Ionic crosslinking strategies for poly(acrylamide) /alginate hybrid hydrogels

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Abstract

Ionically crosslinked alginate hydrogels have been widely studied for application in tissue engineering. Crosslinking of alginates is routinely done by ionic interaction of the carboxyl groups with multivalent anions such as Ca^{2+} and Fe^{3+}. In more complex hydrogel designs, such as interpenetrating network hydrogels and double network hydrogels, alginates have been combined with other synthetic polymers to benefit from the advantages of both worlds. The effect of different ions on the properties of single network alginate hydrogels and double network poly(acrylamide)/alginate hydrogels have been studied extensively. However, past reports offer less in terms of comparing different crosslinking methods or different salt precursors. Here, we explore the influence of ionic crosslinking strategies used for crosslinking alginate in double network P(AAm)/Alginate hybrid hydrogels. A comparison is made between different calcium-based crosslinking methods, including a two-step method using $CaCl_2$, a one-step method using $CaSO_4$, and a one-step method using the combination of $CaCO_3$ with glucono-δ-lactone (GDL). Furthermore, the effect of different metal ions is investigated, including Fe^{3+}, Al^{3+}, Ba^{2+}, Sr^{2+}, and Ca^{2+}. Mechanical properties of the resulting gels are analyzed by tensile and compression testing, revealing a strong correlation between preparation protocol, counter-ion identity, and mechanical response.

Introduction to alginate hydrogels

Alginate, a biopolymer derived from algae, is one of the most commonly used materials in hydrogels [1, 2]. It is an anionic polysaccharide made up of two building blocks: β-D-mannuronate (M) and α-L-guluronate (G). Blocks of M-, G-, and mixed M/G-domains are statistically distributed along the alginate polymer chains (Figure 1a, b & c) [3]. Both building blocks contain carboxylic acid residues that are capable of binding with positively charged counterions. Alginate is often commercially available as a salt, sodium alginate, in which the carboxylic acid groups are deprotonated and countered with Na^+ ions. When the carboxylate groups interact with multivalent cations, links between different polymer chains are readily formed, resulting in an ionically crosslinked network at sufficiently high ion concentrations. Calcium crosslinked alginate is by far the most used ionically crosslinked hydrogel and has been studied for applications in many different areas [4] Crosslinking of the G units in the alginate polymer backbone by calcium ions has been explained in literature by the so-called 'egg-box model' [5-7]. Due to the difference in conformation between M-blocks and G-blocks, the G-blocks participate in ionic crosslinking more effectively, forming stronger connections. The robustness of the network and corresponding mechanical properties of alginate gels are therefore dependent on the G-block/M-block ratio and the length of the G-blocks. Alginates with higher G-block content can form denser networks due to higher degree of ionic crosslinking, leading to higher mechanical strength [8]. Different bacterial sources of alginate naturally possess different G/M ratios in the polymer backbone. This is not often reported by commercial suppliers but can be measured by NMR techniques [9, 10].

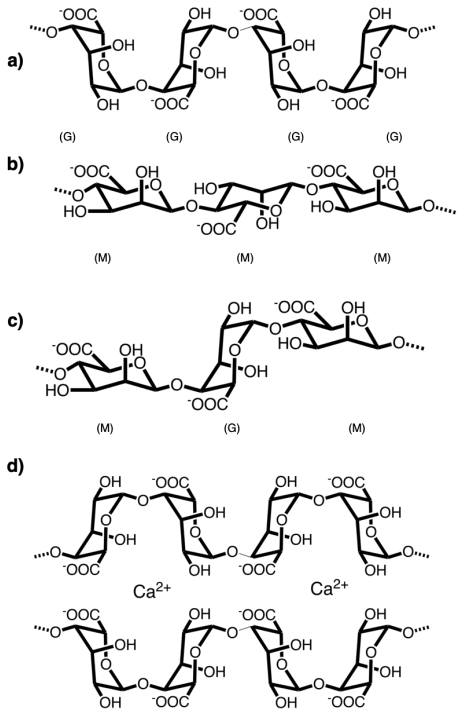

Figure 1: Chemical structure of alginate and ionic crosslinking by calcium ions. a) G-block consisting of repeating guluronate units. b) M-block consisting of repeating mannuronate units. c) G/M-block consisting of a mixture of guluronate and mannuronate units. d) Crosslinking of G-block in the alginate backbone by bivalent calcium ions, commonly referred to as the 'egg-box' model.

The type of ionic crosslinking agent has a drastic influence on the mechanical properties of alginate gels. Several strategies for crosslinking alginate have been demonstrated in literature, including using cations such as calcium, iron, barium, zinc, aluminium, and strontium [11-15]. Whereas bivalent alkaline earth cations (Ca^{2+}, Mg^{2+} and Sr^{2+}) form ionic bonds exclusively with G-units in alginate, bivalent transition metal ions (Mn^{2+}; Co^{2+}, Cu^{2+} and Zn^{2+}) and trivalent metal cations (Fe^{3+}, Al^{3+}, Cr^{3+}, Sc^{3+}, Ga^{3+}, and La^{3+}) will complex with both M- and G-blocks [16, 17].

Crosslinking of single network (SN) alginate hydrogels is typically achieved using solutions of highly water-soluble salts. SN alginate hydrogels are most often made in the form of beads by drop-wise addition of a sodium-alginate solution into a salt solution (extrusion-dripping method with highly soluble salts like $CaCl_2$, $FeCl_3$, $AlCl_3$,…) [18]. Alginate chains will crosslink within seconds of exposure to the ions and almost instantaneously form a gel. This rapid gelation process leads to inhomogeneous crosslinking density, with the interaction of the ions with alginate being faster at the surface/interface compared to the inside of the gel, leading to a network structure that is not uniform in terms of either molecular makeup or physical properties.

To obtain objects with more structural complexity than beads, the pre-gel solution should remain fluid for a certain amount of time. Fluidity allows molding, injection, or use in additive manufacturing to make complex 3D hydrogel structures [19]. Therefore, the high crosslinking rate of SN alginate hydrogels results in a limited degree of processability, and determination of their mechanical properties can be difficult as this requires the gels to be made in specific dimensions and shapes (e.g., dogbone shapes for tensile testing and disks for compression testing). While single network, ionically crosslinked alginate hydrogels have found many applications in different areas, their relatively weak mechanical properties and limited cell adhesion require additional modification of the alginate or combination with other non-ionic polymers to obtain a more favorable environment for cells [20]. Functionalization of

alginate with RGD peptides or growth factors has been shown to improve cell viability [2, 21-23].

Alginate has been used in combination with other biopolymers or synthetic polymers to form interpenetrating network hydrogels and double network hydrogels [24-27]. Combining alginate with other polymer networks not only benefits biocompatibility but can also lead to enhanced mechanical integrity. For example, the combination of Ca-Alginate and covalently crosslinked poly(acrylamide) gives rise to a remarkably tough double network hydrogel [24]. The combination of alginate with a covalently crosslinked acrylate network offers opportunities to use different crosslinking strategies for alginate. The generalized preparation procedure for PAAm/Ca-alginate DN hydrogels is illustrated in Figure 2.

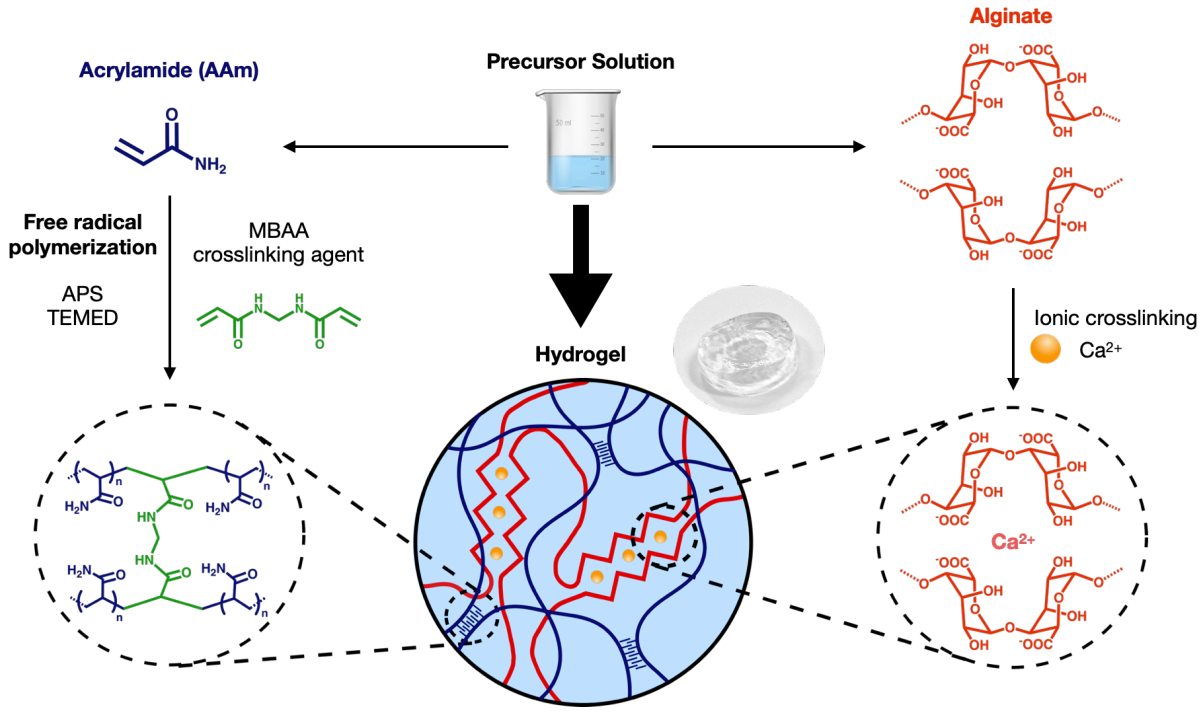

Figure 2: General overview of preparation procedure for P(AAm)/Alginate double network hydrogels.

The precursor solution contains the building blocks for both networks: acrylamide and MBAA for the poly(acrylamide) (PAAm) network and sodium alginate for the other network. The ionic crosslinking agent to form the alginate network can, however, be introduced at a later stage of

the procedure (Figure 3). For the alginate/P(AAm) system, a stable hydrogel can first be formed by polymerization of acrylamide and a covalent crosslinker from a precursor solution containing alginate. The resulting hydrogel has alginate chains entangled within its structure that subsequently can be crosslinked by immersing the hydrogel in a $CaCl_2$ solution, whereupon ion exchange creates the orthogonal network (Figure 3a). With this method, the crosslinking density is difficult to control, and the issue of inhomogeneous crosslinking arises as the surface of the hydrogel will be more densely crosslinked compared to the inside.

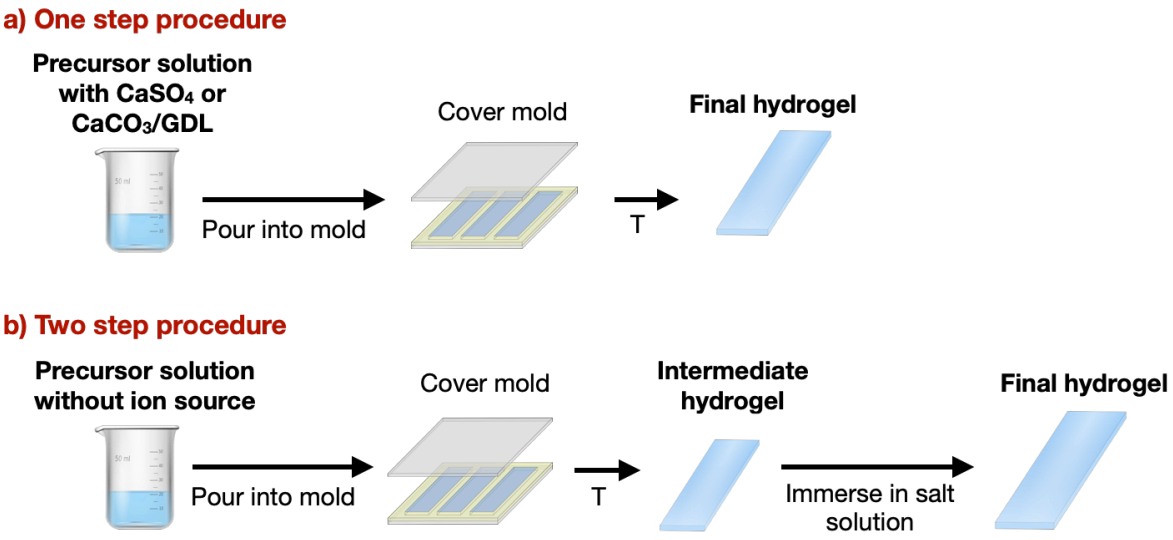

Figure 3: Overview of alginate crosslinking strategies. a) One-step procedure with ionic crosslinking agents present in the precursor solution. b) Two-step procedure with the introduction of the ionic crosslinking agents in a second step after formation of an intermediate hydrogel.

An alternative way to crosslink alginate enables the formation of gels with improved homogeneity. This can be done with the less soluble $CaSO_4$. The lower solubility of the salt results in delayed crosslinking and allows for the alginate/$CaSO_4$ solution to be stirred or otherwise manipulated prior to gelation, leading to more homogeneous crosslinking throughout the hydrogel. Because of the lower crosslinking rate, $CaSO_4$ can be added to the gel precursor solution, eliminating the additional immersing step (Figure 3b). However, the short time window between the addition of $CaSO_4$ and gelation of the alginate solution makes molding or

processing of the hydrogels quite challenging, making this method inconvenient in the sense that you are restricted in the processing window.

Compared with $CaCl_2$ and $CaSO_4$, $CaCO_3$ has very low solubility in water. Combining $CaCO_3$ with D-glucono-δ-lactone (GDL) and alginate creates a much slower gelation process that, as a consequence, generates more uniform hydrogels [11]. Depending on the calcium source and gelation conditions, different gelation rates can be achieved using this strategy [11]. Due to the limited solubility of $CaCO_3$ in water, a uniform distribution of $CaCO_3$ in an alginate solution can easily be obtained. The dissolution of $CaCO_3$ can be controlled by the addition of GDL to the solution, and the crosslinking of alginate can thereby be controlled. Similar to $CaSO_4$, a $CaCO_3$/alginate solution is prepared prior to gelation, leading to a more homogeneous network structure compared with a post-network crosslinking approach.

These calcium-based strategies have all been proven effective in the crosslinking of alginate, but due to the difference in homogeneity of the obtained gels, the physicochemical properties can vary. The effect of different ions on the properties of single network alginate hydrogels and double network poly(acrylamide)/alginate hydrogels have been studied extensively [28, 29]. However, these publications lack the comparison of different crosslinking methods such as a one-step $CaSO_4$ and $CaCO_3$/GDL method or a two-step $CaCl_2$ method. In this manuscript, a comparison is made between the different calcium-based crosslinking strategies in double network PAAm/Alginate hydrogels as well as an expanded comparison between different multivalent cation species. These materials are highly functional, where the carboxylate moieties adorning the alginate form strong associations with counter-ions of various metals. We manipulate these ionic interactions, exploiting the kinetics of ion release and counter-ion strength/size in order to fine tune the properties of hybrid hydrogels containing alginate. The final result is a highly tunable system, with several explicit handles for tuning the mechanical properties uncovered.

Results and discussion

Calcium-based crosslinking strategies for alginate hydrogels

$CaCl_2$ immersion (two step) procedure

Calcium chloride (anhydrous $CaCl_2$) is highly soluble (74.5g/100mL at 20°C) in water. When alginate is added to a $CaCl_2$ solution, crosslinking will occur nearly instantaneously, limiting the ability to mix prior to gelation to obtain a homogeneous solution. For P(AAm)/alginate gels, $CaCl_2$ cannot be added to the precursor solution as this would lead to instantaneous gelation, resulting in a network structure that is not uniform. Instead, a stable hydrogel is first formed by polymerization of the acrylamide network, and the alginate chains are crosslinked after gelation by immersion of the gel in a $CaCl_2$ solution (Figure 2b). The efficiency of the crosslinking is dependent on the diffusion rate of the ions into the gel. The crosslinking density depends on the concentration of the salt solution and the time of immersion, with higher concentrations and longer immersion times leading to higher crosslinking densities.

In practice, controlling the crosslink density is a difficult task. PAAm/alginate hydrogels were immersed for 60 minutes in $CaCl_2$ solutions of different concentrations (0.001M, 0.01M, 0.05M, 0.1M, and 0.3M). From 0.05M upwards, there is no significant differentiation in mechanical properties in the resulting hydrogels, suggesting all the higher salt concentrations lead to full saturation of the gels with Ca^{2+} ions (Figure 4a/b, Table S5). At lower salt concentrations, crosslinking is presumably less extensive, and the resulting gels swell significantly as a result of lower crosslink density (173.5% for 0.001M and 143.9% for 0.01M). At higher concentrations the effect of swelling is less pronounced as the gels are crosslinked faster and more densely, leading to more resistance to swelling. Similarly, when PAAm/alginate hydrogels are immersed in a 0.1M $CaCl_2$ solution, variations in mechanical properties can be observed between different immersion times (Figure 4c/d, Table S6). At this concentration, 1 minute soaking time is insufficient to saturate the hydrogel with Ca^{2+} ions, leading to a tensile modulus lower compared to 5- and 10-minute immersion times (66 kPa

compared to 86kPa and 81 kPa respectively). For immersion times of 10 minutes or longer, the tensile modulus decreases due to extensive swelling of the gels. As the intermediate hydrogel is loosely crosslinked, the gel will quickly swell in an aqueous solution until equilibrium water content is reached. For immersion times of 10 minutes or longer, loss of structural integrity due to additional swelling outweighs the enhancement in mechanical properties as a result of denser crosslinking, leading to a lower tensile modulus. For immersion times of 10 minutes or longer, water absorption outpaces the ionic crosslinking process to the extent that the gels swell significantly during this immersion stage. For immersion times lower than 10 minutes, ionic crosslinking outpaces water absorption.

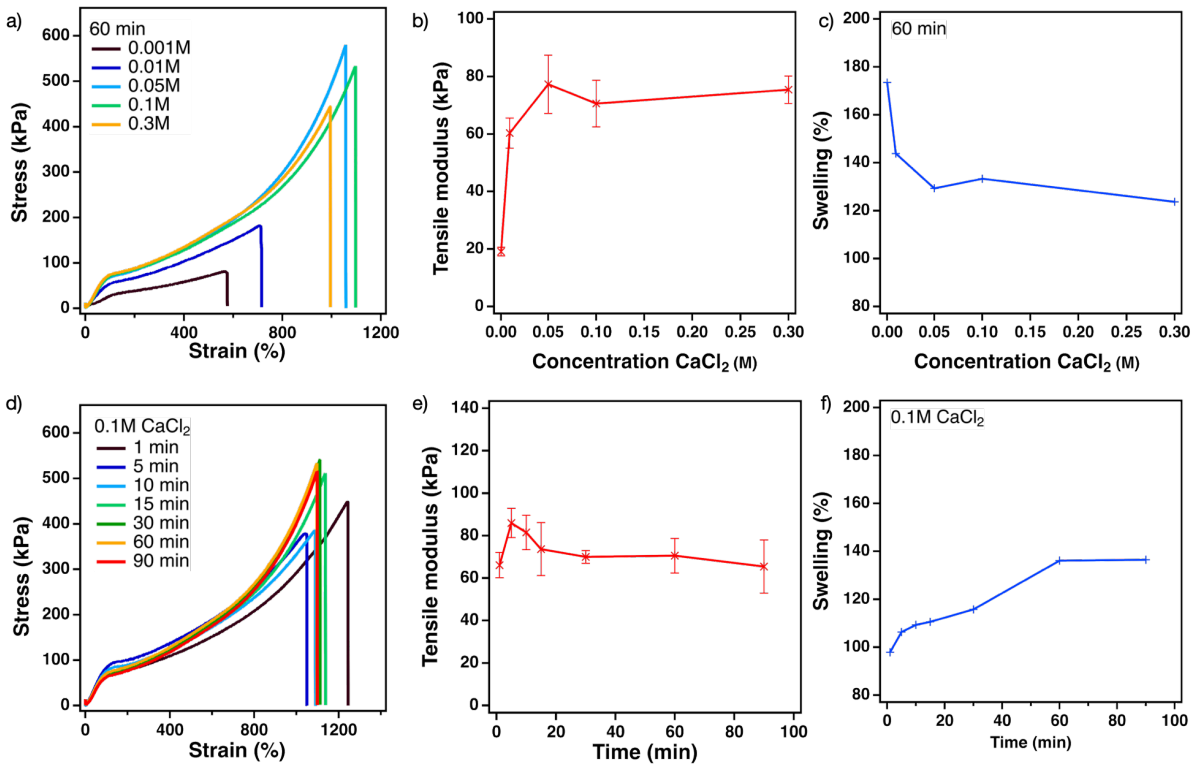

Figure 4: mechanical properties of $CaCl_2$ crosslinked P(AAm)/Alginate hydrogels. a/b/c) Comparison of tensile properties of P(AAm)/alginate hydrogels soaked for 60 minutes in different concentrations of $CaCl_2$ in water and their resulting swelling ratios. d/e/f) Comparison of tensile properties of P(AAm)/alginate hydrogels soaked for different durations in a 0.1M $CaCl_2$ solution and their resulting swelling ratios. Note: the lowest concentration of $CaCl_2$ in part b and c is 0.001 M; the lowest time in part e and f is 1 min.

$CaSO_4$ (one step) procedure

The solubility of calcium sulfate ($CaSO_4 \cdot 2H_2O$, 260 mg/L) is considerably lower than that of $CaCl_2$. At low $CaSO_4$ concentrations, alginate can be added to the precursor solution and stirred to obtain a homogeneous solution. At higher $CaSO_4$ concentrations, the time between the addition of alginate to the solution and gelation becomes too short to allow stirring, resulting in an inhomogeneous solution and non-uniform network structure.

A series of P(AAm)/Alginate hydrogels were made containing 86% water, 12.6% PAAm and 1.4% alginate using a one-step procedure. Different amounts of $CaSO_4$ were added to the precursor solution and the mechanical properties of the resulting hydrogels were determined by tensile and compression testing to determine the upper limit of $CaSO_4$. The amount of $CaSO_4$ added is calculated as a weight percentage relative to alginate. Homogeneous hydrogels were obtained for weight percentages of $CaSO_4$ up to 15 wt %. For the lower concentrations (8 wt % and 10 wt %) the precursor solution could be stirred for more than 2 minutes prior to molding, while for the higher concentrations (12 wt % and 15 wt %) the stirring time becomes significantly shorter, with 60 s and 30 s of stirring respectively. The precursor solution containing 20 wt % $CaSO_4$ gelled instantly and no test samples could be made for this concentration. The resulting hydrogels showed increasing tensile and compression modulus with increased $CaSO_4$ content caused by a higher crosslink density (Figure 5 c/d, Tables S1 and S2).

Similarly, for precursor solutions containing equal amounts of $CaSO_4$, larger amounts of alginate will lead to faster gelation and a less homogeneous network structure. To determine the upper limit of alginate content, a series of P(AAm)/Alginate hydrogels were made containing 86% water and a variation in P(AAm) and alginate content using a one-step procedure. The amount of $CaSO_4$ added to the precursor solution was fixed at 10 wt % relative to alginate. Homogeneous hydrogels were obtained for weight percentages of alginate up to 2.8%. For precursor solutions containing 3.5% alginate or more, no test samples could be

made due to rapid gelation. The resulting hydrogels showed increasing tensile and compression modulus with increased alginate content, presumably owing to more energy dissipation provided by the alginate network (Figure 5 a/b, Table S1 and Table S2).

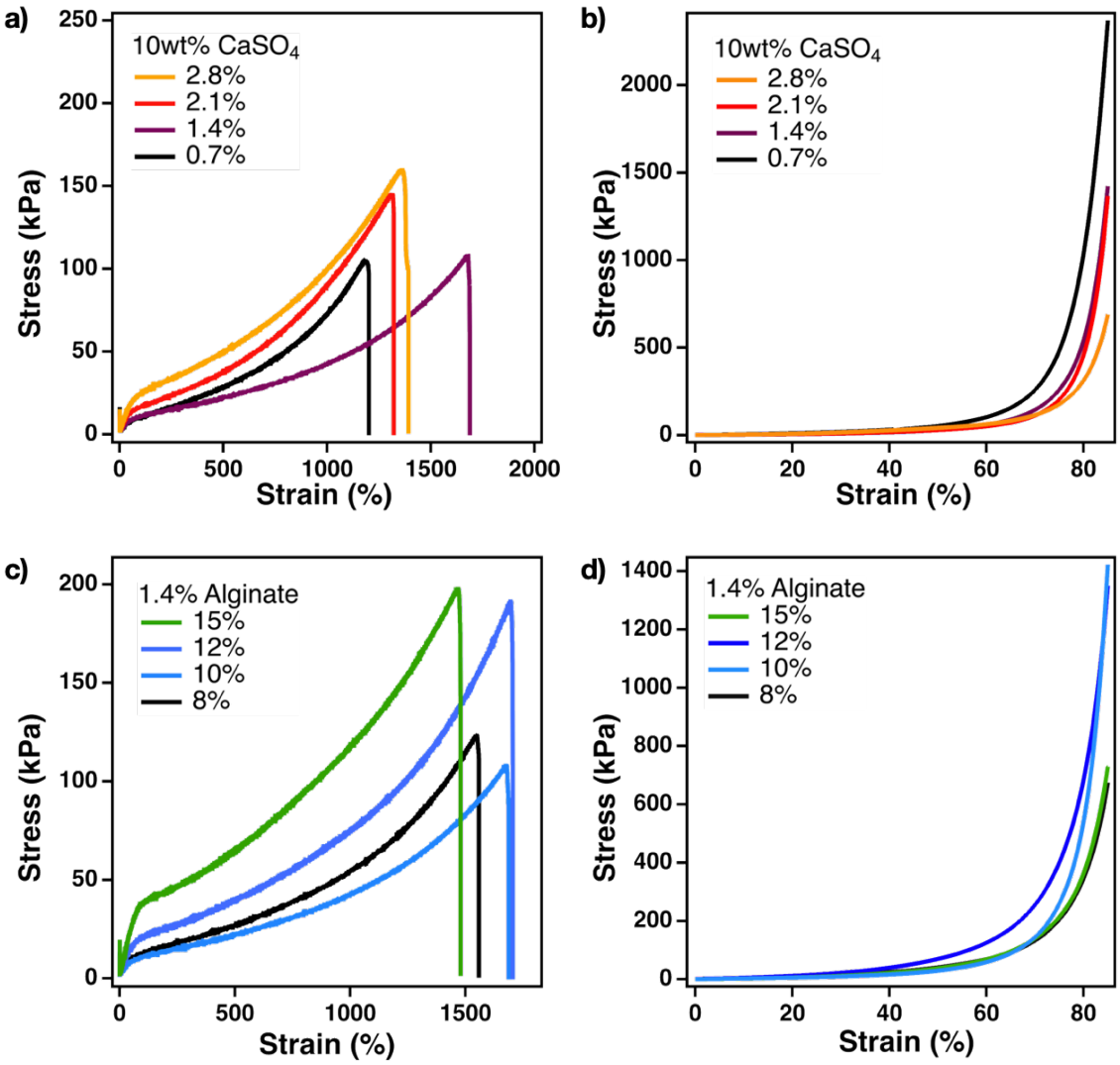

Figure 5: Mechanical properties of CaSO4 crosslinked P(AAm)/Alginate hydrogels. a/b) Tensile /compression stress-strain curves for P(AAm)/Ca-Alginate hydrogels with 86% water content. Legend contains the weight percentage alginate (x%), the corresponding weight percentage PAAm can be calculated as (14-x)%. As a calcium source, 10% $CaSO_4$ (relative to alginate) was added. c/d) Tensile /compression stress-strain curves for P(AAm)/Ca-Alginate hydrogels with 86% water content, 12.6% AAm and 1.4% Alginate. Legend contains the weight percent of $CaSO_4$ (relative to alginate).

$CaCO_3$ / GDL time delayed gelation (one step) procedure

Calcium carbonate alone does not lead to crosslinking of alginate due to its limited solubility (13 mg/L). A solution of alginate and $CaCO_3$ can be stirred until a homogeneous solution/slurry is obtained. To prevent sedimentation of $CaCO_3$, the solution was sonicated for 5 minutes before pouring into a mold. Without extra stimuli, no crosslinking occurs. Only upon contact with protons will $CaCO_3$ react and disintegrate into Ca^{2+} ions, CO_2, and water.

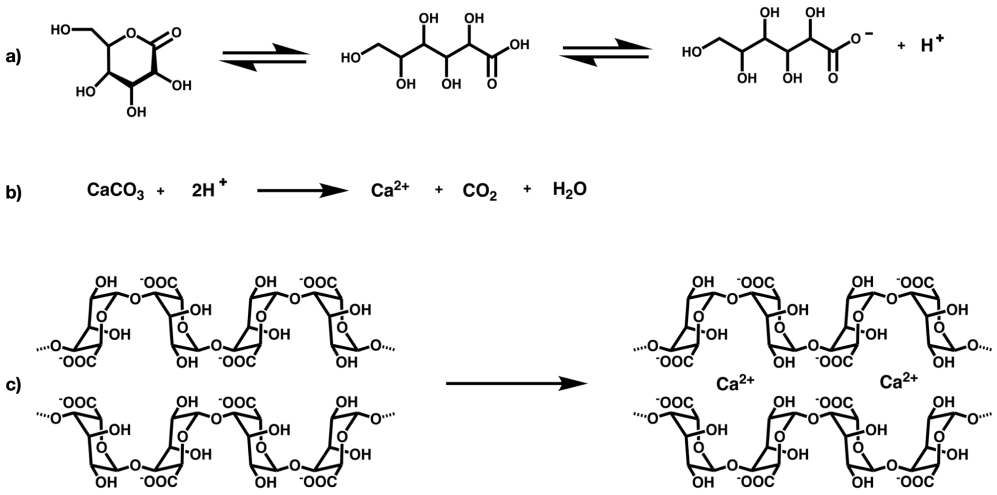

Figure 6: Mechanism behind the $CaCO_3$/GDL method. a) hydrolysis of glucono-d-lactone to gluconic acid and its acid-base equilibrium. b) disintegration of calcium carbonate in acidic medium. c) Crosslinking of alginate by Ca^{2+} ions.

Glucono-δ-lactone, a food additive commonly used as an acidifier, can be used to drive this disintegration. GDL will undergo partial hydrolysis to gluconic acid upon addition to water (Figure 6a) [30, 31]. This will lead to a gradual release of H^+ and the subsequent disintegration of $CaCO_3$ (Figure 6b). Subsequently, the released Ca^{2+} ions will interact with the carboxylic acid groups of alginate, resulting in crosslinking (Figure 6c). The rate of hydrolysis of GDL determines the rate at which alginate is crosslinked.

Two protons are required per molecule of $CaCO_3$ and therefore a 2:1 molar ratio between GDL/$CaCO_3$ is necessary to ensure complete conversion of $CaCO_3$. Using less than 2 equivalents of GDL will result in $CaCO_3$ particles trapped inside the hydrogel network. Using

more than 2 equivalents will lead to faster gelation due to the dependence of the rate of crosslinking on the concentration of GDL, with a higher concentration leading to faster hydrolysis and faster release of Ca^{2+} ions [11].

A series of P(AAm)/Alginate hydrogels were made containing 86% water, 12.6% PAAm and 1.4% alginate using a one-step procedure. Different amounts of $CaCO_3$ were added to the precursor solution. The amount of GDL added to the precursor solution was fixed at a 2:1 molar ratio between GDL/$CaCO_3$. The presence of $CaCO_3$ turns the precursor solution cloudy (Figure 7c, Table S3 and S4). During gelation, $CaCO_3$ gradually disintegrates, resulting finally in a transparent gel as the product (Figure 7f). Homogeneous hydrogels were obtained for all weight percentages of $CaCO_3$ and alginate. The mechanical properties of the resulting hydrogels were determined by tensile and compression testing (Figure 7 d/e). The amount of $CaCO_3$ added is calculated as a weight percentage relative to alginate.

Similarly, a series of P(AAm)/Alginate hydrogels were made containing 86% water and a variation in P(AAm) and alginate content using a one-step procedure. The amount of $CaCO_3$ added to the precursor solution was fixed at 10 wt % relative to alginate, and the amount of GDL was fixed at a 2:1 molar ratio between GDL/$CaCO_3$. The resulting hydrogels showed increasing tensile and compression modulus with increasing $CaCO_3$ content, consistent with a more densely crosslinked alginate network (Tables S3 and S4, Figure 7 d/e).

Owing to the protocol for preparing the samples, the exact concentration of all components is well-controlled in most cases, and the final substrates molecular make-up is accurately known. The one exception is the method employing $CaCl_2$ as ionic cross linker. In this exceptional case, the pre-formed hydrogels are soaked in an aqueous solution of $CaCl_2$, and the exact amount of Ca^{+2} ions that are absorbed is unknown. Attempts to quantify the calcium content proved inconclusive. However, employing several different solutions with different initial $CaCl_2$ concentration enables us to link initial concentration with final physical (i.e., mechanical) properties.

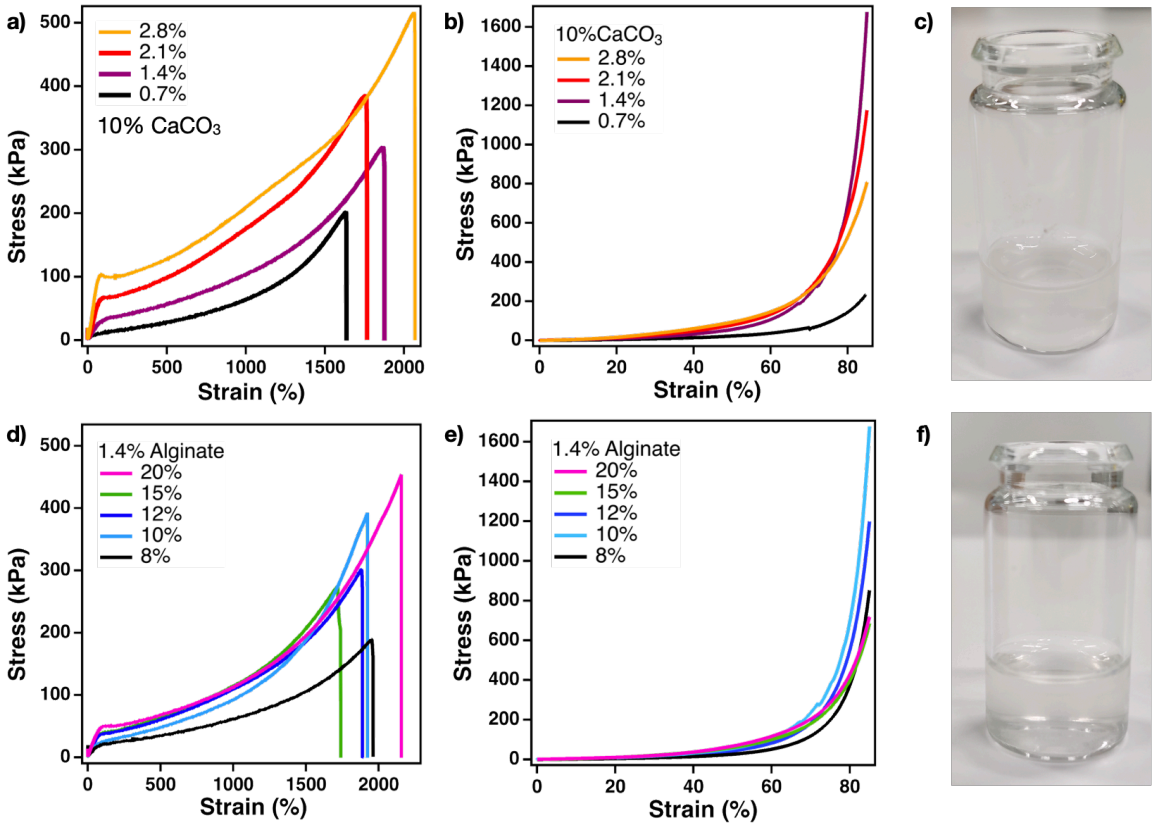

Figure 7: Mechanical properties of $CaCO_3$/GDL crosslinked P(AAm)/Alginate hydrogels. a/b) Tensile /compression stress-strain curves for P(AAm)/Ca-Alginate hydrogels with 86% water content. Legend contains the weight percentage alginate (x%), the corresponding weight percentage PAAm can be calculated as (14-x)%. As a calcium source, 10% $CaCO_3$ (relative to alginate) was added. c) Cloudy pre-gel solution containing AAm, APS, TEMED, alginate, GDL, and $CaCO_3$. Photo taken seconds after addition of GDL. d/e) Tensile /compression stress-strain curves for P(AAm)/Ca-Alginate hydrogels with 86% water content, 12.6% AAm and 1.4% Alginate. Legend contains the weight percent of $CaCO_3$ (relative to alginate). f) Transparent P(AAm)/Ca-Alginate hydrogel made using the $CaCO_3$/GDL method.

Comparison of calcium-based crosslinking strategies

Both $CaSO_4$ and $CaCO_3$/GDL have proven to be effective calcium ion sources for the preparation of PAAm/Ca-Alginate hydrogels in a one-step procedure in the previous sections. Both calcium sources have their advantages and disadvantages. The delay in gelation created using $CaCO_3$/GDL makes the preparation procedure of the gels straightforward, while the procedure with $CaSO_4$ often results in problems during the processing or molding of the gels due to the short time frame between the dissolution of $CaSO_4$ and gelation. This inconveniently short processing window often results in an inhomogeneous network structure, which manifests itself in the tensile moduli of the resulting gels. Gels made with $CaSO_4$ have significantly lower tensile moduli than gels made with $CaCO_3$ at the same calcium and alginate content (Figure 8 b/d). This is a result of the non-uniform network structure, leading to regions that are significantly more susceptible to mechanical failure in the hydrogel. This effect is more pronounced for gels with higher alginate content and cannot be seen in the compression properties (Figure 8 a/c).

The $CaCO_3$/GDL preparation method, therefore, seems to be superior both in terms of preparation simplicity as well as structural uniformity of the resulting gels. One major disadvantage that limits the applicability of this method, is its pH sensitivity. While this is not a problem for PAAm/alginate gels, it might become a problem when the composition of the acrylate networks changes. As the dissolution of $CaCO_3$ relies on the release of protons during the hydrolysis of GDL, acidic groups will interfere, limiting its use in combination with acrylate monomers such as acrylic acid. In addition to that, the $CaCO_3$/GDL method fails to work in buffered solutions such as PBS, highlighting a critical limitation in the field of tissue engineering.

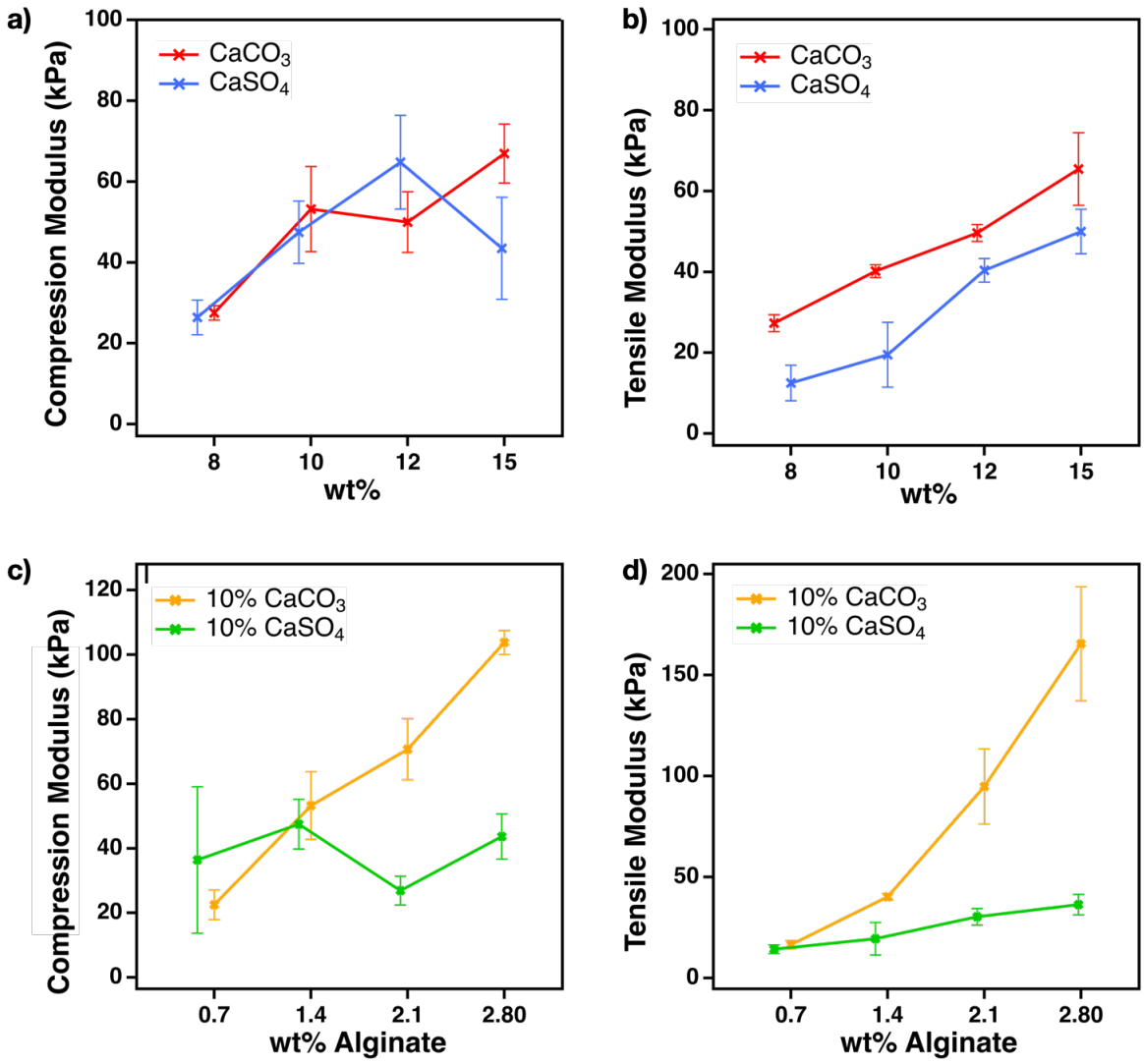

Figure 8: Comparison of calcium-based crosslinking strategies. a) Comparison of compression moduli for PAAm/Alginate gels (9:1 w/w) made with different weight percentages of $CaSO_4/CaCO_3$. b) Comparison of tensile moduli for PAAm/Alginate gels (9:1 w/w) made with different weight percentages of $CaSO_4/CaCO_3$. c) Comparison of compression moduli for PAAm/Alginate gels with variating alginate content containing 10 wt % $CaSO_4/CaCO_3$. d) Comparison of tensile moduli for PAAm/Alginate gels with variating alginate content containing 10 wt % $CaSO_4/CaCO_3$.

Alginate DN hydrogels crosslinked by bivalent and trivalent ions

While poly(acrylamide)/alginate hydrogels are routinely crosslinked by calcium ions, other ions have gained interest as well. By changing the ionic crosslinking agent, the strength of the interaction and corresponding mechanical properties can be tuned [32, 33]. Bivalent cations such as Sr^{2+} and Ba^{2+} are particularly interesting, while Pb^{2+}, $Cu^{2+,}$ and Cd^{2+} are too toxic for tissue engineering applications. Other multivalent ions such as Ti^{3+}, $Al^{3+,}$ and Fe^{3+} have gained attention as well [15, 18, 34]. Yang et. al. reported the influence of Ca^{2+}, Sr^{2+}, Ba^{2+}, Al^{3+}, and Fe^{3+} on the tensile properties of poly(acrylamide)/alginate double network hydrogels [29]. DN hydrogels containing 86% water, 1.56% alginate and 12.44% acrylamide were made and ionic crosslinking of alginate was introduced by soaking the gels in 0.3M salt solution ($CaCl_2$, $SrCl_2.6H_2O$, $BaCl_2.2H_2O$, $AlCl_3.6H_2O$ and $FeCl_3.9H_2O$) for 3 hours. The 3 hours of soaking time was confirmed to be sufficient to ensure complete exchange of Na^+ ions [29]. However, this publication did not study the compression properties or the destabilization effect of alginate-containing gels in a physiological environment. In our present work, similar hydrogels were made, and the effect of different ions was determined by tensile and compression testing, as well as the stability of the gels in physiologically relevant conditions.

Similar to the work of Yang and coworkers, a series of P(AAm)/Alginate hydrogels were made containing 86% water, 12.6% PAAm, and 1.4% alginate using a two-step procedure [29]. In the first step an intermediate P(AAm)/Na-Alginate hydrogel without ionic crosslinker was made. In the second step this intermediate hydrogel was immersed in a 0.1M aqueous salt solution ($CaCl_2$, $FeCl_3$, $AlCl_3$, $BaCl_{2,}$ or $SrCl_2$) for 3 hours to induce ionic crosslinking of the alginate network. All hydrogel variants were transparent, with gels crosslinked with Ca^{2+}, Ba^{2+}, Ca^{2+}, and Al^{3+} being colorless and the gels crosslinked by Fe^{3+} having an orange color (Figure 9). Even though the slight differences in the composition will influence the physicochemical properties, this effect is limited, and the results can be compared regardless. A lower concentration of aqueous salt solutions was used as well, but this difference in concentration

has been proven to have no effect on the mechanical properties as both 0.1 and 0.3M solutions lead to full saturation of the gels. The results are consistent with studies of single network hydrogels [13]. The extent of binding of Ca^{2+}, Sr^{2+}, and Ba^{2+} are significantly different from each other. The extent of binding between metal ions and the GG blocks of alginate increases with higher ionic radius (Ba^{2+}> Sr^{2+}>Ca^{2+}>>Mg^{2+} and Fe^{3+}>Al^{3+}) [32]. This is reflected in the tensile moduli measured for PAAm/Alginate, with higher moduli for the gels made with Ba^{2+} compared to those made with Sr^{2+} or Ca^{2+} (Table S7, Figure 9). Likewise, the tensile modulus for gels made with Fe^{3+} is higher than those made with Al^{3+} (Figure 9). Furthermore, the moduli measured for trivalent cations are much higher than those with divalent cations due to the larger coordination number ($(COO)_3M^{3+}$). When we compare the elastic moduli obtained through tensile testing with the values reported in literature (Table S7), we see a similarity in the gels crosslinked by Fe^{3+} and Al^{3+} [29]. The small deviation could be explained by differences in composition or the method of tensile testing. For Ca^{2+}, Ba^{2+}, and Sr^{2+}, significant differences in tensile modulus are observed. The same trends are seen when comparing compression moduli (Table S8).

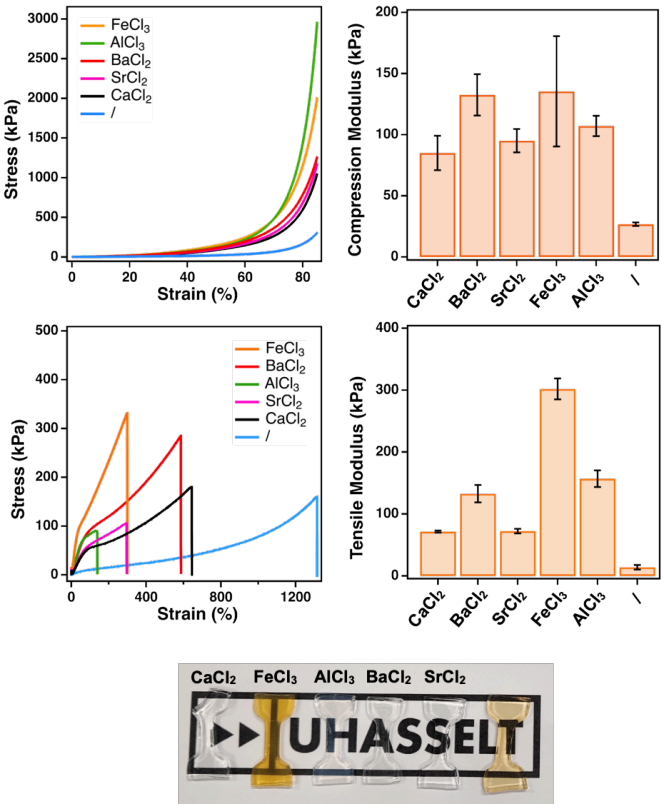

Figure 9: Comparison of tensile and compression properties for DN PAAm/alginate hydrogels crosslinked by bivalent and trivalent ions

Ca-alginate containing hydrogels have low stability in physiological environment; their structure is destabilized by substances such as phosphate and citrate. These substances have a high affinity for Ca^{2+} and essentially scavenge calcium ions, effectively compromising the crosslinking [20]. Interactions of polyvalent ions phosphate groups in PBS buffer solution leads to the formation of insoluble phosphate precipitates and has been proven to have an effect on the swelling and dissolution of single network alginate hydrogels [35]. High concentrations of non-crosslinking ions such as Na^{+} and Mg^{2+} will further destabilize the gel by ion exchange with Ca^{2+} [36].

To test this destabilizing effect, PAAm/alginate DN hydrogels crosslinked by different metal ions were immersed in a PBS solution and the mechanical properties of the gels before and after immersion are compared. For the gels crosslinked by trivalent ions only a small

difference in tensile properties is observed after 3h of immersion in PBS, while a more dramatic decline is seen for the gels crosslinked by bivalent cations. This can be explained by the interactions of divalent cations compared to trivalent cations, leading to a faster release of the bivalent ions, a lower resistance to swelling of the gel and a faster decline in the mechanical properties. Furthermore, the formation of insoluble salts by the interaction of the multivalent ions with phosphate groups in the PBS solutions makes the released ions unavailable for reabsorption into the alginate matrix.

When the immersion time in PBS is increased to 24 hours, the mechanical properties of all gels decrease to values comparable to those of gels made without ionic crosslinking present.

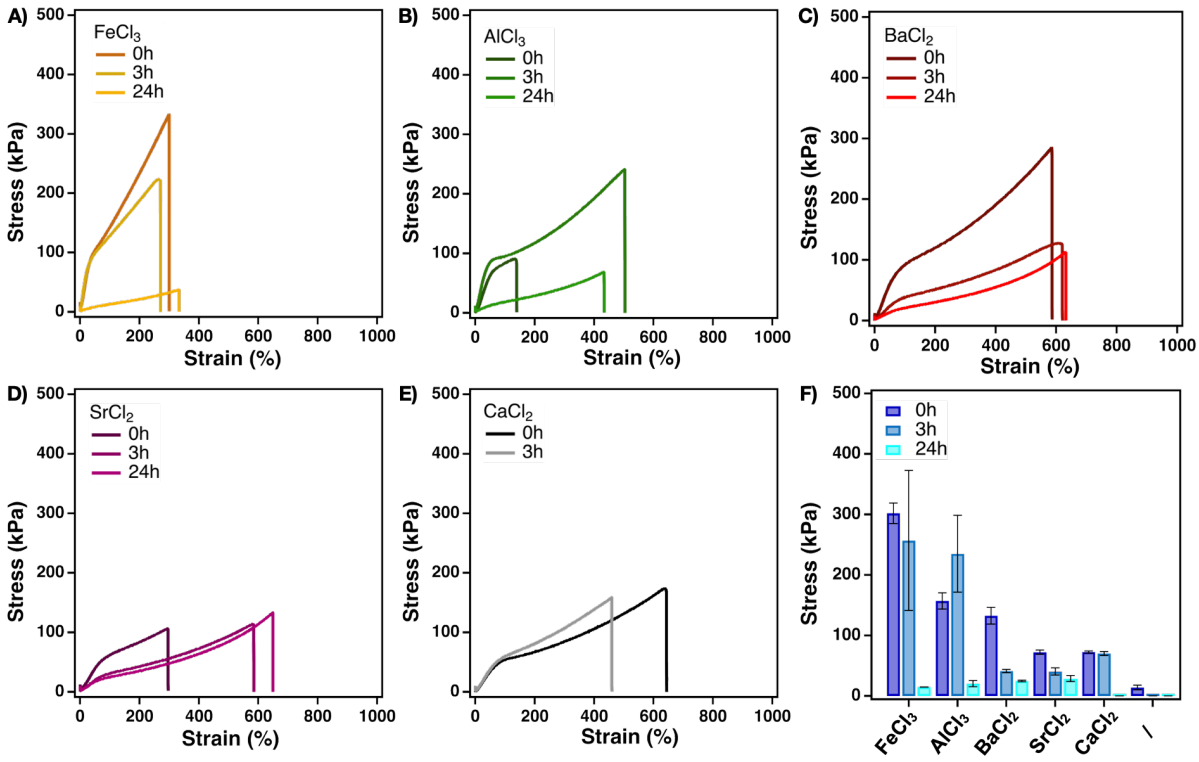

Figure 10: Effect of ion leeching in P(AAm)/M-Alginate hydrogels crosslinked by different metal ions: A) $FeCl_3$ B) $AlCl_3$ C) $BaCl_2$ D) $SrCl_2$ E) $CaCl_2$. Each type of P(AAm)/M-Alginate gel was soaked for 3 and 24 hours in PBS and the resulting effect on the mechanical properties was measured by tensile testing. F) Comparison of tensile moduli measured at different stages of ions leeching for the different ionic crosslinking agents.

Conclusions

Poly(acrylamide)/alginate double network hydrogels are commonly crosslinked through ionic interaction by introducing multivalent cations into the hydrogel. Different ion sources and preparation methods have emerged during the last decade, each with its own advantages and limitations. The mechanical properties of alginate-containing double network hydrogels vary depending on their preparation and crosslinking agent. This effect was studied for poly(acrylamide)/alginate hydrogels using a variety of crosslinking agents ($CaCl_2$, $FeCl_3$, $AlCl_3$, $BaCl_2$, and $SrCl_2$) as well as for preparation methods utilizing different calcium ion sources.

Calcium crosslinked P(AAm)/alginate hydrogels can be prepared by using $CaCl_2$, $CaCO_3$, or $CaSO_4$ as ion sources. With the two-step procedure using $CaCl_2$, the crosslink density and swelling of the gels during immersion are hard to control. Furthermore, this procedure is difficult to apply in 3D printing or injectability due to a second step being necessary to obtain a fully crosslinked hydrogel, which is not the case in the one-step methods using $CaSO_4$ or $CaCO_3$. However, when $CaSO_4$ is used, rapid gelation often limits its applicability. The one-step method using $CaCO_3$ offers the ability to delay gelation in a more controlled manner, and therefore is the most favorable method when looking at 3D printing or injectability. Furthermore, when looking at the mechanical properties of P(AAm)/Ca-Alginate hydrogels with the same composition, those prepared using $CaCO_3$ had better mechanical properties and smaller standard deviations, indicating a more homogeneous network structure. Despite being superior in preparation ease, structural uniformity of the resulting gels, and applicability in 3D printing and injectability, it comes with the major disadvantage of being pH sensitive.

Other ion sources such as $FeCl_3$, $AlCl_3$, $BaCl_2$, and $SrCl_2$ have successfully been used to prepare P(AAm)/M-alginate hydrogels crosslinked by Fe^{3+}, Al^{3}+, $Ba^{2+,}$ and Sr^{2+} ions, respectively. The gels crosslinked by Ba^{2+} had higher compression moduli than those crosslinked by the other bivalent metal ions (Ca^{2+} and Sr^{2+}) due to the higher ionic radius as reported in literature. Similarly, the moduli of gels crosslinked by Fe^{3+} were higher than those

crosslinked by Al^{3+}. Unfortunately, all PAAm/M-Alginate hydrogels were found to have low stability in physiological environment regardless of the nature of the crosslinking ions. After storing the gels in a PBS solution over 24 hours, their mechanical properties all decreased to values comparable of those made without ionic crosslinker present, indicating significant ion leeching.

It is clear that the properties of alginate containing double network hydrogels rely on many different factors. The dependency of the mechanical properties on their preparation method and their stability in physiological environment should be considered when looking at applications of these materials. The limited processability inherent to some of their preparation methods and the possibility of ion leeching may limit the application of these double-network hydrogels in the field of tissue engineering. These factors require more attention in order to fully exploit the outstanding potential of the mechanically tunable hybrid gels described here. Despite some of the possible limitations, we see strong possibilities for printing customized constructs, and the various ionic crosslinking strategies presented here offer some interesting avenues for fine-tuning the systems specifically for additive manufacturing. Constructing tough gels that can be processed via printing techniques remains a challenge [19].

ASSOCIATED CONTENT

Supporting Information

The supporting information is available free of charge at https://

Materials and methods, additional supporting experimental details including stress–strain plots.

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Declaration of Competing Interest

The authors declare no competing financial interests.

Data availability

The processed data required to reproduce these findings will be provided upon request.

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REFERENCES

[1] A.D. Augst, H.J. Kong, D.J. Mooney, Alginate hydrogels as biomaterials, Macromol. Biosci. 6 (2006) 623-633.

[2] J.A. Rowley, G. Madlambayan, D.J. Mooney, Alginate hydrogels as synthetic extracellular matrix materials, Biomaterials 20 (1999) 45-53.

[3] H. Hecht, S. Srebnik, Structural characterization of sodium alginate and calcium alginate, Biomacromolecules 17 (2016) 2160-2167.

[4] J.S. Boateng, K.H. Matthews, H.N.E. Stevens, G.M. Eccleston, Wound healing dressings and drug delivery systems: a review, J. Pharm. Sci. 97 (2008) 2892-2923.

[5] Y. Fang, S. Al-Assaf, G.O. Phillips, K. Nishinari, T. Funami, P.A. Williams, L. Li, Multiple steps and critical behaviors of the binding of calcium to alginate, J. Phys. Chem. B 111 (2007) 2456-2462.

[6] L. Li, Y. Fang, R. Vreeker, I. Appelqvist, E. Mendes, Reexamining the egg-box model in calcium– alginate gels with X-ray diffraction, Biomacromolecules 8 (2007) 464-468.

[7] P. Sikorski, F. Mo, G. Skjåk-Bræk, B.T. Stokke, Evidence for egg-box-compatible interactions in calcium– alginate gels from fiber X-ray diffraction, Biomacromolecules 8 (2007) 2098-2103.

[8] K.I. Draget, G.S. Bræk, O. Smidsrød, Alginic acid gels: the effect of alginate chemical composition and molecular weight, Carbohydr. Polym. 25 (1994) 31-38.

[9] H. Grasdalen, High-field, 1H-nmr spectroscopy of alginate: sequential structure and linkage conformations, Carbohydr. Res. 118 (1983) 255-260.

[10] A. Penman, G.R. Sanderson, A method for the determination of uronic acid sequence in alginates, Carbohydr. Res. 25 (1972) 273-282.

[11] C.K. Kuo, P.X. Ma, Ionically crosslinked alginate hydrogels as scaffolds for tissue engineering: Part 1. Structure, gelation rate and mechanical properties, Biomaterials 22 (2001) 511-521.

[12] D. Ji, J.M. Park, M.S. Oh, T.L. Nguyen, H. Shin, J.S. Kim, D. Kim, H.S. Park, J. Kim, Superstrong, superstiff, and conductive alginate hydrogels, Nat. Commun. 13 (2022) 1-10.

[13] Ý.A. Mørch, I. Donati, B.L. Strand, G. Skjak-Braek, Effect of Ca2+, Ba2+, and Sr2+ on alginate microbeads, Biomacromolecules 7 (2006) 1471-1480.

[14] M. Sarker, M. Izadifar, D. Schreyer, X. Chen, Influence of ionic crosslinkers (Ca2+/Ba2+/Zn2+) on the mechanical and biological properties of 3D Bioplotted Hydrogel Scaffolds, J. Biomater. Sci., Polym. Ed. 29 (2018) 1126-1154.

[15] E.S. Place, L. Rojo, E. Gentleman, J.P. Sardinha, M.M. Stevens, Strontium-and zinc-alginate hydrogels for bone tissue engineering, Tissue Eng. Part A 17 (2011) 2713-2722.

[16] P. Agulhon, V. Markova, M. Robitzer, F.o. Quignard, T. Mineva, Structure of alginate gels: interaction of diuronate units with divalent cations from density functional calculations, Biomacromolecules 13 (2012) 1899-1907.

[17] J. Brus, M. Urbanova, J. Czernek, M. Pavelkova, K. Kubova, J. Vyslouzil, S. Abbrent, R. Konefal, J. Horský, D. Vetchy, Structure and dynamics of alginate gels cross-linked by polyvalent ions probed via solid state NMR spectroscopy, Biomacromolecules 18 (2017) 2478-2488.

[18] D.M. Roquero, A. Othman, A. Melman, E. Katz, Iron (III)-cross-linked alginate hydrogels: a critical review, Mater. Adv. 3 (2022) 1849-1873.

[19] A.A. Aldana, S. Houben, L. Moroni, M.B. Baker, L.M. Pitet, Trends in Double Networks as Bioprintable and Injectable Hydrogel Scaffolds for Tissue Regeneration, ACS Biomater. Sci. Eng. 7 (2021) 4077-4101.

[20] O. Smidsrød, G. Skja, Alginate as immobilization matrix for cells, Trends Biotechnol. 8 (1990) 71-78.

[21] T. Andersen, P. Auk-Emblem, M. Dornish, 3D cell culture in alginate hydrogels, Microarrays 4 (2015) 133-161.

[22] E. Alsberg, K.W. Anderson, A. Albeiruti, R.T. Franceschi, D.J. Mooney, Cell-interactive alginate hydrogels for bone tissue engineering, J. Dent. Res. 80 (2001) 2025-2029.

[23] I. Freeman, S. Cohen, The influence of the sequential delivery of angiogenic factors from affinity-binding alginate scaffolds on vascularization, Biomaterials 30 (2009) 2122-2131.

[24] J.Y. Sun, X.H. Zhao, W.R.K. Illeperuma, O. Chaudhuri, K.H. Oh, D.J. Mooney, J.J. Vlassak, Z.G. Suo, Highly stretchable and tough hydrogels, Nature 489 (2012) 133-136.

[25] S. Houben, A.A. Aldana, A.-S. Huysecom, W. Mpinganzima, R. Cardinaels, M.B. Baker, L.M. Pitet, Hybrid Hydrogels with Orthogonal Transient Cross-linking Exhibiting Highly Tunable Mechanical Properties, ACS Appl. Polym. 5 (2023) 1819-1827.

[26] Y. Yan, M. Li, D. Yang, Q. Wang, F. Liang, X. Qu, D. Qiu, Z. Yang, Construction of Injectable Double-Network Hydrogels for Cell Delivery, Biomacromolecules 18 (2017) 2128-2138.

[27] W.J. Zheng, N. An, J.H. Yang, J. Zhou, Y.M. Chen, Tough Al-alginate/poly (N-isopropylacrylamide) hydrogel with tunable LCST for soft robotics, ACS Appl. Mater. Interfaced 7 (2015) 1758-1764.

[28] G. Liling, Z. Di, X. Jiachao, G. Xin, F. Xiaoting, Z. Qing, Effects of ionic crosslinking on physical and mechanical properties of alginate mulching films, Carbohydr. Polym. 136 (2016) 259-265.

[29] C.H. Yang, M.X. Wang, H. Haider, J.H. Yang, J.-Y. Sun, Y.M. Chen, J. Zhou, Z. Suo, Strengthening alginate/polyacrylamide hydrogels using various multivalent cations, ACS Appl. Mater. Interfaced 5 (2013) 10418-10422.

[30] D.T. Sawyer, J.B. Bagger, The lactone-acid-salt equilibria for D-glucono-δ-lactone and the hydrolysis kinetics for this lactone, J. Am. Chem. Soc. 81 (1959) 5302-5306.

[31] Y. Pocker, E. Green, Hydrolysis of D-glucono-. delta.-lactone. I. General acid-base catalysis, solvent deuterium isotope effects, and transition state characterization, J. Am. Chem. Soc. 95 (1973) 113-119.

[32] C.M. DeRamos, A.E. Irwin, J.L. Nauss, B.E. Stout, 13C NMR and molecular modeling studies of alginic acid binding with alkaline earth and lanthanide metal ions, Inorg. Chim. Acta 256 (1997) 69-75.

[33] Z. Zhang, T. Lin, S. Li, X. Chen, X. Que, L. Sheng, Y. Hu, J. Peng, H. Ma, J. Li, Polyacrylamide/Copper-Alginate Double Network Hydrogel Electrolyte with Excellent Mechanical Properties and Strain-Sensitivity, Macromol. Biosci. 22 (2022) 2100361.

[34] M.C. Straccia, G.G. d'Ayala, I. Romano, P. Laurienzo, Novel zinc alginate hydrogels prepared by internal setting method with intrinsic antibacterial activity, Carbohydr. Polym. 125 (2015) 103-112.

[35] M. Urbanova, M. Pavelkova, J. Czernek, K. Kubova, J. Vyslouzil, A. Pechova, D. Molinkova, J. Vyslouzil, D. Vetchy, J. Brus, Interaction pathways and structure–chemical transformations of alginate gels in physiological environments, Biomacromolecules 20 (2019) 4158-4170.

[36] S.K. Bajpai, S. Sharma, Investigation of swelling/degradation behaviour of alginate beads crosslinked with Ca2+ and Ba2+ ions, React. Funct. Polym. 59 (2004) 129-140.

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