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RESEARCH ARTICLE

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In situ evaluation of alginate-Ca^{2+} gelation kinetics

Ioanna N. Besiri | **Thomas B. Goudoulas** | **Ehsan Fattahi** | **Thomas Becker**

Research Group of Fluid Dynamics, Chair of Brewing and Beverage Technology, TUM School of Life Sciences, Technical University of Munich, Freising, Bayern, Germany

Correspondence
Ehsan Fattahi, Research Group of Fluid Dynamics, Chair of Brewing and Beverage Technology, TUM School of Life Sciences, Technical University of Munich, Weihenstephaner Steig 20, Freising, Weihenstephan D-85354, Germany.
Email: ehsan.fattahi@tum.de

Abstract

A thorough assessment of gelation progress requires real-time recording of biopolymer kinetics. In this study, an in situ investigation with a customized parallel-plate rheometric setup examines how the initial distribution of $CaCl_2$ to alginate through micro-holes affects the process. At constant volume and concentration of reactants, stiffer gels are produced at the same probing time by increasing the number of suppliers (2–12). Due to the oscillation and the different local distribution of the cations into the biopolymer solution, the development of gel front is affected, which is for the first time in situ rheologically investigated by the temporal evaluation of complex modulus, G^*. The alternative configurations of the same number of micro-holes have drastically contribution to this regard, too. Moreover, a two-kernel equation is introduced predicting the alginate-Ca^{2+} gelation kinetics. It represents the initial fast increase in G^*, determined by the concentration of cross-linker, and the slow diffusion of cations to the assembled gelling structures depicted on the successive evolution of G^* in longer times. Finally, a continuous and an intermittent oscillatory time sweep influence the diffusion of the cross-linking solution resulting in considerably different gelation curves for the same concentration of reactants.

KEYWORDS

alginate, cross-linker, gelation, kinetics, reaction, rheology

1 | INTRODUCTION

The cross-linking alginate-Ca^{2+} gelation is implemented in the food industry and bioengineering applications due to the advantageous properties of the polysaccharide, such as biocompatibility and water solubility. As an illustration, it can be utilized in the preparation of complex scaffolds in bone tissue engineering or in the development of biofilms for food packaging.[1,2] The negatively charged long-chain biopolymer, consisting of guluronate (G-) and mannuronate (M-) residues as monomers, reacts with multivalent cations in solutions and forms gels through cross-links.[3,4] The Ca^{2+}-dependent gelation of the polysaccharide is based on the "egg-box" model, where the cations associate the alginate chains to form dimer, trimmer, and consequently, multimer structures.[5–7] In accordance with proposed kinetic models for alginate-Ca^{2+} gelation, the process is developed in two stages.[8,9] An initial fast response in the mechanical properties because of the formation of the first gelling structures is followed by a slow evolution in longer times, where propagation of multimers occurs.[10] Thus, the assessment of the real-time dynamic behavior of these complex biomaterials is crucial to address which parameters control the reaction process.[11]

Advanced rheometric methods record the gelation to control and understand the dynamic response of materials.[12] Customized experimental efforts have been recently developed by modifying the transducer in the head of the rheometer to minimize the time of the rheological characterization,[13] or merging rheology with Fourier transform infrared spectroscopy to in situ monitor the chemical and mechanical behavior of gelation.[14] The fast external alginate-Ca^{2+} reaction, that is, direct mixing of the solutions of reactants,[3] has scarcely been rheologically investigated.[12] The progress of the viscoelastic response of gels needs to be quantified since it is essential information for their applications. To this research framework, a novel custom-made rheometric setup for alginate-Ca^{2+} gelation is recently introduced.[15] The design facilitates the real-time recording of gelation by injecting the cross-linker into the biopolymer solution through micro-holes at the beginning of the rheological experiments. The operation is based on the working principle of an isothermal batch reactor since the system is closed, that is, no inlet and outlet, with a constant volume during the process.[16,17] The setup aids in an effective recording of the external alginate-Ca^{2+} gelation from the initial stages and an accurate and easy control of the volume and the concentration of reactants.[15]

The diffusion of cations into biopolymer chains is controlled by the concentration of the reactants and the molecular characteristics of the polysaccharide.[18–20] This process determines the structure of alginate-Ca^{2+} systems. Skjåk-Bræk *et al.*[21] have observed various degrees of inhomogeneity in cylindrical gel plugs depending on how cations react with the biopolymer network. For example, when Ca^{2+} ions diffuse through dialysis membranes placed at both ends of a plastic cylinder containing alginate, the concentration profile of gel is decreased at the center, especially at low calcium concentration. In addition, by adding one droplet of alginate in different Ca^{2+} baths and mixing them for 1–4000 s, Lee and Rogers[22] have reported that there is an osmotic gradient between the inner sphere and the bath. Therefore, Ca^{2+} diffuses into the gelled matrix through a driving force until it reaches the area that can form noncovalent bonds with alginate.[22] Estimated values of the diffusion coefficient of Ca^{2+} ions ($D_{Ca^{2+}}$) into alginate solutions are of the order of $10^{-9}\,m^2\,s^{-1}$, as reported in previous research.[21,23]

The recently demonstrated custom-made setup[15] can be used to study how the way the cations are injected into alginate affects the gelation process. In the previous study, the low working gap of the cone-and-plate geometry restricts the volume of reactants. Thus, enough space is not provided to systematically distinguish the impact of the number of micro-holes on the developed hydrogels. This effect is more pronounced considering the limited availability of suppliers (2 and 4) to alginate solution.[15] In the current research, to further study the diffusion of cross-linker to alginate solution, a parallel-plate geometry is utilized at a higher working gap. In addition, the cationic suppliers are increased from 2 and 4 to 8 and 12, with the latter one in two different configurations. The alternate customized designs give the chance to in situ rheologically investigate for the first time the development of gel front, through the temporal evolution of complex modulus, G^*. It critically depends on the number and position of micro-holes when the bulk properties of reactants, that is, volume and concentration, are the same. Different ratios of the concentration of reactants are examined to show how this influences the diffusion of cations to biopolymer network. A two-kernel equation which can fit and predict the progress of cross-linking gelation is introduced. The two-step mechanism of the alginate-Ca^{2+} reaction is adapted to the kinetic model developed by Djabourov *et al.*[24,25] for gelatin gelation, since it presents similar behavior with the temperature dependence of the helical conformation obtained by the gelatin network in aqueous solutions.[26,27] The final part of the study investigates the effect of oscillation on the diffusion of cations to alginate during the reaction process by comparing continuous with intermittent oscillatory time sweeps.

2 | MATERIALS AND METHODS

2.1 | Materials

In the present research, sodium alginate is used as an alginic acid sodium salt from brown algae purchased from Sigma Aldrich (A0682). According to the material documentation, the molecular weight (M_w) is 12–80 kDa and the ratio of monomers is M/G = 61/39. A granular of calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$ 99% min, Alfa Aesar) is utilized as the cross-linker without further purification. To prevent bacterial growth in the biopolymer solution, sodium azide (NaN_3) provided by AppliChem GmbH (UN16875) is selected. For long-term rheological experiments, mineral oil is applied at the rim of parallel-plate geometry to prevent the evaporation of samples.

2.2 | Preparation of solutions

The preparation process for the initial alginate and $CaCl_2$ solutions follows the protocol reported in the previous publication.[15] For the present study, we have prepared 1 and 2 wt.% alginate and a range of concentrations of $CaCl_2$ (37.5–150 mM). The biopolymer solutions are

stored in the refrigerator at 5°C. Before the rheological measurements, the samples are in thermal equilibrium in the room.

2.3 | Custom-made rheometric setup

In the latest research studies, a custom-made rheometric setup is developed for real-time recording of fast crosslinking reactions.[15,28] It consists of two parts that provide an inner cavity when firmly coupled. A volumetric syringe is connected to a side-feed hole, where the crosslinking solution is applied. Then, it fills the internal cavity, and, finally, by an upward flow, it is injected through micro-holes into the biopolymer solution placed on the top of this construction. More details about the design and the operation of the setup can be found in recent publications.[15,28]

In the present study, as Figure 1 shows, different configurations of the upper part (65 mm diameter) and a simpler inner design are conceptualized and constructed to observe how the number and the position of cationic suppliers affect the gelation process. Previously, the configurations were presented with 2 and 4 micro-holes used as injectors of the crosslinking agent to the biopolymer solution.[15,28] In the new configurations, the number of micro-holes is increased to 8 and 12. The 8 micro-holes are quadrilaterals distributed on the top of the setup. The 12 micro-holes are constructed in two arrangements, one where the micro-holes are homogeneously placed (12-H) and one with the micro-holes radially designed (12-R), as Figure 1d,e shows, respectively. The diameter of the micro-holes is approximately 320 μm, as the microscope image shows in Figure S1a. Moreover, the internal channels of the upper part, to inject $CaCl_2$ solution into alginate via the micro-holes,[15,28] have been replaced by a flat inner design, as Figure S1b shows. To provide more micro-holes (>4), a considerable number of internal channels is required, making the design and the construction of the configuration complex. Hence, the inner flat configuration is deemed imperative. This design requires a short central metallic extension of the upper part of the setup to enable the simultaneous filling of all micro-holes by an upward flow when $CaCl_2$ solution is injected at constant pressure (see Figure S1b). The previously used inner central hole cannot fill simultaneously all micro-holes (>4) in the flat inner design, since the suppliers located close to the central hole will be filled

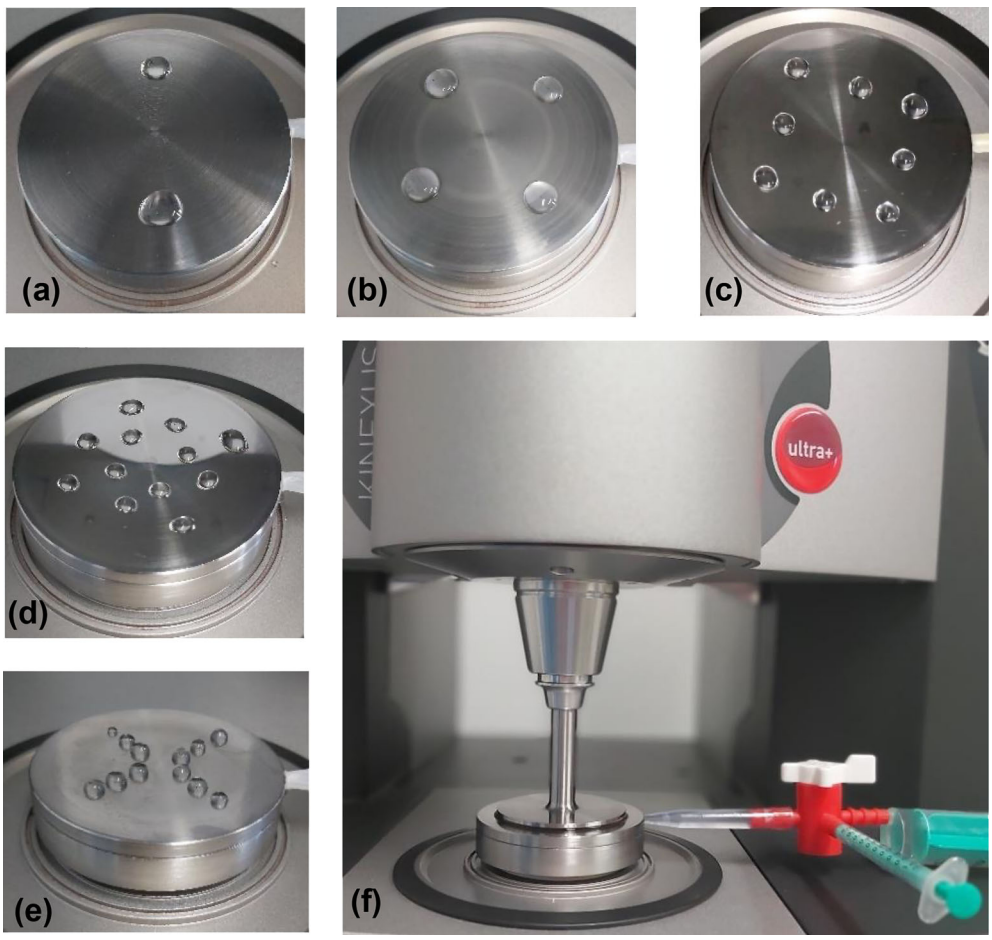

FIGURE 1 (a–e) Available configurations of the rheometric custom-made setup: (a) 2, (b) 4, (c) 8, (d) 12-H, and (e) 12-R micro-holes. (f) Final configuration on the Kinexus Ultra + rheometer during the experiments. The three-way valve is used to inject the crosslinking solution into the biopolymer fluid. [Color figure can be viewed at wileyonlinelibrary.com]

first. As an additional improvement in the current study, we use a three-way-valve as an inlet (ø 5–7 mm), utilizing one way to fill the cavity of the construction with the cross-linking agent and the other way to inject the $CaCl_2$ solution into the biopolymer by a smaller and more precise syringe. The final configuration on the rheometer during the experimental measurement is presented in Figure 1f. The construction has been designed for Kinexus Ultra+ (Netzsch GmbH) rheometer and, along with a commercial upper parallel plate (PU50 SR1255 SS), comprises the parallel-plate setup.

2.4 | Rheological measurements

The gelation is recorded with the upper plate of 50 mm diameter at a 0.75 mm working gap using the stress-controlled Kinexus Ultra+ (Netzsch GmbH) rheometer. This working gap allows a total volume of 1.8 mL for the two mixed solutions. Thus, all the experiments are performed by injecting 0.6 mL $CaCl_2$ into 1.2 mL alginate, following the biopolymer/cross-linker volume ratio = 2/1. To prevent slip effects during the reaction, sandpaper with a rough surface (Norton Saint Gobain P320) is attached to the upper plate. Before the experiments, the custom-made setup should be firmly placed to the base of the rheometer, and the internal cavity should be filled with the solution of the cross-linking agent. Then the alginate is placed on the top of the setup, the upper plate reaches the working gap and the time sweep measurement starts. While the first data points of mechanical properties are recorded, the $CaCl_2$ solution is injected into the biopolymer fluid at 10 s of each measurement, and the alginate gelation initiates. In Section S2, we present the critical parameters that should be considered to operate the custom-made setup during the rheological experiments effectively and a detailed step-by-step presentation of the rheological procedure.

Small amplitude oscillatory experiments have been conducted at 3% strain (γ%), 1 rad s^{-1} angular frequency (ω) and 25°C. During the preliminary tests and the analysis of the waveforms, we have verified that the applied strain is inside the linear viscoelastic region (LVR). The short-term measurements record the complex modulus, G^*, as a function of time, from the very beginning until 1000 s of the alginate-Ca^{2+} reaction. The process protocol consists of two sequences, which differ in the sampling rate. More specifically, for the first 200 s, the sampling rate is 5 points/s for a detailed initial recording, and for the rest 800 s, the rate is 1 point/s. The data of the short-term experiments presented in Section 3.1 are the average of three repeatable experiments.

Moreover, long-term oscillatory time sweep experiments have been conducted with the 8 micro-holes setup to examine the response of alginate-Ca^{2+} system in longer time scales. A detailed record of the temporal evolution of G^* has been achieved with a sampling rate 1 point/10 s. Furthermore, we record the long-term response of the alginate-Ca^{2+} gelation in an interval manner, without continuous external oscillatory deformation, likewise a typical time sweep (continuous oscillatory flow). We call this measurement intermittent oscillatory flow (Section 3.3). The injection of $CaCl_2$ was performed as before, but the mixture was at rest for the initial 5 min of the reaction. Then, oscillatory recording is applied for 2 min with a sampling rate of 1 point/20 s, followed by a 20 min rest period, where the γ% and ω were for the continuous time sweep experiment. Thus, during 1 h of the intermittent oscillatory flow, the sample underwent only three short oscillatory intervals. These short oscillation intervals were applied for 16 h of the reaction. Details about the data treatment exist in Section S3. Finally, to prevent the evaporation of samples in long-term experiments, we cover the setup with a custom-made plexiglass configuration demonstrated in the previous research,[15] and we apply mineral oil at the rim of the parallel-plate geometry.

3 | RESULTS AND DISCUSSION

3.1 | Effect of micro-holes on short-term alginate-Ca^{2+} reaction

In this section, we present how the in situ initial distribution of cross-linker to biopolymer solution affects the alginate-Ca^{2+} reaction by investigating the dynamic response of the gelling systems. In Figure 2, the temporal evolution of the mechanical properties is observed when 37.5 mM $CaCl_2$ is injected through 8 micro-holes into 1 wt.% alginate. The elastic response of the created hydrogel, that is, storage modulus, G', presents an initial fast increase. However, it does not reach a plateau at 1000 s, although the ascending rate decreases at longer times. On the other hand, the loss modulus, G'', that is, the viscous response, exhibits a considerably slower increase, being almost an order of magnitude lower than G' at 1000 s of the reaction. This observation, along with the fact that G^* is defined as $\sqrt{G'^2 + G''^2}$ justifies that G^* has a similar response with G', as Figure 2 shows. The same behavior of moduli is observed in all the combinations of concentrations of reactants in the current study. For this reason, the effect of the number and the position of suppliers in the alginate-Ca^{2+} gelation process is examined through the temporal evolution of G^*.

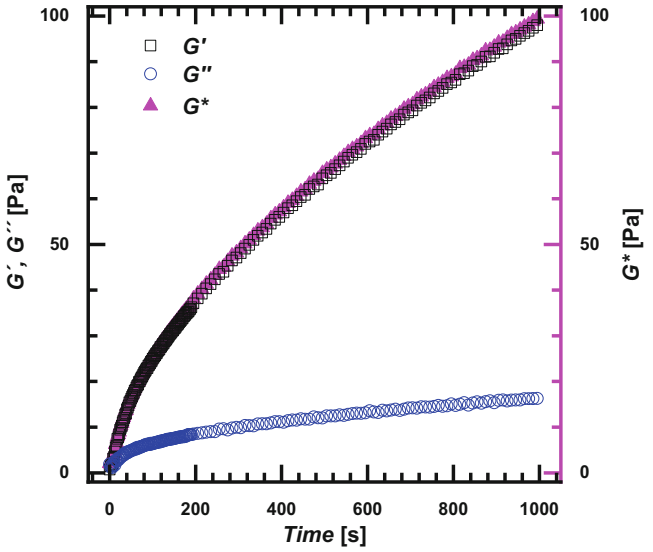

FIGURE 2 Time sweep experiment of 0.6 mL 37.5 mM $CaCl_2$ injected into 1.2 mL 1 wt.% alginate through 8 micro-holes, where the G^* curve (triangles) practically coincides with the G' curve (squares). [Color figure can be viewed at wileyonlinelibrary.com]

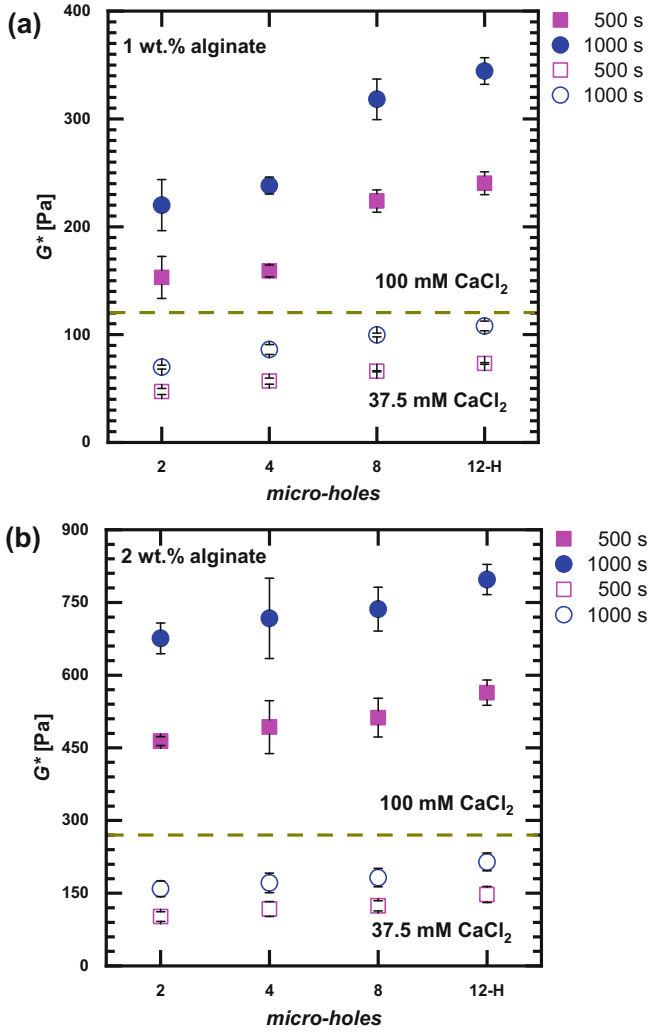

FIGURE 3 G^* as a function of the number of micro-holes at 500 and 1000 s of alginate-Ca^{2+} reaction for (a) 1 wt.% and (b) 2 wt.% alginate. The dashed line designates the two cation concentrations used for these gelation experiments. [Color figure can be viewed at wileyonlinelibrary.com]

Figure 3a,b shows the effect of the number of micro-holes on G^* at 500 and 1000 s of the reaction for two $CaCl_2$ concentrations and alginate of 1 and 2 wt.%, respectively. The entire temporal evolution of these measurements is presented in Section S4. Furthermore, the scales of the axes have been adapted to the magnitude of the moduli to assist the reader. As it is observed, the progress of alginate-Ca^{2+} gelation depends on the number of micro-holes supplying cations to the biopolymer solution when the bulk properties, that is, volume and concentration, of reactants are identical. Comparing the 12-H to 2 micro-holes, we observe 26 Pa higher at 500 s when 37.5 mM $CaCl_2$ is presented in 1 wt.% alginate. A similar response is observed for the other combinations of the concentrations of reactants. As the number of micro-holes increases from 2 to 12-H and a continuous oscillation is applied to the system, the distribution of the cross-linker to the biopolymer solution is enhanced. Finally, as additionally the normalized G^* in Figure S6 shows, the increased percentage of G^* as a function of the number of micro-holes depends on the concentration of reactants.

The injection of the cross-linker into the alginate through micro-holes creates gel fronts, and each develops around the area of each micro-hole. These act as obstacles to the distribution of the ions not crosslinked with the alginate chains. Potter *et al.*[23] previously reported such an obstructing effect by the gradually developing gel matrix on Ca^{2+} diffusion. At a higher number of micro-holes, that is, 8 and 12-H, the smaller volume of $CaCl_2$ solution injected locally in each microhole results in smaller gel fronts at the area around each supplier. This facilitates the reaction of more cations with alginate chains. The formation of more junction zones leads to the propagation of multimers, and hydrogels with higher G^* are obtained.

Bjørnøy *et al.*[20] have shown through dark field microscopy that the gel front in alginate-Ca^{2+} system increases with time as the cations diffuse from the periphery to the center of an alginate disc.[20] In the present study, the increase of the gel front in time through the alginate-Ca^{2+} mixture is enhanced when the number of suppliers is increased. In this way, the mass conversion of reactants to gelling structures is higher, leading to stiffer gels using the 8 and 12 micro-holes.

In addition, the development of the gel front is enhanced by the applied displacement, l, during the oscillatory movement of the parallel-plate. Considering

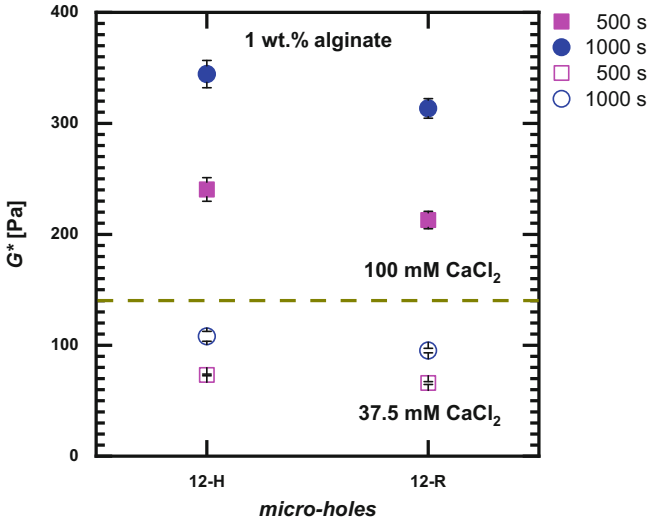

FIGURE 4 G^* as a function of number of micro-holes at 500 and 1000 s of alginate-Ca^{2+} reaction for 1 wt.% using the 12 micro-holes configurations. The dashed line designates the two cation concentrations used for these gelation experiments. [Color figure can be viewed at wileyonlinelibrary.com]

constant strain $\gamma = l/d$, the displacement is higher for microholes that are further away from the center of the parallel-plate. This is the maximum for the 12-H configuration. The effect of the displacement of all gel fronts due to the oscillation is further investigated in Figure 4, which shows how the arrangement of 12 microholes affects the progress of alginate-Ca^{2+} reaction at 500 and 1000 s. It is observed that the use of 12-R micro-holes results in gels with lower mechanical properties compared with the 12-H ones. The reason is that the micro-holes in 12-R are closer to the center, and therefore, 8 micro-holes out of 12 get less effect of the displacement in comparison with the 12-H. The effect of displacement is further investigated in Section 3.3.

In conclusion, as the number of suppliers increases from 2 and 4 to 8, 12-H and 12-R, a more homogeneous distribution of Ca^{2+} ions to alginate chains occurs, leading to more junction zones and, consequently, faster reaction. However, the uniform flow of cross-linker to biopolymer solution is easier to be handled for manual injection of $CaCl_2$ through 8 micro-holes. For this reason, the long-term response of the system in the rest of the study has been investigated with this setup.

3.2 | Long-term alginate-Ca^{2+} kinetics

In this section, oscillatory time sweep experiments are conducted to obtain the long-term temporal evolution of G^*. Figure 5a shows 1 wt.% alginate reacting with $CaCl_2$.

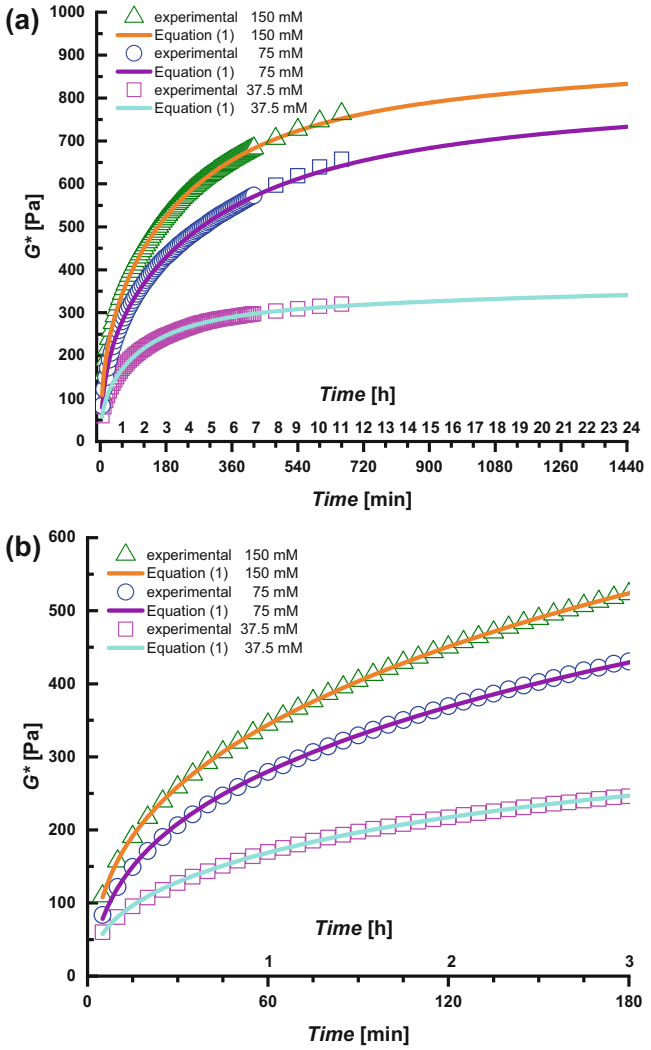

FIGURE 5 (a) Long-term oscillatory time sweep experiments of 1.2 mL 1 wt.% alginate injected by 0.6 mL $CaCl_2$ conducted with 8 micro-holes setup. Each plot represents the different concentrations of cross-linker solution in the measurement: 37.5 (pink squares), 75 (blue circles), and 150 mM (green triangles) $CaCl_2$. The symbols until 7 h are used for the fitting of Equation (1), and the rest are the experimental values of G^* from 8 to 11 h of the reaction. The continuous lines show the extrapolation of Equation (1). (b) The kinetics are presented until 3 h of the reaction for a detailed view of the temporal evolution of G^*. [Color figure can be viewed at wileyonlinelibrary.com]

To evaluate the long-term response of gelation, a systematic increase in the concentration of $CaCl_2$ has been chosen: (x =) 37.5 mM, ($2x$ =) 75 mM, and ($4x$ =) 150 mM. For a better understanding of the results, Figure 5b presents the temporal evolution of the same data until 3 h of the reaction. As it is observed, all the reactions are developed in two stages within 11 h. There is a rapid increase of G^* in the first hours of the process, which is followed by a slow evolution in longer times. For example, when 37.5 mM $CaCl_2$ is present in the system, G^* increases fast

reaching 246 Pa at 3 h of the process, and then it is increased slowly, only by 74 Pa, in the next 8 h, reaching a quasi-steady-state. This follows the observation of Fernández Farrés and Norton[9] who have recorded in situ the temporal evolution of viscosity in a similar alginate-Ca^{2+} system, that is, 22.5 mM $CaCO_3$ reacting with 1 wt.% alginate of relatively low M_w. There, a considerably slow increase of viscoelastic properties was depicted from 3 to approximately 6 h of the reaction.

The increase of G^* in the first hours becomes sharper at the higher availability of cations, that is, 75 mM and 150 mM $CaCl_2$. Therefore, the cationic concentration determines the initial response of kinetics. According to Hashemnejad and Kundu,[29] this stage includes the fast formation of the junction zones, an increase in the length of the previously formed ones, the growth of their diameter, or a combination of these structural events. Thus, the basic multimer structures are created. However, the subsequent slower rate of G^* is attributed to the slow diffusion of still active cations, adopted partially to alginate chains, into the multimers which have been propagated at this stage.[8,30] According to Skjåk-Bræk *et al.*[21] diffusion may be assumed as the rate-determining step in the gelation process. Thus, the initial fast increase of G^* results from the fast reaction between the available free alginate chains and Ca^{2+} cations, while the later slow evolution rate is linked with the slow diffusion process of the alginate monochains crosslinked with cations into the gelling zone.[21]

From the observation of long-term kinetics, a two-kernel equation is required to describe the progress of the reaction. Djabourov *et al.*[25] have conceptualized and proposed a comprehensive kinetic model to understand the mechanism of gelatin gelation, based on optical rotation data. It is based on the helical conformation adopted by gelatin in an aqueous solution and affected by temperature. The chains are interconnected to give triple helixes, and upon propagation and lateral association of the basic structural units, a 3D network is created. The kinetic model is intended to calculate the number of helixes as a function of time and temperature to enlighten the gel conformation.[25] This coil-to-single helix transition is similar to the "egg-box" structures obtained during alginate gelation.

For this reason, we propose an equation adapted to the model of Djabourov *et al.*[25] to study the long-term alginate-Ca^{2+} reaction. To the extent of our knowledge, it has been used to describe other gelatin-alginate systems,[31] but, for the first time, it is applied to the cationic crosslinking alginate gelation. The mathematical expression comprises one exponential and one logarithmic term describing the temporal evolution of the mechanical properties, as follows:

$$G^*(t) = C_1\,[1 - \exp(-t/t_1)] + C_2 \log[1 + (t/t_2)]. \quad (1)$$

The exponential factor is related to the initial fast response of the reaction, while the logarithmic describes the long-term behavior of the gelation. C_1 and C_2 are the constants of each process and are expressed in Pa, matching with the units of $G^*(t)$. More specifically, C_1 is the amplitude of the initial fast response of the system, and C_2 is the slope of the second process as a function of the logarithm of time. Moreover, t_1 is the characteristic time of the fast process, while t_2 is the characteristic time at which the second process appears, and they are expressed in units of min. The physical meaning of constants and times is based on the basic introduction of the equation by Djabourov *et al.*[25] As observed in this equation, the dependent structural parameter is the $G^*(t)$. The number of "egg-box" structures obtained by alginate-Ca^{2+} crosslinking and, therefore, the number of multimers comprising the hydrogel in each time scale is represented by $G^*(t)$, which reveals the total mechanical behavior of the system.

Figure 5a,b shows that the fitting of Equation (1) to the experimental data obtained until 7 h, is successful for all the concentrations of reactants. This is also verified by the R^2 value that reaches 0.99. Details for the fitting process are presented in Section S6. Moreover, the extrapolation of Equation (1) is in a good agreement with the available experimental results from 8 to 11 h in all cases studied here. Thus, this mathematical expression can adequately describe and predict the alginate-Ca^{2+} reaction. In addition, the prediction of the process until 24 h shows that the alginate-Ca^{2+} gelation is in a quasi-steady-state, and it is still evolving with a slow rate. As Djabourov *et al.*[25] report, there is no limit on the formation of multimers in physical gelation since gelatin triple helices reformation develops in approx. 42 days, and still, it is not completed.

Table 1 presents the values of constants of Equation (1) describing the experiments in Figure 5a,b. The amplifier of the first fast process, that is, C_1, is higher as the concentration of the $CaCl_2$ increases. This is correlated to the sharper response of gelation, that is, faster reaction, obtained with the higher availability of the cations to the alginate. Blandino *et al.*[32] have reported also an initial fast exponential increase in the gel film thickness during the formation of alginate-Ca^{2+} gel membranes in higher cationic concentrations. Furthermore, the characteristic time of this stage, t_1, is low for 37.5 mM $CaCl_2$, since there is a smaller number of cations which fast occupy the free alginate chains. However, t_1 increases at higher $CaCl_2$ concentrations due to the time needed for the formation of more "egg-box" structures. Remarkably, the time that the exponential term of Equation (1)

TABLE 1 Constants of Equation (1) obtained from the fitting of the experimental data in Figure 5a,b.

Constants of Equation (1)	Concentration of $CaCl_2$ (mM)		
	37.5	75	150
C_1 (Pa)	113.65 ± 2.79	247.40 ± 3.89	306.47 ± 1.86
t_1 (min)	128.75 ± 1.20	321.39 ± 10.93	239.93 ± 1.72
C_2 (Pa)	73.60 ± 1.93	184.53 ± 4.02	183.73 ± 1.55
t_2 (min)	1.17 ± 0.08	3.25 ± 0.15	1.95 ± 0.04

predominates is 80 min less in the 150 mM than in 75 mM $CaCl_2$. This decrease in t_1 can be explained as an effect of the high availability of $CaCl_2$. Since a double number of ions is provided homogeneously to the system, they fast occupy the alginate molecules, forming the basic structures of the alginate-Ca^{2+} mixture in less time. As Nunamaker *et al.*[33] have reported, the required gelation time decreases when more cations participate in the process.

In addition, the evolution of the logarithmic curve of alginate-Ca^{2+} gelation in longer time scales is the slowest in 37.5 mM $CaCl_2$ compared with the higher concentrations since C_2 has the lowest value at this condition. The lower availability of cations leads to fewer multimers diffusing at a slow rate in the system. In 75 mM and 150 mM $CaCl_2$, the slow stage presents higher C_2 values due to the increased number of alginate-Ca^{2+} structures in the slow process. Besides, these values are similar since during the last 7 h of time sweeps, G^* increases in parallel, that is, at a similar rate, for the high $CaCl_2$ concentration (182 and 184 Pa at 75 and 150 mM $CaCl_2$, respectively). The almost identical evolution of G^* in these two different concentrations may be attributed to the possible formation of the same basic multimer structures at high concentrations of $CaCl_2$. Therefore, in the total available volume of 1.8 mL alginate-Ca^{2+} mixture, an equilibrium in multimers is reached. However, the number of monomers occupied by the Ca^{2+} ions is increased in the presence of 150 mM $CaCl_2$ in alginate, and for this reason, the absolute values of G^* are higher compared with those at 75 mM.

Remarkably, these conclusions are supported by the temporal evolution of tan(δ) of the present kinetics presented in Figure S7 (Section S7), where an overlap of 75 and 150 mM is observed, while the 37.5 mM $CaCl_2$ curve deviates. Tan(δ) indicates the viscous to elastic contribution in the structure, irrespectively of the absolute magnitude of moduli. To a great extent, it is the fingerprint of the basic structural unit via the corresponding relaxation time. Therefore, the observed coincidence indicates that the basic structural units are almost the same for the higher concentrations of this study. Finally, as Table 1 shows, the values of t_2 for all the available concentrations of cross-linker reactant are similar since the slow process starts from the beginning of the reaction, that is, the first multimer structures are created.

3.3 | Intermittent versus continuous oscillatory flow

The response of G^* discussed in the previous sections of the study, along with the functional characteristics of the configuration, show that the setup approximates the operation of an isothermal batch reactor. In this kind of reactor, which operates in constant volumes of a closed system, the only independent variable is the time. In addition, the reactants are placed at the beginning of the experiment, and the product is obtained at the end. The continuous oscillatory movement of the parallel-plate geometry assists the local mixing of the reactants.[34–36]

Here, we investigate how continuous oscillation assists the gelation. To achieve this, along with usual time sweeps of continuous oscillation, we propose recording the gelation evolution in an interval fashion, that is, as an intermittent operation, in selected reaction conditions. For this reason, the reaction occurs almost undisturbed by external forces, and the mechanical properties of the creating gelling structures are recorded by the oscillation of the parallel-plate geometry only every 20 min and for a short period of time. Figure 6a,b compares continuous and intermittent oscillatory time sweeps for the cross-linking gelation under a constant alginate concentration and two different concentrations of $CaCl_2$. In Figure 6a, it is observed that in the intermittent oscillatory flow of 1 wt.% alginate reacting with 37.5 mM $CaCl_2$ G^* reaches only 14 Pa at 1 h of gelation, and then it evolves at a slow rate to reach 45 Pa at 16 h of the reaction. This relatively low increase in G^* (approx. 30 Pa in 15 h) for prolonged time is reflected as an almost parallel evolution of mechanical properties as a function of time. By contrast, when continuous oscillation is applied to the alginate-Ca^{2+} system during a typical time sweep, G^* evolves fast in the first 5 h of the process, with the exponential growth discussed in the previous section. At larger times, the rate of increase is lower, and G^*

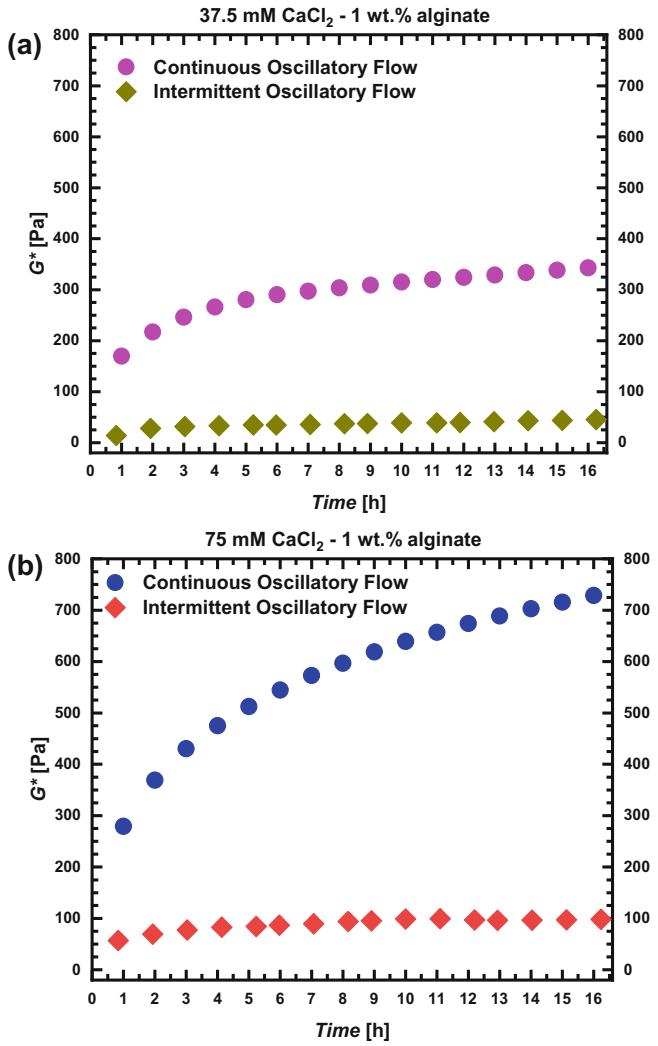

FIGURE 6 Long-term oscillatory time sweep experiments of 1.2 mL 1 wt.% alginate injected by 0.6 mL (a) 37.5 mM and (b) 75 mM $CaCl_2$. The circles represent the kinetics under a continuous oscillatory time sweep on the alginate-Ca^{2+} reaction, while the rhombuses show the intermittent time sweep. (8 microholes setup). [Color figure can be viewed at wileyonlinelibrary.com]

reaches 340 Pa at 16 h. A similar response is observed at the continuous and intermittent oscillatory time sweeps of 1 wt.% alginate reacting with 75 mM $CaCl_2$, as Figure 6b shows. Their difference is approx. 630 Pa at 16 h of the measurement. Therefore, the two different procedures of development affect the structure of the gels and, consequently, the response of G^*, even though the bulk properties of the reaction are the same (i.e., volume and concentration of reactants).

The difference in the scanning mode of oscillation affects the gelation process by influencing the diffusion of the cross-linking solution. The alginate-Ca^{2+} gelation is based on the "egg-box" model extensively studied throughout the years.[37,38] In the continuous time sweep experiment, the oscillatory movement of the upper plate is continuously evolved in the reaction. It enhances the distribution of the cations in the alginate chains and, consequently, the formation of more intermolecular and intramolecular junctions. In this way, the number of "egg-box" structures is increased, and the propagation of multimers is assisted. As a result, the oscillatory flow accelerates the reaction and supports the formation of extended homogeneous robust gels. By contrast, in the intermittent time sweeps, the alginate-Ca^{2+} structures remain mainly at rest for 16 h. The free ions and the alginate chains, which are not occupied by cations or not crosslinked with the remaining biopolymer chains, diffuse almost undisturbed to the system. An initial gelling structure has been formed with the instant injection of cross-linker to biopolymer and further developed during the process. This works as an obstacle for the distribution of cations through the macromolecular structure and, consequently, for the mass conversion of the reactants to products without the application of continuous oscillation. For this reason, the gelling structures obtained with the intermittent time sweeps are less stiff compared with those created by the continuous ones. He *et al.*[39] visualized for the first time the "egg-box" junctions utilizing electron microscopy. It was observed that alginate-Ca^{2+} continues to grow by forming interclusters of multimers with a width of approximately 10 nm.[39] The higher G* values obtained in the continuous oscillatory flow reveal that the constant application of external forces on alginate-Ca^{2+} gelation can increase the rate of intercluster production and, consequently, results in an enhanced propagation of multimers compared with the intermittent oscillation for the same concentration of reactants.

The claim that the oscillation type determines the gelation process can be more robust. The absolute maximum value of displacement at the shear position of 75% is 22.5 μm (see discussion in Section 3.1). This is considerably small compared with the diameter of microholes, that is, 320 μm, but it is constantly applied to alginate-Ca^{2+} system during continuous oscillatory movement and enhances the formation of the gel front. By contrast, in the intermittent flow, the external forces on the system are diminished and there is a slow development of gel front. The recent investigation by Kim *et al.*[40] on the swelling of polymer gels show that the diffusion coefficient of the polymer network includes the polymer−solvent mixing contribution due to the swelling and an elastic contribution from the polymer network. This controls the macromolecule diffusion coefficient in a swelling polymer network.[40] Similarly, the continuous oscillatory displacement enhances the diffusion by adding an extra mixing contribution to the growth of the gel. However, in the intermittent flow, there is no such an

extra mixing effect on the diffusion process, and the developed structures diffuse slower inside the alginate-Ca^{2+} system.

4 | CONCLUSIONS

The present research investigates how the initial distribution of Ca^{2+} into alginate solutions affects the progress of gelation by utilizing different configurations of a customized rheometric setup. In all cases, we can control the bulk properties to be the same, namely the volume and concentration of reactants. In situ short-term oscillatory time sweeps show for the first time that the progress of gel front critically depends on the number and position of micro-holes, which is evaluated through the temporal progress of G^*. Specifically, the mechanical properties of the gels increase systematically in the presence of higher number of micro-holes and, consequently, more structured gels are faster obtained. The arrangement of the 12 micro-holes lead to the formation of different local gel fronts influencing the response of G^* of the developed structures. Between the available configurations of the setup, the 8 micro-holes design is proposed for the cross-linking gelation, where the cations are initially homogeneously distributed to the biopolymer solution, and the injection can easily be handled for manual operation in contrast to the 12 micro-holes configurations.

Moreover, the long-term response of alginate-Ca^{2+} gelation is recorded and evaluated. A two-kernel mathematical expression (Equation 1), consisting of an exponential and a logarithmic term, is proposed to describe the fast initial formation of "egg-box" structures and the slow alginate-Ca^{2+} gelation in longer time scales, respectively. Equation (1), with the constants found for each trial, shows good agreement with the experimental data and can predict the gelation process. The time dependency of the two processes is based on the concentration of cations that participate in the reaction and the structural units formed during the gelation. The continuous and the intermittent time sweeps on alginate-Ca^{2+} gelation shows that the type of oscillation drastically affects the reaction. It is proved that the gelation is faster when it occurs under the continuous oscillation of the parallel-plate geometry, since the diffusion of cations is enhanced compared with the intermittent time sweeps conceptualized to diminish the impact of external forces on the reaction.

As future research, the present in situ rheological investigation can been also applicable with further methods, such as dark field microscopy and small angle X-ray scattering, to correlate the currently discussed mechanical properties to the structural characteristics of the developed structures. The gel front can be visualized and structurally characterized when different local injections of $CaCl_2$ into alginate are achieved, or when external forces, such as a light mixing, accelerate the reaction. In addition, various ionically cross-linking systems, for example, pectin or chitosan, can be studied with the different configurations of the rheometric custom-made setup to enhance the perception of their gelation process.

AUTHOR CONTRIBUTIONS

Ioanna N. Besiri: Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (lead); methodology (equal); validation (equal); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Thomas B. Goudoulas:** Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); supervision (equal); validation (equal); writing – original draft (equal); writing – review and editing (equal). **Ehsan Fattahi:** Conceptualization (equal); formal analysis (equal); methodology (equal); supervision (equal); validation (equal); writing – review and editing (equal). **Thomas Becker:** Supervision (equal); validation (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts to declare.

DATA AVAILABILITY STATEMENT

The data supporting the findings of the current research are available from the corresponding author upon reasonable request.

ORCID

Ioanna N. Besiri https://orcid.org/0000-0002-3598-8843
Thomas B. Goudoulas https://orcid.org/0000-0002-7276-2392
Ehsan Fattahi https://orcid.org/0000-0002-9189-493X
Thomas Becker https://orcid.org/0000-0001-6842-8300

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