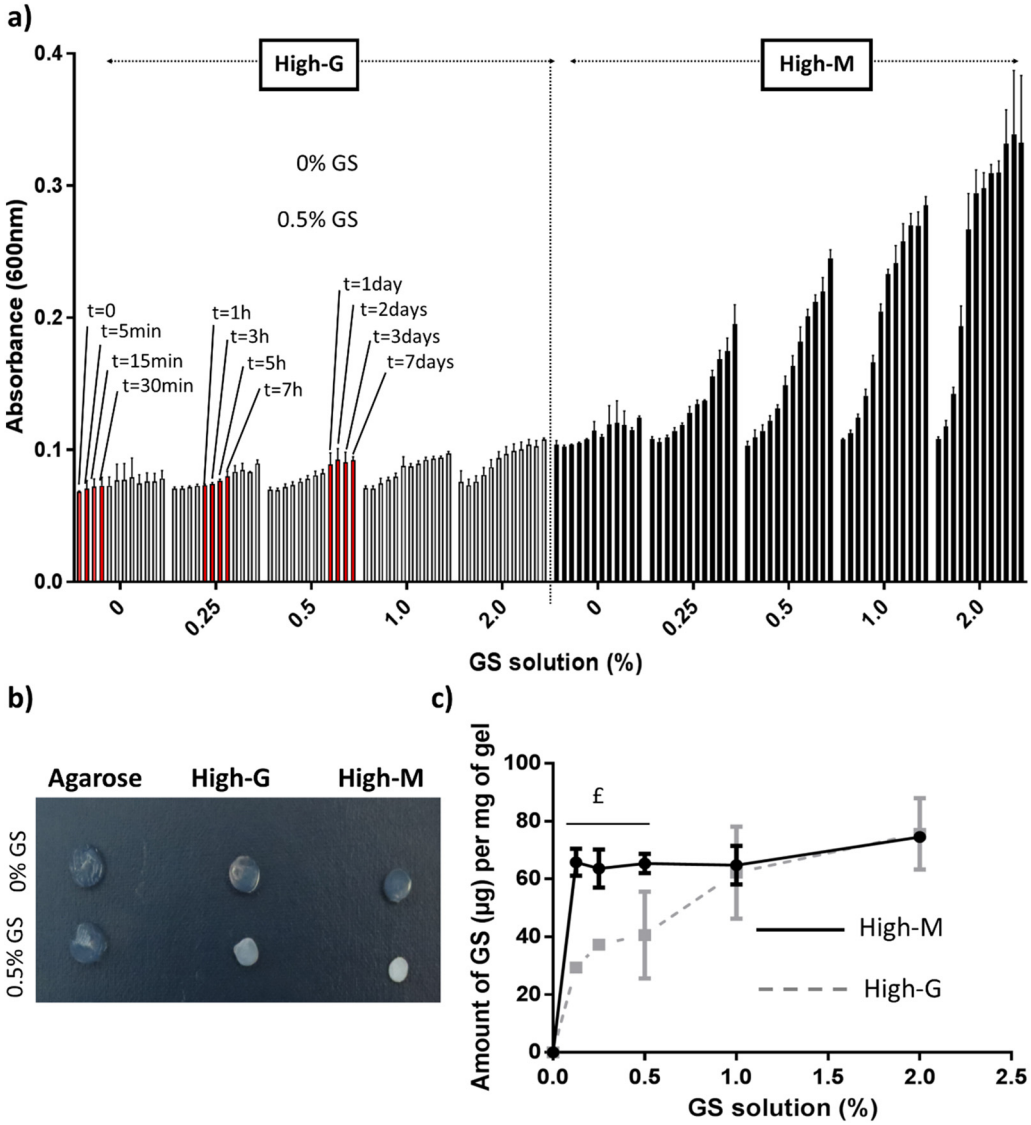

Fig. 1. Evolution of the gels' opacity measured at 600 nm over-time during incubation with GS solutions (the absorbance values registered for the groups incubated with 0% GS correspond to the background absorption of alginate gels, a). The photography shows the macroscopic difference observed between the different alginates before and after incubation with GS, taken at the end of this study. Agarose is shown as control material (b). The amount of GS adsorbed in the gels is depending on the nature of the alginate and on the concentration of GS in the solution (c), with £ denoting significance between the two groups.

$(NH_4^+)_2 + SO_4^{2-}$ did not result in any change of the gel's opacity (SD1). Therefore, the opacity observed is not due to unspecific GS precipitation in presence of polysaccharides or to the presence of only sulfate or ammonium ions, but to specific physico-chemical interactions existing between the alginate and GS. The difference in terms of opacity for the different gels with and without GS is clearly visible macroscopically (Fig. 1b). Fig. 1c shows that GS can diffuse inside the alginate gels, even though to a higher degree for high-M than for High-G when low concentrations of GS are used (from 0.125% up to 0.5%). When GS concentration reaches 1% in the solution, no difference is quantifiable between high-M and high-G alginate, most probably due to a saturation effect. From this investigation, it is clear that alginate does not hamper GS diffusion, which challenges previous reports; [11,12] but the polysaccharide network sequesters GS following internal diffusion. Besides the change in opacity, Fig. 1b shows the alginate network compaction and shrinkage upon addition of GS. This phenomenon was analysed with the swelling/shrinkage experiment reported in Fig. 2. Following o/n incubation with GS, the volume of the gels decreased of 44% and 63% for High-G and High-M respectively (Fig. 2a). 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 (e.g. 0.125%, SD2). Whereas, for High-G, the densification of the gels followed a concentration-dependence pattern, with higher GS concentrations being responsible for higher gels shrinkage (SD2). 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). It rapidly reached a steady state after 1 day of continuous incubation with GS (Fig. 2b). Serial incubation of alginate gels in solutions with and then without GS resulted in repetitive shrinkage and then re-swelling of the gels (Fig. 2c). The cycle was not totally reversible, as mass gained and lost slightly decreased at every cycle. This behaviour can be explained by the GS adsorption in the alginate from the GS-containing medium, triggering gels' shrinkage followed by GS diffusion as soon as the gels are soaked in GS-free medium, with re-swelling of the alginate network. This specific behaviour is particularly meaningful from a clinical perspective. CF-patients are regularly treated via repetitive inhalations of antibiotics, and such a pulsatile administration could have a major impact on biofilm hydration, volume and compaction, with further impact on the embedded microorganisms.

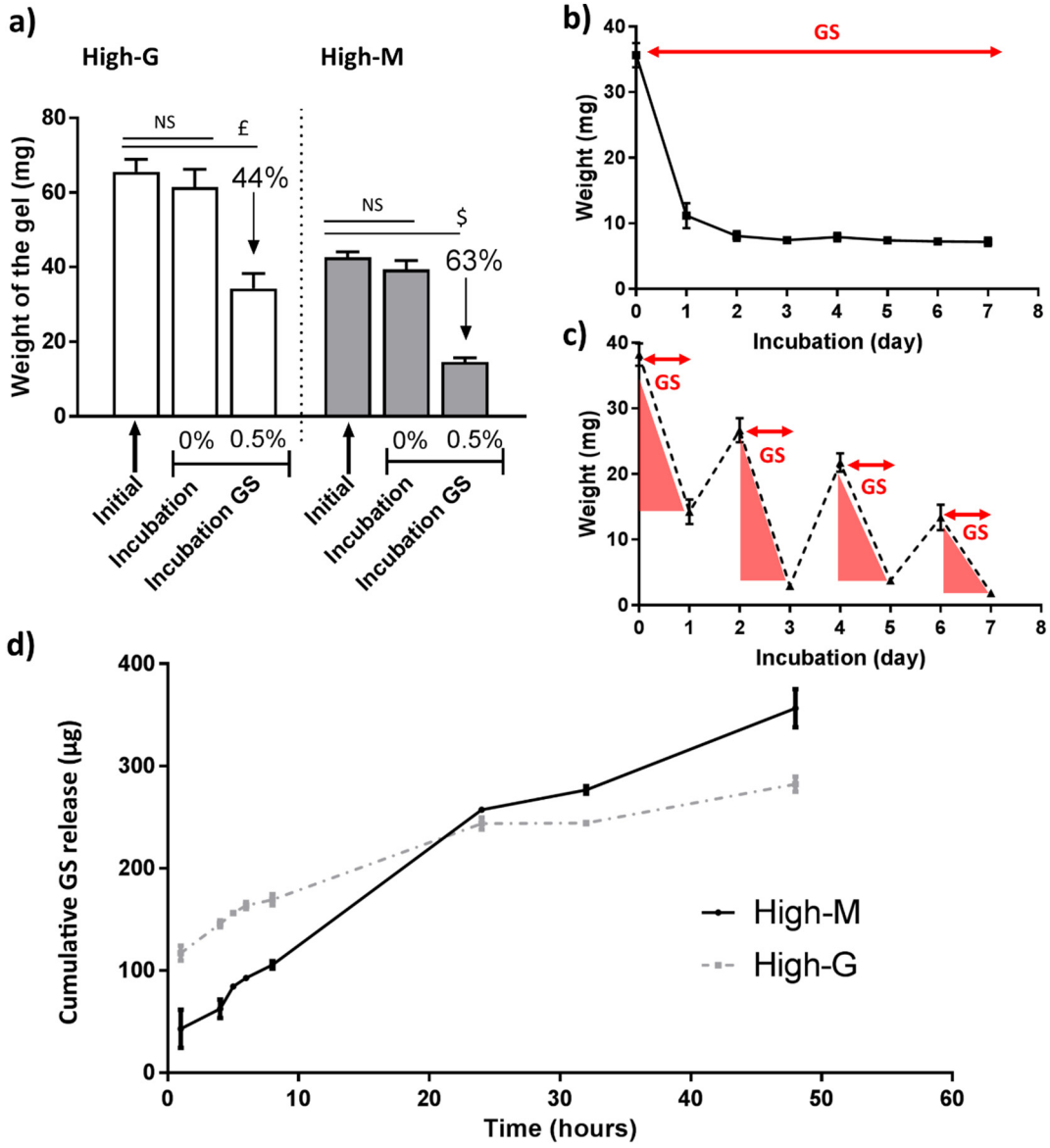

Fig. 2. Weight evolution of alginate gels in absence or presence of GS after 18 h of incubation, a. Differences were statistically significant only for the "incubation GS" group, denoted with £ and $ for High-G and High-M respectively. The difference in gels' weight for High-M alginate comparing a daily refreshing of GS solution (at 0.5% dissolved in PBS with 10% BSA, b) to a discontinuous supply of GS (the presence of GS in the incubating solution is denoted in red, at every other day, c). In vitro cumulative release of GS after incubation with alginate High-M or High-G gels (d).

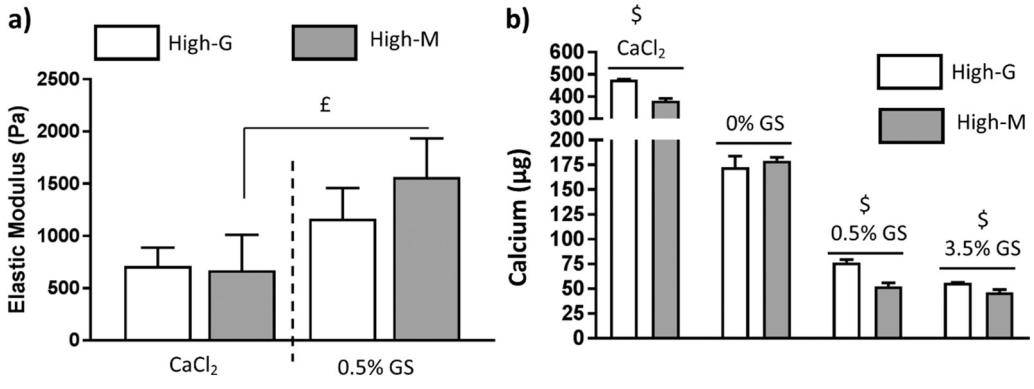

Fig. 3. Influence of GS incubation on the stiffness of alginate gels (£ denotes significance, observed only between High-M group, a) and on the amount of calcium present inside the gels depending on the concentration of GS in the incubating solutions ($ denotes significance between High-G and High-M groups, b).

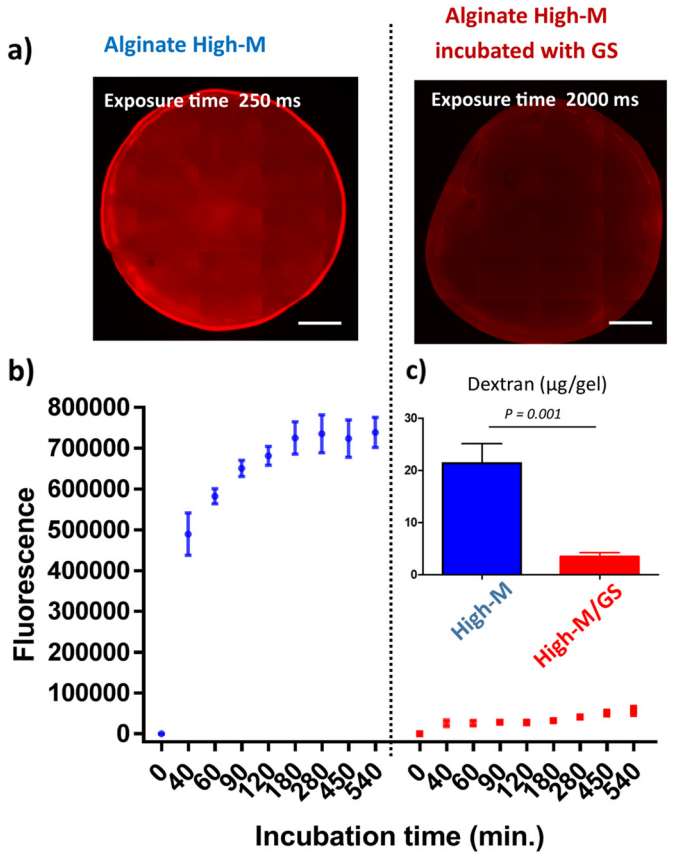

Fig. 4. Investigation of the macromolecular permeability of alginate High-M without and with incubation with GS illustrated using fluorescent microscope (after 6 h of incubation with Dextran-TRITC of 4.4 kDa, scale bars represent 1 mm). The fluorescent laser exposure times had to be optimized for each condition in order to obtain images of good quality due to the large intensity difference between High-M (250 ms) and High-M/GS (2000 ms) (a). The fluorescence intensity of the gels was evaluated by fluorimeter for 540 min (b) and the amount of Dextran-TRITC present inside the gels was subsequently quantified after gels dissolution (c).

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 (Fig. 2d). The GS release was shown to be more sustained in the case of High-M alginate than for High-G alginate gels. Such EPS acting as slow releasing drug reservoir could potentially result in the selection of resistant bacteria if sub-inhibitory concentrations of antibiotics are released locally for extended duration [16].

The network compaction in presence of GS was further investigated by compression tests. GS-laden networks were consistently stiffer than GS-free controls. For High-M, the Young modulus increase was 2.3-fold compared to pre-incubated controls (difference statistically significant, with $p < 0.03$), whereas it reached a 1.6-fold factor for High-G in the same conditions ($p > 0.05$, Fig. 3a). It was already reported that increasing substrate stiffness influences *P. aeruginosa* attachment, growth and decreases it susceptibility to antibiotics. [17] The notion of a drug-dependent stiffening 3D microenvironment could have a remarkable impact on the bacteria embedded in such gel, however the available literature only carries information of bacteria cultivated on 2D surfaces of various stiffnesses [17].

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. [18] Our results challenge such a statement as we did observe a competitive mechanism between GS and calcium electrostatic interaction with the alginate chains (Fig. 3b). Indeed, once GS penetrates the alginate gel, 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 similarly to Ca^{2+} ions, and those ions are expulsed from alginate gels in presence of GS. The differences observed between High-M and High-G, Fig. 3a and b, corroborate previous report showing that Ca^{2+} exhibits preferential binding to G-units, and GS to M-units. [18] This molecular exchange resulting in an enrichment of Ca^{2+} in the ASL and in the surrounding mucus could potentially have a role in the tenacity of the CF-infection. Indeed, increasing the concentration of calcium in medium has been demonstrated to increase the rate and the extend of *P. aeruginosa* biofilm production [19] and to elevate the ASL viscosity [20].

Network swelling or compaction can have a direct effect on the diffusion property of matrices, which can be investigated using tagged-dextrans [40]. Therefore, we employed fluorescent dextran-TRITC as

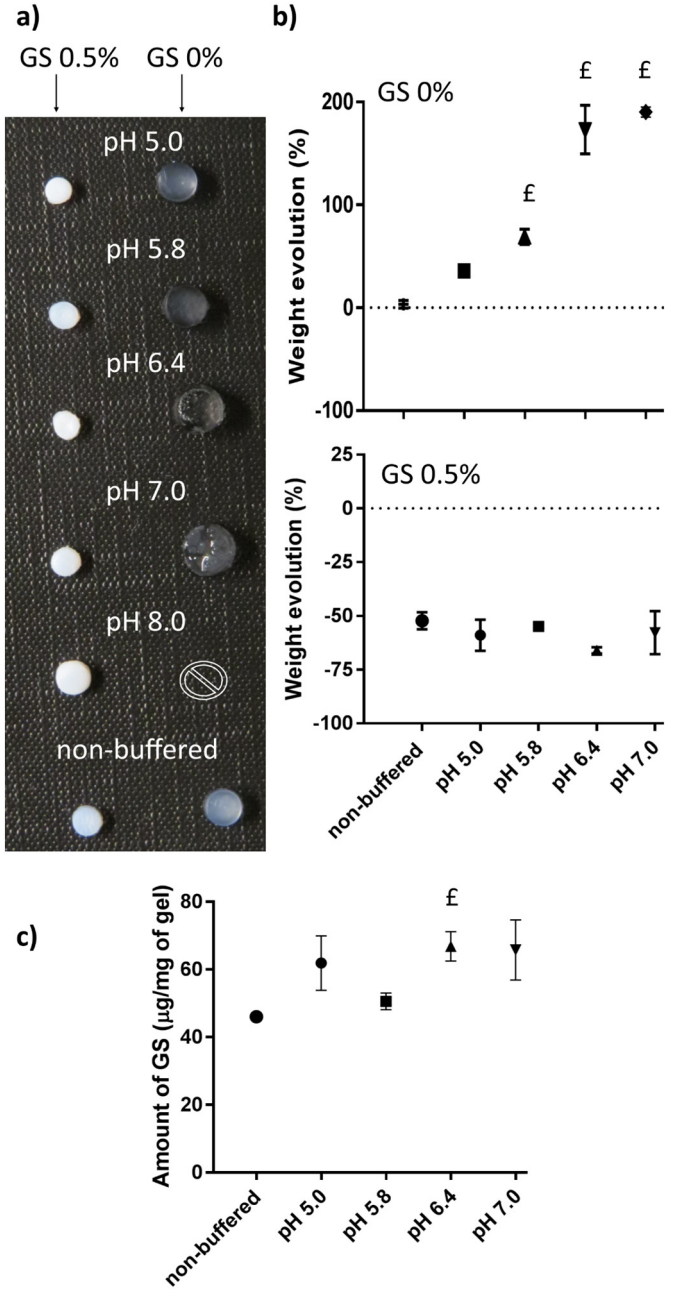

Fig. 5. Influence of the pH microenvironment on the macroscopic aspect of the High-M gels (a), on their swelling/shrinking behaviour depending on the absence or presence of GS in the solutions (b), and on their ability to adsorb GS (c). £ denotes significance compared to non-buffered condition.

macromolecular diffusion probe through High-M gels in presence and absence of GS. The fluorescence microscopy images Fig. 4a illustrate the clear difference in diffusion properties for the gels not exposed to GS in comparison to the gels incubated with GS (even though the exposure time was 8 times longer for High-M with GS). The spectroscopic quantification of the fluorescent evolution showed a fast increase of the fluorescent intensity in the first 40 min of incubation with dextran-TRITC and a plateau was reached after 180 min for the swollen gels in absence of GS in the solution (Fig. 4b). For the samples incubated with dextran-TRITC in combination with GS, a lower fluorescence intensity was measured associated with a lower diffusion of dextran within the gels. The quantification of the dextran-TRITC at the end of the incubation period proved that the presence of GS in the gel's environment was directly responsible for a decrease of the macromolecule diffusion of a factor 6. 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 gels. This result could have significant impact on biofilm physiology and eradication. Following administration of GS in the lung, a nutrient-deprived environment can be formed, which has been shown in vitro to stimulate even further alginate biosynthesis [21]. Macromolecular compaction within the biofilm could also hinder oxygen diffusion, contributing to hypoxic domains formation. This hypothesis deserves particular attention, as *i)* the lungs of CF-patients with biofilm are already characterized by low oxygen diffusion, [22] *ii)* the production of EPS by *P. aeruginosa* increases under anaerobic condition, [22] and *iii)* antimicrobial activity of several antibiotics (including GS) is decreased in environments characterized by low oxygen concentration [23]. Further investigations are necessary to determine the oxygen diffusion in GS-laden alginate matrices, using for instance microelectrode probes [24].

Another important characteristic of CF-lung is the associated local acidity (pH < 6.5), [25,26] therefore, we investigated the influence of the pH on gels' swelling or shrinking. In absence of GS, the pH of the incubating solutions impacted significantly on the swelling behaviour of the gels (Fig. 5a and b). Such phenomenon was completely prevented when the same buffers were supplemented with GS, with a resulting similar shrinkage of ±50%, Fig. 5a and b. Alginate gels were able to adsorb similar amount of GS, independently of the pH values of the media (Fig. 5c). In addition, due to its acidic nature, [27] GS can modify the local pH. Indeed, when a small amount of GS was deposited on top of the alginate gel containing phenol red indicator, a rapid change of the colour was observed, varying from red to yellow (Fig. 6a), whereas no variation was seen on the control gel (Fig. 6b). Such local acidification following antibiotic inhalation could clinically worsen the pharmacological activity of the drugs. Indeed, low pH is associated with a decrease of the aminoglycosides bactericidal activity due to an increase in ionisation degree [28] and to a change in *P. aeruginosa* membrane composition (by secretion of additional phospholipids) affecting its sensitivity to antimicrobial therapies. [29] Along with the acidification, a decrease in volume accompanied by syneresis (expulsion of water) was observed on the GS-supplemented gel (denoted with the blue arrows, Fig. 6a). It is known that *P. aeruginosa* resist to dehydration by secreting more alginate in biofilm due to upregulation of *algD*. [30,31] Therefore, from our experimental results, it can be expected that the

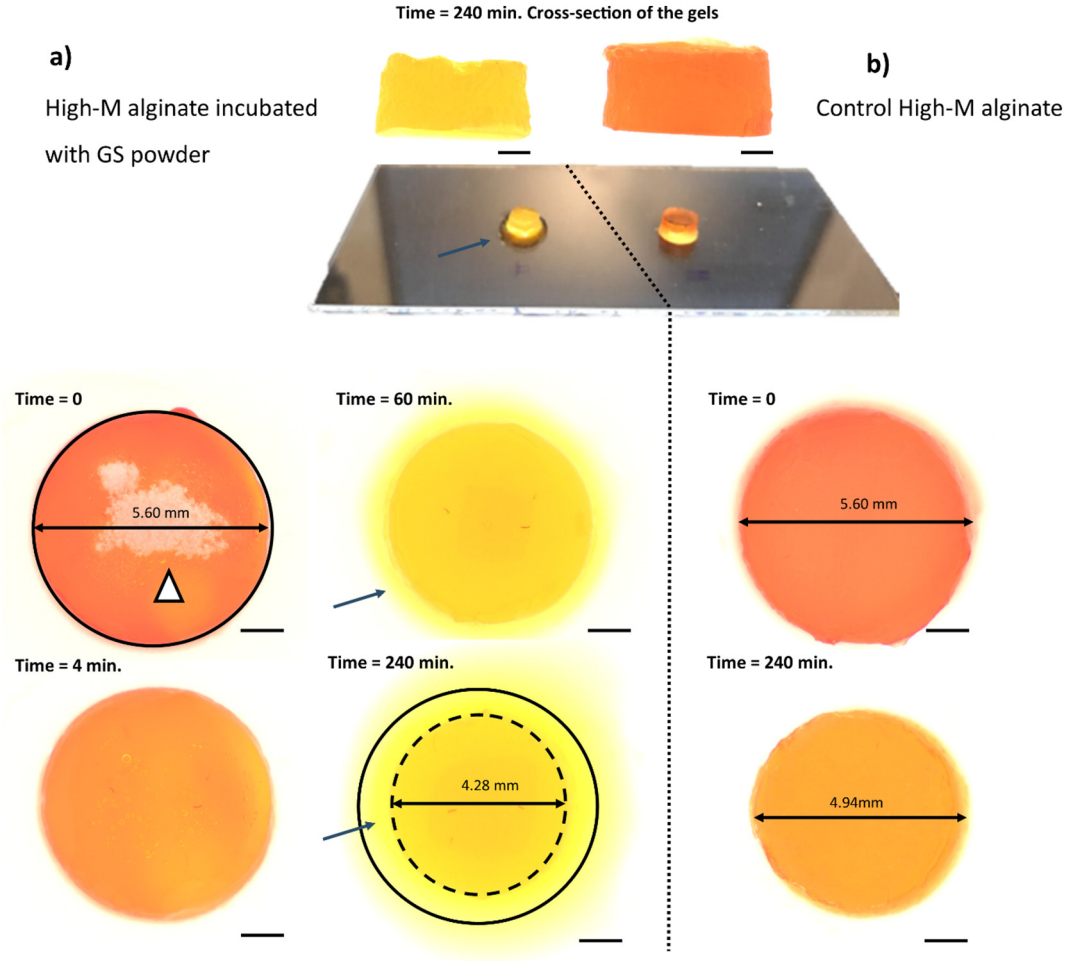

Fig. 6. Acidification of High-M alginate gels containing pH indicator red-phenol following deposition of the GS powder (shown with the white arrow, a) compared to drug-free group (b). Subsequent dehydration and gel shrinkage are denoted by the blue arrows and dash-circle respectively. The scale bars represent 1 mm.

network dehydration that occurs in presence of GS might have a direct effect on the alginate secretion by *P. aeruginosa*.

Thermal analyses were performed on the alginate without and with GS in order to investigate the nature of the water molecules remaining in the gels. TGA results, presented Fig. 7a, showed that the water evaporates at significantly lower temperatures for High-M with GS compared to High-M. 50% of the weight lost was reached at 84 °C for High-M with GS, against 115 °C for High-M without GS. The difference of the remaining dry weights 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, which is equivalent to 0.4 mg per mg of dry alginate. Further experiments conducted using DSC showed a double peak for the water 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 that contains only free molecules of water. This reveals that the water present in High-M without GS is present under at least two molecular states. There is most probably a mixture of free and freezable-bound water, the latter being the portion of the water with thigh interaction with the alginate matrix. [32] Then, the incorporation of GS inside the High-M network is responsible for water displacement, from bound to free-water. This can explain the difference in the profiles of the water evaporation recorded using TGA (Fig. 7a), as free water requires less energy to evaporate compared to bound water. To summarize Fig. 7, 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 their surrounding alginate matrix.

Finally, MRI analyses validated the fact that the degree of interaction of the molecules of water with the alginate matrix depends on the presence or absence of GS (Fig. 7c). 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 water 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 activity of water [33].

Several in vitro observations presented in this manuscript correlate well with pathological signs of CF-patients suffering from chronic infections, such as accumulation of dehydrated mucus in the lung airways, [34] extremely high and abnormal concentration of salts (including Ca^{2+}), [35] acidity [20,26] and decrease of T1 signal in MRI. [36] The in vitro model of alginate-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 the ionic interaction existing between the positively charged ammonium groups in GS and the negatively charged carboxylic groups present in alginate. This strong ionic interaction is responsible for the gel's network reorganization, resulting in the release of calcium ions and of molecules 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 antibiotherapies, 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 structure influence and react to such micro-environmental dynamic evolutions. Indeed, 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 [37–38]. Our study highlights the fact that the monosaccharide composition of alginate in terms of M/G-units has a major impact on its behaviour and must be carefully considered. In addition, further strategies originating from our results could focus on preventing or limiting

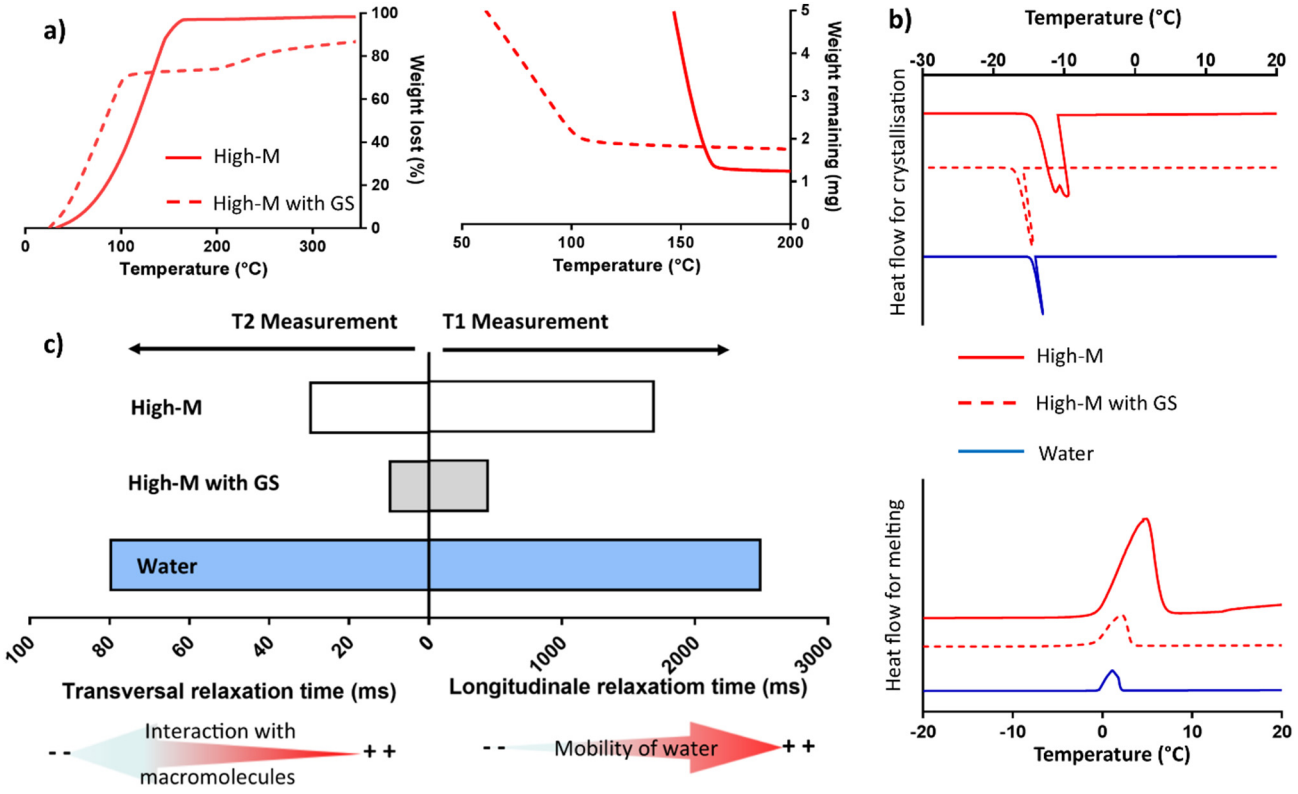

Fig. 7. Thermogravimetric analyses of High-M alginate without and with GS, showing the two different profiles of water evaporation and the difference in residual masses at the end of the experiment (a). Differential scanning calorimetry revealing the different behaviours of the molecules of water present inside the gels containing or not GS, in terms of water crystallization (exothermic peak) and water melting (endothermic peak) (b). Measurement of transversal (T2) and longitudinal (T1) relaxation times of the protons of water inside the gels before and after incubation with GS (c).

the alginate network restructuration occurring during incubation with GS, using novel generation of adjuvants acting for examples as molecular "rigid spacer". Extrapolation of our results to other antibiotics is another important factor to be considered, as other drugs have been described to interact as well with alginate, such as tobramycin (as demonstrated in the preliminary experiment shown SD3) or ciprofloxacin [11,12,38,39].

4. Conclusion

In CF patients, once *Pseudomonas aeruginosa* bacteria have acquired mucoid phenotype, pulmonary infections become extremely difficult to eradicate. The presence of alginate in the biofilm acts as a protective microenvironment for the bacteria from antibiotic therapies. In this study, we showed that the physico-chemical properties of alginate are greatly influenced by gentamicin sulfate (GS). These changes could potentially exacerbate the protective effect of the surrounding EPS on the microorganisms, even though further studies are needed to investigate the influence of *P. aeruginosa* encapsulated within the alginate. In addition, we demonstrated that the chemical structure of alginate in terms of mannuronnate (M) or guluronate (G) motifs impacts strongly on its behaviour once in contact with GS. Our results demonstrate that the selection of an appropriate ratio of M/G motifs in alginate is important in the design of relevant in vitro models of CF-biofilm. By better understanding of how antibiotics interact with alginate and other EPS present in biofilm, the efficacy of existing antibiotics could be improved by altering the EPS binding affinity to antibiotics using novel therapeutic adjuvants.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2018.10.025.

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