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Competitive ligand exchange of crosslinking ions for ionotropic hydrogel formation

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Currently there are limitations to gelation strategies to form ionically crosslinked hydrogels, derived in particular from a lack of control over the kinetics of release of crosslinking ions, which severely restrict applications. To address this challenge, we describe a new approach to form hydrogels of ionotropic polymers using competitive displacement of chelated ions, thus making specific ions available to induce interactions between polymer chains and form a hydrogel. This strategy enables control of ion release kinetics within an aqueous polymer solution and thus control over gelation kinetics across a wide range of pH. The described technique simplifies or facilitates the use of ionotropic hydrogels in a range of applications, such as 3D printing, microfluidic-based cell encapsulation, injectable preparations and large scale bubble and solid free mouldable gels. We investigate a range of chelator-ion combinations and demonstrate this powerful method to form hydrogels across a wide range of pH and µm – cm length scales. We highlight our findings by applying this gelation strategy to some of the more challenging hydrogel application areas using alginate and polygalacturonate as model polymer systems.

Introduction

Ionically crosslinked hydrogels are an important class of materials with applications in food[1], pharmaceutical[2] and biomedical[3] industries. Ionic crosslinking kinetics are difficult to control since they are governed by intrinsic and typically rapid binding of gelling ions by the ionotropic polymer.[4] As a consequence it is difficult to make homogenous gels or delay gelling after free cross-linking ions (CI) have been introduced to the polymer solution, therefore making some applications requiring controlled, on demand or bulk gelation difficult or not feasible. By containing the CI within the polymer solution either as a chelate (e.g. Ca-Ethylenediaminetetraacetic acid - Ca-EDTA or Ca-ethylene glycol tetraacetic acid, Ca-EGTA)[5, 6] or as a solid (e.g. $CaCO_{3(s)}$)[7], some degree of control over gelling kinetics can be achieved. CI may be released by lowering pH either rapidly by introduction of an acid, resulting in essentially instantaneous gelation, or gradually via the hydrolysis of d-glucono-δ-lactone (GDL), which can result in a hydrogel taking several hours to fully crosslink.[7] Current "slow" methods allow injectable or mouldable preparations to be achieved but have several limitations. When Ca-EDTA/EGTA complexes are used, the pH must be reduced below~pH 5 to release Ca^{2+} ions which may be prohibitive for cell or protein encapsulation. The use of $CaCO_3$/GDL in the correct proportions allows for gelling to occur at neutral pH, however, the use of a solid source of calcium restricts certain applications e.g. microfluidics.[8] Furthermore, a solid component prohibits filter sterilisation and may also introduce structural anisotropy in the final gel due to sedimentation or inhomogeneous distribution of the particles. Some more recent strategies to partially circumvent these limitations include the use of freeze-dried, CI saturated hydrogels, which when hydrated and mixed with polymer solution will slowly rehydrate and release gelling ions.[9, 10] Bulk gels can be formed with limited control over gelling kinetics, however this method still relies on the use of a solid component. CI may also be chelated by a photo-labile protecting group, therefore allowing gelation upon exposure to light (typically near UV) enabling rapid, triggerable gelation kinetics, however release is dependent on the penetration of light making injection into deep or inaccessible areas not possible.[11] Despite profound efforts, no simple and versatile method to control the kinetics of ionotropic gel formation exists. For 3D printing of ionotropic hydrogels, this limitation has been partially alleviated through the use of a sacrificial support gel structure, but the need to control gelling kinetics still remains.[12]

To address this significant gap in scientific knowledge, we propose a novel route to control the release of CI into an aqueous solution of ionotropic polymer to form a hydrogel, that we term competitive ligand exchange crosslinking (CLEX). We demonstrate that this can be achieved using reactants present entirely in the aqueous phase, under biocompatible conditions and can be designed to allow control over gelation

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time, from seconds to minutes, thereby technically supporting implementation in many processes and applications. We focus on alginate as the key example due to widespread use and contemporary interest in this polyelectrolyte.[13-15] However, CLEX could be applied to other ionotropic hydrogel forming biopolymers or synthetic polymers and to demonstrate this we successfully form hydrogels of polygalacturonate using CLEX.

Experimental

Materials

All chemicals were purchased from Sigma Aldrich (Norway), unless otherwise stated. De-ionized water (DIW, with a resistivity of 10-15 MΩ cm) was used to prepare all aqueous solutions, dissolved gases were removed by bubbling argon gas for 20 mins. Alginate (source *L. hyperborea* stipe) with a guluronic acid residue fraction of F_G = 0.68, corresponding to 68 % (PROTONAL LF 2005, FMC Biopolymer, Haugesund, Norway) and a M_W of 275 kg mol^{-1} was dissolved in DIW to a final concentration of 3 wt%. Polygalacturonic acid, sodium salt (from citrus fruit) was dissolved in DIW to a final concentration of 3 wt%. 1 M aqueous stock solutions of $CaCl_2$, $Zn(CH_3CO_2)_2$, $FeSO_4$ and $MnCl_2$ were prepared. Crosslinking ion chelator (CIC) solutions were prepared by mixing $CaCl_2$ with either 0.5 M ethylenediaminetetraacetic acid (EDTA) (UltraPure™, pH 8.0 Thermo Scientific), 1 M ethylenediamine-N,N'-disuccinic acid (EDDS), 0.4 M propylenediamine-N,N,N',N'-tetraacetic acid (PDTA) or 0.4 M 1,2-cyclohexanedinitrilotetraacetic acid (CDTA) with 3 % hydrogel and a suitable buffer to give a final concentration of 60 mM Ca-CIC, 60 mM buffer and 1 % hydrogel. Exchange ion chelator (EIC) solutions were prepared by mixing $Zn(CH_3CO_2)_2$, $FeSO_4$ or $MnCl_2$ with 0.909 M ethylenediamine-N,N'-diacetic acid (EDDA) (Alfa Aesar), 1 M EDDS, or 1 M glycine with 3 % hydrogel and a suitable buffer to give a final concentration of 60 mM EI-EIC, 60 mM buffer and 1 % hydrogel. Buffers were selected from acetate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-Morpholinopropane-1-sulfonic acid (MOPS) and 3-(Cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO) depending on pH. pH of all solutions was adjusted using HCl and NaOH. Alginate gels were also formed following the method of Draget et al.[7] Briefly, a solution containing 1.5 wt/vol% alginate and 45 mM Ca-EDTA at pH 7.0 was mixed in a 2:1 ratio with a freshly prepared 180mM aqueous solution of d-glucono-δ-lactone (GDL)

Electron Paramagnetic Resonance (EPR) Spectroscopy

EPR spectroscopy was performed using a JEOL JES-FR30 EPR spectrophotometer (JEOL, Japan). 100 µL samples were placed in a quartz dip cell and spectra were collected between 250-400 mT. The spectra were subjected to analysis using EasySpin in MatLab (version 7.8), using the *chili* program[16], in order to obtain the EPR parameters of the various complexes. In *chili* the stochastic Liouville equation is used to solve the tumbling motion of the molecules and is followed by a fitting routine to obtain the EPR parameters of the sample.

Rheology

Rheological characterisation was performed using a Paar Physica MCR 300 Rheometer. A parallel plate geometry with serrated plate surfaces (PP50 serrated plate, diameter = 50 mm) which provided minimal wall slip was used. Storage and loss moduli at a measuring gap of 1 mm were recorded as a function of time at an angular frequency (ω) of 1 rad s^{-1}, amplitude of 1 mrad and temperature of 25 °C. Frequency sweeps were made in the range ω = 0.1 – 10 rad s^{-1} at γ = 10 %. Equal volumes (1.75 mL) of 2 component gels were measured onto the rheometer plate using a 5 mL pipette. The first component was placed directly onto the bottom plate, then the other was pipetted onto it just prior to starting the measurement. In experiments above 30 min in duration, low viscosity silicone oil (200/10 cS fluid, Dow Corning, USA) was introduced following setting of the gap to seal the gel sample from the atmosphere and to prevent evaporation. Using this approach a lag time of approximately 30s resulted from the time of delivery of the second polymer solution to the start of data collection. For all measurements of CLEX hydrogels, the final concentration of Ca^{2+}, Zn^{2+} and chelators was 30 mM except glycine which was 60 mM. pH was maintained by the use of pH buffers, pH 5 = acetate, pH 6 = MES, pH 7 & 7.4 = MOPS, pH 8 = HEPES, pH 9 = CAPSO, all used at a concentration of 30mM. For Ca-EDTA / GDL crosslinked alginate, the final concentration of Ca^{2+} was also 30 mM and was prepared as described above. All measurements were repeated a minimum of three times.

Microfluidic gelling and cell encapsulation

Polydimethylsiloxane (PDMS) microfluidic devices were fabricated using standard soft lithography.[17] In short, moulds were produced by (1) spinning photoresist (SU8-3050, MicroChem Corp.) onto silicon wafers (University Wafers) at 3000 rpm (~50 µm channel heights) (2) pre-exposure baking at 95 °C for 15 min (3) Exposing photoresist with UV light (360nm, 250 mJ cm^{-2}) through emulsion films with CAD designs of microfluidic geometries (4) post-exposure baking at 65 °C for 1 min and at 95 °C for 5 min (5) developing (mr-Dev600, Micro Resist Technology GmbH, Germany) for 8 min. PDMS and initiator (Sylgard 184 kit, Dow Corning, USA) at a ratio of 10:1 was cast onto the silicon wafers with resist patterns and cured for 3 h at 65 °C. The PDMS was peeled, punched to form channel inlets and outlets, plasma treated for 30 s at 50 W (Femto, Diener Electronics, Germany) and bonded to a slab of PDMS. The MF channels were rendered fluorophilic with 1 % (v/v) fluorosilane (1*H*,1*H*,2*H*,2*H*- perfluorooctyl) in HFE7500 (3M®, USA) for 5 min after plasma treatment. Encapsulation of cells in microbeads in the microfluidic devices was carried out using flow focusing designs. We used a hydrofluoroether (HFE7500) as the carrier fluid to form monodisperse emulsion droplets. 1 % (v/v) of triblock-biocompatible fluorinated surfactant with two oligomeric perfluorinated polyethers (Krytox® FSH 157, DuPont) attached to polyethelyneglycol (ED-900 Jeffamine®, Huntsman, USA) synthesized as described by Holze and coworkers[18] was added to the carrier fluid. Two alginate solutions (1: 0.6 wt% Alginate 84 mM Ca-EDTA and 2: 0.6 wt% Alginate 84 mM Zn-EDDA) were mixed in a co-flow region prior to emulsification by the carrier fluid. For alginate microfiber production, the carrier fluid was replaced by a

10 wt% dextran solution (from *Leuconostoc spp*. M_W 450 – 650 kg mol^{-1}) with 84 mM Zn-EDDA and only one alginate solution was used (0.6 wt% Alginate 84 mM Ca-EDTA). Cell samples suspended in media prior to mixing with alginate and were introduced via the aqueous inlet(s) in both microfluidic systems.

Hydrogel moulding

To create the mould, a CAD model was designed using blender (v2.77, Blender Institute, Amsterdam) and 3D printed using a Wanhao duplicator i3 v2 (Wanhao USA, Miami, Florida) in acrylonitrile butadiene styrene. Prior to use the printed mould was treated with 1 % (v/v) fluorosilane (1*H*,1*H*,2*H*,2*H*- perfluorooctyl,) in HFE7500 (3M®) for 5 min. CLEX alginate hydrogel was introduced to the mould via a dual syringe (BD Medical, New Jersey) fitted with a mixing nozzle. One syringe contained 2 % alginate, 60 mM Ca-EDTA-MOPS and the other contained 2 % alginate, 60 mM Zn-EDDA-MOPS both adjusted pH 7.4. Green food colourant was added to the first solution to aid visualisation of mixing. A metal NTNU tie pin was introduced to the setting gel and encapsulated in the gel.

Cell toxicity measurements

Cell viability was measured by toludine blue infiltration. Murine calvarial pre-osteoblast cells, MC3T3-E1 subclone 4 (ATCC® CRL-2593™) were cultured to 80 % confluency in α-MEM supplemented with 1 µm ml^{-1} gensumycin, 2 mM glutamine and 10 % foetal calf serum. Cell were detached and then dispersed in 2 mL of the following solutions at a concentration of 25,000 cells mL^{-1}: culture medium (CM) CM diluted 40 % with water, CM diluted 40 % to give final concentration of 60 mM Ca-EDTA, Zn-EDTA, Zn-EDDA, EDTA, EDDA, and 120 mM glycine. All solutions were buffered to pH 7.4 using 60 mM MOPS. Negative control group was CM containing 0.1 % triton-X. The cell suspensions were incubated at 37 °C for 2 hours (typical length of cell encapsulation experiment) in the test solutions and then centrifuged and washed in CM twice before resuspending and counting. Toluidine blue was added and stained cells determined as dead using a Countess cell counter. Each condition was repeated in triplicate.

Results and Discussion

The relative ionic affinity and ability to form gels has been extensively studied for alginate and a range of divalent cations. In their seminal paper, Haug and Smidsrød[19] established the following series for the concentration of divalent cations required to bring about gelation of alginate:

Ba < Pb < Cu < Sr < Cd < Ca < Zn < Ni < Co < Mn,Fe < Mg

By comparing this series to the affinity series of cation chelators, e.g. EDTA, see **Table 1**, it becomes apparent that differences with the alginate series can be exploited to control the release of ions. For example, Zn^{2+}, Mn^{2+} and Fe^{2+} bind weakly to alginate, but are strongly bound by EDTA (Log K = 16.5, 13.9 and 14.3 respectively[20]). In contrast, Ca^{2+} shows a reversed trend: it binds more weakly to EDTA (Log K = 10.6) but more strongly to alginate than all these ions. Therefore, upon introduction of Zn^{2+}, Mn^{2+} or Fe^{2+} to a solution of Ca-EDTA, Ca^{2+} will be exchanged and rendered free. In the presence of alginate, the liberated Ca^{2+} is therefore available to crosslink the polymer. This principle of ionic displacement forms the basis of CLEX. We demonstrate this by preparing a 1 % alginate solution containing Zn^{2+}, Fe^{2+} or Mn^{2+} exchange ions (EI) at a concentration low enough to not form a gel (here we determined critical gel concentrations of > 9 mM for Zn^{2+} and > 12 mM for Fe^{2+} and Mn^{2+} for our 1 % alginate). When this solution was mixed in equal volume with a 1 % alginate solution containing 20 mM Ca-EDTA, a stable gel was formed within a few seconds (see **ESI Fig 1** and **Table 2**). This observation was also repeated when a Ca-EDTA containing alginate solution was gelled upon addition of EI or when aqueous Ca-EDTA was added to EI-alginate solutions at concentrations below the critical gel concentration of the EI, but above that of Ca^{2+} (measured to be > 3mM for 1 % alginate). We note that for many applications, gelation by combining two polymer solutions rather than by the addition of a diluting aqueous gelling agent is advantageous since working solutions with increased polymer concentrations are problematic due to the power law dependence of viscosity as a function of concentration.[21] This has particular implications for applications involving polymer flows in narrow channels, such as microfluidics, 3D printing and delivery through a needle.

Hydrogels formed using this approach gel rather rapidly, and are also not fully cross-linked since the maximum concentration of CI available for crosslinking is limited by the concentration of the EI (see **Table 2**). To increase the concentration of EI, a second chelator was considered that would need a higher affinity for the EI than the alginate, but also a lower affinity to the EI than the crosslinking ion chelator (CIC), so that the reaction could still proceed. We term this second chelator the exchange ion chelator (EIC). The overall CLEX mechanism is described diagrammatically in **Fig 1.**

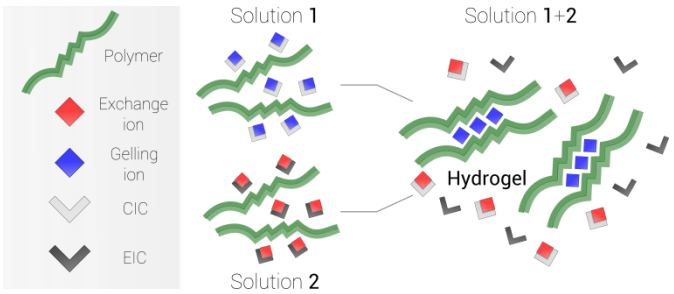

Figure 1: Schematic illustration of the competitive ligand exchange crosslinking (CLEX) mechanism, inspired by the egg-box model often used to describe alginate gelation. A preferred example is illustrated where a similar ionotropic polymer is present both in the aqueous crosslinking ion chelator (CIC) solution (solution 1) and the aqueous exchange ion chelator (EIC) solution (solution 2). Upon mixing the two solutions, the exchange ion displaces the gelling ion which is rendered free to crosslink the polymer.

Table 1: Log K values of selected cations and ligands at 25°C, background electrolyte concentration (μ) of 0.1M and equilibrium quotient of [ML]/[M][L], extracted from [20] unless otherwise indicated.

Ion	Alginate	EDTA	EDDA	EGTA	EDDS	PDTA	CDTA	TRIS	Glycine	Tricine
Ca^{2+}	~4.0[22]	10.6	2.9[a]	10.5	4.6[a]	11.55	13.1	0.25[a]	2.39[a23]	2.4[a24]
Fe^{2+}	<<2.27[b]	14.3	8.63	11.8	-	15.5	18.9	-	4.3[25]	-
Mn^{2+}	<<2.27[b]	13.9	7.0	12.2	8.6	15.0	17.5	-	3.41[23]	2.7[24]
Zn^{2+}	<2.27[b]	16	11.1	12	13.4	17.5	19.3	2.27	3.04[23]	5.59[26]

[a] The strength of this complex is weaker than or similar to Ca-alginate, therefore gelation will likely occur if this cation-ligand combination occurs in combination with alginate at pH 7. [b]At the time of writing we are unaware of any reliable published data, therefore these values represent an estimate of the magnitude of Log K based on our experimental observations and the well established affinity series of alginate to bivalent cations.

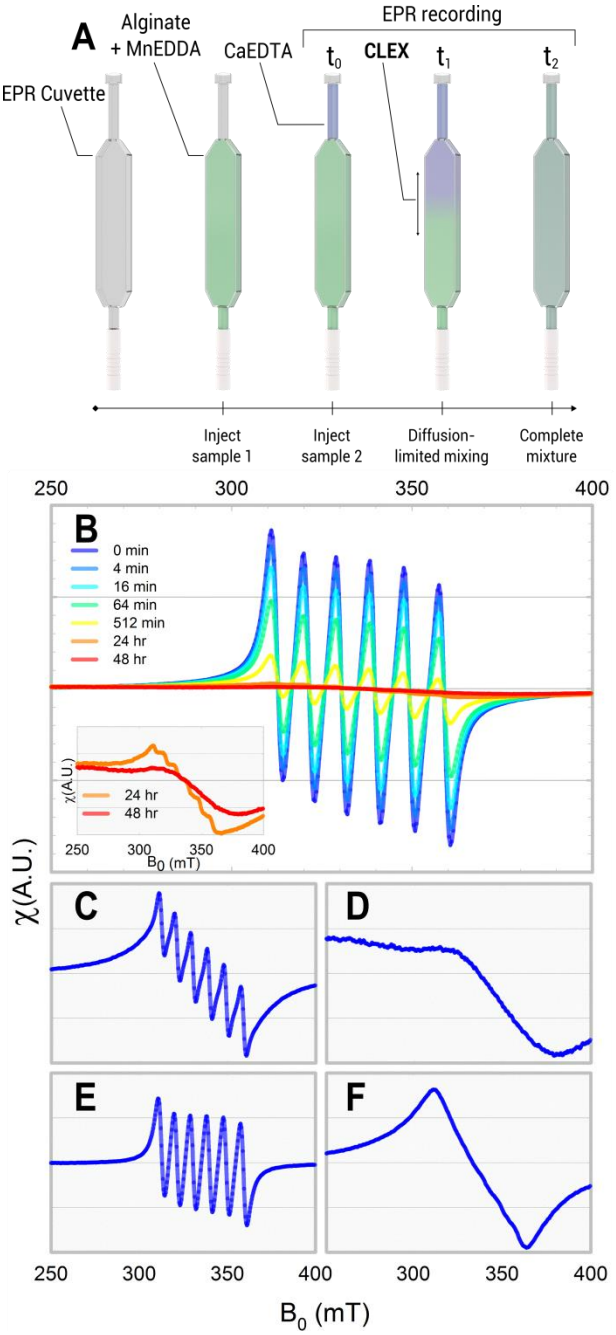

Figure 2: Characterisation of the CLEX mechanism using EPR. **A:** Diagram showing the design of EPR measurements. A Mn-EDDA alginate was injected into an EPR cuvette to completely fill the resonance cavity, then a Ca-EDTA solution was injected into the remaining volume of the tube (t_0). CLEX was initiated at the interface of the two solutions and the gelled region grew with time (t_1) **B:** Evolution of the Mn^{2+} EPR spectra at indicated timepoints, inset shows spectral detail at 24 and 48hr. All relevant ligand environments of Mn^{2+} are shown in control spectra **C:** Mn-EDDA, **D:** Mn-EDTA, **E:** Mn-alginate. **F:** High molar ratio of EDDA to Mn^{2+} (20:1).

The gelling experiment described above was improved by using Ethylenediamine-N,N'-diacetic acid (EDDA) as an EIC, since at pH 7 it has a low affinity for Ca^{2+} (Log K = 2.9[20]) which is less than alginate (estimated to be approximately Log K = 4.0[22]). Using an EIC, a higher concentration of EI (e.g. Zn^{2+}, Mn^{2+} or Fe^{2+}) could now be applied in solution with alginate without forming a gel. Concentrations several factors greater than that required to theoretically fully crosslink our 1 % alginate samples could now be made; exact concentrations were dependant on the EI-EIC combination and pH. Upon mixing an alginate solution containing Zn-EDDA as the EI-EIC with an alginate solution containing Ca-EDTA as the CI-CIC of excess Ca^{2+} concentration to fully crosslink the total amount of alginate (30 mM) and at pH 7, a strong gel was formed (**Table 2**). Crosslinking occurs due to the relative affinities between the individual inorganic ions, chelators and alginate (see **Table 1**). In this example the relative affinities (log K values in parentheses) between the ions and ligands are:

Ca^{2+}: EDTA (10.6) > Alginate (~4.0) > EDDA (2.9)

Zn^{2+}: EDTA (16.0) > EDDA (11.1) > Alginate (<2.27)

Therefore Zn^{2+} is unable to crosslink alginate in the presence of either chelator, and Ca^{2+} is unable to crosslink alginate in the presence of EDTA alone. However Ca^{2+} may crosslink alginate in the presence of EDDA alone or in the presence of both Zn^{2+} and EDTA.

To experimentally verify the CLEX mechanism, Electron Paramagnetic Spectroscopy (EPR) was used to monitor the reaction between Ca-EDTA and Mn-EDDA in the presence of alginate (**Fig 2**). In the geometry used for EPR data collection, CLEX was initiated at the interface of the two solutions within the quartz EPR cuvette and the gelled region grew with time, thus the diffusion of reactants was gradually retarded as the gelled region grew, substantially slowing diffusion of chelated ions between the liquid phases (**Fig 2A**). This allowed the reaction to be monitored over a long timescale (up to 48 hours for the reaction to complete) and characterise changes in the ligand environment of Mn^{2+} that were otherwise too rapid to observe clearly in the absence of alginate or by mixing prior to analysis. By comparing the time series recorded in **Fig 2B** it is evident that the experimental spectra evolved from resembling the control spectra recorded for Mn-EDDA (**2C**), to that of Mn-EDTA (**2D**) and not to that of Mn-alginate (**2E**). This observation verified that Mn^{2+} was exchanged from EDDA to EDTA and was not associated with crosslinking the alginate gel

(See also **ESI Fig 2** and **ESI Table 1**). Since Mn^{2+} was not associated with alginate, we verify that the crosslinking of alginate was a consequence of liberated Ca^{2+} and not free or released Mn^{2+} from the ionic exchange events. As well as Zn^{2+}, Fe^{2+} and Mn^{2+}, this approach should also be possible to perform using other ions which also have a lower binding affinity than the target CI for the polymer of interest, such as Co^{2+} and Ni^{2+} with Ca^{2+} as the CI for alginate, however these were not tested in our experiments due to toxicity issues which may negate their suitability for biomedical applications. For further discussion of the EPR results, please see the ESI.

As well as strictly controlling the amount of CI available, use of an EIC also allows for precise pH control of the crosslinking reaction. Solutions of free (i.e. unchelated) EI such as Zn^{2+}, Fe^{2+} and Mn^{2+} tend to be acidic and will likely precipitate hydroxide salts upon an increase in pH. However this is perturbed when the ion is chelated, which allows the alginate solution to be buffered at a higher desired pH. Also the rate and extent of the CLEX reaction, which in turn determines the kinetics of gel formation, may be tuned since the relative affinities between the ions, chelators and alginate vary as a function of pH. To investigate this we performed a series of gelation experiments under controlled conditions of pH using additional buffers (**Fig 3**).

Fig 3A shows the kinetics of gel formation, as measured by rheology, of CLEX formed gels with precisely defined pH in the neutral to alkaline range. For each reactant combination and given pH, the point of gel formation was defined by the time-point at which G'=G'', and measured gelation times are given in **Table 1**. At pH 7.0 the alginate achieved a plateau storage modulus of 731 ± 58 Pa which was less than that attained by fully crosslinked control samples made by the CaEDTA/GDL method (1015 ± 7 Pa, **Fig 3B**), therefore full crosslinking was not achieved. As the pH increased to pH 7.4 and 8.0 the ultimate storage modulus reduced to 574 ± 115 and 52 ± 2 Pa respectively, indicating that crosslinking decreased further with increasing pH. This apparent restriction in the availability of the CI is likely due to the EIC competing with the alginate for Ca^{2+} as equilibrium between the two is approached. Increasing the pH from 7.4 to 8.0 also resulted in a lengthened time to gelation from 28 to 328 s

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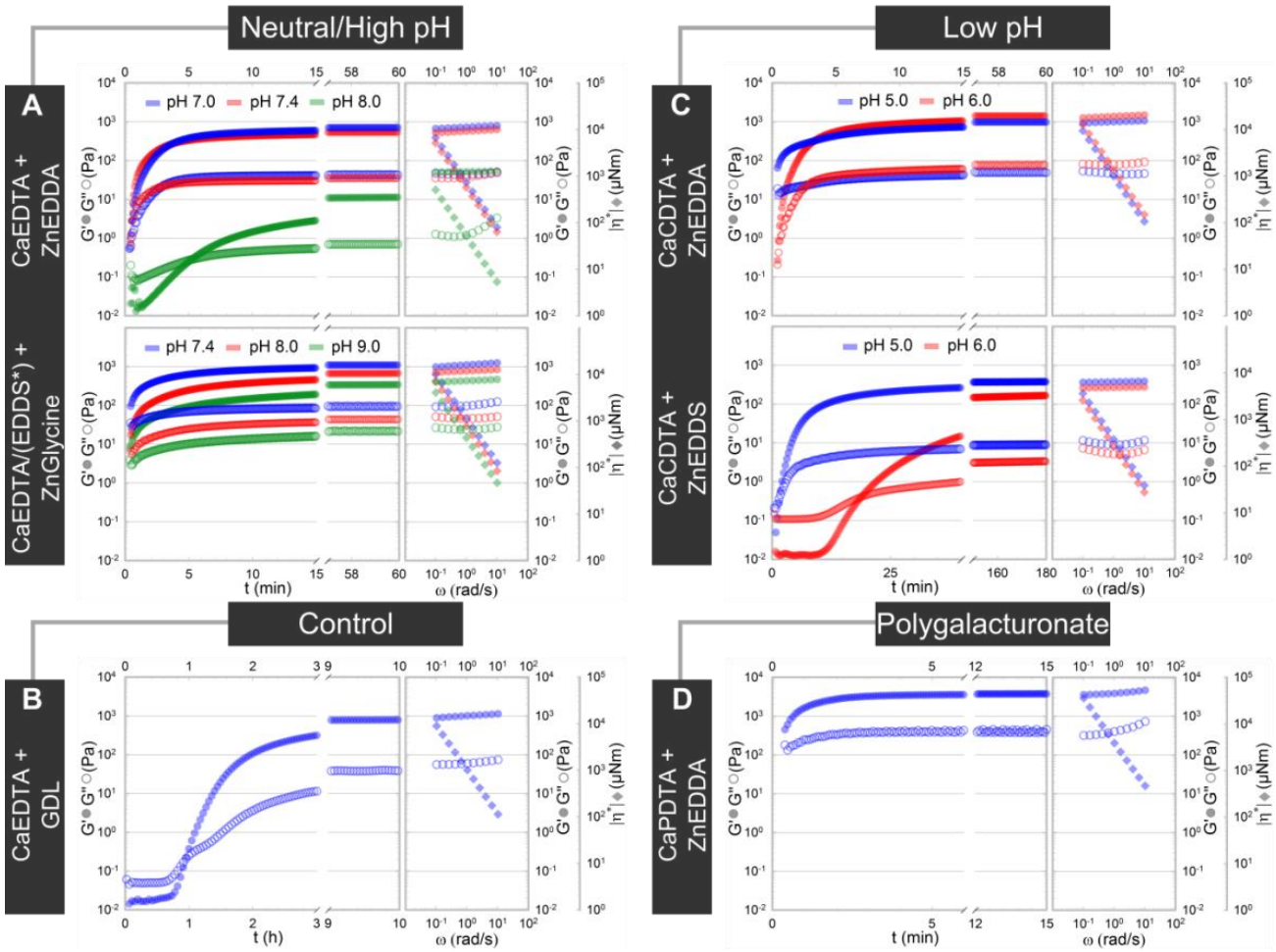

Figure 3: Rheological characterisation of the temporal change in mechanical properties and frequency dependence of 1% alginate and polygalacturonate hydrogels. **A**: Alginate gels formed by CLEX at neutral to alkaline pH using EDTA or EDDS as the CIC and EDDA or glycine as the EIC as indicated. **B**: Alginate gels formed by Ca-EDTA - GDL hydrolysis. **C**: Alginate gels formed by CLEX at acidic pH using CDTA as the CIC and EDDA or EDDS as the EIC as indicated. **D**: Poly(galacturonate) gel formed by CLEX. The near frequency independence of G' and G''/G' >10 after completion of the CLEX reaction indicates the dominance of the elastic character in the resulting gels. The absolute value of complex viscosity, $|\eta^*|$ is inversely proportional to oscillation frequency, ω, also reflecting these rheological features of the gels.

Table 2: Summary of gel strengths and gelling times for 1% hydrogels made by CLEX at the indicated conditions as measured by rheology. Final Ca^{2+} concentration = 30mM.

Solution 1: Polymer [CI] (CIC)	Solution 2: Polymer [EI] (EIC)	pH	Gel strength (Pa)	Gel time (s)
Alg [Ca^{2+}] (EDTA)	Alg [Zn^{2+}][a]	7.0	18.4 ± 2.3	NM[b]
Alg [Ca^{2+}] (EDTA)	Alg [Fe^{2+}][a]	7.0	212 ± 11	2.7 ± 1.8
Alg [Ca^{2+}] (EDTA)	Alg [Mn^{2+}][a]	7.0	227 ± 7	NM[b]
Alg [Ca^{2+}] (EDTA)	Alg [Zn^{2+}] (EDDA)	7.0	731 ± 58	23.4 ± 0.4
Alg [Ca^{2+}] (EDTA)	Alg [Zn^{2+}] (EDDA)	7.4	575 ± 115	28.4 ± 1.5
Alg [Ca^{2+}] (EDTA)	Alg [Zn^{2+}] (EDDA)	8.0	52.3 ± 2.1	328 ± 60
Alg [Ca^{2+}] (EDTA)	Alg [Zn^{2+}] (Glycine)[c]	7.4	1124 ± 203	5.2 ± 2.8
Alg [Ca^{2+}] (EDTA)	Alg [Zn^{2+}] (Glycine)[c]	8.0	787 ± 120	13.3 ± 2.1
Alg [Ca^{2+}] (EDDS)	Alg [Zn^{2+}] (Glycine)[c]	9.0	441 ± 134	8.4 +/- 3.6
Alg [Ca^{2+}] (CDTA)	Alg [Zn^{2+}] (EDDA)	5.0	1002 ± 167	10.3 ± 5.9
Alg [Ca^{2+}] (CDTA)	Alg [Zn^{2+}] (EDDA)	6.0	1385 ± 92	44.7 ± 4.9
Alg [Ca^{2+}] (CDTA)	Alg [Zn^{2+}] (EDDS)	5.0	381 ± 13	99.2 ± 11.8
Alg [Ca^{2+}] (CDTA)	Alg [Zn^{2+}] (EDDS)	6.0	273 ± 24	1161 ± 118
Alg [Ca^{2+}] (EDTA)	GDL[d]	7.0 – 4.5	1015 ± 7	3420 ± 226
PolyG [Ca^{2+}] (PDTA)	PolyG [Zn^{2+}] (EDDA)	7.0	3870 ± 170	17.8 ± 0.1

[a]Concentrations of unchelated EI were the maximum that could be added without initiating gelation, (i.e. Zn^{2+} = 9mM, Mn^{2+}, Fe^{2+}= 12mM), [b]Not measurable as gelation occurred before accurate measurements could be obtained, [c]Glycine concentration was 120mM, [d]GDL concentration was 180mM

By way of visual demonstration of the effect of pH, comparisons of alginate gelation behaviours at two different pH values for a CLEX reactant combination of Ca-EDTA and Zn-EDDA and conventional ionic gelling using $CaCl_{2(aq)}$ are shown in **ESI videos 1-3**.

By selecting an alternative EIC, with weaker binding to Ca^{2+}, the equilibrium could be shifted to enable gelling at a higher range of pH. Several chelators were found to be effective in this regard including TES, TRIS and tricine. However, glycine was found to be particularly useful since it was possible to chelate Zn^{2+} ions up to pH 11 without precipitation. Gels formed using glycine as the EIC at pH 7.4 and 8.0 gelled quickly (5 and 13 s respectively) and appeared to be fully crosslinked at pH 7.4 (plateau storage modulus of (1124 **±** 203). At pH 8.0, the final storage modulus achieved was 787 ± 120, indicating incomplete crosslinking. Although uncrosslinked, stable EI solutions of alginate and Zn-glycine could be formed in the pH range of 6.5 - 11, gelling with CaEDTA was only achievable up to pH 8.5. By substituting EDTA with EDDS as the CIC, which has a lower affinity to Ca^{2+}, gels could be formed at higher pH. Increasing the pH resulted in a further loss in strength, indicating the approach of another equilibria with the EIC (glycine) and alginate.

Under acidic conditions (pH < 6), EDTA is unsuitable as a CIC for Ca^{2+} with alginate since binding of the ion is too weak compared to the polymer and crosslinking occurs. Therefore a stronger chelator, such as 1,2-cyclohexanedinitrilotetraacetic acid (CDTA), is required. Strong, fully crosslinked gels were formed at pH 5 and 6 using a combination of Ca-CDTA and Zn-EDDA (**Fig 3C & Table 2**).

By applying EDDS, this time as the EIC in combination with CDTA as the CIC, partial gelation could be achieved at pH 5 and 6 and gelation times were greatly extended compared to samples made using EDDA as the EIC (**Fig 3C & Table 2**). When CLEX was applied to form hydrogels of poly(galacturonate) at neutral pH, a CIC with a strong affinity to Ca^{2+} (propylenediamine-N,N,N',N'-tetraacetic acid (PDTA)) was also necessary (**Fig 3D**). This is because poly(galacturonate) has a higher affinity for Ca^{2+} than alginate, (Log K = c.4.5-5.4[27]).

One particularly interesting application of CLEX is for microfluidic based cell encapsulation. Existing gelation strategies typically rely on pH triggered release rendering free ions for crosslinking. Although homogenous gelation is obtainable with these strategies, the pH reduction is often highly detrimental to cells[28], and gelation in stagnation points of the microchannels due to rapid gelling kinetics or use of solid components in the aqueous phases often results in clogging over time.[8] CLEX was achieved in a microfluidic device by using dual aqueous flow devices to encapsulate cells in either droplets or fibres of alginate (**Fig 4A**). Reliable device operation and excellent cell viability was noted post encapsulation for both conditions, which is highly encouraging for the application of CLEX to encapsulate cells. A more rigorous analysis of the application of CLEX to the microencapsulation of both eukaryotic and prokaryotic cells is described in detail elsewhere.[29]

By way of demonstration, CLEX was also applied to form larger scale bulk gels by casting (**Fig 4B**) into an injection mould using a dual syringe to deliver equal volumes of alginate solution. A homogenous, bubble free gel was obtained.

To evaluate potential toxicity, component chemicals involved in CLEX at physiological pH were assessed and found to be non-toxic to cells following an exposure duration (2 hours) and chemical concentrations relevant to those used in cell encapsulation experiments (**Fig 4C**). This is a significant finding, since free Zn^{2+} ions are known to be acutely toxic to mammalian cells in the nanomolar range.[30] Speciation is important when considering the toxic effects of inorganic ions and serum albumin is mainly responsible for binding physiologically occurring Zn^{2+}, with Log K values reported in the range of 6.1–7.5.[31] It has been recently shown that in the

presence of albumin in cell culture, the toxicity of Zn^{2+} was reduced by several orders of magnitude.[32] In our experiments, high concentrations of chelated Zn^{2+} were well tolerated by the cells, which indicates that the chelators were effective in preventing free Zn^{2+} exposure to the cells in sufficient concentrations to cause toxic effects.

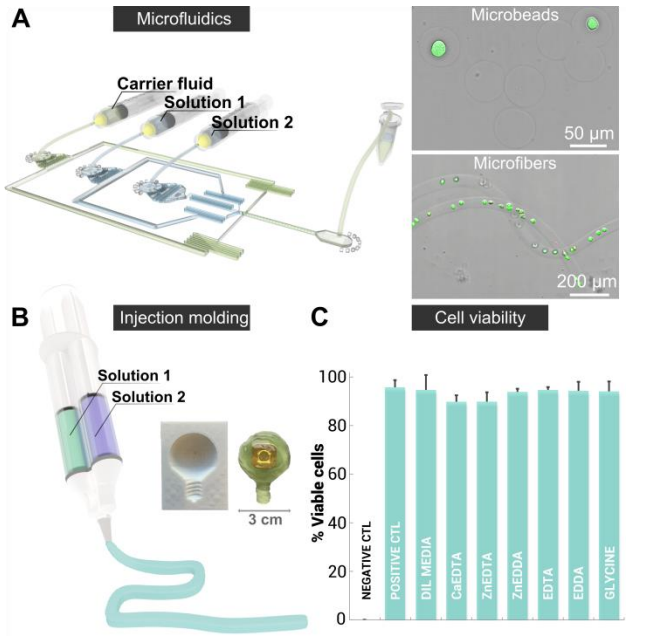

Figure 4: **A**: Demonstration of CLEX applied in microfluidic channels to encapsulate cells. Here murine pre-osteoblast cells were encapsulated in alginate hydrogel in the form of beads and fibres using dual aqueous flow microfluidic devices. Encapsulated cells have been stained using Calcien –AM (live, green) and Ethidium homodimer (dead, red). **B**: CLEX used to cast a replica model following extrusion though a two component syringe. **C**: Cell viability of murine pre-osteoblast cells as determined by toluidine blue infiltration following 2hr exposure to 60mM concentration of the indicated CLEX components in cell media. No significant difference ($P < 0.05$) in cell viability was observed for all experimental groups tested compared to positive (untreated) control cells. n=3, one way ANOVA with Holm-Sidak post hoc test.

Conclusions

In summary, we have developed a new approach to control the release of gelling ions that now enables previously challenging applications of hydrogels to be realised. This gelation strategy allows hitherto unattainable extents of control over the kinetics of ionotropic hydrogel formation over a wide range of pH. We believe this approach will have far reaching impact, particularly for cell encapsulation and biomedical applications such as injectable tissue fillers and 3D printed tissue scaffolds.

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