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REVIEW

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Preparation of alginate hydrogel microparticles by gelation introducing cross-linkers using droplet-based microfluidics: a review of methods

Cheng Zhang, Romain Grossier, Nadine Candoni and Stéphane Veessler*

Abstract

This review examines the preparation of alginate hydrogel microparticles by using droplet-based microfluidics, a technique widely employed for its ease of use and excellent control of physicochemical properties, with narrow size distribution. The gelation of alginate is realized “on-chip” and/or “off-chip”, depending on where cross-linkers are introduced. Various strategies are described and compared. Microparticle properties such as size, shape, concentration, stability and mechanical properties are discussed. Finally, we consider future perspectives for the preparation of hydrogel microparticles and their potential applications.

Keywords: Alginate, Hydrogel, Microparticle, Crosslinking, Droplet-based microfluidics

Introduction

Hydrogel microparticles are widely used today, especially in biological and pharmaceutical applications. They are usually used as a matrix to encapsulate bioactive agents such as drugs, proteins, cells, etc. [2, 12, 30] in applications like drug delivery [1], cell culture and tissue engineering [49]. In addition, fluorescence-encoded hydrogel microparticles are extensively employed in multiplex bioassays [42, 62, 64]. Another important use is as cell-mimicking microparticles with similar size, shape, deformability and mechanical properties [18, 32, 59]. Hydrogels can be made of various biopolymers such as gelatine, agarose, alginate, pectin, etc. Alginate stands out because of its low cost, non-toxicity and ease of crosslinking [25].

With the increasing interest in alginate hydrogel microparticles, various preparation methods have been reported in the literature [27], including conventional

emulsification [8], spray-drying [40], extrusion dripping [4, 24], microfluidics [39, 56, 60] and soft lithography [36]. The huge diversity of techniques and strategies can make it confusing to choose the right method. The present review focuses on a microparticle-producing technique widely used for its efficacy in controlling physicochemical properties: droplet-based microfluidics. After basic recalls on alginate chemistry and droplet-based microfluidics, the various strategies applied within this technique and the properties of the microparticles obtained are described in this review. We expect this paper useful for researchers who want to know what is possible to do with droplet-based microfluidics for the preparation of alginate hydrogel microparticles by introducing cross-linkers.

Alginate hydrogel microparticles

Hydrogels are described as hydrophilic polymeric networks which can absorb and retain large amounts of water within the structure. The hydrogel network is formed by polymer crosslinking. When crosslinking is

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realized by molecular entanglement, ionic, H-bonding or hydrophobic forces, hydrogels are called physical or reversible gels. Otherwise, when covalent forces intervene, they are called chemical or permanent gels [6, 19].

Alginate

Alginate is a natural polysaccharide. Although it can also be synthesized by several bacteria, all the commercially available alginate is produced from the extraction of brown algae [11]. Alginate is widely used in the biomedical field because it is biocompatible and non-toxic [25].

Sodium alginate (Na-alginate) is the most widely used alginate salt. It dissolves in water to a viscous solution. Alginate is a linear copolymer containing β -D-mannuronate (M) and α -L-guluronate (G) residues (Fig. 1).

Gelation of alginate

Alginate hydrogel is produced by gelation which is caused by covalent [14] or ionic crosslinking [15, 51]. Ionic crosslinking is more commonly used because of its simplicity and mild conditions. It can be carried out at room temperature or up to 100 °C, usually with divalent cations as cross-linkers. Calcium chloride is the most widely used [25], due to its non-toxicity [1] and availability.

Only G-blocks (Fig. 1a) made of consecutive G residues can participate in ionic crosslinking because of their favorable spatial structure [15, 25]. Ionic crosslinking of alginate is described by the “egg-box” model [16] (Fig. 2).

In this review, we present various two-step methods of producing alginate hydrogel microparticles. First, sodium alginate droplets are generated using droplet-based

microfluidics. Second, internal or external gelation transforms droplets into alginate hydrogel microparticles via different strategies. It should be noted that only ionic crosslinking is discussed herein. In internal gelation, the cross-linkers are inside the alginate droplet whereas in external gelation, the cross-linkers come from outside the alginate droplet.

Droplet-based microfluidics

Principle of droplet generation

Microfluidics is a technique used to manipulate fluids in channels of micrometric dimensions. Fluids are mixed by adding junctions that connect the channels. When immiscible or partially miscible fluids are mixed in the junction, microdroplets can be generated: this is called droplet-based microfluidics.

The principle is similar to that of conventional emulsification, which consists of blending two immiscible liquids. The advantage of droplet-based microfluidics is monodispersity and repeatability of droplets due to precise control over experimental conditions such as channel geometry, flow rates and viscosities of fluids, etc. [41, 46]. Furthermore, monodisperse droplets can be generated without using surfactant [28, 48, 59], which is impossible with conventional emulsification.

The droplets generated in droplet-based microfluidics can serve as microreactors to carry out physical, chemical or biological reactions [65]. Being small (nL to μ L volume), they require a small quantity of reactants. As droplet composition can be made identical, numerous identical experiments can be performed, enabling a reliable statistical approach to data.

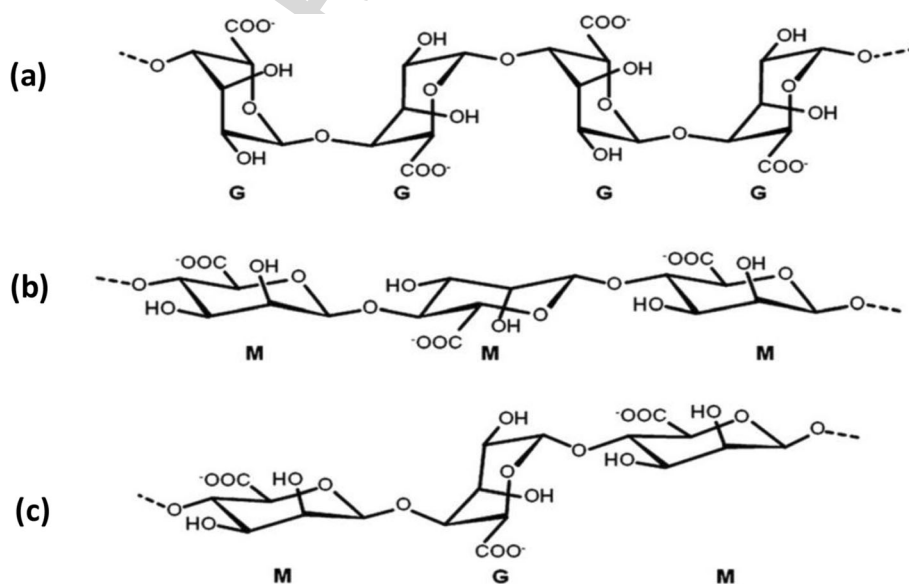


Fig. 1 Chemical structures of **a** G-block, **b** M-block and **c** alternating G and M-blocks in alginate. Figure reprinted with permission from Reference [25]

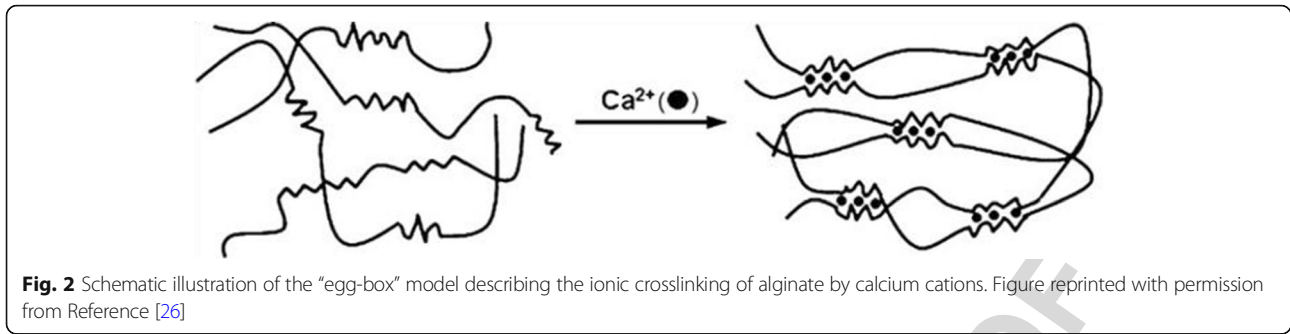


Fig. 2 Schematic illustration of the “egg-box” model describing the ionic crosslinking of alginate by calcium cations. Figure reprinted with permission from Reference [26]

Flow properties

In droplet-based microfluidics, a continuous fluid and a dispersed fluid are injected separately and then mixed in a junction. Fluids are Newtonian and droplets of the dispersed fluid (D) are generated in the flow of the continuous fluid (C). The physicochemical properties influencing droplet formation are density, dynamic viscosity, surface tension between the continuous and the dispersed fluids, velocity of the flows and characteristic dimensions of the microfluidic system, such as the diameter of channels (w) for cylindrical microfluidic systems. Based on these properties, fluid dynamics is characterized as follows:

- 1- Inertial forces and viscous forces are compared through the Reynolds number, calculated using the continuous fluid properties: density (ρ_C), dynamic viscosity (μ_C) and flow velocity (v_C).

$$Re = \frac{\rho_C \times v_C \times w}{\mu_C} \quad (1)$$

Typically for microfluidics, values of Re are lower than 1: the flow is laminar and the effect of inertia can be ignored. Thus, the average velocity v of a flow is evaluated from its volumetric flow rate Q and w as follows:

$$v = \frac{Q}{\pi(w/2)^2} \quad (2)$$

- 2- The generation of droplets in a microfluidic junction creates a free interface between the two fluids, characterized by the interfacial energy γ_{CD} . The corresponding capillary effects are in competition with gravity effects. The length above which gravity effects dominate capillary effects is the capillary length l_c :

$$l_c = \sqrt{\frac{\gamma_{CD}}{\Delta\rho \times g}} \quad (3)$$

with g the gravity acceleration and $\Delta\rho$ the difference in density between the two fluids. For instance, with fluorinated oil FC70 as the continuous fluid and ethanol as the dispersed fluid, l_c is equal to 2.4 mm [63]. Hence gravity does not influence the deformation of the interfaces in millimetric or sub-millimetric channels.

- 3- Shear stress and interfacial energy are compared through the capillary number Ca . When generating droplets of a dispersed fluid in a continuous fluid, Ca is usually calculated using v_C and μ_C of the continuous fluid, and γ_{CD} of the interface between the continuous and the dispersed fluid:

$$Ca = \frac{\mu_C \times v_C}{\gamma_{CD}} \quad (4)$$

Microfluidic geometry

Microfluidic devices can be in the form of either chips with microchannels and junctions produced by soft lithography, or an assembly of capillaries and junctions [37]. In terms of materials, polydimethylsiloxane (PDMS) is the most commonly used for microfluidic chips [28, 60]. For capillaries, both glass [5, 20] and fluoropolymer can be used [48, 59].

The channel geometry of a microfluidic device influences droplet generation. Three frequently used geometries are “cross-flow”, “co-flow” and “flow-focusing” (Fig. 3).

Cross-flowing

For cross-flowing geometry, continuous fluid and dispersed fluid mix with an angle θ ($0^\circ < \theta \leq 180^\circ$) at the

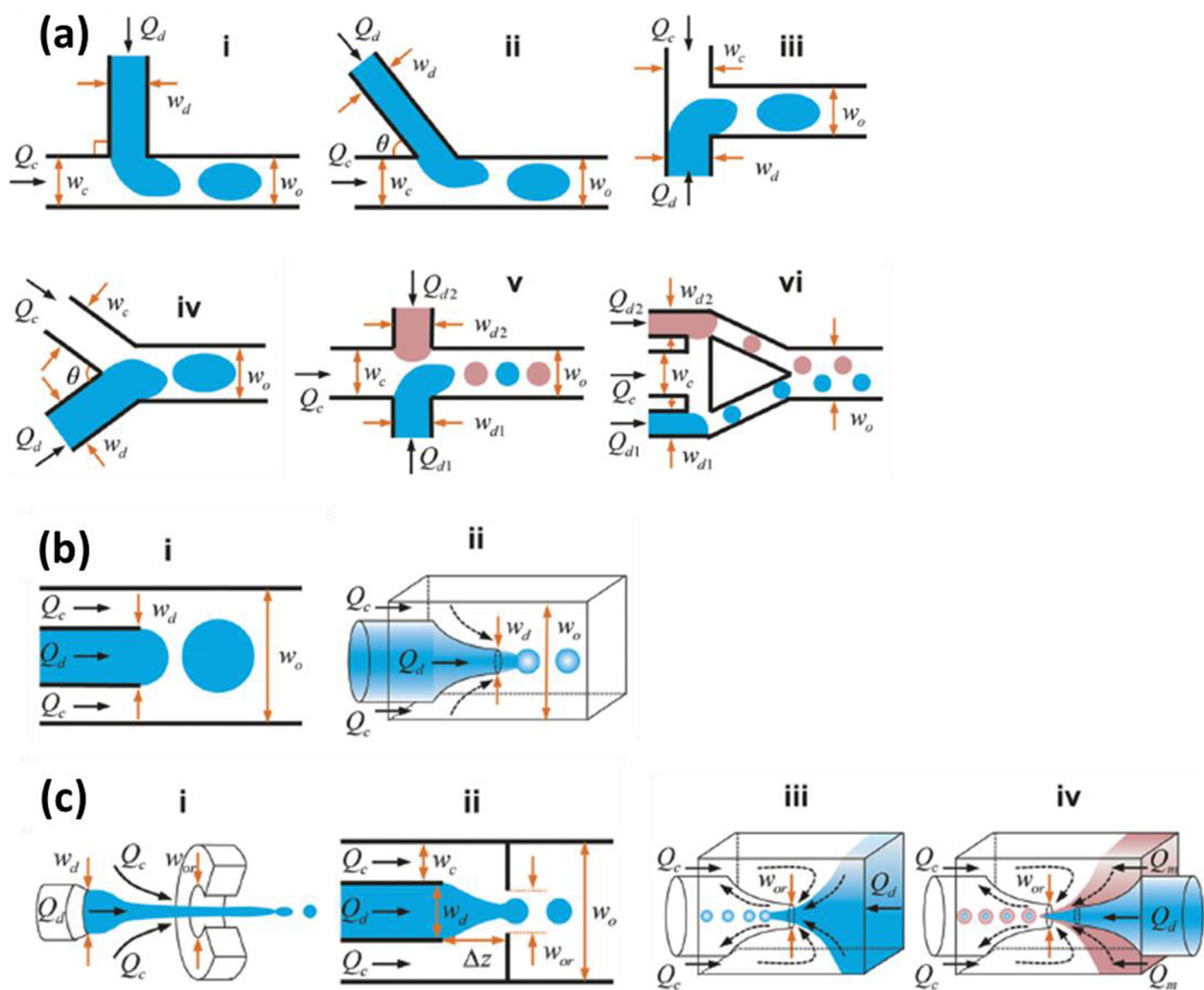


Fig. 3 Schematic illustrations **a** “cross-flowing”, **b** “co-flow” and **c** “flow-focusing” geometries for a microfluidic device. Q and w denote respectively flow rate and channel width. Subscripts d , c , o and or denote respectively dispersed fluid, continuous fluid, outlet channel and orifice. Figure reprinted with permission from Reference [65]

junction (Fig. 3a). Where the two fluids meet, first an interface is formed due to the immiscibility of the two fluids. Shear force then pushes the head of the dispersed fluid into the continuous fluid until a part breaks off: the droplet is formed. Then it circulates in the channel of the continuous fluid [46].

Cross-flowing geometry is often called T-junction geometry, where two fluids flow orthogonally (Fig. 3a i). However, other shapes of junctions can also be used, such as a junction with an arbitrary angle θ (Fig. 3a ii), or a Y-shaped junction (Fig. 3a iv). For two fluids facing each other ($\theta = 180^\circ$, Fig. 3a iii), the geometry is called “head-on”. A combination of two junctions (Fig. 3a v, vi) can also be used to introduce two different dispersed fluids and one continuous fluid. Cross-flowing geometry is widely used due to its ease of assembly and handling [41, 65].

Flow-focusing

For co-flow geometry, two immiscible fluids flow in two concentric channels (Fig. 3b). Droplets are formed at the outlet of the inner channel. Flow-focusing geometry is actually similar to co-flow geometry. The distinction presented in the literature is somewhat ambiguous [65], leading some to consider flow-focusing as a special co-flow geometry [41]. For flow-focusing geometry (Fig. 3c), two immiscible fluids are focused through an orifice, which allows smaller droplets to be generated than with co-flow geometry.

Droplet generation regime

For each geometry, droplets can be generated following three different break-off mechanisms. The transition from one mechanism to another can be achieved by varying capillary

F4 204 numbers Ca [65]. Figure 4 shows an example of three mech-
205 anisms for a cross-flowing geometry.

206 **Squeezing**

207 As Fig. 4a shows, as it is injected into the principal chan-
208 nel, the dispersed fluid is pushed forward by the con-
209 tinuous fluid. A thin “neck” is thus formed. Because the
210 continuous fluid applies weak shear force, the forming
211 droplet reaches the opposing channel wall without
212 breaking off. The neck becomes thinner until it breaks,
213 so that a plug-shaped droplet confined by channel wall
214 is formed. Squeezing mechanism appears when Ca is
215 low ($Ca \leq 0.01$) [10].

216 **Dripping**

217 As Fig. 4b shows, the shear force applied is now higher.
218 The forming droplet breaks off before touching the op-
219 posing channel wall. A spherical droplet is formed with
220 a diameter smaller than that of the channel. This drip-
221 ping mechanism appears at a higher Ca ($Ca \geq 0.02$) [10].

222 **Jetting**

223 As Fig. 4c shows, a liquid jet is emitted from the dis-
224 persed fluid channel. It flows and remains attached to
225 the channel wall, due to a strong shear force from the
226 continuous fluid [9]. The jet breaks up into droplets at
227 the end because of Rayleigh-Plateau instability [65].
228 Droplets of polydisperse sizes are formed. This jetting
229 mechanism appears at the highest Ca ($Ca \approx 0.2$).

Gelation

Internal gelation

For internal gelation, cross-linkers come from inside the
alginate droplets and are either soluble or insoluble/
slightly soluble in water. In this approach, cross-linkers
are always introduced in the microfluidic device.

Water-soluble cross-linkers

With water-soluble cross-linkers such as barium chlor-
ide ($BaCl_2$) and calcium chloride ($CaCl_2$), alginate is
crosslinked directly at the interior of droplets. These
agents can be mixed with Na-alginate before or after
droplet generation.

Mixing cross-linkers before droplet generation A first
category of strategies is based on mixing water-soluble
cross-linkers with Na-alginate before droplet generation.
The cross-linkers used in these studies are $BaCl_2$ and
 $CaCl_2$; these strategies are summarized in Table 1.

Trivedi et al. worked on cell encapsulation by alginate
hydrogel microparticles [48]. For the preparation of mi-
croparticles, an aqueous solution of cell-containing Na-
alginate (1%) and a solution of $BaCl_2$ (50 mM) were
injected into the capillary and mixed via a Y-shaped
junction. At the exit from the mixing region, highly vis-
cous silicone oil (10 cSt) without surfactant was injected
by flow-focusing in order to generate droplets. However,
the mixing of Na-alginate and barium cations triggered
ionic crosslinking, causing gelation in the mixing region
which impacted droplet generation. Finally, instead of
generating droplets as expected, a jet of gel was

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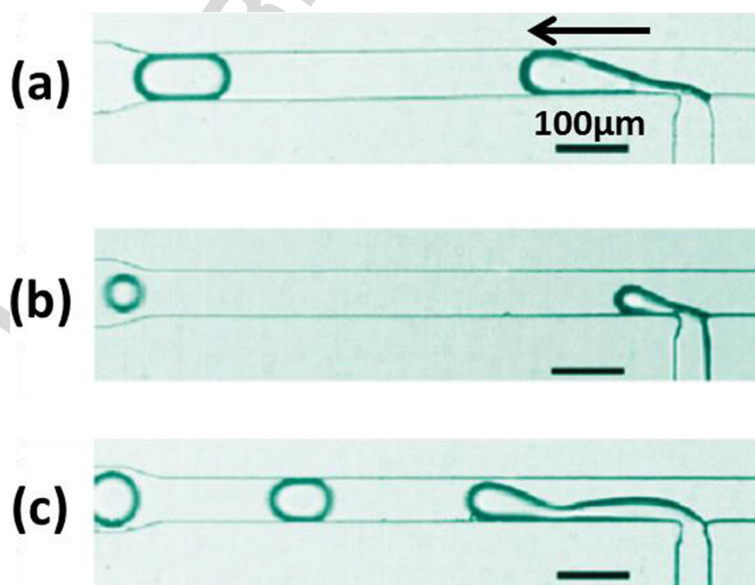


Fig. 4 Three break-off mechanisms of droplet generation with a cross-flowing geometry: **a** squeezing, **b** dripping and **c** jetting. The arrow indicates the droplet flow direction. Figure reprinted with permission from Reference [57]. Copyright 2010 American Chemical Society

Table 1 Internal gelation with water-soluble cross-linkers: mixing cross-linkers with Na-alginate before droplet generation

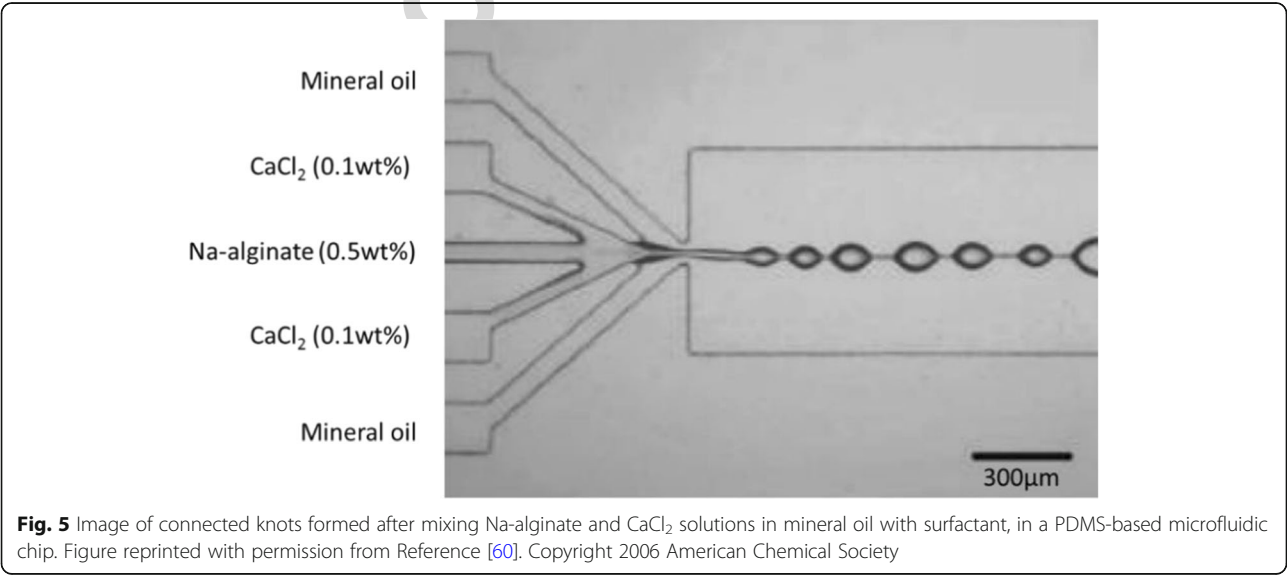
References		Trivedi et al., 2009 [48]	Zhang et al., 2006 [60]	Rondeau and Cooper-White, 2008 [39]	Present review	Present review
Droplet generation	Concentration of Na-alginate	1 wt%	0.5 wt%	0.5 wt%	0.06 wt%	0.006 wt%
	Continuous fluid	Silicone oil	Mineral oil	DMC ^a	DMC ^a	DMC ^a
	Use of surfactant	NO	Span 80	Not mentioned	NO	NO
	Geometry	Flow-focusing	Flow-focusing	Flow-focusing	Cross-flowing	Cross-flowing
	Microfluidic material	Fluoropolymer tubing and junctions	PDMS chip	PDMS chip	Fluoropolymer tubing and junctions	Fluoropolymer tubing and junctions
Internal gelation by mixing Na-alginate and water-soluble cross-linker	Cross-linker	BaCl ₂ (50 mM)	CaCl ₂ (0.1 wt%)	CaCl ₂ (0.25 wt%)	CaCl ₂ (0.06 wt%)	CaCl ₂ (0.002 wt%)
	Geometry	Flow-focusing	Cross-flowing	Flow-focusing	–	Cross-flowing
	Mixing	Before droplet generation	During droplet generation	Before droplet generation	Off-line, before droplet generation	During droplet generation

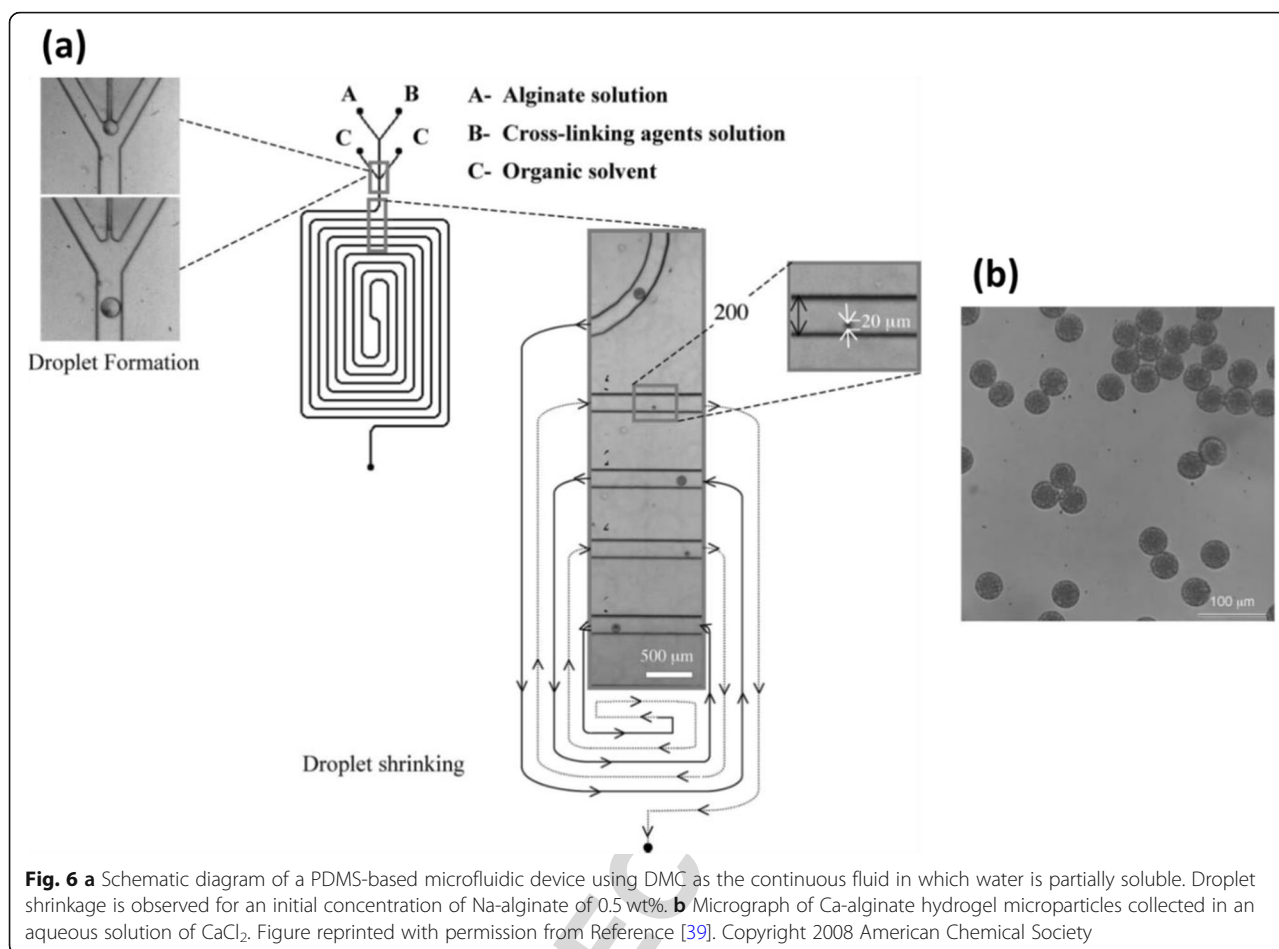
^aContinuous and dispersed fluids partially miscible - DMC (Dimethyl Carbonate)

produced with a partially formed droplet head and a long gelatinous tail.

To deal with this issue, the mixing region can be reduced before droplet generation, as Zhang et al. did [60]. Using a 5-channel microfluidic device, they mixed Na-alginate fluid (0.5 wt%), CaCl₂ fluids (0.1 wt%) and mineral oil fluids with a surfactant (Span 80, no concentration mentioned) as shown in Fig. 5. Droplets were generated by co-flow. However, instead of producing discrete droplets, a line of knots connected with each other was formed. This phenomenon persisted with a wide range of flow rates of oil due to viscosity which increased instantly when Na-alginate and CaCl₂ were mixed, because of rapid gelation. It was therefore impossible to generate droplets at the junction, despite the use of surfactant.

The problem can be solved by using low concentrations of Na-alginate and CaCl₂ solutions. In this case, gelation proceeds after droplet generation and is enhanced by using partially miscible fluids. Rondeau and Cooper-White used Dimethyl carbonate (DMC) as the continuous fluid [39] (Fig. 6). The solubility of water in DMC is about 3 wt% at room temperature [43]. Aqueous solutions of Na-alginate (0.5 wt%) and CaCl₂ (0.25 wt%) were injected respectively from inlets A and B (Fig. 6a). After a short pre-gelation channel, DMC was injected from inlet C. Na-alginate/CaCl₂ droplets were generated in DMC (no mention of surfactant usage) by flow-focusing. Along the serpentine channel, because of the low solubility of water in DMC, water diffused gradually from droplets into DMC, causing the shrinkage of droplets along the channel. Internal gelation occurred at the





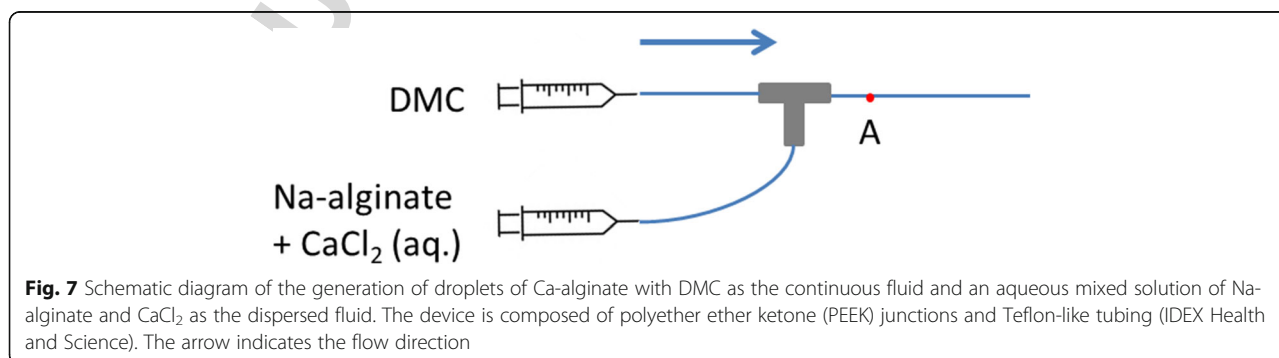
f6.1
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f6.3
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291 same time. Microparticles with a diameter of 20 μm were
292 observed at the outlet of the channel and collected in an
293 aqueous solution of CaCl_2 (2 N) to reinforce the gelation
294 (Fig. 6b). The diameter of Ca-alginate hydrogel micro-
295 particles was influenced by the experimental parameters
296 such as the initial concentration of Na-alginate, flow
297 rates of fluids and channel size. To be precise, smaller
298 Ca-alginate hydrogel microparticles can be obtained by
299 using a less concentrated Na-alginate solution, a higher
300 flow rate ratio between the continuous fluid and the

dispersed fluid, or a narrower channel. However, DMC 301
is also slightly soluble in water, with a solubility of 12.7 302
wt% at 20 $^{\circ}\text{C}$ [43]. Thus, during diffusion of water from 303
droplets into DMC, DMC can also diffuse into droplets. 304
This means that, after gelation, DMC can be captured 305
inside alginate hydrogel microparticles. Additional work 306
measuring the amount of DMC residue within micropar- 307
ticles could open the way to further applications. 308

Following the work of Rondeau and Cooper-White, we 309
tested, in a T-junction (Fig. 7), the direct generation of 310

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droplets of Ca-alginate in DMC without surfactant from a mixture of more diluted Na-alginate and CaCl_2 solutions (both at 0.06 wt% after mixing). However, this solution was not clear and local gelation was occasionally observed with the naked eye. When these gels entered the channel, droplets were generated in a discontinuous way. This indicates that, even at very low concentrations, thorough mixing of Na-alginate and CaCl_2 solutions leads to gelation, disturbing droplet generation.

F8 In a microfluidic device (Fig. 8) of similar design to Zhang et al., we were able to generate discrete droplets by using extremely diluted solutions of Na-alginate (0.006 wt%) and CaCl_2 (0.002 wt%). The continuous fluid was DMC without surfactant. Droplets were observed after the cross-junction (point A in Fig. 8a). Since they were relatively close to each other in the channel, causing coalescence at the outlet (point B in Fig. 8a), a second flow of DMC was introduced as a spacer using a T-junction. When the second DMC flow rate was relatively low, the generation of droplets upstream was not disturbed, so that droplets were uniform (Fig. 8b). However, the coalescence at the outlet persisted. Thus, high second DMC flow rates were applied to sufficiently increase the distance between droplets. Nevertheless, this quickly disturbed the generation of droplets upstream, as indicated by heterogeneities in droplet size and frequency (Fig. 8c). Using surfactant would prevent droplet coalescence.

To summarize (Table 1), authors mixed highly concentrated solutions of Na-alginate and water-soluble

cross-linkers before droplet generation to make them gelate. However, droplet generation was hindered by rapid gelation and microparticles were difficult to obtain. To delay gelation, less concentrated solutions of Na-alginate and water-soluble cross-linkers were mixed before droplet generation, in the microfluidic device or offline. Then, concentrations were increased after droplet generation by diffusion of water from the droplets to the continuous fluid, due to their partial miscibility with water. Hence, gelation proceeded slowly with droplet shrinkage. However, mixing cross-linkers with Na-alginate before droplet generation led to heterogeneities in droplet size and frequency.

Mixing cross-linkers after droplet generation To delay gelation, water-soluble cross-linkers need to be mixed with Na-alginate only after droplet generation. Studies doing so, and which also use BaCl_2 and CaCl_2 as cross-linkers, are summarized in Table 2.

Xu et al. prevented rapid gelation by delaying the direct contact between Na-alginate and calcium cations [54]. In a first cross-junction, two face-to-face channels were used to introduce CaCl_2 (2 wt%) and Na-alginate (2 wt%) solutions (Fig. 9a) perpendicularly to a flow of water. Thus, after the first cross-junction, a flow of water (acting as a buffer) separates the flows of Na-alginate and CaCl_2 . Then octyl alcohol oil (no mention of surfactant) was injected at a second cross-junction. Droplets of Na-alginate/ CaCl_2 were generated by flow-focusing. In the “synthesizing channel” (Fig. 9a), within each droplet,

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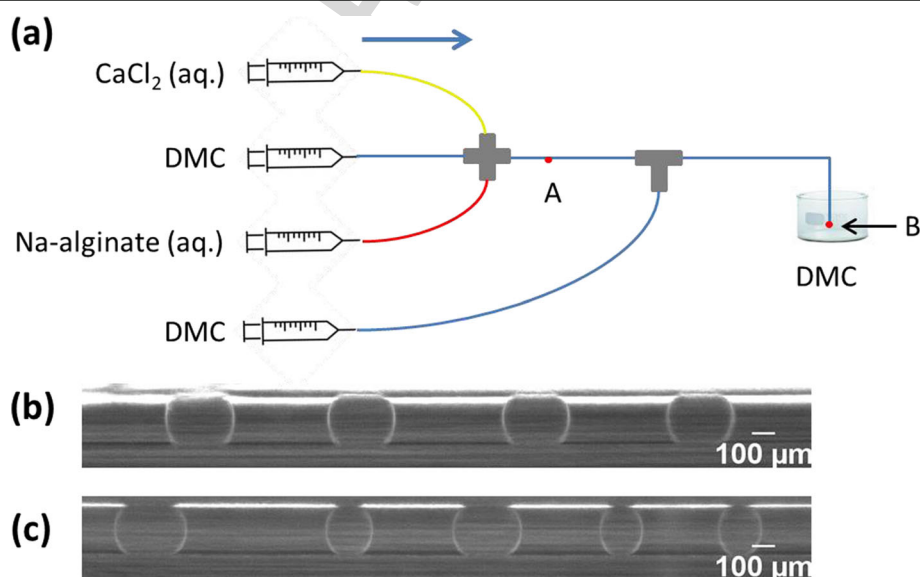


Fig. 8 a Schematic diagram of the generation of Ca-alginate droplets in DMC using a cross-junction. The T-junction served to introduce DMC as a spacer to increase the distance between droplets. The device is composed of polyether ether ketone (PEEK) junctions and Teflon-like tubing (IDEX Health and Science). Micrograph of droplets observed at point A when **b** droplet generation was not disturbed by introducing the spacer and **c** when it was disturbed

Table 2 Internal gelation with water-soluble cross-linkers: mixing cross-linkers with Na-alginate after droplet generation

Reference		Xu et al., 2008 [54]	Liu et al., 2006 [28]	Trivedi et al., 2010 [47]
Droplet generation	Concentration of Na-alginate	2 wt%	2 wt%	1 wt%
	Continuous fluid	octyl alcohol oil	soybean oil	silicone oil
	Use of surfactant	Not mentioned	NO	NO
	Geometry	Flow-focusing	Flow-focusing	Flow-focusing
	Microfluidic material	PMMA chip	PDMS chip	Fluoropolymer tubing and junctions
Internal gelation by mixing Na-alginate and water-soluble cross-linker	Cross-linkers	CaCl ₂ (2 wt%)	CaCl ₂ (2 wt%)	BaCl ₂ (50 mM)
	Geometry	Cross-flowing	Expansion chamber	Cross-flowing
	Mixing	Coalescence of flows	Coalescence of droplets	Coalescence of droplets with flow

mixing Na-alginate and CaCl₂ induced internal gelation. In this way droplets were transformed into Ca-alginate hydrogel microparticles (Fig. 9b). For this strategy, the size of Ca-alginate hydrogel microparticles is entirely dependent on the experimental conditions, such as flow rates of fluids and channel size. Manipulation of microparticles is difficult if their diameter is smaller than 10 μm.

Another strategy to delay gelation was carried out by Liu et al. [28] involving coalescence of Na-alginate droplets with CaCl₂ droplets generated separately. First, on a microfluidic chip (Fig. 10a), Na-alginate (2 wt%) droplets (Fig. 10b) and CaCl₂ (2 wt%) droplets (Fig. 10c) were generated in soybean oil without surfactant by flow-focusing using two independent cross-junctions. Then droplets converged via a T-junction (Fig. 10d) followed by two successive circular expansion chambers (Fig. 10d, e). Thus, droplets could collide either at the T-junction

or in circular chambers. Within the coalesced droplets, Na-alginate was crosslinked by calcium cations forming Ca-alginate hydrogel microparticles. With different flow rates and channel geometries, various shapes and sizes of microparticles could be produced (Fig. 10f). Nevertheless, the design of circular expansion chambers gives rise to local changes in flow velocity. Droplet circulation can be disturbed, thereby affecting homogeneity in droplet shape, size and frequency.

Droplets could also be coalesced by exploiting physicochemical parameters between the continuous fluid and the dispersed fluid. In the work of Trivedi et al., droplets of Na-alginate (1 wt%) containing cells were generated upstream in a highly viscous silicone oil (10 centistoke) by flow-focusing without surfactant [47, 48]. An aqueous solution of BaCl₂ (50 mM) was injected downstream by a T-junction. With the help of dye, observations at the T-junction indicated that BaCl₂ fluid

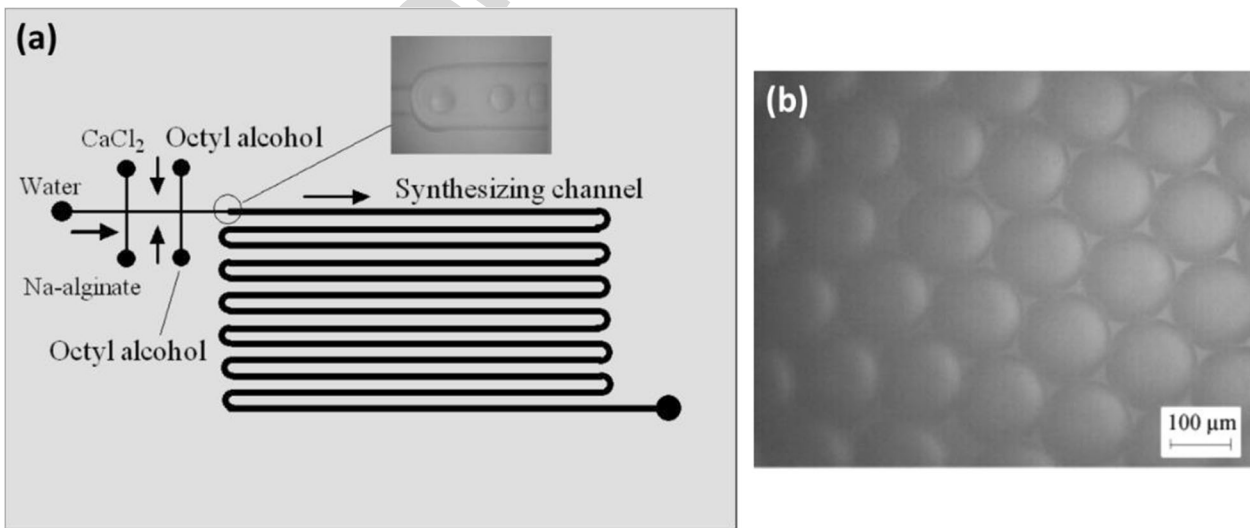


Fig. 9 **a** Schematic diagram of Ca-alginate hydrogel microparticles prepared in a PMMA based microfluidic device. **b** Micrographs of Ca-alginate hydrogel microparticles. Figure reprinted with permission from Reference [54]

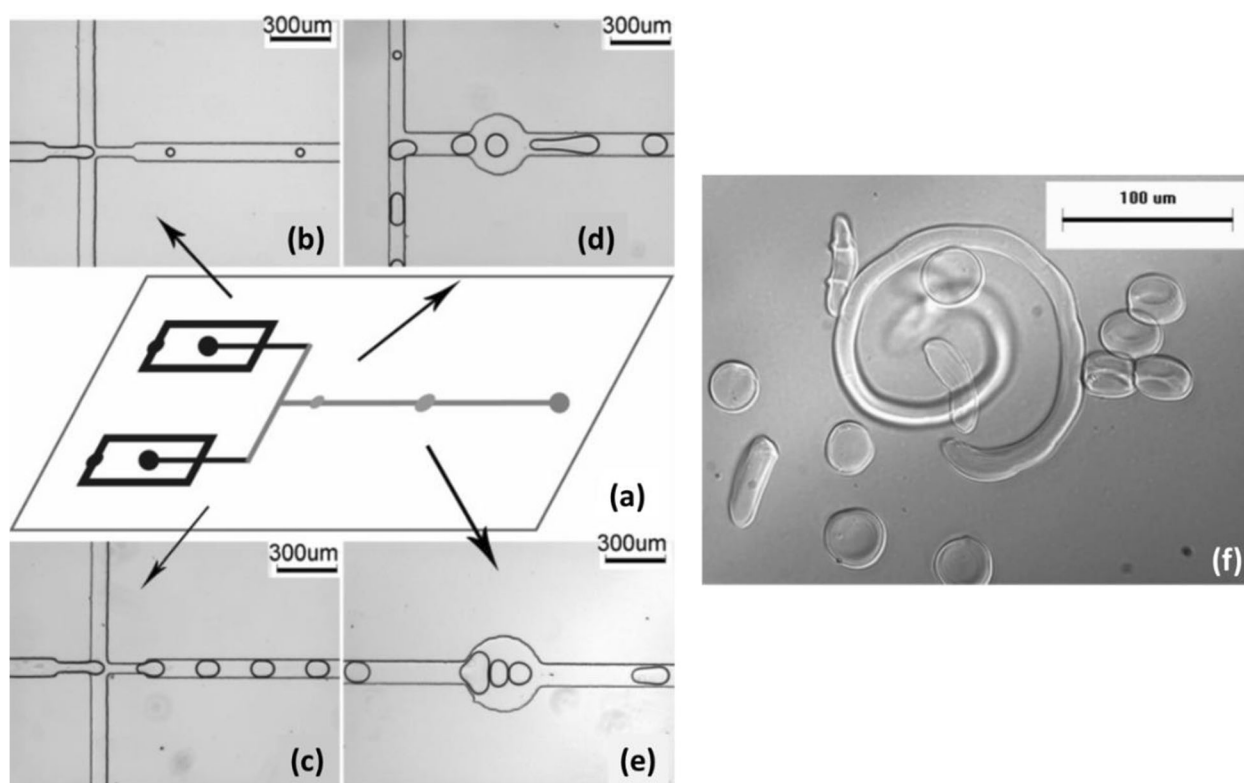


Fig. 10 **a** Schematic diagram of the PDMS-based microfluidic device. **b** Flow-focusing channel to generate alginate droplets. **c** Flow-focusing channel to generate CaCl_2 droplets. **d** T-junction followed by a first circular expansion chamber. **e** A second circular expansion chamber. **f** Ca-alginate hydrogel microparticles of different shapes and sizes. Figure reprinted with permission from Reference [28]. Copyright 2006 American Chemical Society

merged spontaneously with Na-alginate/cells droplets, instead of forming independent BaCl_2 droplets. However, this strategy lacks flexibility. The expected coalescence happens only when appropriate solvents are used. For instance, when using low-viscosity and low-interfacial energy γ_{CD} soybean oil, independent droplets of BaCl_2 were observed. They coalesced downstream with Na-alginate/cells droplets. This implied that successful coalescence of droplets could only take place with appropriate interfacial energy and viscosity [48].

Water-insoluble or weakly soluble cross-linkers

To delay gelation, Na-alginate can be mixed with water-insoluble or weakly soluble cross-linkers, in water. This will not lead to instant gelation since there are no available cations in water. In the case of cross-linkers which are pH-sensitive, such as calcium carbonate (CaCO_3) and calcium-ethylenediaminetetraacetic acid (Ca-EDTA) complex, an acid is used in the continuous fluid to release the cations from inert cross-linkers. Therefore, gelation by the available cations happens after droplet generation. These strategies are summarized in Table 3.

In the work of Zhang et al. [61], fine particles of CaCO_3 (0.1 wt%) were dispersed in an aqueous solution

of Na-alginate (2 wt%). Soybean oil with a surfactant (Span 80, 3 wt%) and containing acetic acid (5 wt%) was used as the continuous fluid (Fig. 11a). Droplets of Na-alginate/ CaCO_3 were generated by flow-focusing in soybean oil/acetic acid (Fig. 11b). Droplets pH decreased because of the acetic acid in the oil. As a result, calcium cations were released from CaCO_3 , causing internal gelation of the alginate. Finally, Ca-alginate hydrogel microparticles were collected in oil (Fig. 11c). However, when collected on a substrate, they had a “pancake” shape and were soluble in aqueous solution owing to insufficient gelation. No improvement was observed from increasing the concentration of acetic acid or that of CaCO_3 . Moreover, a higher concentration of CaCO_3 particles would give rise to their aggregation in the channel [61]. The mechanical properties of the microparticles could not therefore be improved.

The same principle was also applied by Akbari and Pirbodaghi to prepare cell-encapsulating microparticles (Fig. 12) [3]. At a first T-junction, a fluid of Na-alginate (1.5 wt%) containing cells flowed into the middle channel (Fig. 12a), while the Na-alginate fluid (1.5 wt%) containing CaCO_3 nanoparticles (35 mM) was introduced by two side channels (Fig. 12b). This geometry was used to

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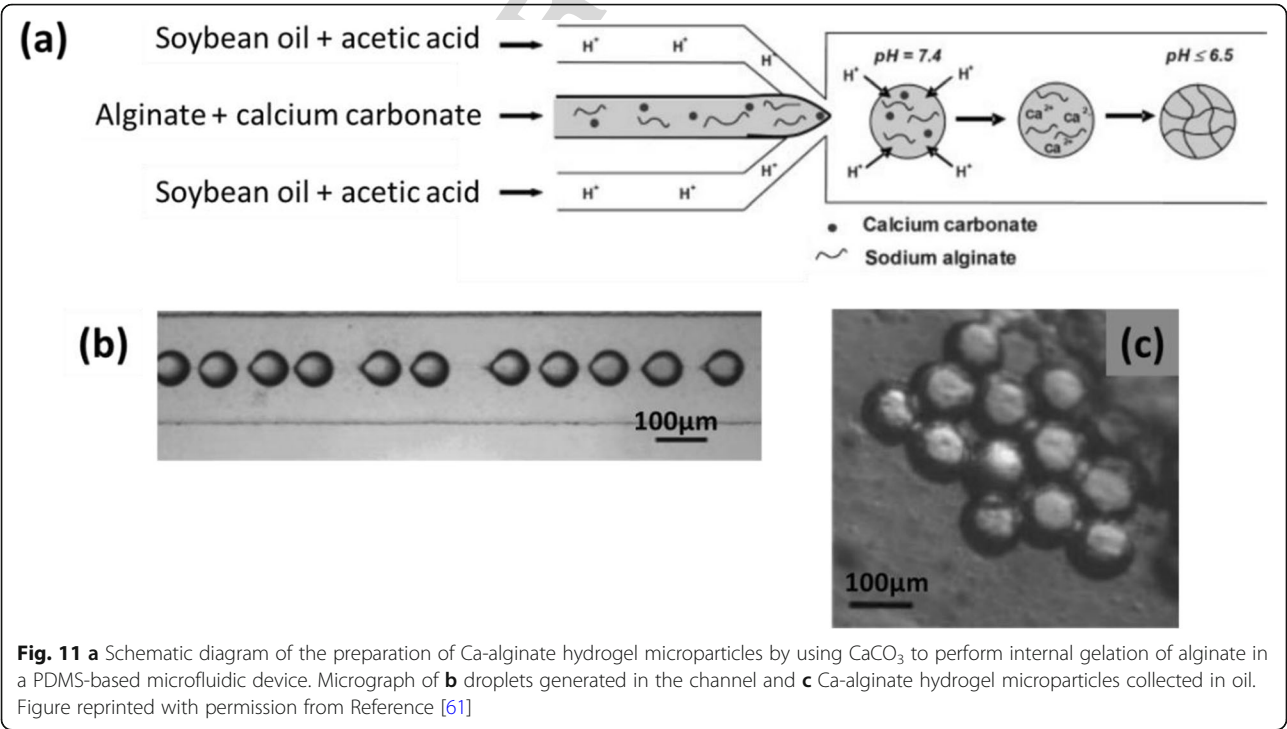
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Table 3 Internal gelation with water-insoluble or weakly soluble cross-linkers which are pH sensitive

References		Zhang et al., 2007 [61]	Akbari and Pirbodaghi, 2014 [3]	Yu et al., 2019 [56]	Utech et al., 2015 [49]
Droplet generation	Concentration of Na-alginate	2 wt%	1,5 wt%	2 w/v%	2 wt%
	Continuous fluid	Soybean oil	Fluorocarbon oil	Mineral oil	Fluorinated carbon oil
	Use of surfactant	Span 80	Fluorinated surfactant	Span 80	Biocompatible surfactant
	Geometry	Flow-focusing	Flow-focusing	Flow-focusing	Flow-focusing
	Microfluidic material	PDMS chip	PDMS chip	PDMS chip	PDMS chip
Internal gelation by mixing Na-alginate and water-insoluble or weakly soluble cross-linkers	Cross-linkers	CaCO ₃ (0,1 wt%)	CaCO ₃ (35 mM)	CaCO ₃ (200 mM)	Ca-EDTA (50 mM)
	Mixing	Off-line, before droplet generation	Before droplet generation	Off-line, before droplet generation	Off-line, before droplet generation
	Gelation by acid addition	In continuous fluid	In continuous fluid	In collecting fluid	In continuous fluid

create a coaxial stream while avoiding direct mechanical contact between cells and the potentially damaging CaCO₃ particles. At a second T-junction, fluorocarbon oil with surfactant (fluorinated surfactant, 1 wt%) was injected. Droplets of Na-alginate/cells/CaCO₃ were then generated by flow-focusing. After droplet collection, acetic acid (0.1 vol%) dissolved in oil was added to release calcium cations within droplets, causing gelation of alginate. Droplets were thus transformed into Ca-alginate hydrogel microparticles, some with cells encapsulated (Fig. 12c). However, the mixture of CaCO₃ and Na-alginate was not homogeneous, which can be seen

from Fig. 12b. Thus, the varying amounts of CaCO₃ influenced the degree of gelation in each droplet, yielding Ca-alginate hydrogel microparticles with different mechanical properties. This issue is not discussed by Akbari and Pirbodaghi [3]. Furthermore, not all microparticles encapsulated cells, for reasons not explored in the article. Sorting is therefore required after the preparation of microparticles, which complicates the procedure. Combining the strategy of Zhang et al. and Akbari and Pirbodaghi to conduct gelation both in the microfluidic device and in the collection bath, Yu et al. [56] produced Ca-alginate hydrogel microparticles for protein



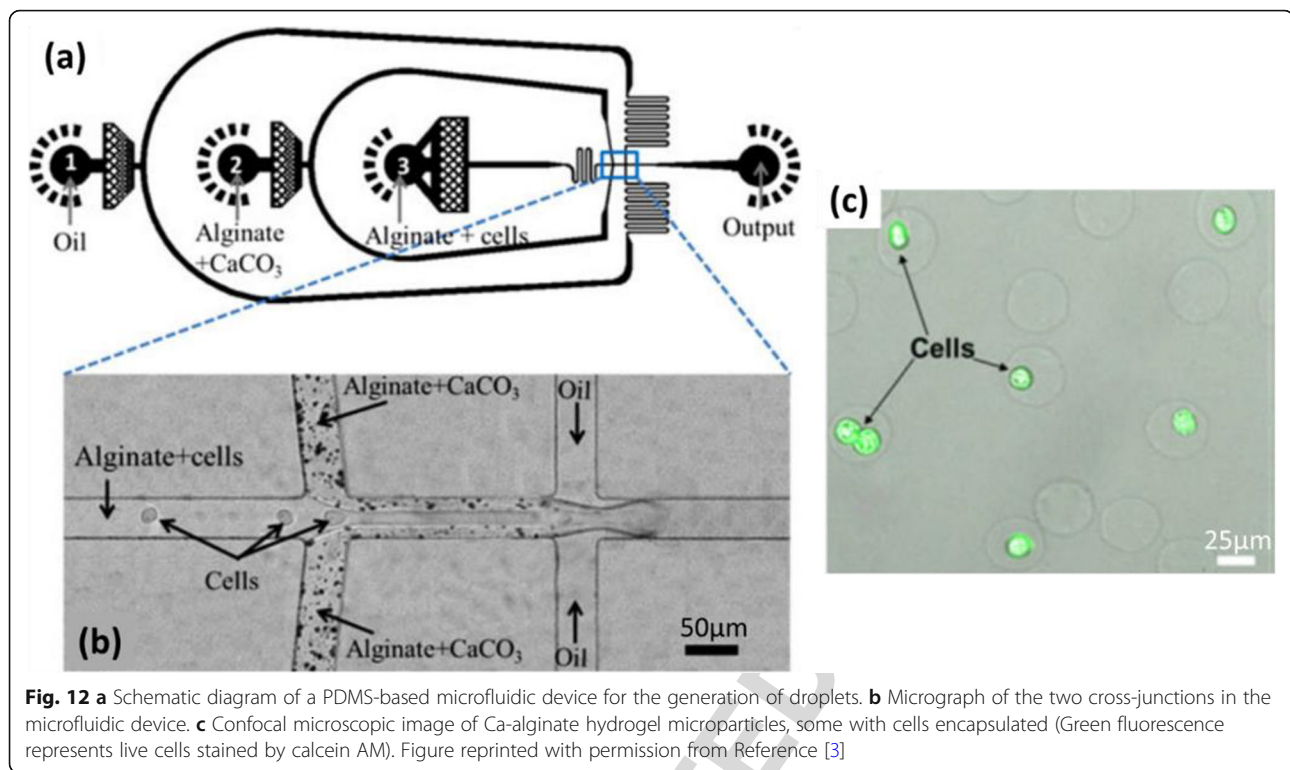


Fig. 12 **a** Schematic diagram of a PDMS-based microfluidic device for the generation of droplets. **b** Micrograph of the two cross-junctions in the microfluidic device. **c** Confocal microscopic image of Ca-alginate hydrogel microparticles, some with cells encapsulated (Green fluorescence represents live cells stained by calcein AM). Figure reprinted with permission from Reference [3]

encapsulation. First, from inlet 4 (Fig. 13a), an aqueous solution of antigen or protein was injected. It co-flowed with another aqueous solution of alginate (2 w/v%) mixed with CaCO_3 particles (200 mM) and injected from inlet 3. Mineral oil with Span 80 added was injected from inlet 2 as a continuous fluid. In the flow-focusing channel, droplets containing alginate, CaCO_3 and protein were formed. From inlet 1, another continuous fluid, mineral oil containing Span 80 and acetate acid, was introduced. When the acetate acid diffused into droplets, calcium cations were released. The alginate was then crosslinked, leading to preliminary gelation. The droplets were collected in an aqueous solution of CaCl_2 (0.27 M) to enhance gelation. In the end, spherical hydrogel microparticles were formed, with protein encapsulated (Fig. 13b-c). According to the authors, the preliminary gelation in the microchannel prevented the deformation that occurs when droplets are collected directly in an aqueous solution of CaCl_2 . As mentioned above, however, since CaCO_3 is not soluble in water, a high concentration of CaCO_3 will clog the microchannel. Thus, the scope for preliminary gelation is limited. Moreover, it takes time (in this case, overnight) to obtain a mixture where CaCO_3 particles are well dispersed.

In order to obtain a homogeneous internal structure of hydrogel microparticles, Utech et al. used a slightly water-soluble calcium-ethylenediaminetetraacetic acid (Ca-EDTA) complex as the cross-linker [49]. An aqueous solution of Na-alginate (2 wt%) mixed with Ca-

EDTA (50 mM) was first prepared. This homogeneous mixture was used as the dispersed fluid for the microfluidic system. The continuous fluid was a fluorinated carbon oil with a biocompatible surfactant (1 wt%) containing acetic acid (0.05 vol%). Droplets of Na-alginate/Ca-EDTA were generated in oil/acetic acid by flow-focusing (Fig. 14a). Due to the use of acetic acid, calcium cations were released from Ca-EDTA in each droplet (Fig. 14b), causing internal gelation of the alginate. The Ca-alginate hydrogel microparticles formed (Fig. 14c) had a homogeneous internal structure and were stable in an aqueous medium without dissolution. It should be noted that, the solubility of Ca-EDTA in water being low (0.26 M at 20 °C), the concentration of Ca-EDTA in the Na-alginate solution was limited in order to keep the solution homogeneous. Thus, this strategy is not appropriate when microparticles need to be highly crosslinked. Furthermore, care should be taken with Ca-EDTA, as EDTA is used to dissolve alginate hydrogel microparticles in the literature [29, 56].

In conclusion, internal gelation of alginate can be realized by using cross-linkers that are soluble or insoluble/slightly soluble in water. When water-soluble cross-linkers are used, the instant gelation disturbs droplet generation. The problem can be solved by using partially miscible fluids with limited mixing prior to droplet generation, and/or by using extremely diluted solutions and surfactant (Table 1). Mixing cross-linkers and Na-alginate after droplet generation involves merging

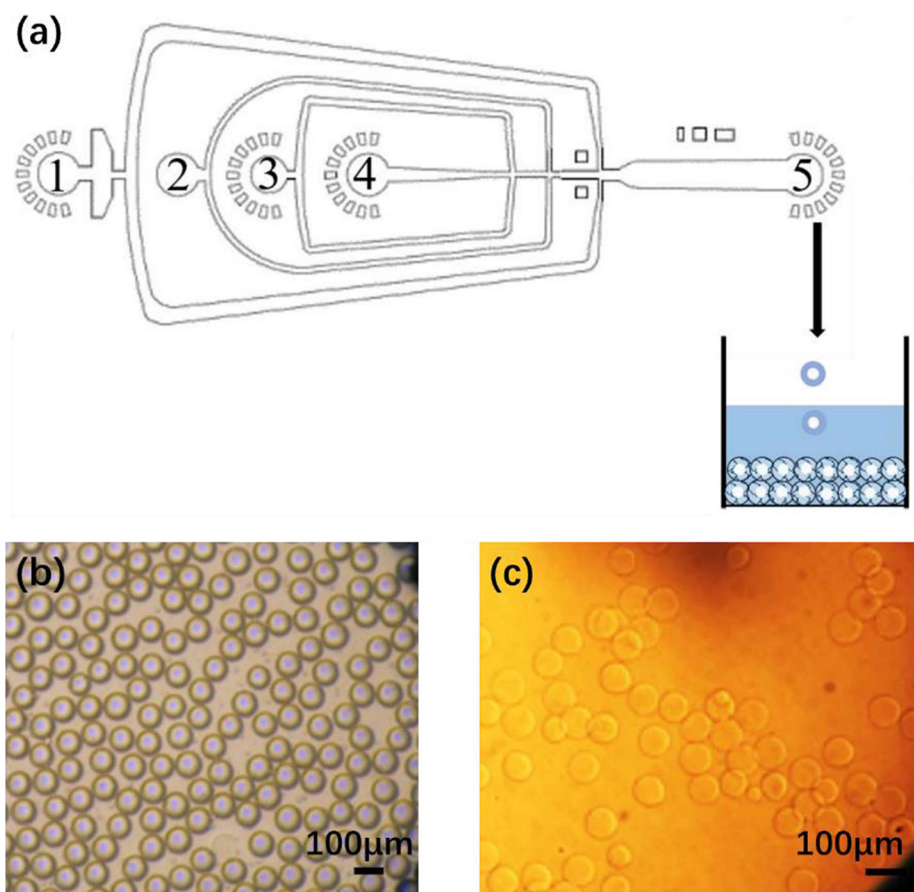


Fig. 13 **a** Schematic diagram of a PDMS-based microfluidic device for the preparation of antigen-core alginate-shell microparticles. Inlet 1: Mineral oil with 3 wt% Span 80 and 0.2 vol% acetic acid; Inlet 2: Mineral oil with 3 wt% Span 80; Inlet 3: 2 w/v% alginate solution containing 200 mM CaCO_3 ; Inlet 4: an antigen or protein aqueous solution. Micrographs of Ca-alginate hydrogel microparticles in **b** oil and **c** water. Figure reprinted with permission from Reference [56]

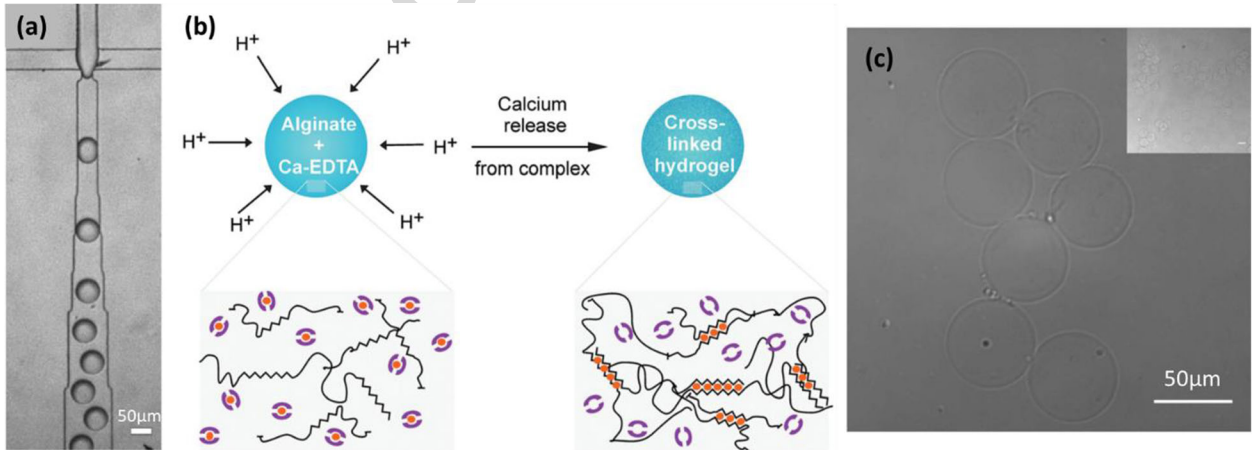


Fig. 14 **a** Micrograph of the T-junction in a microfluidic device, where droplets of Na-alginate/Ca-EDTA were generated in oil/acetic acid. **b** Schematic illustration of the crosslinking process in each droplet. **c** Micrograph of Ca-alginate hydrogel microparticles in an aqueous medium. Figure reprinted with permission from Reference [49]

droplets or flows of Na-alginate and water-soluble cross-linkers (Table 2). The resulting droplets are dependent on physicochemical properties like viscosity and interfacial energy. If water-insoluble/slightly soluble cross-linkers are used, they are mixed with alginate before droplet generation. For pH-sensitive cross-linkers, acid is then needed to release cations, after which internal gelation takes place (Table 3).

A homogeneous microparticle internal structure can be achieved by choosing appropriate cross-linkers. However, because of low solubility in water, it is important to limit the concentration of cross-linkers to avoid precipitates in the channel.

External gelation

In external gelation, cross-linkers come from outside the alginate droplets and are diffused into the alginate droplets or the microparticles formed, inducing crosslinking. Unlike internal gelation, in which cross-linkers are always introduced “on-chip” (in the microfluidic device), in external gelation, cross-linkers can be introduced both “on-chip” and/or “off-chip” (outside the microfluidic device).

On-chip introduction of cross-linkers

For external gelation, several authors introduced cross-linkers “on-chip”. They used calcium acetate ($\text{Ca}(\text{CH}_3\text{COO})_2$) or CaCl_2 as cross-linkers, as summarized in Table 4.

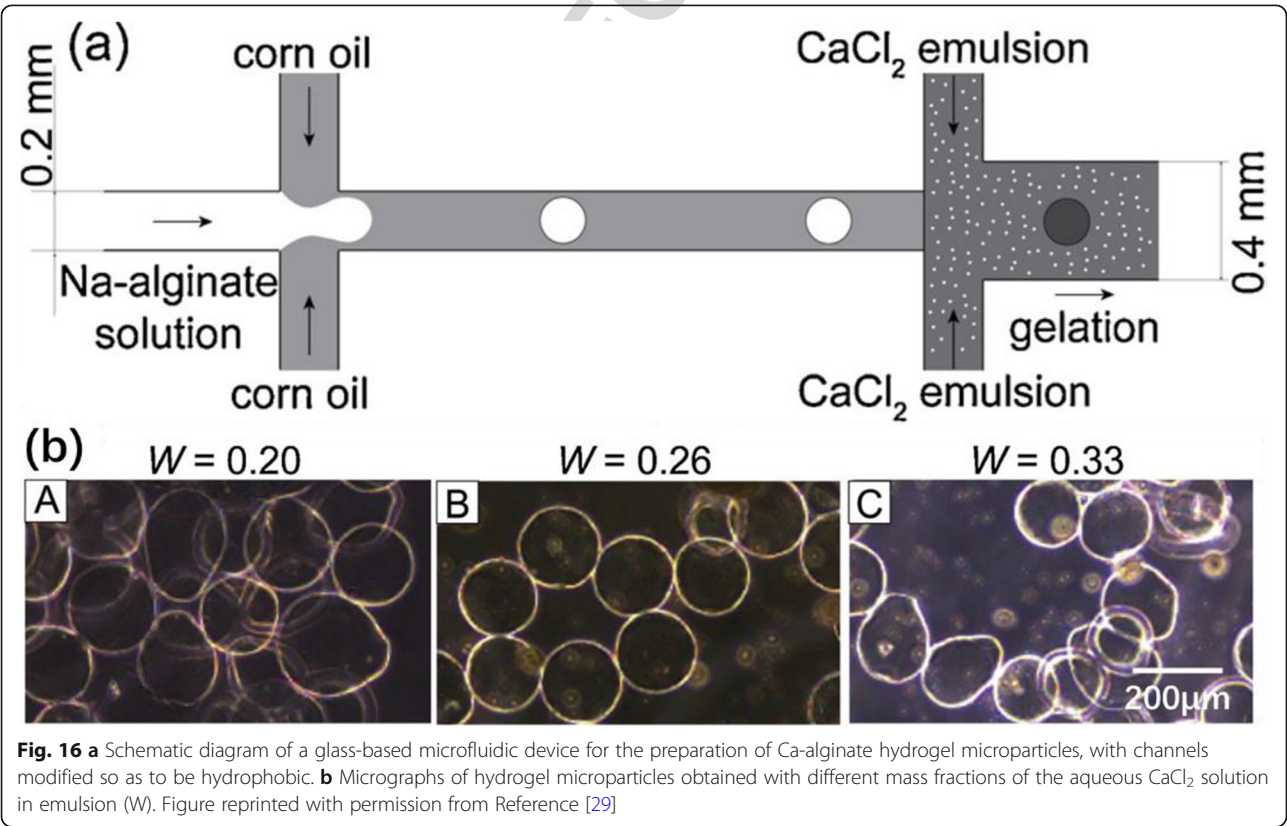
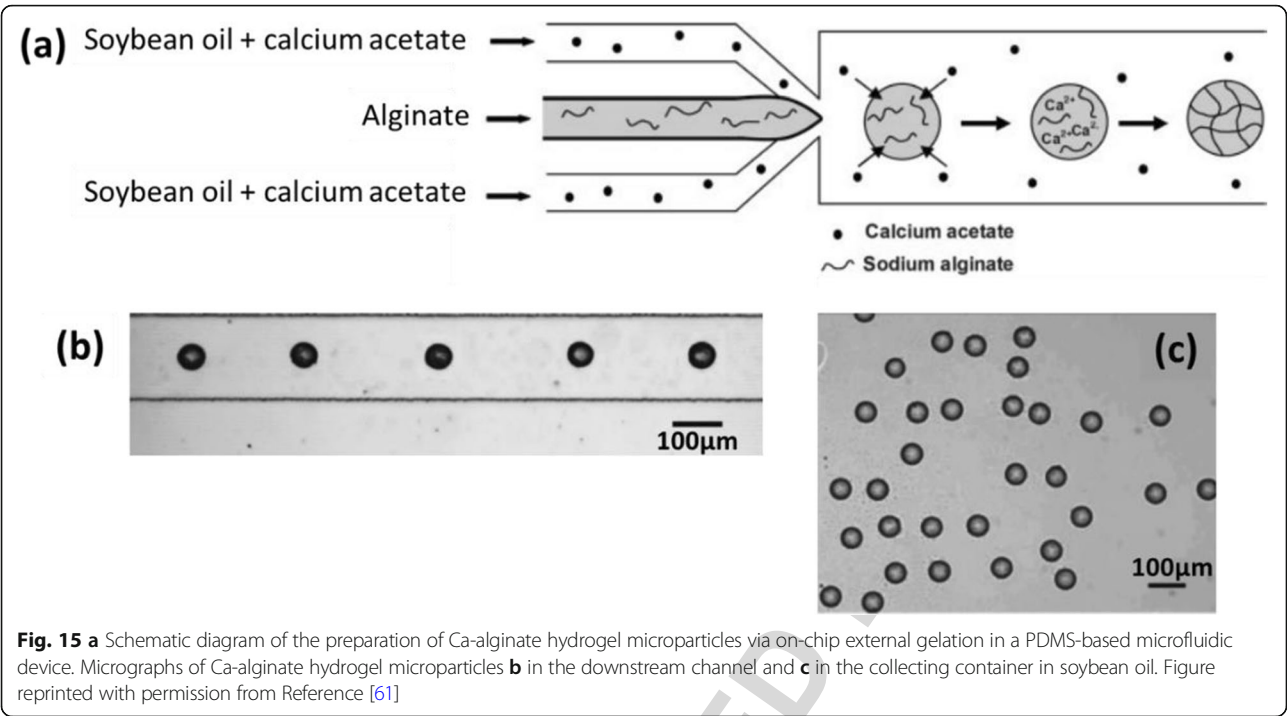
Cross-linkers can be contained in the continuous fluid, as described in Zhang et al. [61] $\text{Ca}(\text{CH}_3\text{COO})_2$ (2 wt%)

was dissolved in soybean oil, the continuous fluid. In the microfluidic device detailed previously (III.1.2.), Na-alginate (2 wt%) droplets were generated by flow-focusing (Fig. 15a) in oil/ $\text{Ca}(\text{CH}_3\text{COO})_2$, with surfactant (Span 80, 3 wt%). $\text{Ca}(\text{CH}_3\text{COO})_2$ diffused and dissolved in Na-alginate droplets along the channel (Fig. 15b), causing external gelation on-chip. Finally, Ca-alginate hydrogel microparticles were collected in oil (Fig. 15c). They showed better stability in an aqueous medium and had a higher Young’s modulus compared with those produced by internal gelation (III.1.2.). Consequently, stronger gelation was achieved by external gelation. However, increasing the concentration of $\text{Ca}(\text{CH}_3\text{COO})_2$ in soybean oil caused clogging in the microchannel [61]. Thus, it is difficult to vary the rate of gelation of microparticles.

One way to limit channel clogging is to make the cross-linkers diffuse slowly in Na-alginate droplets. Thus, Liu et al. used emulsion fluids to introduce cross-linker s[29]. A glass-based microfluidic device was used, with channels modified so as to be hydrophobic. Droplets of Na-alginate (3 wt%) were first generated in corn oil at the first flow-focusing channel (Fig. 16a). The emulsion of CaCl_2 , containing CaCl_2 droplets in corn oil (with surfactant SY-Glyster CRS-75), was injected downstream of the cross-junction. The contact between CaCl_2 and Na-alginate droplets caused ionic crosslinking, leading to gelation. Ca-alginate hydrogel microparticles were obtained. However, it was found that the microparticles could easily be deformed (Fig. 16b-A, b-C) by several

Table 4 External gelation with on-chip introduction of cross-linkers

Reference		Zhang et al., 2007 [61]	Liu et al., 2019 [29]	Sugaya et al., 2011 [44]	Pittermannová et al., 2016 [34]
Droplet generation	Concentration of Na-alginate	2 wt%	3 wt%	0.025–0.15 wt%	1 w/v%
	Continuous fluid	Soybean oil	Corn oil	Methyl acetate	1-undecanol
	Use of surfactant	Span 80	SY-Glyster CRS-75	Not mentioned	Abil Em 90
	Geometry	Flow-focusing	Flow-focusing	Flow-focusing	Flow-focusing
	Microfluidic material	PDMS chip	Glass chip	PDMS chip	PDMS chip
External gelation by introducing Na-alginate and cross-linkers on chip before collection	Cross-linkers	$\text{Ca}(\text{CH}_3\text{COO})_2$ (2 wt%) in the continuous fluid	Emulsion of CaCl_2 in the continuous fluid	CaCl_2 (1 M) in the dispersed fluid	Emulsion of CaCl_2 (2 wt%) droplets in the continuous fluid
	Geometry	–	Flow-focusing	Cross-flowing	Flow-focusing
	Mixing	During droplet generation	After droplet generation	After droplet generation	After droplet generation



parameters, such as the mass fraction of the aqueous CaCl_2 solution in emulsion (W). Deformation occurred when the value of W was too high or too low, so that an optimal value of W was required for homogeneous spherical microparticles (Fig. 16b-B). The morphology and homogeneity of microparticles also varied with flow rates and surfactant concentrations. Lacking flexibility, this strategy is thus not appropriate for producing spherical hydrogel microparticles. Moreover, generating small particles requires reducing the channel size, involving a risk of droplet coalescence before gelation in CaCl_2 emulsion.

To avoid reducing the channel size, partially miscible fluids can be used as the dispersed and continuous fluids. Sugaya et al. used methyl acetate as the continuous fluid [44]. Na-alginate (0.025–0.15 wt%) droplets were generated in methyl acetate (no mention of

surfactant usage) by flow-focusing. In the following channel, because of the solubility of water in methyl acetate (8 wt%), water dissolved gradually from the droplets into methyl acetate. Thus, the droplets shrank and became more concentrated downstream. CaCl_2 solution (1 M) was then injected by side channels and flowed with the droplets by co-flow. Calcium cations diffused into the droplets, inducing on-chip external gelation of alginate. Finally, spherical Ca-alginate hydrogel microparticles with a diameter of less than $20\text{ }\mu\text{m}$ were obtained. In this strategy, after CaCl_2 fluids were introduced, two competing processes occurred simultaneously in each droplet: gelation and shrinkage of droplets. The competition between gelation and shrinkage is not discussed in this article. However, the results indicate that extremely small droplets tend to approach the channel wall after shrinkage. With the CaCl_2 fluid,

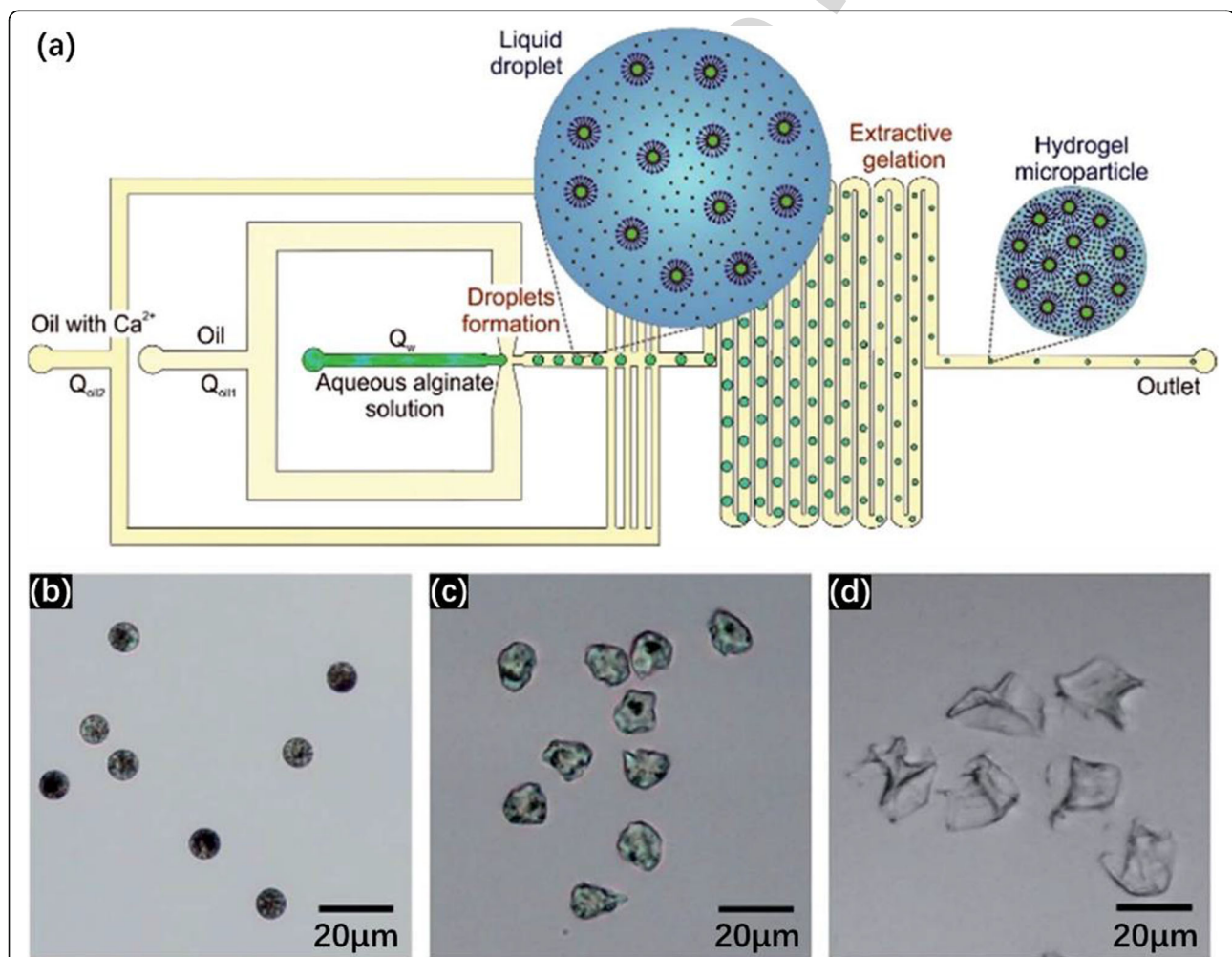


Fig. 17 a Schematic diagram of a PDMS-based microfluidic device for the preparation of Ca-alginate hydrogel microparticles. Micrographs of microparticles with a **b** spherical, **c** slightly deformed and **d** collapsed morphology obtained using different experimental flow rates and calcium concentrations. Figure reprinted with permission from Reference [34]

after gelation, Ca-alginate hydrogel microparticles adhere to the channel wall.

Adhesion to the channel and coalescence of microparticles can be avoided thanks to progressive addition of the cross-linker. Pittermannová et al. used as continuous fluid 1-undecanol, whose water-solubility is 2.7 vol% [34]. The experiment was carried out in a PDMS-based microfluidic device (Fig. 17a). An aqueous alginate solution (1 wt%) was first injected. After 1-undecanol (with 5 wt% surfactant Abil Em 90), shown as “oil” in Fig. 17a, was injected into the flow-focusing channel, droplets of alginate were formed. CaCl_2 (2 wt%) was dispersed in another fluid, 1-undecanol with 5 wt% surfactant Abil Em 90, yielding an emulsion. This emulsion was injected after droplet generation, through successive channels (Fig. 17a). Hence, the droplets were increasingly separated from each other, avoiding coalescence. Moreover, they were surrounded by more and more CaCl_2 , increasing gelation, and by more and more 1-undecanol, increasing diffusion of water. Thus, gelation and shrinkage of droplets occurred gradually and simultaneously. According to the authors, this procedure avoids the droplet generation instability caused by pre-gelation. However, spherical hydrogel microparticles (Fig. 17b) were only obtained using certain flow rates and calcium concentrations. Otherwise, the microparticles were slightly deformed (Fig. 17c) or collapsed (Fig. 17d), which was explained using a core-shell model [34]. Unfortunately, this explanation does not take into account the change in alginate concentration in the droplets due to water extraction, a factor which is bound to impact deformation. Simply prolonging the water extraction process before introducing cross-linkers, as done by Sugaya et al., could avoid deformation [44].

Off-chip introduction of cross-linkers

Other strategies of external gelation introduce cross-linkers “off-chip”, i.e., during droplet collection. Ca $(\text{CH}_3\text{COO})_2$ or CaCl_2 are used as cross-linkers and the collection bath procedure depends on the strategy, as summarized in Table 5.

Hu et al. [20] studied the influence of external gelation conditions on the shape of microparticles. Na-alginate (1.5 wt%) droplets were first generated in n-decanol with surfactant (Span 80, 5 wt%), using concentric glass capillaries in co-flow geometry (Fig. 18A). For off-chip external gelation, droplets were collected in a two-phase gelation bath: the upper phase of n-decanol with surfactant (Span 80, 5 wt%) containing CaCl_2 (15 wt%) allowed for pre-gelation of alginate; the bottom phase, an aqueous solution of barium acetate (15 wt%), strengthened the gelation. Glycerol (0–70 wt%) was added to the bottom phase to regulate viscosity. Ca-alginate hydrogel particles of different shapes (Fig. 18B) were obtained by varying gelation conditions such as the interfacial energy γ_{CD} , the concentration and type of surfactant, the height h between the end of the capillary and the surface of the gelation bath, and the viscosity of the bottom phase in the gelation bath. The shape of microparticles was shown to depend on forces applied to the surface of droplets when they passed through the interface in the gelation bath. The force from γ_{CD} maintains the spherical form of droplets, while the viscous force causes deformation. The final shape resulted from the overall effect of these two forces [20]. As can be seen in this strategy, droplet collection is accompanied by the consumption of the two different cations. Thus, to obtain a large quantity of microparticles, these cations should be replenished to ensure that each droplet undergoes

Table 5 External gelation with off-chip introduction of cross-linkers

References		Hu et al., 2012 [20]	Zhang et al., 2020 [59]	Present review
Droplet generation	Na-alginate	1.5 wt%	0.006–1 wt%	0.006–1 wt%
	Continuous fluid	N-decanol	DMC ^a	DMC ^a
	Use of surfactant	Span 80	NO	NO
	Geometry	Flow-focusing	Cross-flowing	Cross-flowing
	Microfluidic material	Glass chip	Fluoropolymer tubing and junctions	Fluoropolymer tubing and junctions
External gelation by introducing Na-alginate and cross-linkers off-chip during collection	Number of phases or steps	2 phases - 1 collection bath		2 phases - 2 successive collection baths
	Description of phases or steps	Phase 1 CaCl_2 (15 wt%) in n-decanol	CaCl_2 (0.1–1 wt%)	Step 1 DMC and evaporation of DMC
		Phase 2 Ba $(\text{CH}_3\text{COO})_2$ (15 wt%) in water and glycerol		Step 2 CaCl_2 (0.5–10 wt%) in water
	Surfactant	Span 80	NO	NO

^aContinuous and dispersed fluids partially miscible

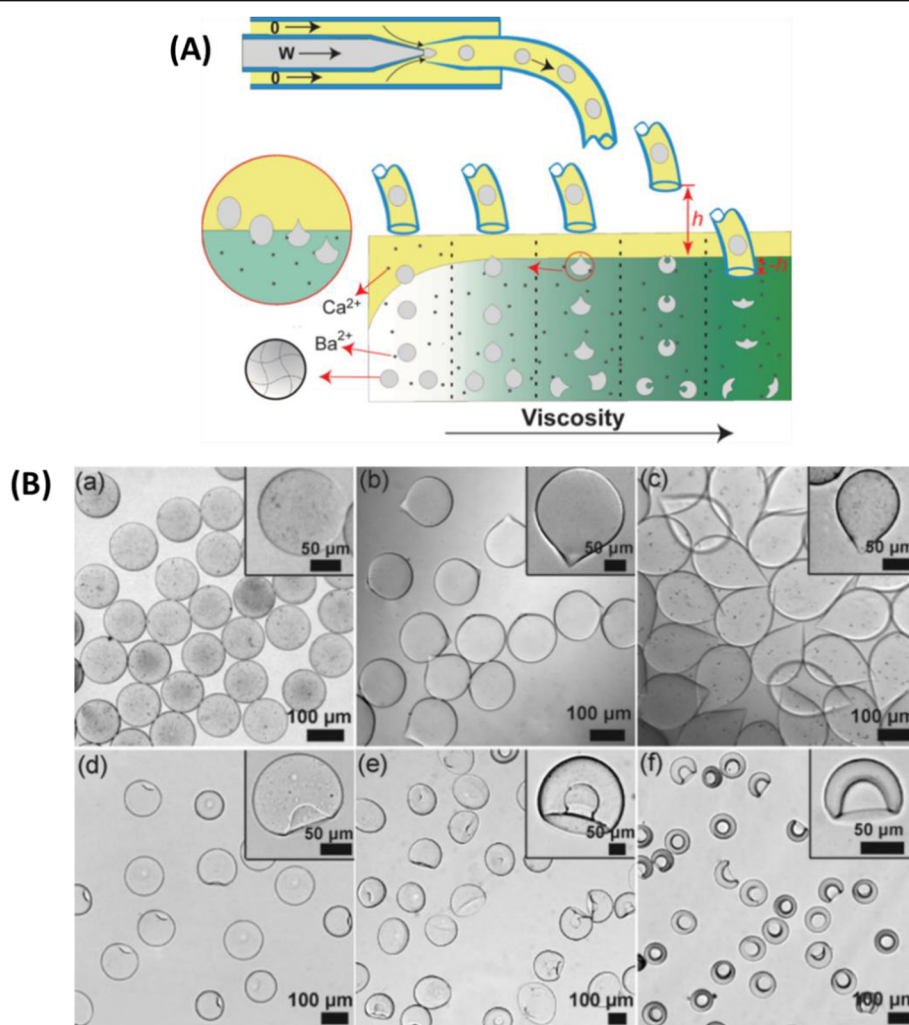


Fig. 18 A Schematic diagram of the preparation of Ca-alginate hydrogel microparticles using a microfluidic device constructed with glass capillaries, and off-chip gelation in a two-phase gelation bath. **B** Micrographs of Ca-alginate hydrogel microparticles of different shapes prepared under different experimental conditions. Figure reprinted with permission from Reference [20]

sufficient gelation. However, in practice, when and how to replenish them remains an issue.

To avoid the problem of replenishing the bath with the two cations, we collected Na-alginate droplets in an aqueous solution containing CaCl_2 (Fig. 19a) without pre-gelation. Na-alginate (0.006–1 wt%) droplets were first generated in DMC in a T-junction and Teflon-like capillaries (IDEX Health and Science), without using surfactant [59]. Because water is slightly soluble in DMC, 3 wt%, water diffused gradually from droplets into DMC, causing the droplets to shrink as they passed through the channel (point A to B in Fig. 19a). Thus, droplet size reduced to below 100 μm . Furthermore, since alginate dissolution in the continuous fluid is negligible [39], with the loss of water, the alginate concentration in droplets increased. Then, the channel outlet (point B in Fig. 19a) was immersed in an aqueous

solution of CaCl_2 (0.1–1 wt%). An interface was created at the channel outlet (Fig. 19b) because of the non-total miscibility between DMC and water. Na-alginate droplets passed through the interface and entered the CaCl_2 solution, leading to off-chip external gelation. After gelation, Ca-alginate hydrogel microparticles were droplet-shaped (Fig. 19c) and tadpole-shaped (Fig. 19d), as in Fig. 18B, b-c. The shape of the microparticles varied with the flow rates, the concentration of Na-alginate and that of CaCl_2 . It is likely that the deformation mechanism involved the forces applied to droplets at the interface, as explained by Hu et al. [20].

To improve the spherical shape of microparticles, we collected droplets in a bath of the continuous fluid, i.e., DMC. Hence, the droplets continued to shrink and were finally transformed into spherical condensed Na-alginate microparticles, not yet gelated. For the gelation of the

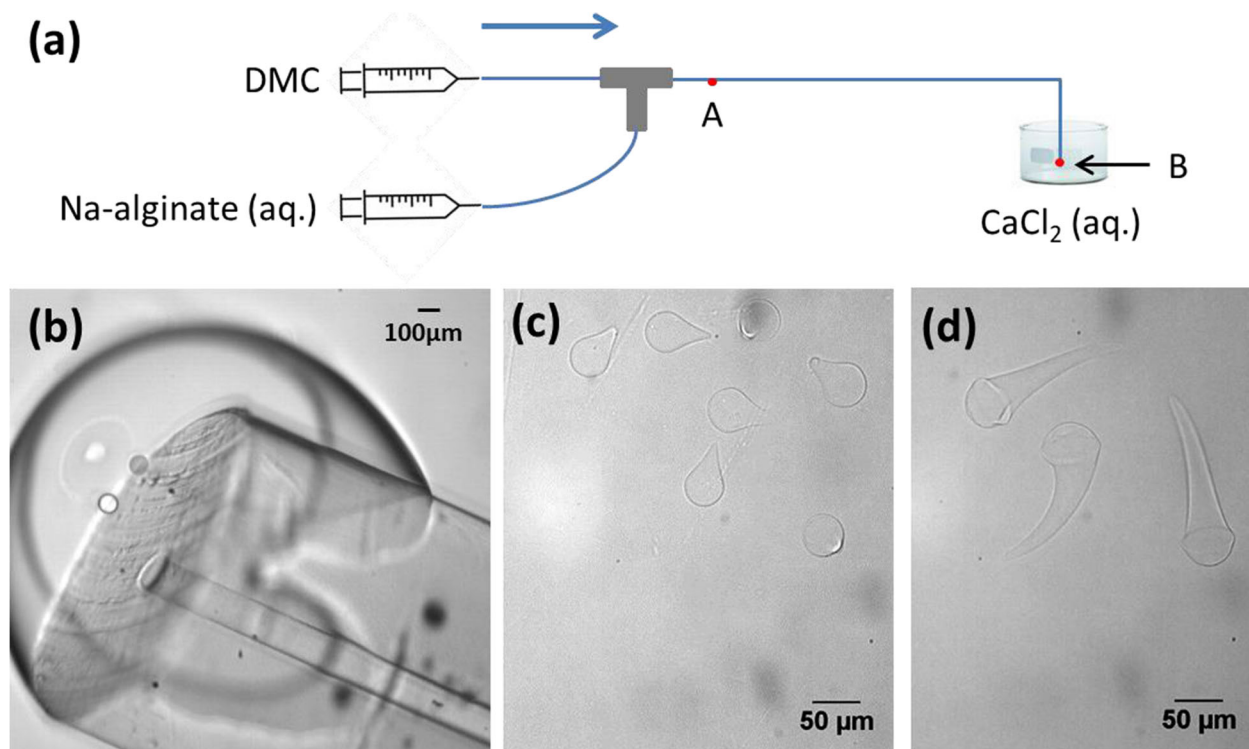


Fig. 19 a Schematic diagram of the preparation of Ca-alginate hydrogel microparticles by off-chip external gelation without pre-gelation. Droplets were generated using a microfluidic device assembled from fluoropolymer capillaries and a T-junction. Micrographs of **b** the channel outlet immersed in an aqueous solution of CaCl₂; Ca-alginate hydrogel microparticles prepared by collecting droplets in an aqueous solution of CaCl₂ at concentrations of **c** 1 wt% and **d** 0.1 wt%

microparticles, the bath of DMC was first evaporated. Then, an aqueous solution of CaCl₂ (0.5–10 wt%) was added to the dried Na-alginate microparticles, inducing off-chip external gelation. Observations showed that this process was accompanied by the swelling of the microparticles without deformation (Fig. 20b-c). In the end, spherical Ca-alginate hydrogel microparticles were obtained. They were insoluble in water, indicating efficient gelation. Moreover, the concentration of CaCl₂ had no significant effect on the size of the Ca-alginate microparticles. Since no surfactant is used in this method, no surfactant-removing step is needed, which simplifies the process. However, the quantity of microparticles produced is limited by the need to avoid droplet coalescence.

In conclusion, external gelation of alginate can be performed both on-chip and off-chip. For on-chip external gelation (Table 4), cross-linkers can be added to the continuous fluid, i.e., the oil. However, only limited concentrations can be used, since most are slightly soluble in oil. Therefore, introducing cross-linkers in emulsion form increases the quantity of alginate available for gelation. However, the particles are large and deformed. On the other hand, if partially miscible fluids are used, an aqueous solution of cross-linkers can be injected after

droplet shrinkage. Particle size is reduced but the gelation is too rapid. Things can be improved by dissolving cross-linkers in an oil-based emulsion, introduced in small quantities but repeatedly.

For off-chip external gelation (Table 5), cross-linkers are introduced into the collection bath. A two-phase collection bath permits pre-gelation of Na-alginate droplets before gelation. However, the particles are large. Droplet size can be reduced to below the channel diameter by using partially miscible fluids for droplet generation, and the droplets can then be collected directly in the dispersed phase containing the cross-linker. However, the microparticles are deformed. To further reduce particle size and improve gelation, our solution is to perform two-step collection. Thus, off-chip external gelation can be used to produce shape-controlled and size-controlled microparticles.

Properties of alginate hydrogel microparticles

After preparation, alginate hydrogel microparticles should be characterized to obtain better knowledge of their properties, which will determine their further applications. This section discusses characterization approaches and factors influencing particle properties.

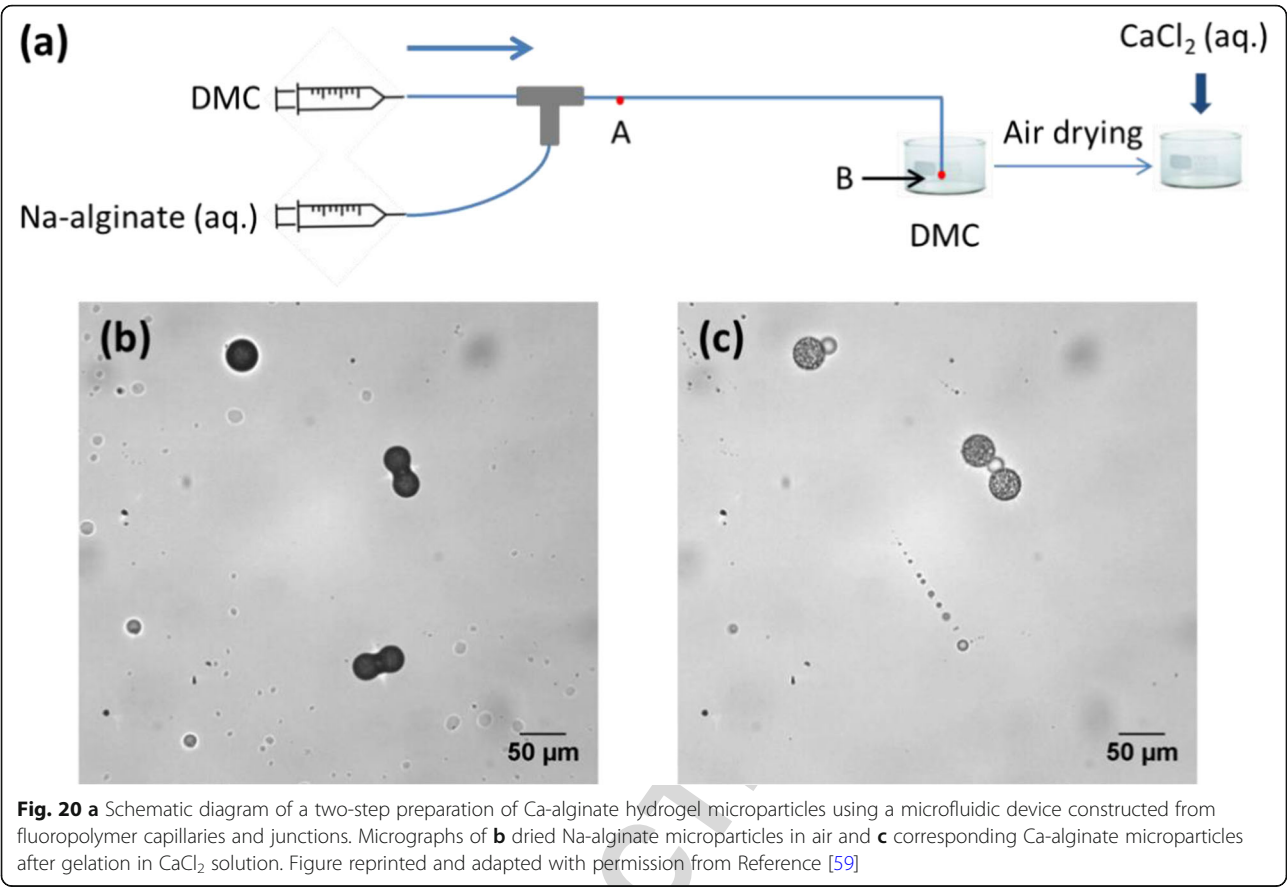


Table 6 Average size of alginate microparticles prepared using droplet-based microfluidics with different gelation methods		
Average size of microparticles	Gelation method	Reference
1–50 μm	Internal gelation with water-soluble cross-linkers mixed with Na-alginate before droplet generation	Rondeau and Cooper-White 2008 ^a [39]
10–300 nm		
50–300 μm		Xu et al. 2008 [54]
20–50 μm	Internal gelation with water-soluble cross-linkers mixed with Na-alginate after droplet generation	Liu et al. 2006 [28]
22–42 μm		Trivedi et al. 2009 [48]
60–100 μm		Zhang et al. 2007 [61]
26 μm	Internal gelation with water-insoluble cross-linkers mixed with Na-alginate after droplet generation	Akbari and Pirbodaghi 2014 [3]
50–100 μm		Yu et al. 2019 [56]
10–50 μm		Utech et al. 2015 [49]
50–70 μm	External gelation with on-chip introduction of cross-linkers	Zhang et al. 2007 [61]
147–176 μm		Liu et al. 2019 [29]
5–10 μm		Pittermannová et al. 2016 ^a [34]
6–10 μm	External gelation with off-chip introduction of cross-linkers	Sugaya et al. 2011 ^a [44]
100–200 μm		Hu et al. 2012 [20]
7–40 μm		Zhang et al. 2020 ^a [59]

^aPartially miscible fluids were used

Size

Size is one of the most important properties of alginate hydrogel microparticles. For example, in drug delivery, microparticle size and size distribution affect drug release kinetics [50]. Size can be measured by optical or light-scattering (sub-micrometer range) microscopy [21], or using microgrippers [58].

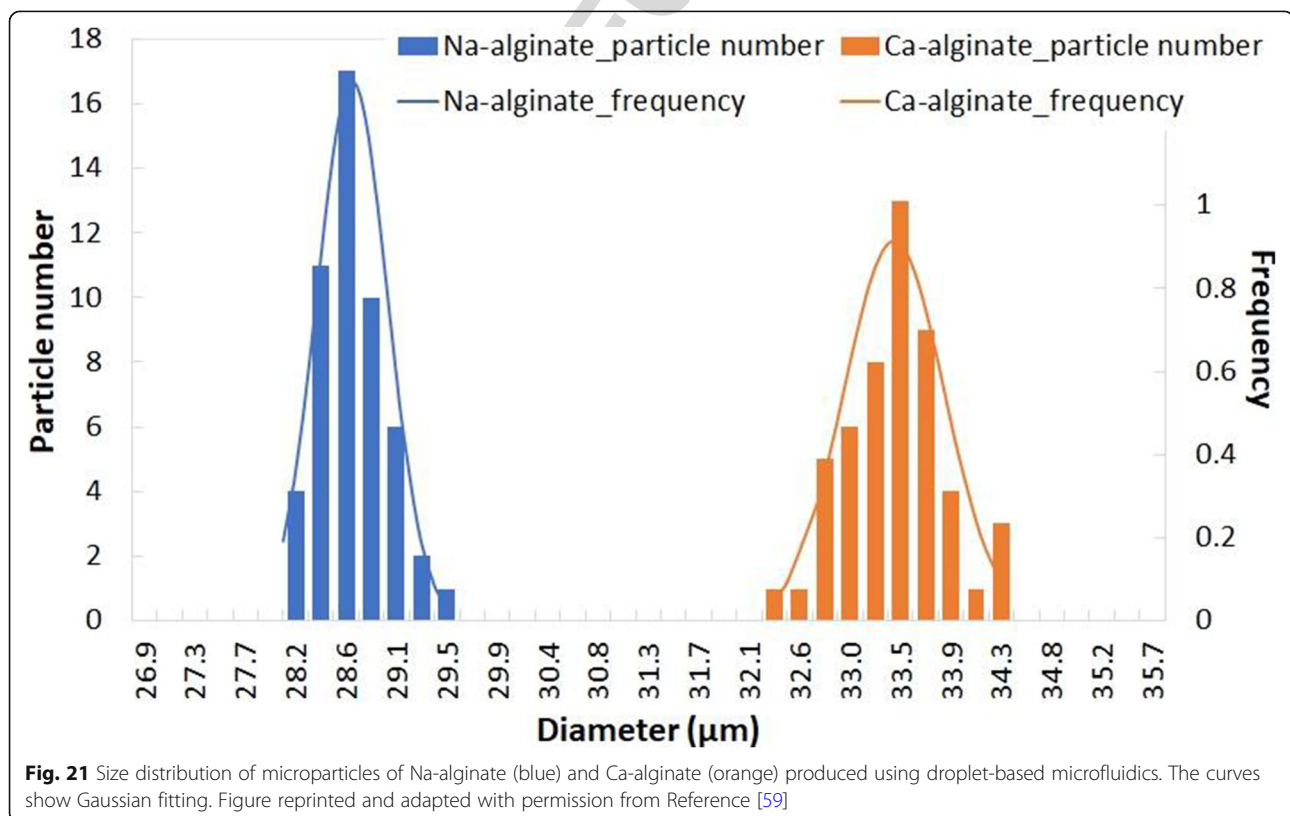
Droplet-based microfluidics allows monodisperse microparticles to be produced with accurate control of size and size distribution. Table 6 shows the average size attained under droplet-based microfluidics using different gelation methods.

Figure 21 shows an example of the narrow size distribution of Na-alginate and Ca-alginate microparticles produced by droplet-based microfluidics [59] (part III.2.2.), indicating the monodispersity of the particle size. This is an advantage compared to conventional emulsification, which yields a broader size distribution [55].

With droplet-based microfluidics, the size of alginate hydrogel microparticles is influenced by several factors linked to the fluids used to generate them. When immiscible fluids are used for the dispersed and the continuous fluids, the size of alginate hydrogel microparticles is completely dependent on the size of the droplets first generated. Droplet size is influenced by channel size, and smaller droplets can be generated by using narrower channels. Other important factors are flow rates, alginate

concentration [39, 59], and fluid viscosities [41, 46]. However, reducing channel size increases hydraulic resistance, as well as the pressure required to generate droplets. Moreover, it should be noted that in most cases, the Na-alginate solution used is relatively viscous. Therefore, high pressure may cause leakage or even destruction of the microfluidic device [3, 49]. Thus, even when channel diameter is decreased and/or the flow rate of the continuous fluid is increased, producing droplets of a diameter below 10 μm remains challenging.

Droplets of this size, below 10 μm , can be obtained without applying high pressure (Table 6), by using partially miscible fluids with low solubility in each other [39, 44, 59]. The dispersed fluid is an aqueous solution containing Na-alginate; the continuous fluid is an organic solvent that is partially miscible with water and in which water has low solubility. The partial miscibility between the continuous and the dispersed fluids should be slight enough so that interfacial energy γ_{CD} still allows the generation of droplets. The low solubility of water in the continuous fluid allows water diffusion from droplets into it, causing the droplets to shrink. As a result, the initially obtained diluted large droplets are transformed into concentrated small droplets or microparticles. Thus, their size is no longer dependent on the size of droplets initially generated but varies with the interaction between water and the continuous fluid.



Shape

The shape of microparticles is another important property. A specific shape is sometimes needed; for example, red blood cell-mimicking microparticles are often required in a biconcave shape [32]. In drug delivery, the shape of microparticles has an impact on the drug-release profile [13]. The overall shape of microparticles can be observed by using optical microscopy. Confocal microscopy of fluorescent samples can be used to form a spatial 3D image [21]. Better resolution can be obtained by using atomic force microscopy (AFM) or scanning electron microscopy (SEM) [59].

With droplet-based microfluidics, the spherical droplets initially generated can be transformed into spherical alginate hydrogel microparticles after gelation. Non-spherical microparticles can also be obtained. For example, as presented previously, Liu et al. first generated droplets of Na-alginate and CaCl_2 separately [28]. Then the droplets were fused in a specifically designed microfluidic device, leading to gelation. By varying the channel geometry and controlling the flow rates of fluids, Ca-alginate microparticles of different shapes were obtained (Fig. 10f). A different method was presented by Hu et al. [20] Na-alginate droplets were first generated and then collected in a two-phase gelation bath. Spherical droplets were deformed via interfacial energy derived from surfactant and viscous force. Thus, different shapes were produced (Fig. 18B) by controlling the surfactant used and the viscosity of the gelation bath.

Concentration

After preparation, the concentration of alginate in the microparticles can be calculated approximately. For instance, Zhang et al. used partially miscible fluids [59]. An aqueous solution of Na-alginate was prepared with a known concentration. After droplet generation, droplet shrinkage occurred during passage through the channel due to water diffusion into the continuous fluid. Droplets were hence transformed into microparticles. As the diffusion of Na-alginate into the continuous fluid is negligible [39], the quantity of Na-alginate is constant. It can be calculated by multiplying the droplet volume and initial concentration. Finally, by measuring microparticle size, the concentration of Na-alginate can be calculated. The final concentration of Na-alginate varies from 20 to 100 wt%, depending on the initial concentration and diameter of the droplets generated [59]. Furthermore, as presented in Utech et al., the homogeneity of composition of microparticles can be determined with the help of fluorescence technology [49].

Stability

In most cases, surfactant is added in the continuous fluid [48, 49, 61] to lower interfacial energy between the

continuous and the dispersed fluids γ_{CD} . Note that for each of the above studies, use or non-use of surfactant is mentioned when indicated by the authors (Tables 1, 2, 3, 4, 5). Surfactant facilitates the creation of a new interface, and thus the formation of droplets. It also stabilizes the formed droplets by preventing their coalescence [41]. Before the application of microparticles, the surfactant should be dissolved [3], except for biocompatible surfactant [49], although protocols for removing surfactant are rarely reported in the literature. To remove the oil used during the preparation, microparticles should be washed several times with an aqueous solution, followed by centrifugation [61].

However, despite its advantages, the use of surfactant may be undesirable. Surfactant has been shown to impact the surface properties of microparticles, such as morphology [45] and surface hydrophobicity [22]. Additionally, if rinsing is insufficient, the traces of surfactant on microparticles can damage the devices during application. In this situation, microparticles should be prepared without surfactant, which is possible with droplet-based microfluidics. In the microchannel, the coalescence of droplets can be avoided by enlarging the distance between droplets, which can be achieved by regulating flow rates. Furthermore, gelation, either on-chip or off-chip, solidifies droplets and thus helps to avoid coalescence as well. Another strategy consists of using partially miscible fluids. This means that the droplets shrink and become more and more condensed during passage through the channel. At the outlet, either gelation [39] or a final shrinkage [59] can help avoid coalescence.

Moreover, for their stability, alginate hydrogel microparticles should be insoluble in water. This can be achieved by adopting proper gelation methods using a sufficient quantity of cross-linkers for effective gelation.

Mechanical properties

Mechanical properties of alginate hydrogel microparticles are usually characterized by measuring the Young's modulus, which varies with several factors. According to the type of bond between alginate and cross-linkers, covalent crosslinking results in a higher Young's modulus than ionic crosslinking in microparticles [6]. For ionic crosslinking, different cations present different affinities, i.e. different forces with alginate, thus different Young's moduli [33]. In addition, the Young's modulus increases with the concentration of alginate [31]. To measure the Young's modulus of a microparticle, it needs to be deformed under a known force, which can be either compressive or tensile [17]. The techniques used in the literature include micropipette aspiration [23], compression [7, 52] or Atomic Force Microscopy (AFM) [59, 61].

938 *Micropipette aspiration technique*

939 In the micropipette aspiration technique, controlled
940 pressure is used to pull on the sample surface. When
941 this pressure is high enough, the sample behaves like a
942 viscoelastic fluid flowing inside the micropipette [17].
943 With a known pressure applied, the Young's modulus is
944 calculated by applying the homogenous half-space model
945 as described by Kleinberger et al. [23].

946 *Compression technique*

947 This technique consists in compressing a microparticle
948 between two parallel plates [7] or between the flat end
949 of a glass fiber and a glass surface [52]. A force trans-
950 ducer is connected to the equipment to measure the
951 force applied. By varying the force, microparticle de-
952 formation can be recorded. Finally, according to the
953 force-deformation curve and equations based on theor-
954 etical models, the Young's modulus is calculated.

955 However, both the micropipette aspiration technique
956 and the compression technique are unsuitable for micro-
957 particles with high resistance to deformation, like the
958 Na- and Ca-alginate microparticles generated by Zhang
959 et al. [59]. In this case, Atomic Force Microscopy was
960 used to measure resistance to deformation.

961 *Atomic force microscopy*

962 Measurement of the Young's modulus of a microparticle
963 with Atomic Force Microscopy (AFM) involves indent-
964 ing it. The indentation depth (order of 100 nm) is gener-
965 ally about 100 times less than the diameter of the
966 microparticle (order of 10 μ m). Hence the Young's
967 modulus represents the local mechanical property on
968 the surface, depending on the measuring point, as in
969 Zhang et al. [59]. As the surface of their microparticles
970 was smooth, variation in the local mechanical property
971 was explained by the porous inner structure observed by
F22372 Scanning Electron Microscopy (SEM) (Fig. 22).

973 *Conclusion*

974 This review focuses on the preparation of alginate
975 hydrogel microparticles via droplet-based microfluidics.
976 Various strategies are presented and classified within
977 categories that represent the full range of methods used
978 in the literature. Thus, readers will find that any strategy
979 encountered can fit into one of the categories we present
980 here.

981 To summarize, gelation is indispensable to transform
982 alginate microdroplets into alginate hydrogel microparti-
983 cles. It is realized by crosslinking, which requires cross-
984 linkers to be introduced either inside or outside the
985 microdroplets, causing respectively internal gelation or
986 external gelation. For internal gelation, cross-linkers are
987 introduced "on-chip" (in the microfluidic device). For
988 external gelation, cross-linkers can be introduced both

"on-chip" and/or "off-chip" (outside the microfluidic de- 989
vice). The review describes the various strategies applied 990
under the microfluidic technique, and the size, shape, 991
concentration, stability and mechanical properties of the 992
alginate hydrogel microparticles obtained. 993

Lastly, we wish to stress the ease of constructing a 994
microfluidic device with Fluoropolymer tubing and junc- 995
tions compared to a chip, whatever its composition: 996
PDMS, PMMA or glass. A microfluidic device's geom- 997
etry can be tuned flexibly, whereas in a chip, the geom- 998
etry of channels is fixed. Chip fabrication is laborious, 999
time-consuming and expensive [12]. Clean room facil- 1000
ities are indispensable and the products used for the 1001
photolithography are toxic. 1002

Thus, in terms of flexibility, cost and efficiency, we 1003
highly recommend using a microfluidic device with 1004
Fluoropolymer tubing and junctions, especially for 1005
proof-of-concept demonstrations. 1006

1007 *Outlook*

1008 There is scope for several future improvements in the
1009 preparation of alginate hydrogel microparticles via
1010 droplet-based microfluidics.

1. When preparing microparticles using droplet-based 1011
microfluidics, surfactant is usually used. Unfortu- 1012
nately, surfactant has been shown to affect particle 1013
morphology [34]. Moreover, surfactant can damage 1014
the equipment involved in subsequent applications 1015
[59]. It obviously needs to be removed; however, 1016
the literature contains little information on how to 1017
remove surfactant. Moreover, despite its import- 1018
ance, the microparticle purifying process is barely 1019
touched on [34]. Future work could therefore use- 1020
fully provide more details of the full preparation 1021
process, including surfactant removal. 1022
2. In practice, a large number of microparticles usually 1023
needs to be produced. However, the throughput of 1024
microparticles fabricated by microfluidics is still 1025
limited. Exploring ways to scale up microparticle 1026
production would therefore be a welcome 1027
contribution. 1028
3. Currently, most publications concern proof-of- 1029
concepts or preliminary demonstrations. Little work 1030
is available on microparticles in real biological or 1031
biomedical applications [53]. Apart from biological 1032
barriers [35], the physiological environment is quite 1033
complex in terms of composition and rheological 1034
characteristics, which makes it extremely unlikely 1035
that microparticles can provide the functions re- 1036
quired. The feasibility of using alginate hydrogel mi- 1037
croparticles in real-life applications needs to be 1038
assessed. 1039

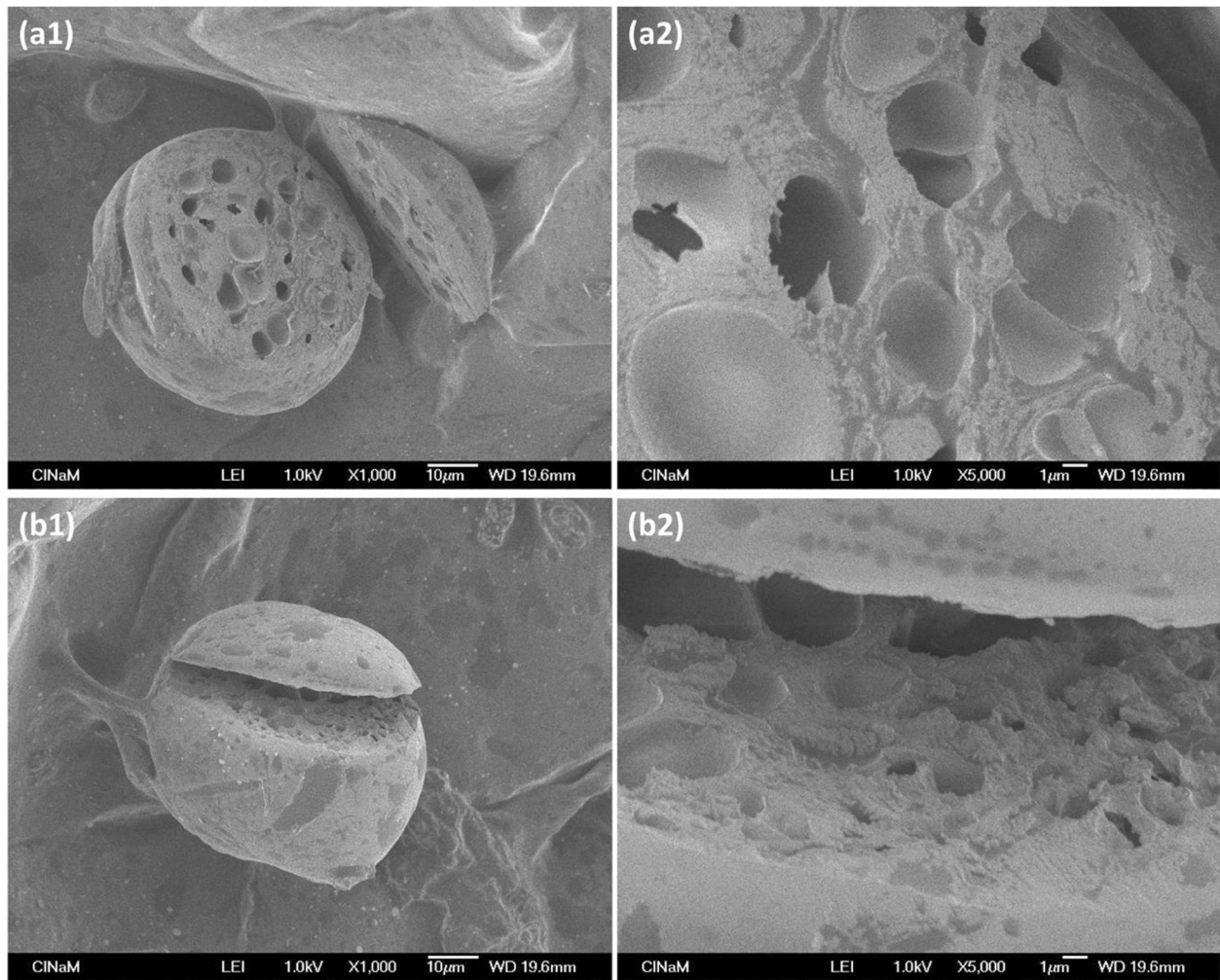


Fig. 22 SEM photographs of 2 Na-alginate microparticles (a) and (b), magnified 1000x (a1 and b1) and 5000x (a2 and b2). Na-alginate microparticles were prepared following the method mentioned in the publication [59]. Figure reprinted and adapted with permission from Reference [59]

4. The relation between degree of gelation and mechanical properties is rarely discussed in the literature. In fact, the degree of gelation itself is barely mentioned although, from a microscopic point of view, it influences mechanical properties. Thus, we believe it is worth assessing degree of gelation by measuring the concentration of cross-linkers inside the final hydrogel microparticles. Subsequently, the relation between degree of gelation and mechanical properties should also be addressed.
5. In practical applications, the surface of microparticles usually needs to be modified. For example, one way to detect antigens is to graft antibodies onto the surface of microparticles [38]. We note that the surface modification is always an extra step after the preparation of microparticles, thus complicating the process. The feasibility of an “all-in-one” process should be studied: can all the

steps be performed within one single microfluidic device, combining microparticle production and surface modification?

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Authors’ contributions

CZ, RG, NC and SV searched references and collected information. CZ and NC established the structure of whole manuscript. All authors wrote and reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

1073 **Declarations**1074 **Ethics approval and consent to participate**

1075 Not applicable.

1076 **Consent for publication**

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1078 **Competing interests**

1079 The authors declare no conflict of interest.

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