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# Gelation kinetics of alginate chains through covalent bonds

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ABSTRACT

Gelation kinetics of polymer chains through covalent bonds depends on many variables and affects many applications. Here we study the gelation kinetics of alginate chains through crosslinkers adipic acid dihydrazide (AAD), with coupling reagents 1-ethyl-3-(3-dimethylaminopropyl) (EDC) and N-hydroxysuccinimide (NHS). We use the rate equation of the reaction to calculate the extent of reaction, and use the Flory–Stockmayer theory to calculate the gelation time. The model predicts the gelation time as a function of the concentration of alginate, crosslinkers, and coupling reagents, as well as the functionality of an alginate chain and the rate constant of the reaction. Given an aqueous solution, we conduct rheological tests to measure the storage and loss moduli as functions of time, and determine the gelation time by the time when the storage modulus equals the loss modulus. The gelation times so determined for solutions of various compositions agree well with those predicted by the model. This combination of the model and experiments provide a practical approach to the design of gelation kinetics for applications.

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Alginate is a type of polysaccharide abundant in nature. It has been widely used in a range of applications in the food industry [1,2], pharmaceutics [3,4], cosmetics [5], textile printing [6], and animal feed [7]. In particular, due to its biocompatibility, low toxicity, and ease of gelation [4], alginate hydrogels have been applied in many emerging biomedical applications, including drug delivery [8], wound dressing [9], and tissue regeneration [10].

Alginate chains can form hydrogels through either ionic or covalent crosslinks. Ionic crosslinks use multivalent cations (e.g., $Ca^{2+}$, $Al^{3+}$) in the aqueous solution to bind COOH on the alginate chains [4,11,12]. However, because the ionic crosslinking is too rapid and difficult to control, these hydrogels usually have poor mechanical properties [13]. Furthermore, the ionic crosslinks dissociate readily under physiological conditions as the multivalent cations exchange with single-valent cations (e.g., $Na^{+}$ and $K^{+}$). For example, calcium-crosslinked alginate hydrogels lose more than 60% of their initial mechanical strength within 15 h of exposure to physiological fluids [14]. Covalent crosslinks can circumvent the abovementioned issues, owing to the slower reaction and stable covalent bonds. Polymers and difunctional molecules such as poly(ethylene glycol)-diamines [15,16], poly(acrylamide-co-hydrazide) [17], methyl ester L-lysine [16], and adipic acid dihydrazide [16–19] have been used to covalently crosslink alginate chains. In addition, alginate chains can be chemically modified with methacrylate, and covalently crosslinked under an argon laser or UV irradiation [20–22].

Alginate hydrogels covalently crosslinked by AAD have been broadly used in biomedical applications due to the simple reaction, mild reaction condition, and controllable mechanical properties [16,19]. Gelation kinetics of alginate chains is often tuned to meet specific applications. For example, gelation should be fast when used as hemostatic dressings [23], but slow when used for cell encapsulation [24]. Recently, this crosslinking system has been employed in topological adhesion between two preexisting hydrogels [25]. In this application, an aqueous solution of alginate chains, crosslinkers AAD, and coupling reagents EDC and NHS is first placed in between two hydrogels, and then a covalent alginate network forms *in situ*, in topological entanglement with the polymer networks of the two preexisting hydrogels. Strong adhesion forms even when the alginate network entangles with each preexisting hydrogel network by a single polymer mesh size, so that diffusion of the alginate chains into the preexisting hydrogel networks takes negligible time, and the gelation time determines the time needed to form strong adhesion [26].

Here, we study the gelation kinetics of alginate chains crosslinked by AAD, in the presence of the coupling reagents EDC and NHS. We use the rate equation of the reaction to calculate the extent of reaction, and use the Flory–Stockmayer theory to calculate the gelation time. The model predicts the gelation time as a function of the concentration of the ingredients (alginate, AAD, EDC), the functionality of an alginate chain, and the rate constant of the reaction. We conduct rheological tests to measure

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the storage and loss moduli as functions of time, and determine the gelation time by the time when the storage modulus equals the loss modulus. The measured gelation times of solutions of various compositions agree well with those predicted by the model.

This approach to study gelation kinetics has been used for other polymer systems. Gelation kinetics of polymers has been studied for polymerization in a homogeneous solution [27]. The gelation time is calculated using a combination of the rate equation of the reaction and the Flory–Stockmayer gelation theory [28,29], which has been applied for tetra-poly(ethylene glycol) hydrogels [30], peptides crosslinked PEG hydrogels [31], and pentaerythritol propoxylate tris(3-(furfurylthiol)propionate) gels [32].

Forming a crosslink between two alginate chains through AAD, in the presence of EDC and NHS, involves three main reactions [33]. First, a COOH group on an alginate chain reacts with an EDC to form an O-acylisourea intermediate (Fig. 1a). The O-acylisourea intermediate is unstable in the aqueous solution and may have many side reactions [34,35]. Second, the O-acylisourea intermediate reacts with an NHS to create a stable NHS-ester intermediate and release a urea [36] (Fig. 1b). Third, the NHS-ester intermediates on two alginate chains react with an AAD to crosslink the alginate chains through two amide bonds (Fig. 1c). Such crosslinking process propagates among all alginate chains to form a three-dimensional alginate network. The first reaction is much slower than the second and third reactions [35,37,38]. The rate constant $k_1$ is on the order of $10^{-3}$ $M^{-1}$ $s^{-1}$ [37], $k_3$ is on the order of 10 $M^{-1}$ $s^{-1}$ [30], and $k_2$ is comparable to $k_3$ [39]. Therefore, so long as the NHS is in a sufficient amount and AAD is not scarce to inhibit gelation, the time for the second and the third reactions can be neglected, and the first reaction is the rate-limiting step that determines the overall rate of gelation.

The reaction between COOH and EDC is first-order in both COOH and EDC [37,40]. We use the concentration [COOH] as a function of time $t$ to measure the extent of the reaction. In a homogeneous solution, the rate of reaction is proportional to the concentrations of COOH and EDC:

$$\frac{d[\mathrm{COOH}]}{dt} = -k_1 [\mathrm{COOH}][\mathrm{EDC}] \tag{1}$$

where $k_1$ is the reaction constant. Let $[\mathrm{COOH}]_0$ and $[\mathrm{EDC}]_0$ be the initial concentrations in the solution. The reaction consumes COOH and EDC in equal numbers; let the number of each species that has reacted be $m = [\mathrm{COOH}]_0 - [\mathrm{COOH}] = [\mathrm{EDC}]_0 - [\mathrm{EDC}]$. Eq. (1) becomes that

$$\frac{dm}{dt} = k_1 ([\mathrm{COOH}]_0 - m)([\mathrm{EDC}]_0 - m) \tag{2}$$

Integrating equation (2), we obtain that

$$k_1 t = \frac{1}{[\mathrm{COOH}]_0 - [\mathrm{EDC}]_0} \ln \frac{([\mathrm{COOH}]_0 - m)[\mathrm{EDC}]_0}{([\mathrm{EDC}]_0 - m)[\mathrm{COOH}]_0} \tag{3}$$

Given the initial concentrations $[\mathrm{COOH}]_0$ and $[\mathrm{EDC}]_0$, Eq. (3) determines the extent of reaction $m$ at time $t$. When $[\mathrm{EDC}]_0 = 0$ (no coupling reagent) or $[\mathrm{COOH}]_0 = 0$ (no alginate), the above solution diverges, as the reaction cannot proceed without them. When $[\mathrm{COOH}]_0 = [\mathrm{EDC}]_0$, Eq. (3) is inapplicable, but we can still integrate equation (2) and obtain that

$$k_1 t = \frac{m}{([\mathrm{EDC}]_0 - m)[\mathrm{EDC}]_0} \tag{4}$$

This equation can also be obtained from Eq. (3) in the limit as $[\mathrm{COOH}]_0$ approaches $[\mathrm{EDC}]_0$.

We next use the Flory–Stockmayer theory to calculate the gelation time [28,29]. The theory makes the following assumptions: (i) there are no intramolecular reactions on the same polymer chain, (ii) functional groups that participate in reaction have equal reactivity, (iii) there are no chain-entanglements that may serve as apparent crosslinks. Gelation is defined as the extent of reaction that leads to an infinite network. We assume that each alginate chain has the same number $n$ of COOH groups. Each AAD molecule has two amines, so that $[\mathrm{NH_2}] = 2[\mathrm{AAD}]$. We further assume that AAD molecules crosslink different alginate chains, but do not intralink the same alginate chain (Fig. 2). This figure only shows one crosslink between two alginate chains. Other possibilities include multiple crosslinks between two alginate chains and an AAD molecule grafts on one alginate chain without connecting to another. Because the critical condition of gelation is the formation of an infinite network, all polymer chains are required to be crosslinked at the gelation point. To ensure gelation, an alginate chain that is connected with a previous alginate chain must at least make one crosslink to the next alginate chain, so that every alginate chain at least has two crosslinks. Gelation begins with an alginate chain reacting with one amine on an AAD molecule. The other amine on the AAD molecule then reacts with another alginate chain to create a crosslink. Subsequently, successive crosslinks are made among more alginate chains until all alginate chains are crosslinked. At this point, the alginate solution has become an alginate network.

The number fraction of reacted COOH groups is $m/[\mathrm{COOH}]_0$, and the number fraction of reacted $\mathrm{NH_2}$ groups is $m/[\mathrm{NH_2}]_0$. The probability of forming a crosslink is the product of the two number fractions:

$$\alpha = \frac{m^2}{[\mathrm{COOH}]_0 [\mathrm{NH_2}]_0} \tag{5}$$

After each connection, because an alginate chain has one COOH group reacted from a prior connection, there are $n - 1$ opportunities for this alginate chain to make the next connection (there are $n-1$ COOH groups that can be used to react with AAD). To ensure at least one connection to the next alginate chain, at least one COOH group among the $n-1$ COOH groups should react with an AAD, therefore, $\alpha \geq 1/(n-1)$ [29]. The critical condition for gelation is when every previously connected alginate chain has exactly one connection to the next alginate chain, namely, $\alpha(n-1) = 1$. This condition, along with Eq. (5), gives the extent of reaction at gelation, which is calculated as

$$m_g = \sqrt{\frac{[\mathrm{COOH}]_0 [\mathrm{NH_2}]_0}{n-1}} \tag{6}$$

Substituting Eq. (6) into Eqs. (3) and (4), we obtain the gelation time of alginate, $t_g$.

The gelation time relates $k_1$, $[\mathrm{COOH}]_0$, $[\mathrm{NH_2}]_0$, $[\mathrm{EDC}]_0$, and $n$. The rate constant $k_1$ is fixed for a specific chemical reaction (Fig. 1a). The gelation time decreases with the increase of COOH (Fig. 3a), because more COOH provides more reaction sites for gelation and a higher opportunity to form an alginate network. In experiments, this can be done by increasing either the molecular weight or the concentration of alginate. However, if AAD is abundant compared to alginate, AAD may occupy all reaction sites on an alginate chain and thus prevent the connection of alginate to form a network. As a result, the gelation time increases with $\mathrm{NH_2}$concentration (Fig. 3b). Moreover, higher EDC concentration promotes gelation (Fig. 3c), as more EDC increases the rate of chemical reaction between COOH and $\mathrm{NH_2}$. The effect of EDC concentration on the carbodiimide reaction kinetics has also been studied and observed in other systems [41,42]. The functionality of alginate affects the chance of branching. Smaller functionality indicates fewer reaction sites on the alginate chain and thus a lesser chance to create a connection. Gelation becomes delayed. We show that the gelation time $k_1 t_g$ is 0.246 $M^{-1}$ at a functionality of 10,000 and significantly increases to 19.7 $M^{-1}$ at a

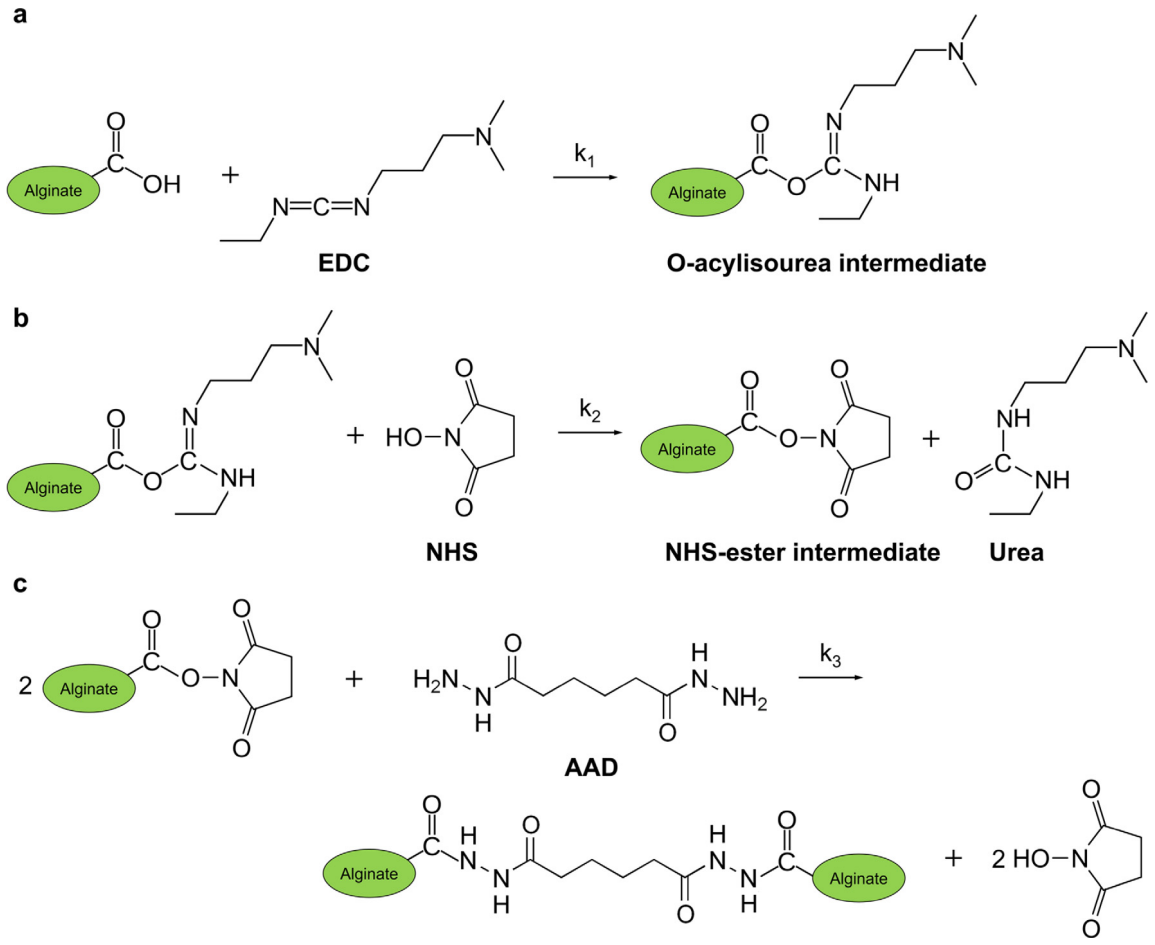


**Fig. 1.** The formation of a covalent crosslink between two alginate chains. a, A COOH group on an alginate chain reacts with an EDC to form an O-acylisourea intermediate. b, An O-acylisourea intermediate reacts with an NHS to form an NHS-ester intermediate and an EDC-derived urea. c, An AAD reacts with two NHS-ester intermediates on two alginate chains to form amide bonds, thus creating a crosslink between the two alginate chains.

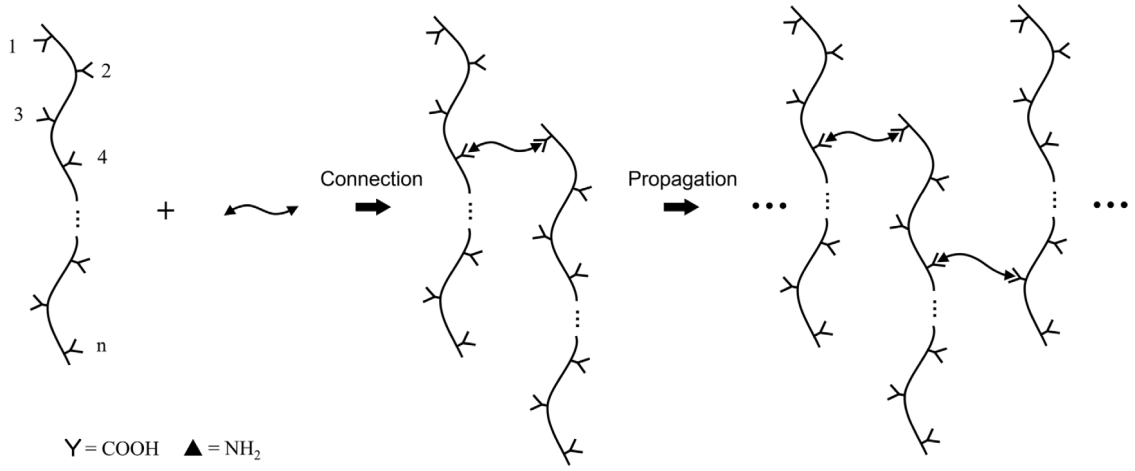


**Fig. 2.** Crosslink alginate chains into a network. An alginate chain of $n$ functionalities crosslink with another alginate chain via a bifunctional AAD molecules. Such crosslinking propagates until all alginate chains are crosslinked. Here one crosslink between two alginate chains is shown, multiple crosslinks between two alginate chains are also possible.

functionality of 10 (Fig. 3d). In the limiting case, when alginate has a functionality of 2, gelation is impossible.

Next we test the model by rheological measurements. Aqueous solutions are mixed from the following ingredients: sodium alginate (Manugel GMB, molecular weight = 170–240 kDa, FMC Biopolymer, Philadelphia, PA), AAD (Sigma Aldrich, A0638), EDC (Sigma Aldrich, E1769), and NHS (Sigma Aldrich, 130672). The mix time is typically several seconds, which is not included in the total gelation time. Oscillatory tests are carried out at room temperature under 2% oscillation strain at angular frequency of 1 rad/s using a rheometer (TA Instruments Discovery HR-3 Hybrid Rheometer) with a cone and plate geometry. The storage and loss

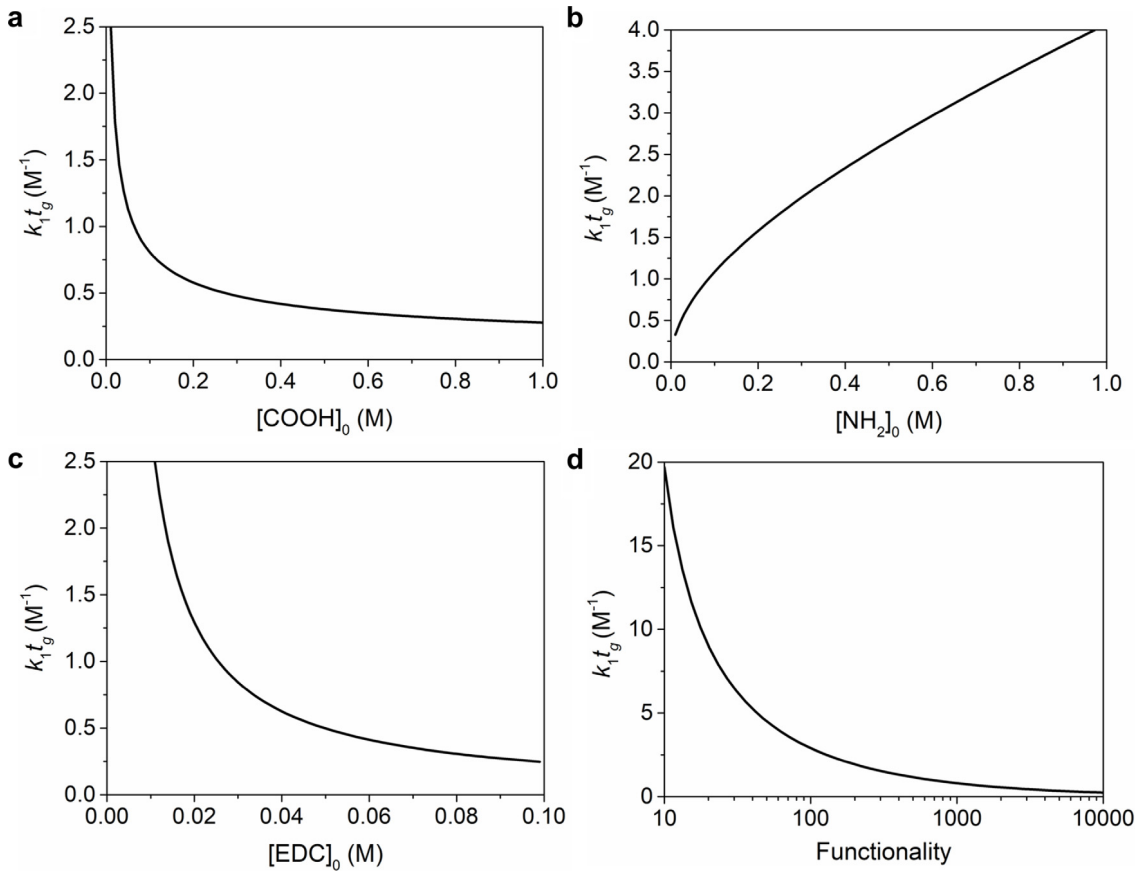


**Fig. 3.** The predicted gelation time as functions of various parameters. a, $[COOH]_0$, with $[NH_2]_0 = 0.0574$ M, $[EDC]_0 = 0.0313$ M, and $n = 1000$. b, $[NH_2]_0$, with $[COOH]_0 = 0.1$ M, $[EDC]_0 = 0.0313$ M, and $n = 1000$. c, $[EDC]_0$, with $[COOH]_0 = 0.1$ M, $[NH_2]_0 = 0.0574$ M, and $n = 1000$. d, Functionality of alginate, with $[COOH]_0 = 0.1$ M, $[NH_2]_0 = 0.0574$ M, and $[EDC]_0 = 0.0313$ M.

moduli are recorded as functions of time (Fig. 4a). In all tests, to ensure the second and the third reactions are fast compared to the first reaction, the mass of NHS is kept the same as the mass of EDC, and the mass of AAD is varied in a range of sufficient amount for gelation. Following a common practice, we define the gelation time when the storage and loss moduli coincide. For example, for an alginate solution prepared by dissolving 0.06 g alginate, 0.0125 g AAD, 0.03 g EDC and 0.03 g NHS in 3 ml DI water, the gelation time is measured to be about 1112 s (Fig. 4b). To measure $k_1$, we prepare 3 ml alginate solution with $n = 1000$ [43] ($n$ has a distribution of 787 – 1111, here we choose $n = 1000$ as a representative value), $[COOH]_0 = 0.1$ M (alginate = 0.06 g), $[NH_2]_0 = 0.0574$ M (AAD = 0.015 g), and varied $[EDC]_0$ of 0.093 M (0.054 g), 0.0626 M (0.036 g), and 0.0469 M (0.027 g). We measure the gelation time as a function of EDC and fit the data using our model with $k_1$ as the fitting parameter. The fitted curve agrees well with the experimental data when $k_1 \sim 0.0006$ $M^{-1}$ $s^{-1}$(Fig. 4c). We then use this value of $k_1$ to predict the gelation time as a function of $NH_2$ and COOH concentration. We also use the rheometer to experimentally measure the gelation time and show that our theoretical prediction agrees well with experiment data (Fig. 4d, e).

This value of the reaction constant, $k_1 = 0.0006$ $M^{-1}$ $s^{-1}$, is somewhat smaller than that reported in the literature, which is on the order of 0.001 $M^{-1}$ $s^{-1}$ [37,39]. The discrepancy may be attributed to the fundamental difference between gelation kinetics and reaction kinetics. The gelation kinetics involves not only the local reaction kinetics between two functional groups but also the infinite connection of alginate chains into a network. Since each connection requires one alginate chain to find another alginate chain, it takes a longer time than that of local chemical reaction facilitated by the collision of molecules. Furthermore, the formation of loops in the alginate network may delay gelation, and thus decrease the measured rate constant. For example, the connection of an alginate chain to itself creates a loop, which does not contribute to the gelation of the network.

The model of gelation can guide the design of the gelation kinetics of alginate in practical applications. In the design of alginate hemostatic dressing, alginate precursor should have high concentrations of alginate and EDC (within biological tolerance) and high molecular weight to ensure fast gelation. By contrast, in the design of alginate cell culture, lower concentration of EDC is preferred to ensure slow and uniform gelation. In addition, because the crosslink density determines the mechanical properties of a hydrogel, one can tune the AAD concentration in alginate precursor to correlate the gelation time with the elastic modulus, stretchability, strength, and fracture energy of alginate hydrogels. Furthermore, the model of gelation provides a foundation to study the alginate enabled topological adhesion, where the gelation kinetics and the diffusion kinetics are coupled.

In summary, we adopt rate equations of chemical reaction and Flory–Stockmayer theory to study the gelation kinetics of AAD crosslinked alginate. The model predicts the gelation time as a function of experimental variables. Rheological tests are carried out to determine the rate constant. By using the measured rate constant, the gelation times predicted by the model agree well with those measured by the rheological tests. The combined model and experiments provide an approach to the design of gelation kinetics in practical applications.

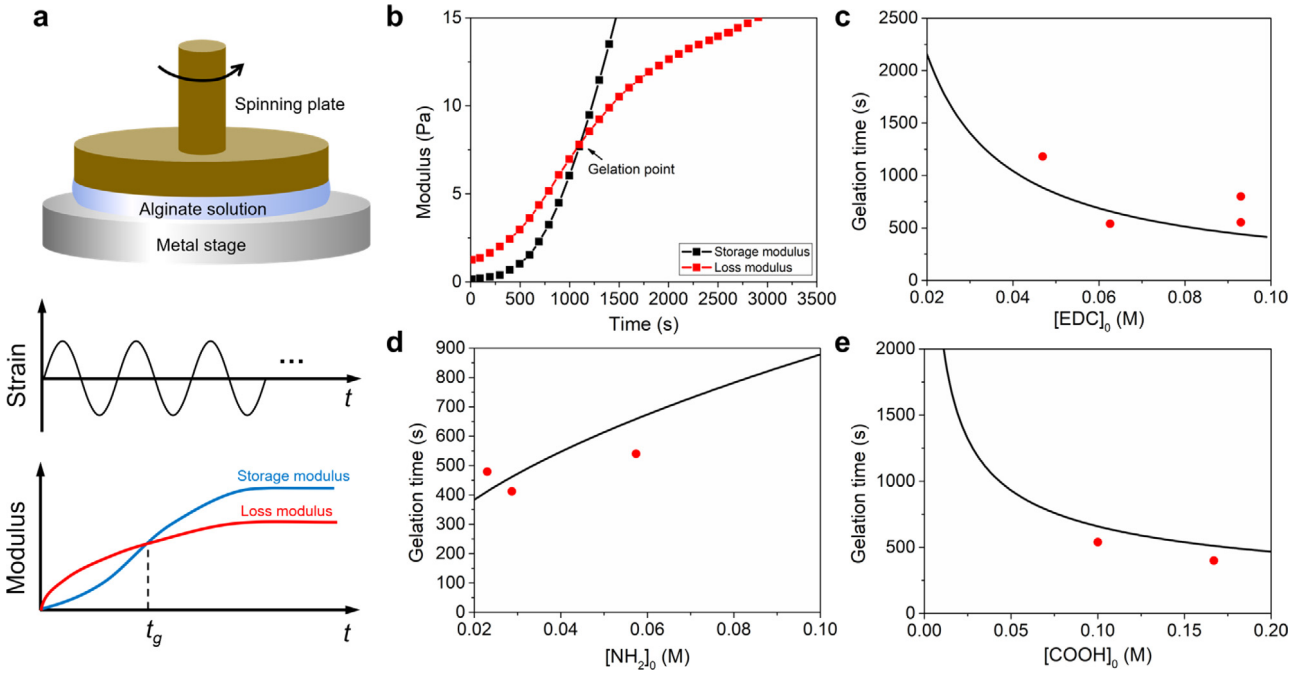


**Fig. 4.** Comparing rheological data with the model. a, Experiment setup of rheological tests. Oscillatory strain is applied, and storage and loss moduli are measured as functions of time. The gelation time is defined when the storage and loss moduli equal. b, An example of the storage and loss moduli as functions of time. This example shows a gelation time of 1112 s. c, Gelation time is measured as a function of the EDC concentration. Rate constant $k_1$ is determined by fitting the experimental data to the model of gelation, giving $k_1 = 0.0006\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$. The prediction by the model agrees well with experiment data with varied concentration of d, $[NH_2]_0$ and e, $[COOH]_0$, using the same value of $k_1$. In c, d, e, the black curves represent theoretical curves and red dots are experimental data.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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