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

### 57 Alginate

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

63 Sodium alginate (Na-alginate) is the most widely used  
 64 alginate salt. It dissolves in water to a viscous solution. Alginate  
 65 is a linear copolymer containing  $\beta$ -D-mannuronate (M) and  $\alpha$ -L-guluronate (G) residues (Fig. 1).  
 F1 66

### 67 Gelation of alginate

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

76 Only G-blocks (Fig. 1a) made of consecutive G residues  
 77 can participate in ionic crosslinking because of their favorable  
 78 spatial structure [15, 25]. Ionic crosslinking of alginate  
 79 is described by the “egg-box” model [16] (Fig. 2).  
 F2 80

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

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

### 90 Droplet-based microfluidics

#### 91 Principle of droplet generation

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

98 The principle is similar to that of conventional emulsification,  
 99 which consists of blending two immiscible liquids. The  
 100 advantage of droplet-based microfluidics is monodispersity  
 101 and repeatability of droplets due to precise control over  
 102 experimental conditions such as channel geometry, flow  
 103 rates and viscosities of fluids, etc. [41, 103  
 104 46]. Furthermore, monodisperse droplets can be gener-  
 105 ated without using surfactant [28, 48, 59], which is im-  
 106 possible with conventional emulsification.

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

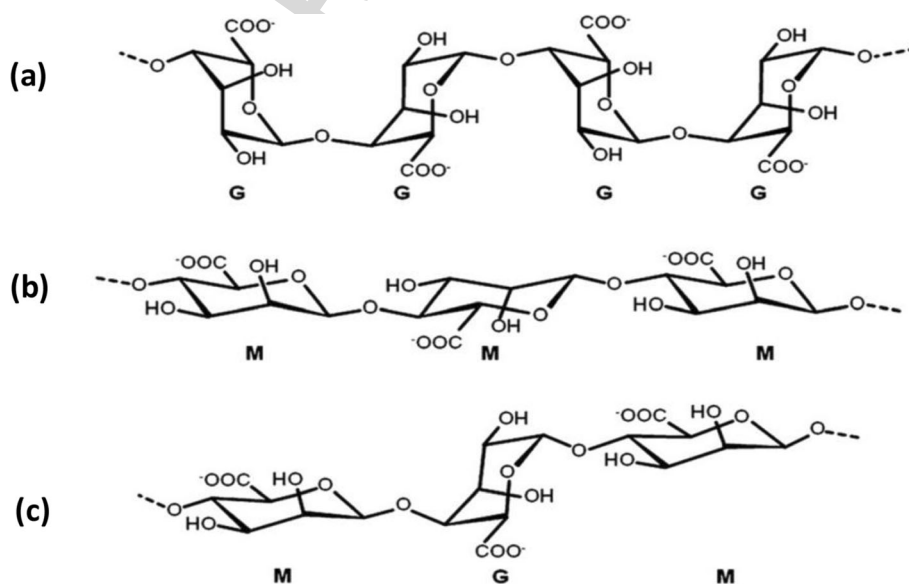
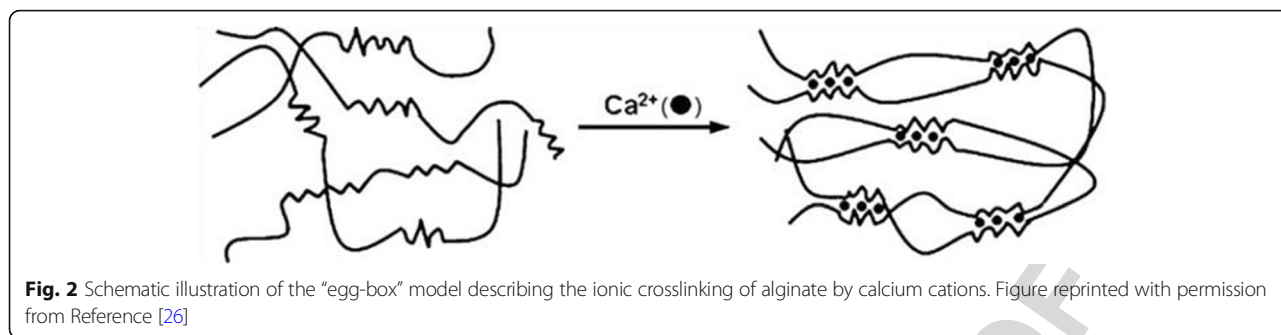


Fig. 1 Chemical structures of a G-block, b M-block and c alternating G and M-blocks in alginate. Figure reprinted with permission from Reference [25]



**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]

f2.1  
f2.2  
f2.3

114 **Flow properties**

143

115 In droplet-based microfluidics, a continuous fluid and a  
 116 dispersed fluid are injected separately and then mixed in a  
 117 junction. Fluids are Newtonian and droplets of the dis-  
 118 persed fluid (D) are generated in the flow of the continu-  
 119 ous fluid (C). The physicochemical properties influencing  
 120 droplet formation are density, dynamic viscosity, surface  
 121 tension between the continuous and the dispersed fluids,  
 122 velocity of the flows and characteristic dimensions of the  
 123 microfluidic system, such as the diameter of channels (w)  
 124 for cylindrical microfluidic systems. Based on these prop-  
 125 erties, fluid dynamics is characterized as follows:

126 1- Inertial forces and viscous forces are compared  
 127 through the Reynolds number, calculated using the  
 128 continuous fluid properties: density ( $\rho_C$ ), dynamic  
 129 viscosity ( $\mu_C$ ) and flow velocity ( $v_C$ ).

130

$$Re = \frac{\rho_C \times v_C \times w}{\mu_C} \tag{1}$$

131 Typically for microfluidics, values of Re are lower than  
 132 1: the flow is laminar and the effect of inertia can be ig-  
 133 nored. Thus, the average velocity  $v$  of a flow is evaluated  
 134 from its volumetric flow rate  $Q$  and  $w$  as follows:

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

135

136 2- The generation of droplets in a microfluidic  
 137 junction creates a free interface between the two  
 138 fluids, characterized by the interfacial energy  $\gamma_{CD}$ .  
 139 The corresponding capillary effects are in  
 140 competition with gravity effects. The length above  
 141 which gravity effects dominate capillary effects is  
 142 the capillary length  $l_c$ :

$$l_c = \sqrt{\frac{\gamma_{CD}}{\Delta\rho \times g}} \tag{3}$$

with  $g$  the gravity acceleration and  $\Delta\rho$  the difference in  
 density between the two fluids. For instance, with fluori-  
 nated 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 inter-  
 faces 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  $\mu_C$  of the  
 continuous fluid, and  $\gamma_{CD}$  of the interface between  
 the continuous and the dispersed fluid:

$$Ca = \frac{\mu_C \times v_C}{\gamma_{CD}} \tag{4}$$

**Microfluidic geometry**

Microfluidic devices can be in the form of either chips  
 with microchannels and junctions produced by soft litho-  
 graphy, 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 influ-  
 ences droplet generation. Three frequently used geom-  
 etries are “cross-flow”, “co-flow” and “flow-focusing”  
 (Fig. 3).

**Cross-flowing**

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

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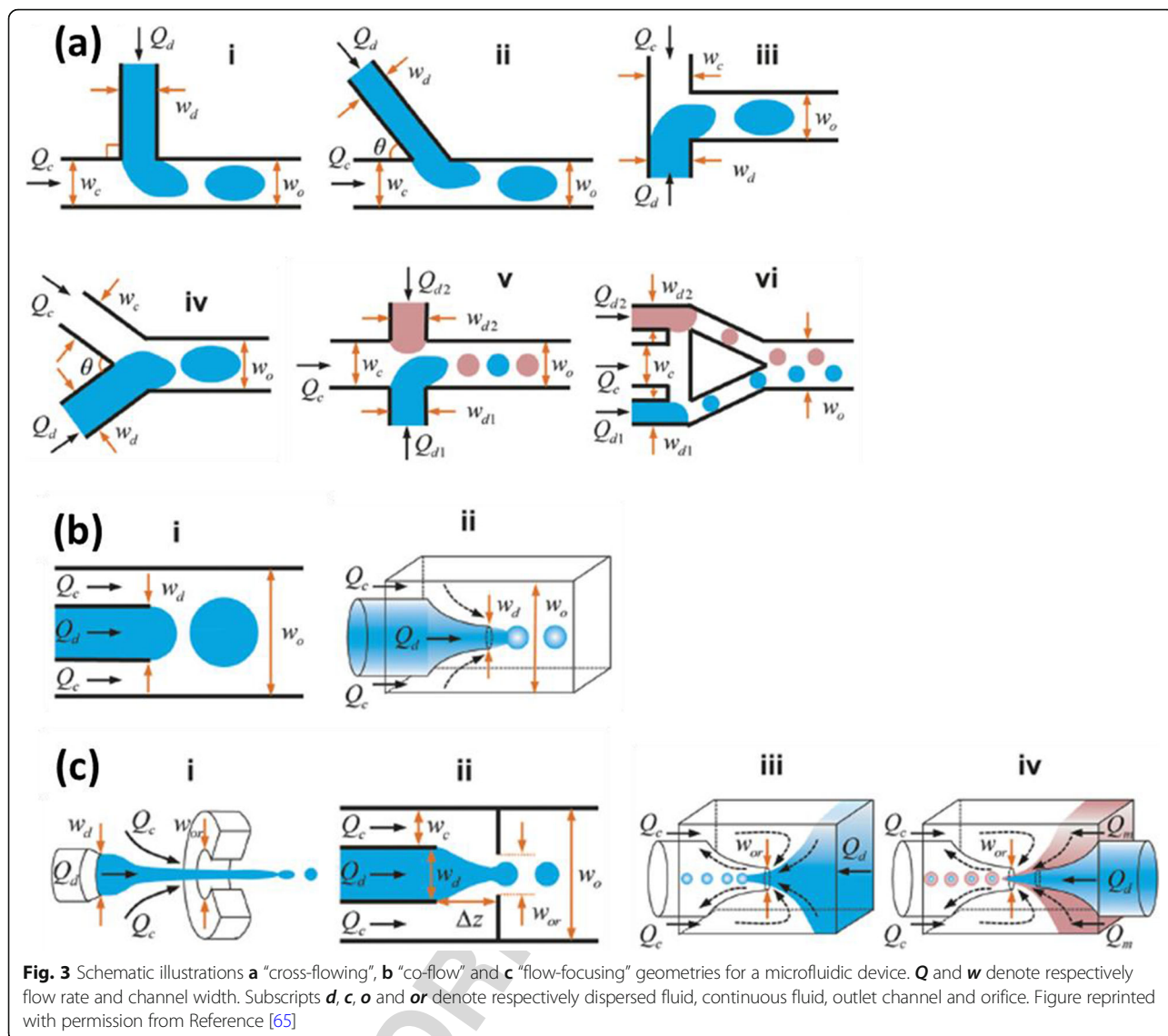
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**Fig. 3** Schematic illustrations **a** “cross-flowing”, **b** “co-flow” and **c** “flow-focusing” geometries for a microfluidic device. **Q** and **w** denote respectively flow rate and channel width. Subscripts **d, c, o** and **or** denote respectively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

f3.1  
f3.2  
f3.3  
f3.4

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

178 Cross-flowing geometry is often called T-junction  
179 geometry, where two fluids flow orthogonally (Fig. 3a i).  
180 However, other shapes of junctions can also be used,  
181 such as a junction with an arbitrary angle  $\theta$  (Fig. 3a ii),  
182 or a Y-shaped junction (Fig. 3a iv). For two fluids facing  
183 each other ( $\theta = 180^\circ$ , Fig. 3a iii), the geometry is called  
184 “head-on”. A combination of two junctions (Fig. 3a v, vi)  
185 can also be used to introduce two different dispersed  
186 fluids and one continuous fluid. Cross-flowing geometry  
187 is widely used due to its ease of assembly and handling  
188 [41, 65].

**Flow-focusing**

189 For co-flow geometry, two immiscible fluids flow in two  
190 concentric channels (Fig. 3b). Droplets are formed at the  
191 outlet of the inner channel. Flow-focusing geometry is  
192 actually similar to co-flow geometry. The distinction  
193 presented in the literature is somewhat ambiguous [65],  
194 leading some to consider flow-focusing as a special co-  
195 flow geometry [41]. For flow-focusing geometry (Fig. 3c),  
196 two immiscible fluids are focused through an orifice,  
197 which allows smaller droplets to be generated than with  
198 co-flow geometry. 199

**Droplet generation regime**

200 For each geometry, droplets can be generated following three  
201 different break-off mechanisms. The transition from one  
202 mechanism to another can be achieved by varying capillary  
203



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 ( $Ca \approx 0.2$ ).

230 **Gelation**

231 **Internal gelation**

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

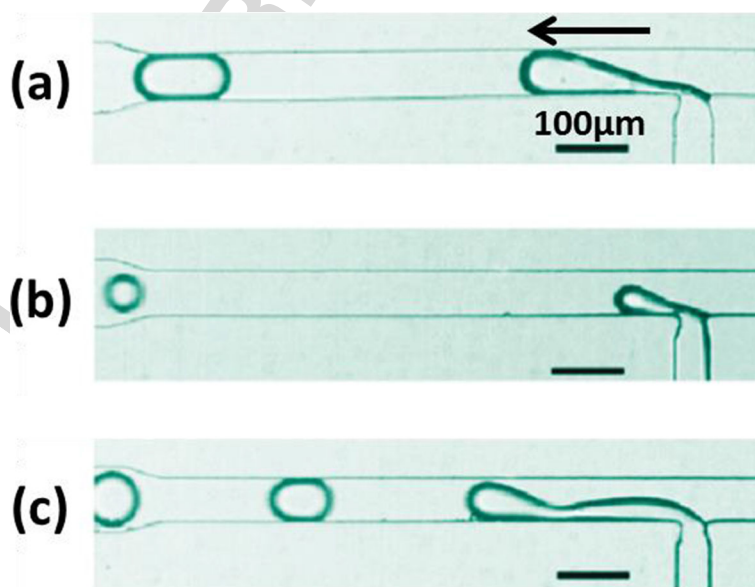
236 **Water-soluble cross-linkers**

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

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

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

T1



**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

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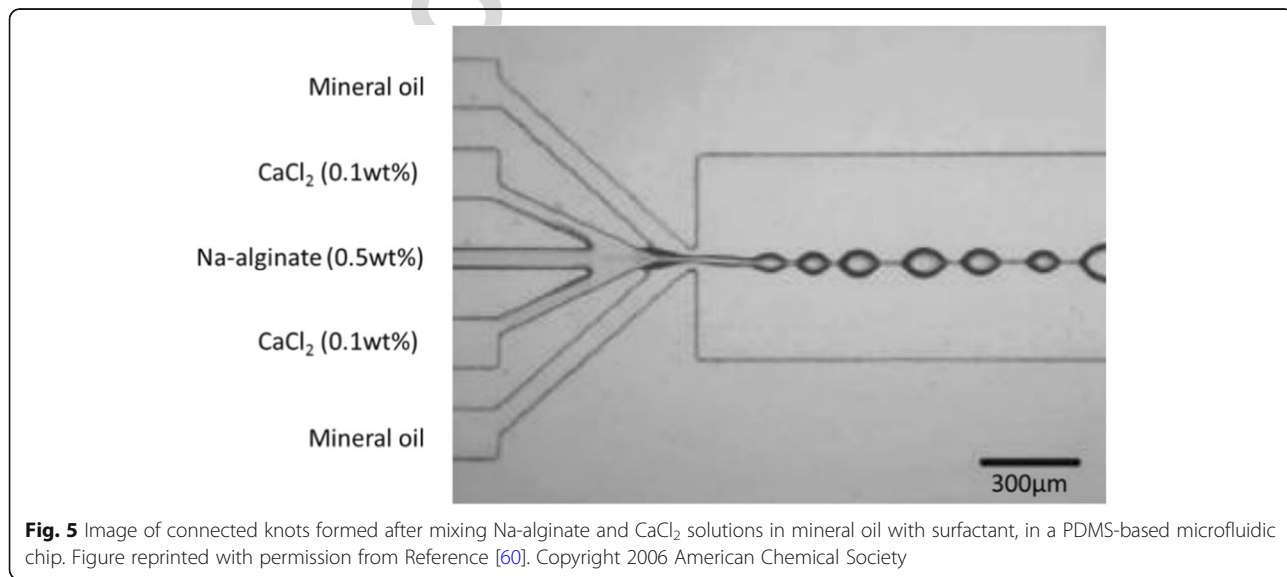
t1.1 **Table 1** Internal gelation with water-soluble cross-linkers: mixing cross-linkers with Na-alginate before droplet generation

t1.2	References		Trivedi et al., 2009 [48]	Zhang et al., 2006 [60]	Rondeau and Cooper-White, 2008 [39]	Present review	Present review
t1.3	<b>Droplet generation</b>	<b>Concentration of Na-alginate</b>	1 wt%	0.5 wt%	0.5 wt%	0.06 wt%	0.006 wt%
t1.4		<b>Continuous fluid</b>	Silicone oil	Mineral oil	DMC <sup>a</sup>	DMC <sup>a</sup>	DMC <sup>a</sup>
t1.5		<b>Use of surfactant</b>	NO	Span 80	Not mentioned	NO	NO
t1.6		<b>Geometry</b>	Flow-focusing	Flow-focusing	Flow-focusing	Cross-flowing	Cross-flowing
t1.7		<b>Microfluidic material</b>	Fluoropolymer tubing and junctions	PDMS chip	PDMS chip	Fluoropolymer tubing and junctions	Fluoropolymer tubing and junctions
t1.9	<b>Internal gelation by mixing Na-alginate and water-soluble cross-linker</b>	<b>Cross-linker</b>	BaCl <sub>2</sub> (50 mM)	CaCl <sub>2</sub> (0.1 wt%)	CaCl <sub>2</sub> (0.25 wt%)	CaCl <sub>2</sub> (0.06 wt%)	CaCl <sub>2</sub> (0.002 wt%)
t1.10		<b>Geometry</b>	Flow-focusing	Cross-flowing	Flow-focusing	–	Cross-flowing
t1.11		<b>Mixing</b>	Before droplet generation	During droplet generation	Before droplet generation	Off-line, before droplet generation	During droplet generation

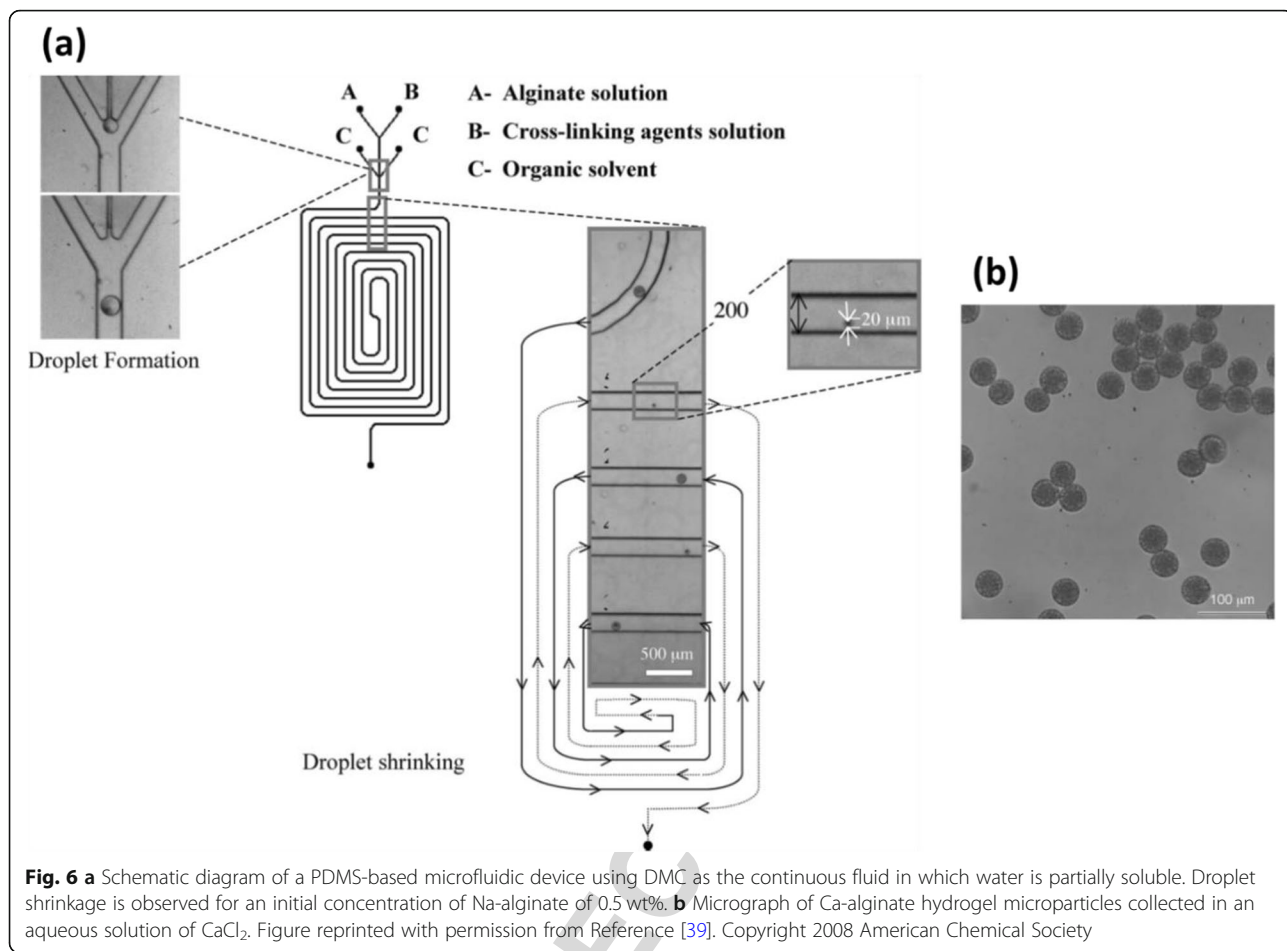
t1.16 <sup>a</sup>Continuous and dispersed fluids partially miscible - DMC (Dimethyl Carbonate)

259 produced with a partially formed droplet head and a  
 260 long gelatinous tail.  
 261 To deal with this issue, the mixing region can be re-  
 262 duced before droplet generation, as Zhang et al. did [60]  
 263 Using a 5-channel microfluidic device, they mixed Na-  
 264 alginate fluid (0.5 wt%), CaCl<sub>2</sub> fluids (0.1 wt%) and min-  
 265 eral oil fluids with a surfactant (Span 80, no concentra-  
 F5 266 tion mentioned) as shown in Fig. 5. Droplets were  
 267 generated by co-flow. However, instead of producing  
 268 discrete droplets, a line of knots connected with each  
 269 other was formed. This phenomenon persisted with a  
 270 wide range of flow rates of oil due to viscosity which in-  
 271 creased instantly when Na-alginate and CaCl<sub>2</sub> were  
 272 mixed, because of rapid gelation. It was therefore impos-  
 273 sible to generate droplets at the junction, despite the use  
 274 of surfactant.

The problem can be solved by using low concentra- 275  
 tions of Na-alginate and CaCl<sub>2</sub> solutions. In this case, 276  
 gelation proceeds after droplet generation and is en- 277  
 hanced by using partially miscible fluids. Rondeau and 278  
 Cooper-White used Dimethyl carbonate (DMC) as the 279  
 continuous fluid [39] (Fig. 6). The solubility of water in 280 **F6**  
 DMC is about 3 wt% at room temperature [43]. Aqueous 281  
 solutions of Na-alginate (0.5 wt%) and CaCl<sub>2</sub> (0.25 wt%) 282  
 were injected respectively from inlets A and B (Fig. 6a). 283  
 After a short pre-gelation channel, DMC was injected 284  
 from inlet C. Na-alginate/CaCl<sub>2</sub> droplets were generated 285  
 in DMC (no mention of surfactant usage) by flow- 286  
 focusing. Along the serpentine channel, because of the 287  
 low solubility of water in DMC, water diffused gradually 288  
 from droplets into DMC, causing the shrinkage of drop- 289  
 lets along the channel. Internal gelation occurred at the 290



f5.1 **Fig. 5** Image of connected knots formed after mixing Na-alginate and CaCl<sub>2</sub> solutions in mineral oil with surfactant, in a PDMS-based microfluidic  
 f5.2 chip. Figure reprinted with permission from Reference [60]. Copyright 2006 American Chemical Society  
 f5.3

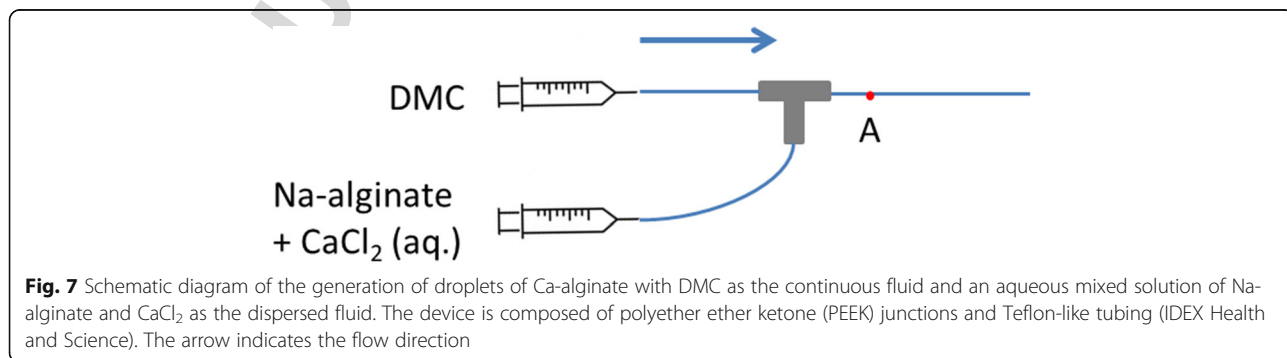


f6.1  
 f6.2  
 f6.3  
 f6.4

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<sub>2</sub> (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 °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 **F7**



f7.1  
 f7.2  
 f7.3  
 f7.4



311 droplets of Ca-alginate in DMC without surfactant from  
 312 a mixture of more diluted Na-alginate and CaCl<sub>2</sub> solu-  
 313 tions (both at 0.06 wt% after mixing). However, this solu-  
 314 tion was not clear and local gelation was occasionally  
 315 observed with the naked eye. When these gels entered  
 316 the channel, droplets were generated in a discontinuous  
 317 way. This indicates that, even at very low concentrations,  
 318 thorough mixing of Na-alginate and CaCl<sub>2</sub> solutions  
 319 leads to gelation, disturbing droplet generation.

F8 320 In a microfluidic device (Fig. 8) of similar design to  
 321 Zhang et al., we were able to generate discrete droplets  
 322 by using extremely diluted solutions of Na-alginate  
 323 (0.006 wt%) and CaCl<sub>2</sub> (0.002 wt%). The continuous fluid  
 324 was DMC without surfactant. Droplets were observed  
 325 after the cross-junction (point A in Fig. 8a). Since they  
 326 were relatively close to each other in the channel, caus-  
 327 ing coalescence at the outlet (point B in Fig. 8a), a sec-  
 328 ond flow of DMC was introduced as a spacer using a T-  
 329 junction. When the second DMC flow rate was relatively  
 330 low, the generation of droplets upstream was not dis-  
 331 turbed, so that droplets were uniform (Fig. 8b). How-  
 332 ever, the coalescence at the outlet persisted. Thus, high  
 333 second DMC flow rates were applied to sufficiently in-  
 334 crease the distance between droplets. Nevertheless, this  
 335 quickly disturbed the generation of droplets upstream,  
 336 as indicated by heterogeneities in droplet size and fre-  
 337 quency (Fig. 8c). Using surfactant would prevent droplet  
 338 coalescence.

339 To summarize (Table 1), authors mixed highly con-  
 340 centrated solutions of Na-alginate and water-soluble

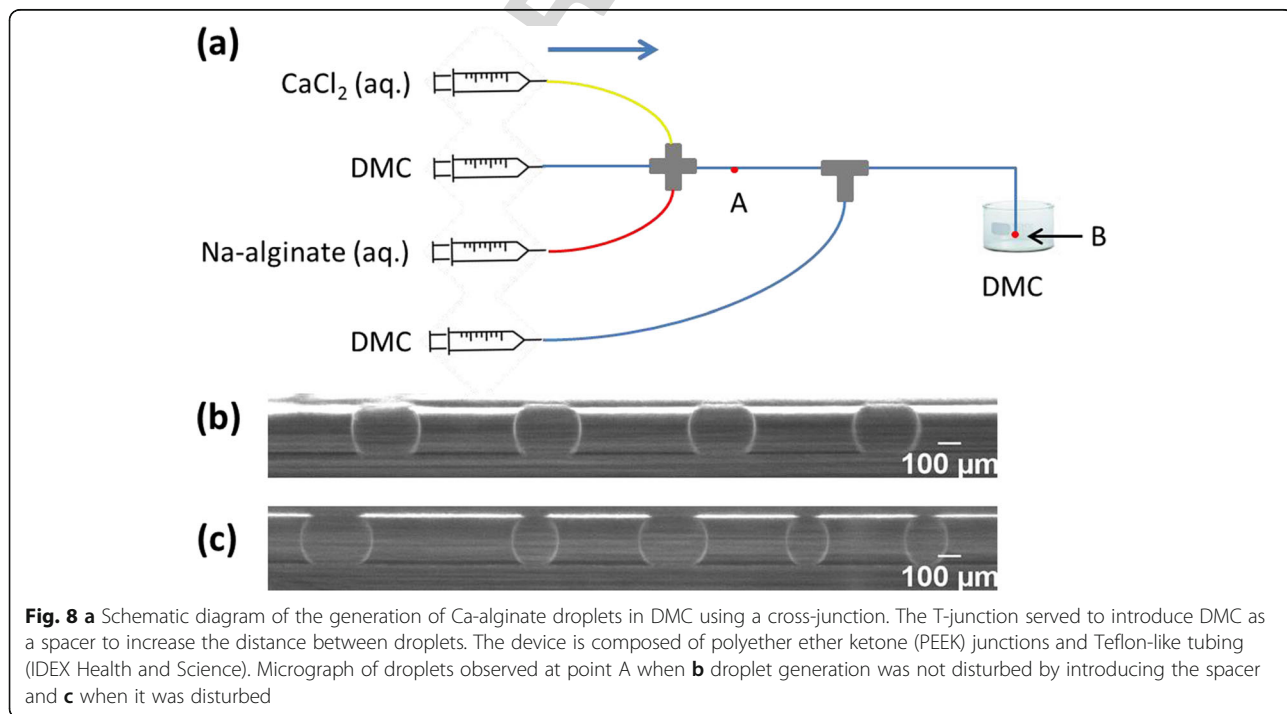
341 cross-linkers before droplet generation to make them 341  
 342 gelate. However, droplet generation was hindered by 342  
 343 rapid gelation and microparticles were difficult to obtain. 343  
 344 To delay gelation, less concentrated solutions of Na- 344  
 345 alginate and water-soluble cross-linkers were mixed be- 345  
 346 fore droplet generation, in the microfluidic device or off- 346  
 347 line. Then, concentrations were increased after droplet 347  
 348 generation by diffusion of water from the droplets to the 348  
 349 continuous fluid, due to their partial miscibility with 349  
 350 water. Hence, gelation proceeded slowly with droplet 350  
 351 shrinkage. However, mixing cross-linkers with Na- 351  
 352 alginate before droplet generation led to heterogeneities 352  
 353 in droplet size and frequency. 353

**Mixing cross-linkers after droplet generation** To 354  
 355 delay gelation, water-soluble cross-linkers need to be 355  
 356 mixed with Na-alginate only after droplet generation. 356  
 357 Studies doing so, and which also use BaCl<sub>2</sub> and CaCl<sub>2</sub> as 357  
 358 cross-linkers, are summarized in Table 2. 358

T2

359 Xu et al. prevented rapid gelation by delaying the direct 359  
 360 contact between Na-alginate and calcium cations 360  
 361 [54]. In a first cross-junction, two face-to-face channels 361  
 362 were used to introduce CaCl<sub>2</sub> (2 wt%) and Na-alginate 362  
 363 (2 wt%) solutions (Fig. 9a) perpendicularly to a flow of 363  
 364 water. Thus, after the first cross-junction, a flow of water 364  
 365 (acting as a buffer) separates the flows of Na-alginate 365  
 366 and CaCl<sub>2</sub>. Then octyl alcohol oil (no mention of surfac- 366  
 367 tant) was injected at a second cross-junction. Droplets of 367  
 368 Na-alginate/CaCl<sub>2</sub> were generated by flow-focusing. In 368  
 369 the “synthesizing channel” (Fig. 9a), within each droplet, 369

F9



f8.1  
 f8.2  
 f8.3  
 f8.4  
 f8.5

t2.1 **Table 2** Internal gelation with water-soluble cross-linkers: mixing cross-linkers with Na-alginate after droplet generation

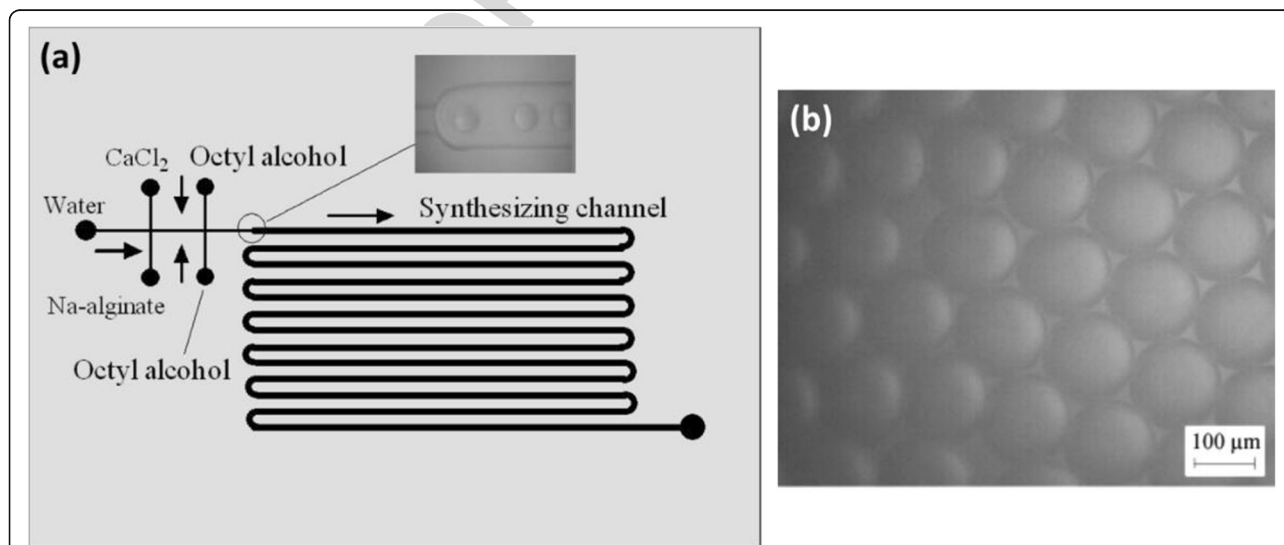
t2.2	Reference	Xu et al., 2008 [54]	Liu et al., 2006 [28]	Trivedi et al., 2010 [47]
t2.3	<b>Droplet generation</b>			
	<b>Concentration of Na-alginate</b>	2 wt%	2 wt%	1 wt%
t2.4	<b>Continuous fluid</b>	octyl alcohol oil	soybean oil	silicone oil
t2.5	<b>Use of surfactant</b>	Not mentioned	NO	NO
t2.6	<b>Geometry</b>	Flow-focusing	Flow-focusing	Flow-focusing
t2.7	<b>Microfluidic material</b>	PMMA chip	PDMS chip	Fluoropolymer tubing and junctions
t2.8	<b>Internal gelation by mixing Na-alginate and water-soluble cross-linker</b>			
t2.9		<b>Cross-linkers</b>	CaCl <sub>2</sub> (2 wt%)	CaCl <sub>2</sub> (2 wt%)
t2.10		<b>Geometry</b>	Cross-flowing	Expansion chamber
t2.11				Cross-flowing
t2.12		<b>Mixing</b>	Coalescence of flows	Coalescence of droplets
			Coalescence of droplets	Coalescence of droplets with flow

370 mixing Na-alginate and CaCl<sub>2</sub> induced internal gelation.  
 371 In this way droplets were transformed into Ca-alginate  
 372 hydrogel microparticles (Fig. 9b). For this strategy, the  
 373 size of Ca-alginate hydrogel microparticles is entirely  
 374 dependent on the experimental conditions, such as flow  
 375 rates of fluids and channel size. Manipulation of micro-  
 376 particles is difficult if their diameter is smaller than  
 377 10 μm.

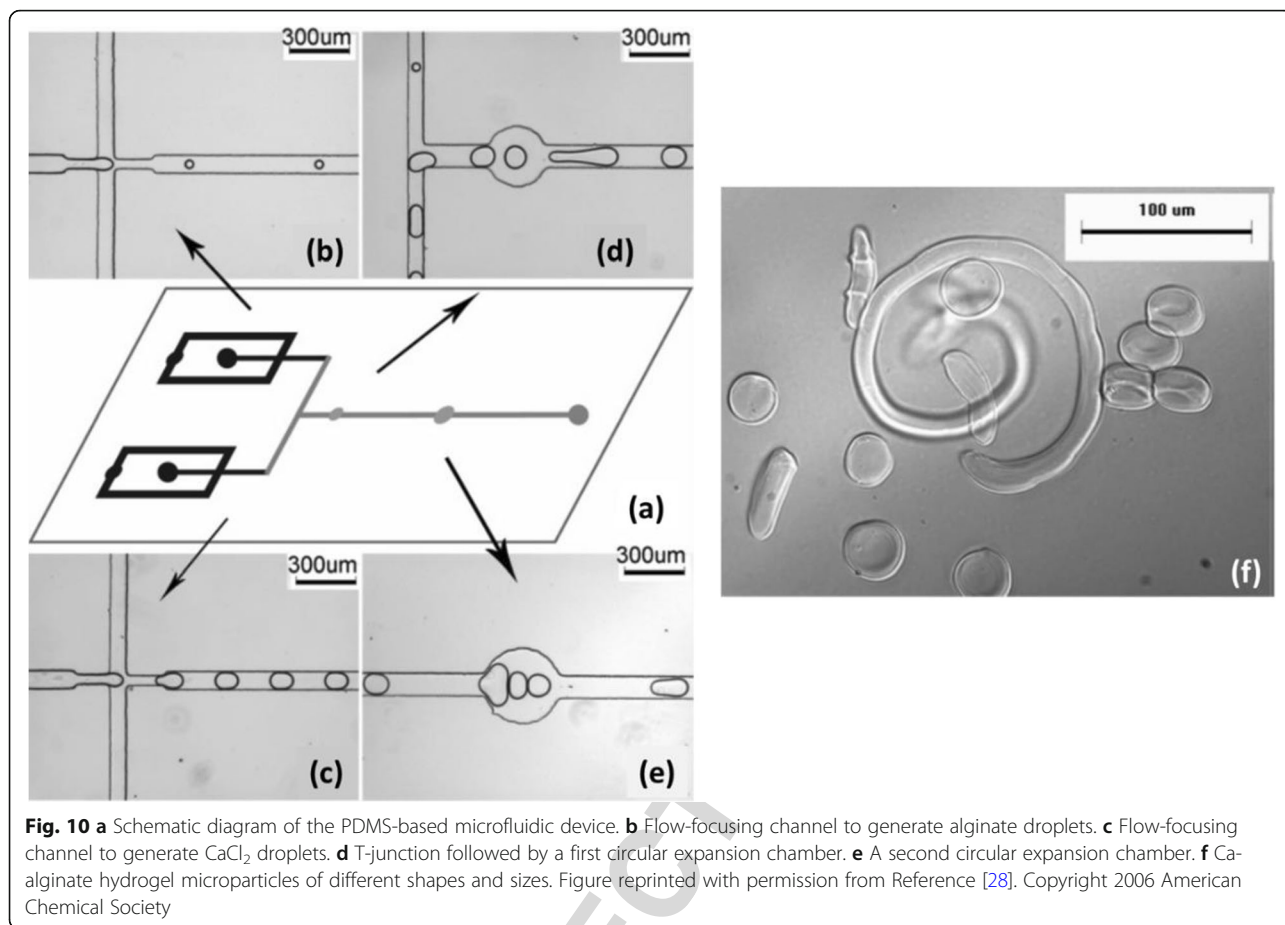
378 Another strategy to delay gelation was carried out by  
 379 Liu et al. [28] involving coalescence of Na-alginate drop-  
 380 lets with CaCl<sub>2</sub> droplets generated separately. First, on a  
 F10381 microfluidic chip (Fig. 10a), Na-alginate (2 wt%) droplets  
 382 (Fig. 10b) and CaCl<sub>2</sub> (2 wt%) droplets (Fig. 10c) were  
 383 generated in soybean oil without surfactant by flow-  
 384 focusing using two independent cross-junctions. Then  
 385 droplets converged via a T-junction (Fig. 10d) followed  
 386 by two successive circular expansion chambers (Fig. 10d,  
 387 e). Thus, droplets could collide either at the T-junction

388 or in circular chambers. Within the coalesced droplets, 388  
 Na-alginate was crosslinked by calcium cations forming 389  
 Ca-alginate hydrogel microparticles. With different flow 390  
 rates and channel geometries, various shapes and sizes 391  
 of microparticles could be produced (Fig. 10f). Neverthe- 392  
 less, the design of circular expansion chambers gives rise 393  
 to local changes in flow velocity. Droplet circulation can 394  
 be disturbed, thereby affecting homogeneity in droplet 395  
 shape, size and frequency. 396

397 Droplets could also be coalesced by exploiting physio- 397  
 chemical parameters between the continuous fluid 398  
 and the dispersed fluid. In the work of Trivedi et al., 399  
 droplets of Na-alginate (1 wt%) containing cells were 400  
 generated upstream in a highly viscous silicone oil (10 401  
 centistoke) by flow-focusing without surfactant [47, 48]. 402  
 An aqueous solution of BaCl<sub>2</sub> (50 mM) was injected 403  
 downstream by a T-junction. With the help of dye, obser- 404  
 vations at the T-junction indicated that BaCl<sub>2</sub> fluid 405



f9.1 **Fig. 9** a Schematic diagram of Ca-alginate hydrogel microparticles prepared in a PMMA based microfluidic device. b Micrographs of Ca-alginate  
 f9.2 hydrogel microparticles. Figure reprinted with permission from Reference [54]  
 f9.3



f10.1 **Fig. 10 a** Schematic diagram of the PDMS-based microfluidic device. **b** Flow-focusing channel to generate alginate droplets. **c** Flow-focusing  
 f10.2 channel to generate CaCl<sub>2</sub> droplets. **d** T-junction followed by a first circular expansion chamber. **e** A second circular expansion chamber. **f** Ca-  
 f10.3 alginate hydrogel microparticles of different shapes and sizes. Figure reprinted with permission from Reference [28]. Copyright 2006 American  
 f10.4 Chemical Society  
 f10.5

406 merged spontaneously with Na-alginate/cells droplets,  
 407 instead of forming independent BaCl<sub>2</sub> droplets. How-  
 408 ever, this strategy lacks flexibility. The expected coales-  
 409 cence happens only when appropriate solvents are used.  
 410 For instance, when using low-viscosity and low-  
 411 interfacial energy  $\gamma_{CD}$  soybean oil, independent droplets  
 412 of BaCl<sub>2</sub> were observed. They coalesced downstream  
 413 with Na-alginate/cells droplets. This implied that suc-  
 414 cessful coalescence of droplets could only take place  
 415 with appropriate interfacial energy and viscosity [48].

416 **Water-insoluble or weakly soluble cross-linkers**

417 To delay gelation, Na-alginate can be mixed with water-  
 418 insoluble or weakly soluble cross-linkers, in water. This  
 419 will not lead to instant gelation since there are no avail-  
 420 able cations in water. In the case of cross-linkers which  
 421 are pH-sensitive, such as calcium carbonate (CaCO<sub>3</sub>)  
 422 and calcium-ethylenediaminetetraacetic acid (Ca-EDTA)  
 423 complex, an acid is used in the continuous fluid to re-  
 424 lease the cations from inert cross-linkers. Therefore, gel-  
 425 ation by the available cations happens after droplet  
 T3 426 generation. These strategies are summarized in Table 3.

427 In the work of Zhang et al. [61], fine particles of  
 428 CaCO<sub>3</sub> (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<sub>3</sub> were generated by flow-focusing in soy-  
 bean 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<sub>3</sub>, causing internal gel-  
 ation of the alginate. Finally, Ca-alginate hydrogel micro-  
 particles 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<sub>3</sub>. More-  
 over, a higher concentration of CaCO<sub>3</sub> 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 chan-  
 nel (Fig. 12a), while the Na-alginate fluid (1.5 wt%) con-  
 taining CaCO<sub>3</sub> nanoparticles (35 mM) was introduced by  
 two side channels (Fig. 12b). This geometry was used to

F11

F12

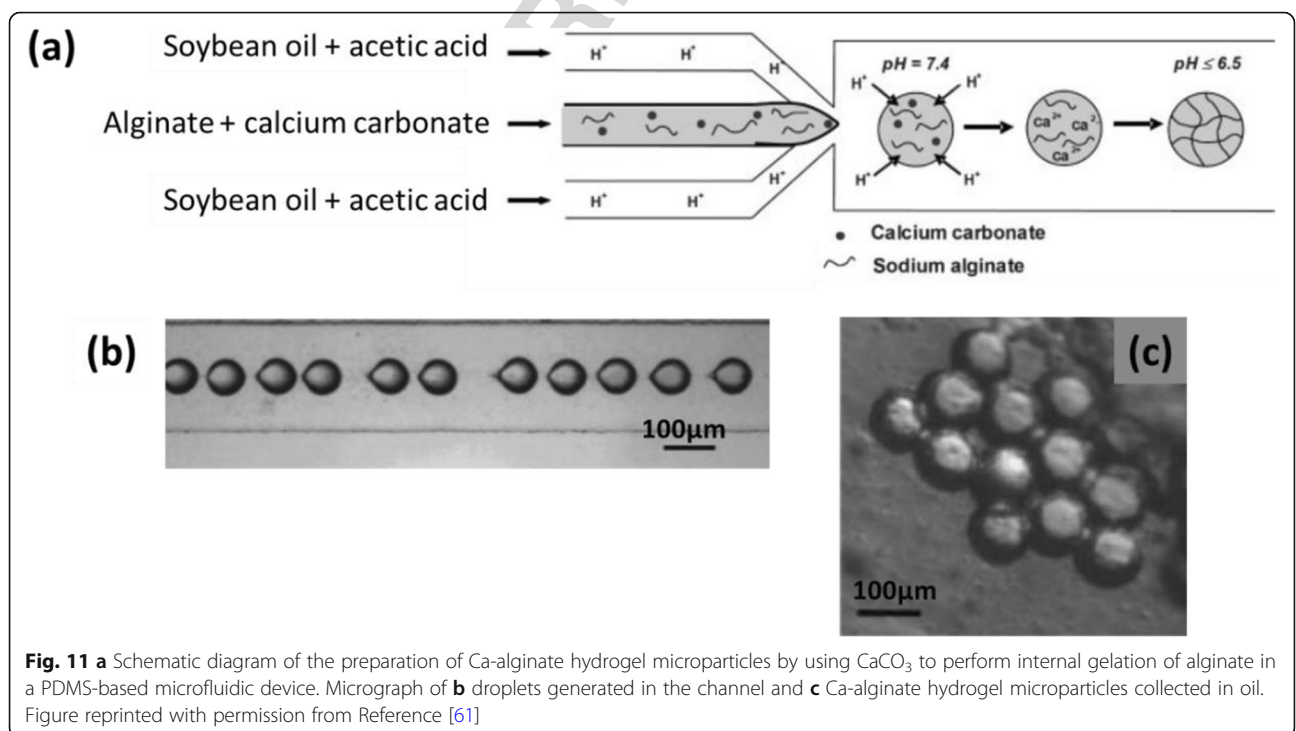
t3.1 **Table 3** Internal gelation with water-insoluble or weakly soluble cross-linkers which are pH sensitive

t3.2	References	Zhang et al., 2007 [61]	Akbari and Pirbodaghi, 2014 [3]	Yu et al., 2019 [56]	Utech et al., 2015 [49]	
t3.3	<b>Droplet generation</b>	<b>Concentration of Na-alginate</b>	2 wt%	1,5 wt%	2 w/v%	2 wt%
t3.4		<b>Continuous fluid</b>	Soybean oil	Fluorocarbon oil	Mineral oil	Fluorinated carbon oil
t3.5		<b>Use of surfactant</b>	Span 80	Fluorinated surfactant	Span 80	Biocompatible surfactant
t3.6		<b>Geometry</b>	Flow-focusing	Flow-focusing	Flow-focusing	Flow-focusing
t3.7		<b>Microfluidic material</b>	PDMS chip	PDMS chip	PDMS chip	PDMS chip
t3.8						
t3.9	<b>Internal gelation by mixing Na-alginate and water-insoluble or weakly soluble cross-linkers</b>	<b>Cross-linkers</b>	CaCO <sub>3</sub> (0,1 wt%)	CaCO <sub>3</sub> (35 mM)	CaCO <sub>3</sub> (200 mM)	Ca-EDTA (50 mM)
t3.10		<b>Mixing</b>	Off-line, before droplet generation	Before droplet generation	Off-line, before droplet generation	Off-line, before droplet generation
t3.11		<b>Gelation by acid addition</b>	In continuous fluid	In continuous fluid	In collecting fluid	In continuous fluid
t3.12						
t3.13						
t3.14						

453 create a coaxial stream while avoiding direct mechanical  
 454 contact between cells and the potentially damaging  
 455 CaCO<sub>3</sub> particles. At a second T-junction, fluorocarbon  
 456 oil with surfactant (fluorinated surfactant, 1 wt%) was  
 457 injected. Droplets of Na-alginate/cells/CaCO<sub>3</sub> were then  
 458 generated by flow-focusing. After droplet collection,  
 459 acetic acid (0.1 vol%) dissolved in oil was added to re-  
 460 lease calcium cations within droplets, causing gelation of  
 461 alginate. Droplets were thus transformed into Ca-  
 462 alginate hydrogel microparticles, some with cells encapsu-  
 463 lated (Fig. 12c). However, the mixture of CaCO<sub>3</sub> and  
 464 Na-alginate was not homogeneous, which can be seen

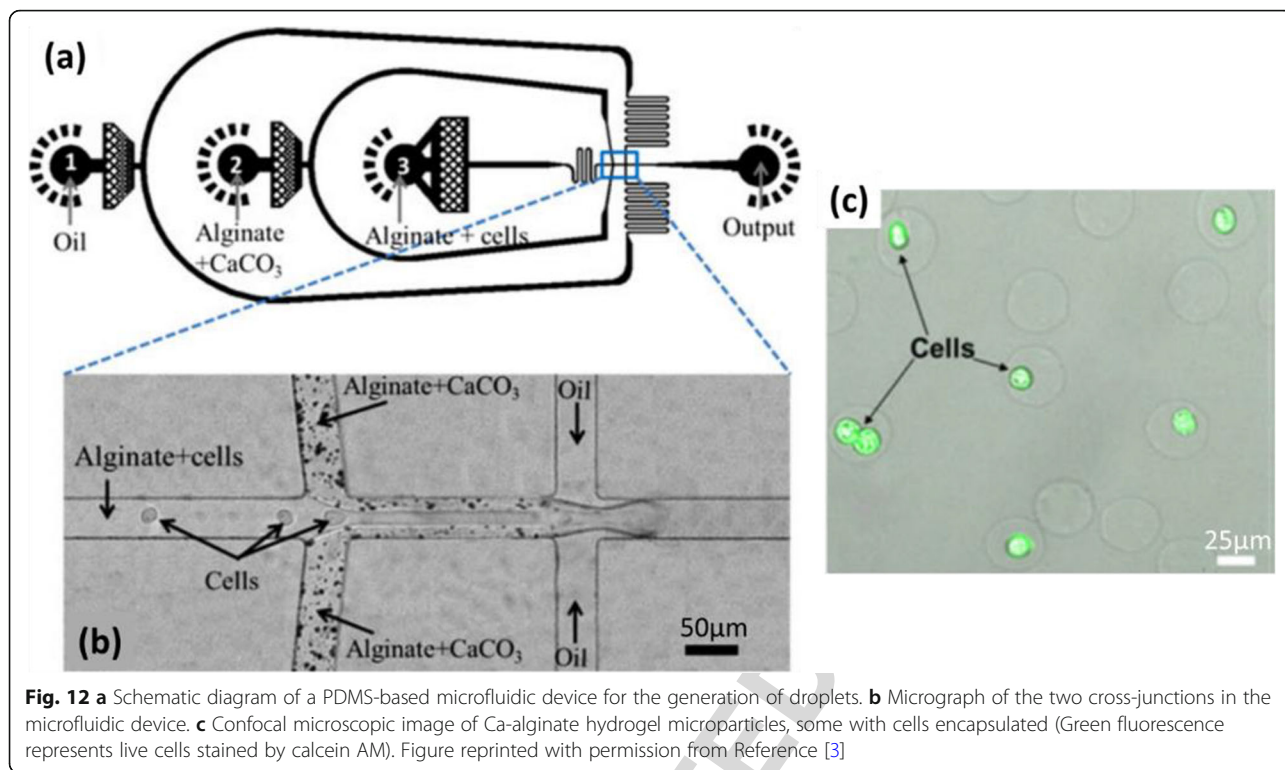
from Fig. 12b. Thus, the varying amounts of CaCO<sub>3</sub> in-  
 465 fluenced the degree of gelation in each droplet, yielding  
 466 Ca-alginate hydrogel microparticles with different mech-  
 467 anical properties. This issue is not discussed by Akbari  
 468 and Pirbodaghi [3]. Furthermore, not all microparticles  
 469 encapsulated cells, for reasons not explored in the arti-  
 470 cle. Sorting is therefore required after the preparation of  
 471 microparticles, which complicates the procedure.

Combining the strategy of Zhang et al. and Akbari and  
 473 Pirbodaghi to conduct gelation both in the microfluidic  
 474 device and in the collection bath, Yu et al. [56] produced  
 475 Ca-alginate hydrogel microparticles for protein 476



f11.1 **Fig. 11 a** Schematic diagram of the preparation of Ca-alginate hydrogel microparticles by using CaCO<sub>3</sub> to perform internal gelation of alginate in  
 f11.2 a PDMS-based microfluidic device. Micrograph of **b** droplets generated in the channel and **c** Ca-alginate hydrogel microparticles collected in oil.  
 f11.3 Figure reprinted with permission from Reference [61]  
 f11.4





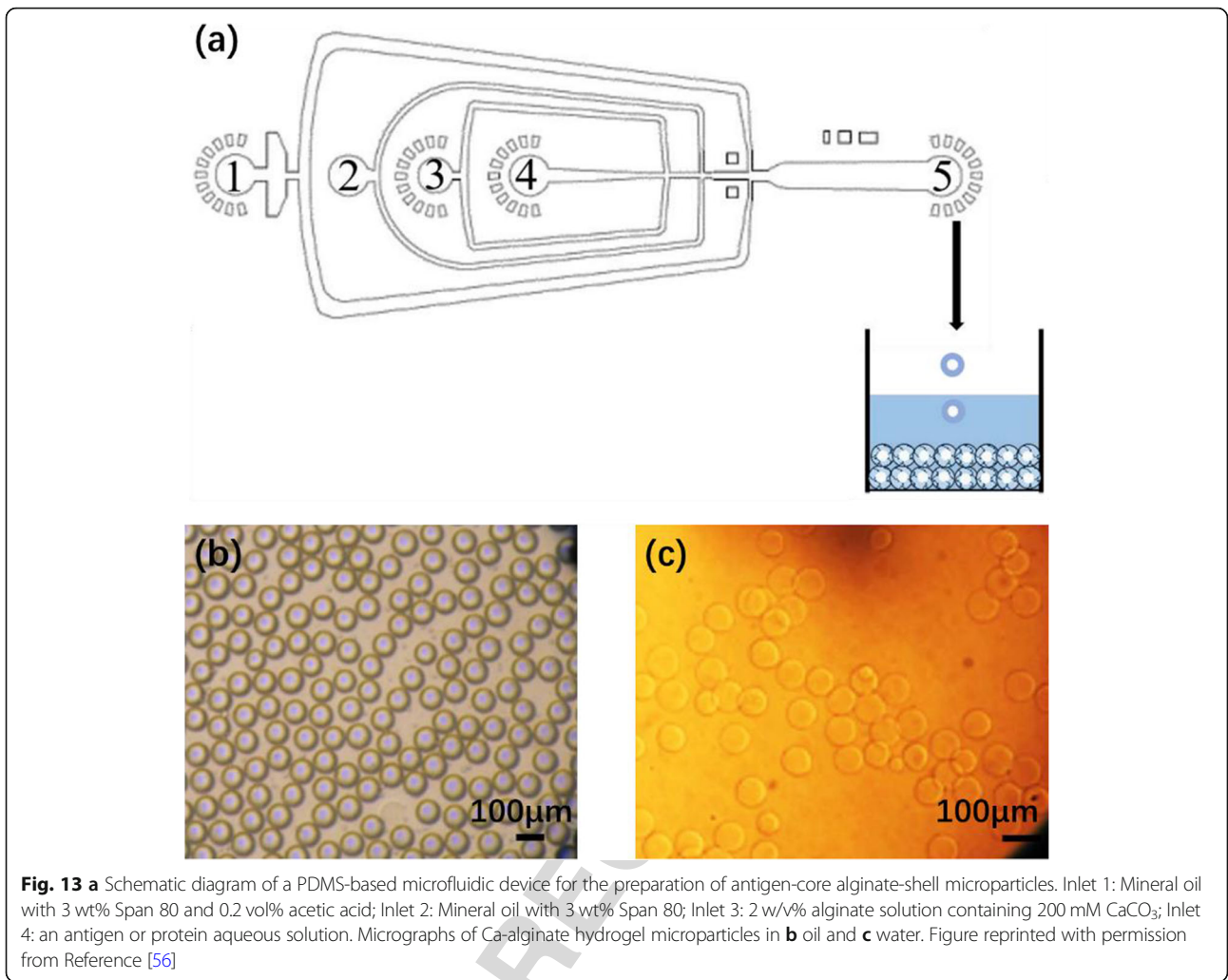
f12.1 **Fig. 12 a** Schematic diagram of a PDMS-based microfluidic device for the generation of droplets. **b** Micrograph of the two cross-junctions in the  
 f12.2 microfluidic device. **c** Confocal microscopic image of Ca-alginate hydrogel microparticles, some with cells encapsulated (Green fluorescence  
 f12.3 represents live cells stained by calcein AM). Figure reprinted with permission from Reference [3]  
 f12.4

477 encapsulation. First, from inlet 4 (Fig. 13a), an aqueous solu-  
 478 tion of antigen or protein was injected. It co-flowed with  
 479 another aqueous solution of alginate (2 w/v%) mixed with  
 480 CaCO<sub>3</sub> particles (200 mM) and injected from inlet 3. Mineral  
 481 oil with Span 80 added was injected from inlet 2 as a  
 482 continuous fluid. In the flow-focusing channel, droplets  
 483 containing alginate, CaCO<sub>3</sub> and protein were formed.  
 484 From inlet 1, another continuous fluid, mineral oil con-  
 485 taining Span 80 and acetate acid, was introduced. When  
 486 the acetate acid diffused into droplets, calcium cations  
 487 were released. The alginate was then crosslinked, leading  
 488 to preliminary gelation. The droplets were collected in an  
 489 aqueous solution of CaCl<sub>2</sub> (0.27 M) to enhance gelation.  
 490 In the end, spherical hydrogel microparticles were formed,  
 491 with protein encapsulated (Fig. 13b-c). According to the  
 492 authors, the preliminary gelation in the microchannel pre-  
 493 vented the deformation that occurs when droplets are col-  
 494 lected directly in an aqueous solution of CaCl<sub>2</sub>. As  
 495 mentioned above, however, since CaCO<sub>3</sub> is not soluble in  
 496 water, a high concentration of CaCO<sub>3</sub> will clog the micro-  
 497 channel. Thus, the scope for preliminary gelation is lim-  
 498 ited. Moreover, it takes time (in this case, overnight) to  
 499 obtain a mixture where CaCO<sub>3</sub> particles are well dis-  
 500 persed.  
 501 In order to obtain a homogeneous internal structure  
 502 of hydrogel microparticles, Utech et al. used a slightly  
 503 water-soluble calcium-ethylenediaminetetraacetic acid  
 504 (Ca-EDTA) complex as the cross-linker [49]. An aque-  
 505 ous solution of Na-alginate (2 wt%) mixed with Ca-

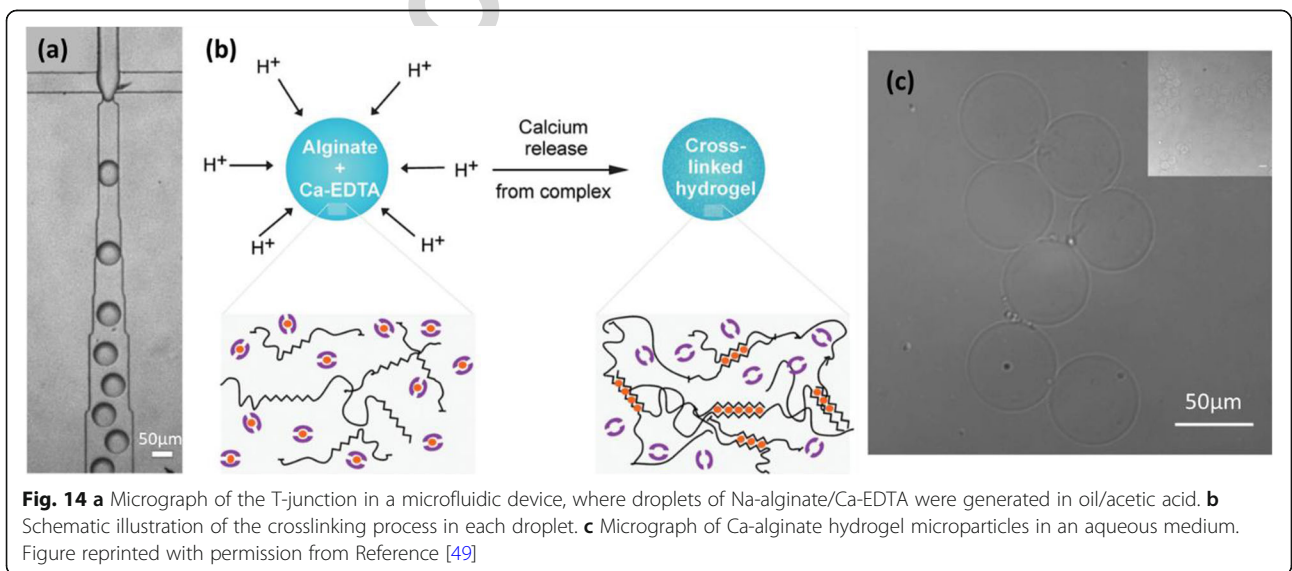
EDTA (50 mM) was first prepared. This homogeneous  
 mixture was used as the dispersed fluid for the micro-  
 fluidic system. The continuous fluid was a fluorinated  
 carbon oil with a biocompatible surfactant (1 wt%) con-  
 taining acetic acid (0.05 vol%). Droplets of Na-alginate/  
 Ca-EDTA were 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 appro-  
 priate when microparticles need to be highly crosslinked.  
 Furthermore, care should be taken with Ca-EDTA, as  
 EDTA is used to dissolve alginate hydrogel microparti-  
 cles in the literature [29, 56].  
 In conclusion, internal gelation of alginate can be real-  
 ized 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 gen-  
 eration, and/or by using extremely diluted solutions and  
 surfactant (Table 1). Mixing cross-linkers and Na-  
 alginate after droplet generation involves merging

F14





f13.1  
f13.2  
f13.3  
f13.4  
f13.5



f14.1  
f14.2  
f14.3  
f14.4

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  $\text{CaCl}_2$  as cross-linkers, as summarized in Table 4.

T4

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.

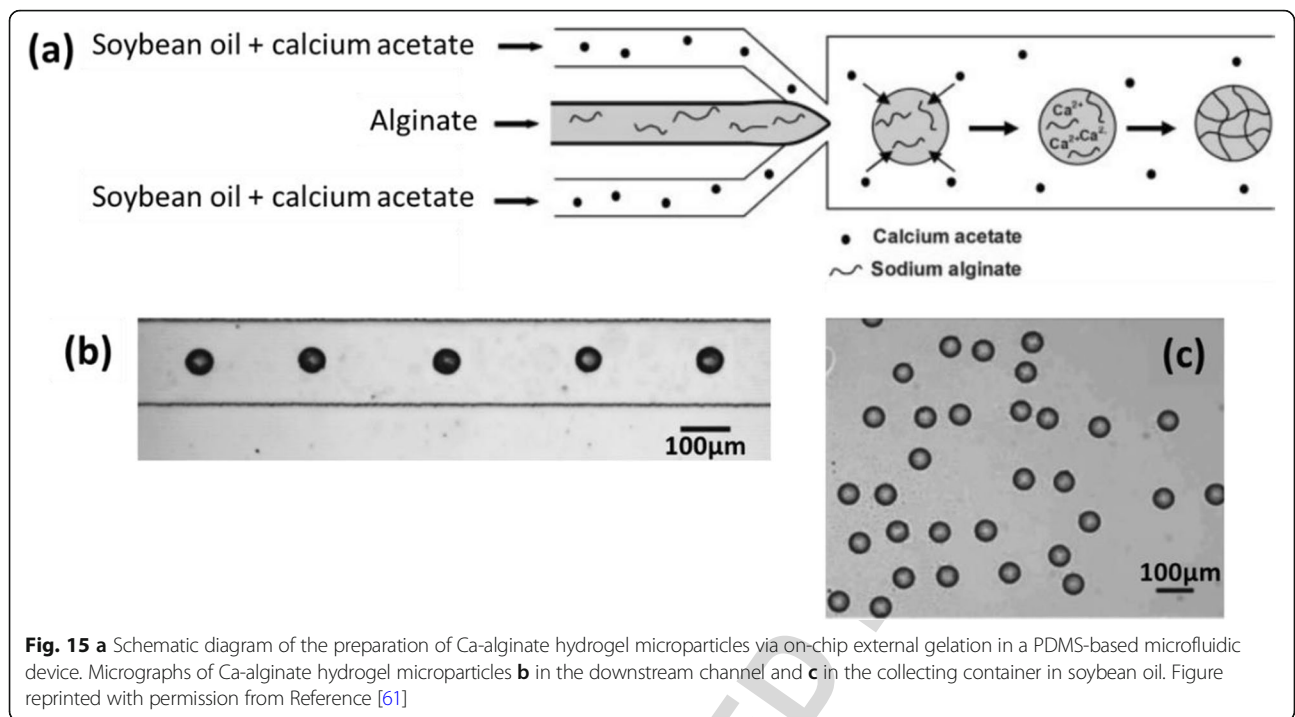
One way to limit channel clogging is to make the cross-linkers diffuse slowly in Na-alginate droplets. Thus, Liu et al. used emulsion fluids to introduce cross-linkers [29]. A glass-based microfluidic device was used, with channels modified so as to be hydrophobic. Droplets of Na-alginate (3 wt%) were first generated in corn oil at the first flow-focusing channel (Fig. 16a). The emulsion of  $\text{CaCl}_2$ , containing  $\text{CaCl}_2$  droplets in corn oil (with surfactant SY-Glyster CRS-75), was injected downstream of the cross-junction. The contact between  $\text{CaCl}_2$  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

F15

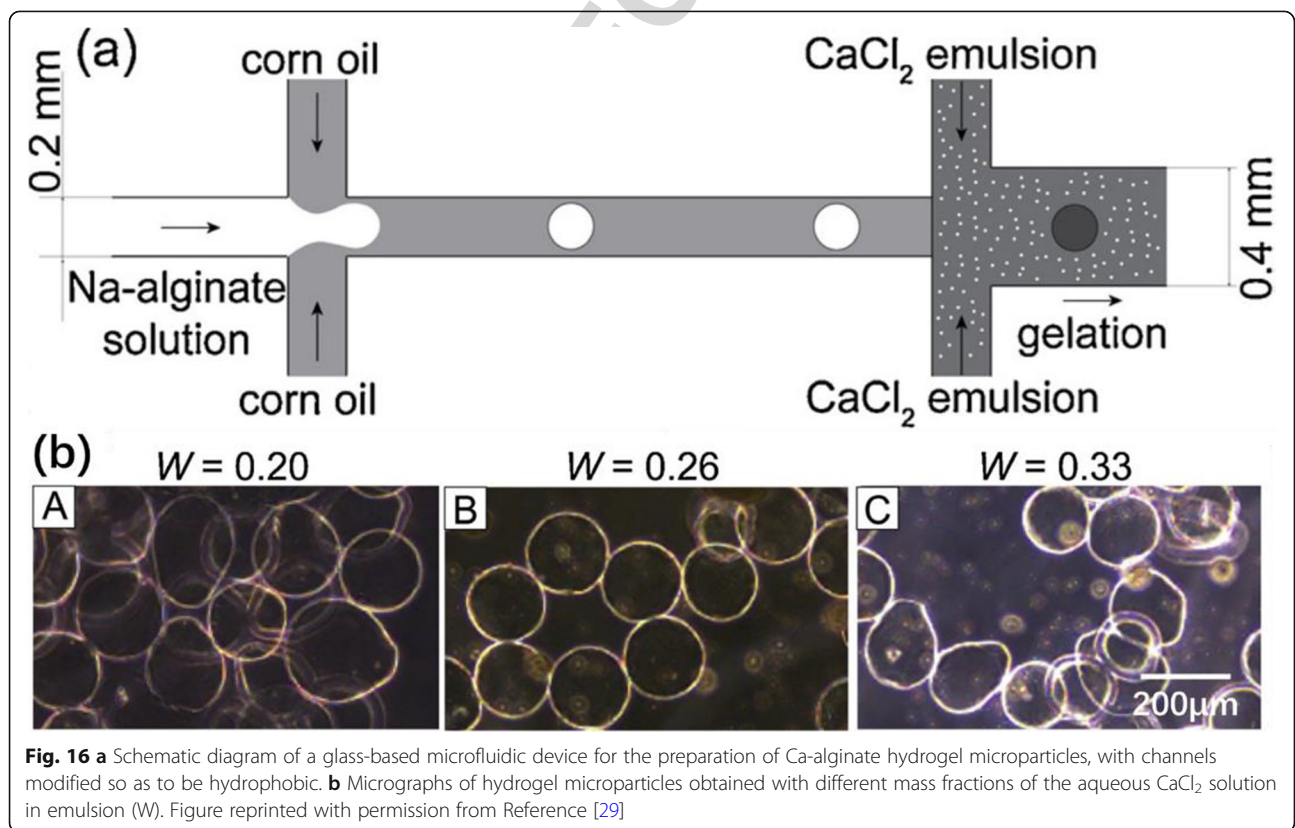
F16

**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]	
t4.3	<b>Droplet generation</b>	<b>Concentration of Na-alginate</b>	2 wt%	3 wt%	0.025–0.15 wt%	1 w/v%
t4.4		<b>Continuous fluid</b>	Soybean oil	Corn oil	Methyl acetate	1-undecanol
t4.5		<b>Use of surfactant</b>	Span 80	SY-Glyster CRS-75	Not mentioned	Abil Em 90
t4.6		<b>Geometry</b>	Flow-focusing	Flow-focusing	Flow-focusing	Flow-focusing
t4.7		<b>Microfluidic material</b>	PDMS chip	Glass chip	PDMS chip	PDMS chip
t4.8		<b>Cross-linkers</b>	$\text{Ca}(\text{CH}_3\text{COO})_2$ (2 wt%) in the continuous fluid	Emulsion of $\text{CaCl}_2$ in the continuous fluid	$\text{CaCl}_2$ (1 M) in the dispersed fluid	Emulsion of $\text{CaCl}_2$ (2 wt%) droplets in the continuous fluid
t4.9	<b>External gelation by introducing Na-alginate and cross-linkers on chip before collection</b>	<b>Geometry</b>	–	Flow-focusing	Cross-flowing	Flow-focusing
t4.10		<b>Mixing</b>	During droplet generation	After droplet generation	After droplet generation	After droplet generation
t4.11						
t4.12						
t4.13						
t4.14						
t4.15						



f15.1  
 f15.2  
 f15.3  
 f15.4

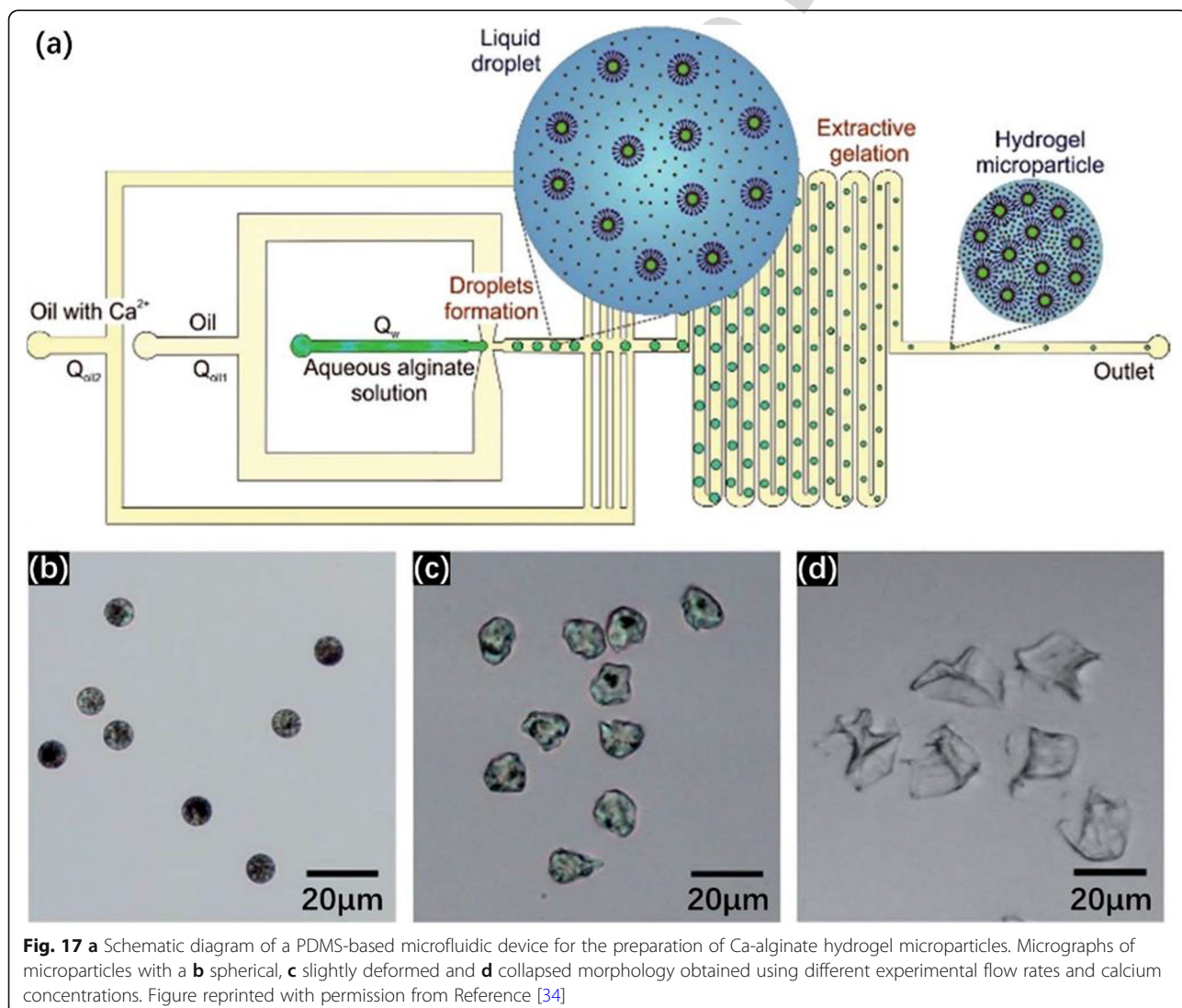


f16.1  
 f16.2  
 f16.3  
 f16.4

595 parameters, such as the mass fraction of the aqueous  
 596  $\text{CaCl}_2$  solution in emulsion (W). Deformation occurred  
 597 when the value of W was too high or too low,  
 598 so that an optimal value of W was required for  
 599 homogeneous spherical microparticles (Fig. 16b-B).  
 600 The morphology and homogeneity of microparticles  
 601 also varied with flow rates and surfactant concentra-  
 602 tions. Lacking flexibility, this strategy is thus not  
 603 appropriate for producing spherical hydrogel micro-  
 604 particles. Moreover, generating small particles requires  
 605 reducing the channel size, involving a risk of droplet  
 606 coalescence before gelation in  $\text{CaCl}_2$  emulsion.

607 To avoid reducing the channel size, partially miscible  
 608 fluids can be used as the dispersed and continuous  
 609 fluids. Sugaya et al. used methyl acetate as the continu-  
 610 ous fluid [44]. Na-alginate (0.025–0.15 wt%) droplets  
 611 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 drop-  
 lets into methyl acetate. Thus, the droplets shrank and  
 became more concentrated downstream.  $\text{CaCl}_2$  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 micro-  
 particles with a diameter of less than  $20\ \mu\text{m}$  were  
 obtained. In this strategy, after  $\text{CaCl}_2$  fluids were intro-  
 duced, two competing processes occurred simultane-  
 ously in each droplet: gelation and shrinkage of  
 droplets. The competition between gelation and shrink-  
 age is not discussed in this article. However, the results  
 indicate that extremely small droplets tend to approach  
 the channel wall after shrinkage. With the  $\text{CaCl}_2$  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]

f17.1  
 f17.2  
 f17.3  
 f17.4



629 after gelation, Ca-alginate hydrogel microparticles ad-  
630 here to the channel wall.

631 Adhesion to the channel and coalescence of micropar-  
632 ticles can be avoided thanks to progressive addition of  
633 the cross-linker. Pittermannová et al. used as continuous  
634 fluid 1-undecanol, whose water-solubility is 2.7 vol%  
635 [34]. The experiment was carried out in a PDMS-based  
F17 636 microfluidic device (Fig. 17a). An aqueous alginate solu-  
637 tion (1 wt%) was first injected. After 1-undecanol (with  
638 5 wt% surfactant Abil Em 90), shown as “oil” in Fig. 17a,  
639 was injected into the flow-focusing channel, droplets of  
640 alginate were formed.  $\text{CaCl}_2$  (2 wt%) was dispersed in  
641 another fluid, 1-undecanol with 5 wt% surfactant Abil  
642 Em 90, yielding an emulsion. This emulsion was injected  
643 after droplet generation, through successive channels  
644 (Fig. 17a). Hence, the droplets were increasingly sepa-  
645 rated from each other, avoiding coalescence. Moreover,  
646 they were surrounded by more and more  $\text{CaCl}_2$ , increas-  
647 ing gelation, and by more and more 1-undecanol, increas-  
648 ing diffusion of water. Thus, gelation and shrinkage  
649 of droplets occurred gradually and simultaneously. Ac-  
650 cording to the authors, this procedure avoids the droplet  
651 generation instability caused by pre-gelation. However,  
652 spherical hydrogel microparticles (Fig. 17b) were only  
653 obtained using certain flow rates and calcium concentra-  
654 tions. Otherwise, the microparticles were slightly de-  
655 formed (Fig. 17c) or collapsed (Fig. 17d), which was  
656 explained using a core-shell model [34]. Unfortunately,  
657 this explanation does not take into account the change  
658 in alginate concentration in the droplets due to water  
659 extraction, a factor which is bound to impact deforma-  
660 tion. Simply prolonging the water extraction process  
661 before introducing cross-linkers, as done by Sugaya  
662 et al., could avoid deformation [44].

#### Off-chip introduction of cross-linkers

663 Other strategies of external gelation introduce cross-  
664 linkers “off-chip”, i.e., during droplet collection. Ca  
665  $(\text{CH}_3\text{COO})_2$  or  $\text{CaCl}_2$  are used as cross-linkers and the  
666 collection bath procedure depends on the strategy, as  
667 summarized in Table 5. 668

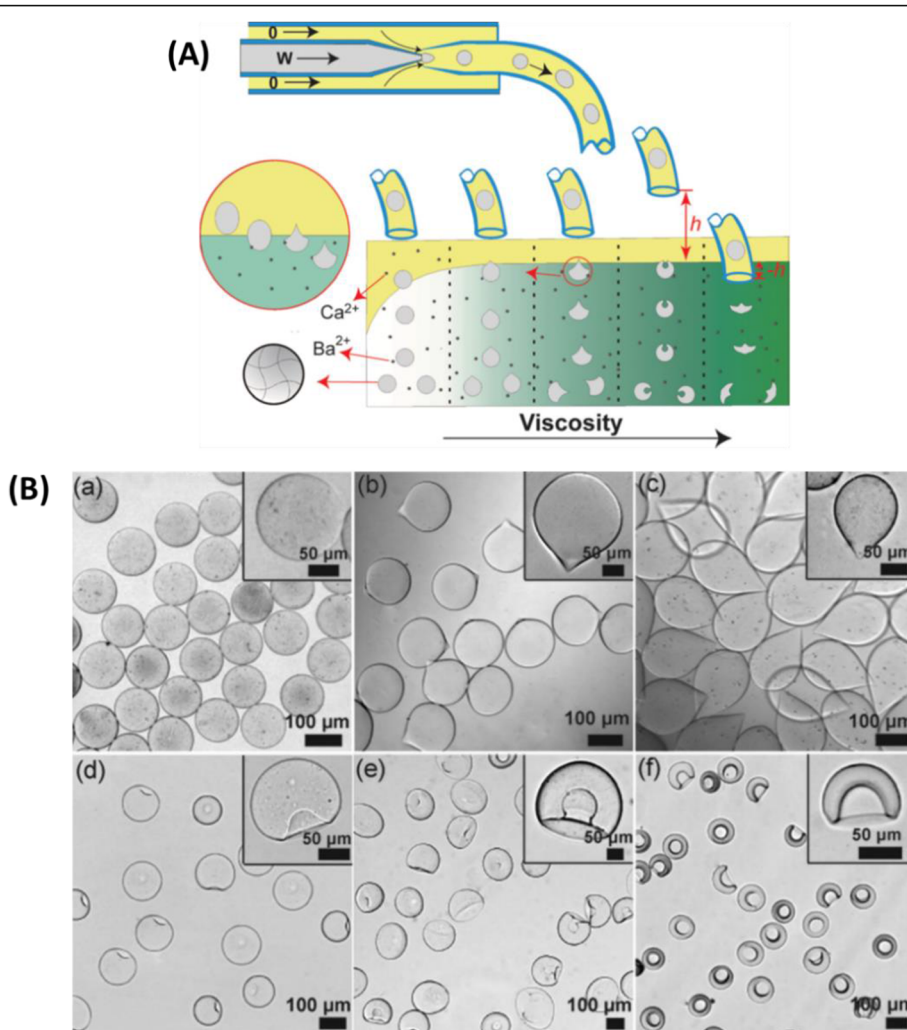
669 Hu et al. [20] studied the influence of external gelation  
670 conditions on the shape of microparticles. Na-alginate  
671 (1.5 wt%) droplets were first generated in n-decanol with  
672 surfactant (Span 80, 5 wt%), using concentric glass capil-  
673 laries in co-flow geometry (Fig. 18A). For off-chip exter-  
F18 674 nal gelation, droplets were collected in a two-phase  
675 gelation bath: the upper phase of n-decanol with surfac-  
676 tant (Span 80, 5 wt%) containing  $\text{CaCl}_2$  (15 wt%) allowed  
677 for pre-gelation of alginate; the bottom phase, an aque-  
678 ous solution of barium acetate (15 wt%), strengthened  
679 the gelation. Glycerol (0–70 wt%) was added to the bot-  
680 tom phase to regulate viscosity. Ca-alginate hydrogel  
681 particles of different shapes (Fig. 18B) were obtained by  
682 varying gelation conditions such as the interfacial energy  
683  $\gamma_{\text{CD}}$ , the concentration and type of surfactant, the height  
684 h between the end of the capillary and the surface of the  
685 gelation bath, and the viscosity of the bottom phase in  
686 the gelation bath. The shape of microparticles was  
687 shown to depend on forces applied to the surface of  
688 droplets when they passed through the interface in the  
689 gelation bath. The force from  $\gamma_{\text{CD}}$  maintains the spheri-  
690 cal form of droplets, while the viscous force causes de-  
691 formation. The final shape resulted from the overall  
692 effect of these two forces [20]. As can be seen in this  
693 strategy, droplet collection is accompanied by the consump-  
694 tion of the two different cations. Thus, to obtain a  
695 large quantity of microparticles, these cations should be  
696 replenished to ensure that each droplet undergoes

t5.1 **Table 5** External gelation with off-chip introduction of cross-linkers

t5.2	References		Hu et al., 2012 [20]	Zhang et al., 2020 [59]	Present review	
t5.3	<b>Droplet generation</b>	<b>Na-alginate</b>	1.5 wt%	0.006–1 wt%	0.006–1 wt%	
t5.4		<b>Continuous fluid</b>	N-decanol	DMC <sup>a</sup>	DMC <sup>a</sup>	
t5.5		<b>Use of surfactant</b>	Span 80	NO	NO	
t5.6		<b>Geometry</b>	Flow-focusing	Cross-flowing	Cross-flowing	
t5.7		<b>Microfluidic material</b>	Glass chip	Fluoropolymer tubing and junctions	Fluoropolymer tubing and junctions	
t5.8	<b>External gelation by introducing Na-alginate and cross-linkers off-chip during collection</b>	<b>Number of phases or steps</b>	2 phases - 1 collection bath	1 phase - 1 collection bath	2 phases - 2 successive collection baths	
t5.9		<b>Description of phases or steps</b>	Phase 1	$\text{CaCl}_2$ (15 wt%) in n-decanol	$\text{CaCl}_2$ (0.1–1 wt%)	Step 1 DMC and evaporation of DMC
t5.10			Phase 2	Ba $(\text{CH}_3\text{COO})_2$ (15 wt%) in water and glycerol		Step 2 $\text{CaCl}_2$ (0.5–10 wt%) in water
t5.11		<b>Surfactant</b>	Span 80	NO	NO	

t5.12 <sup>a</sup>Continuous and dispersed fluids partially miscible

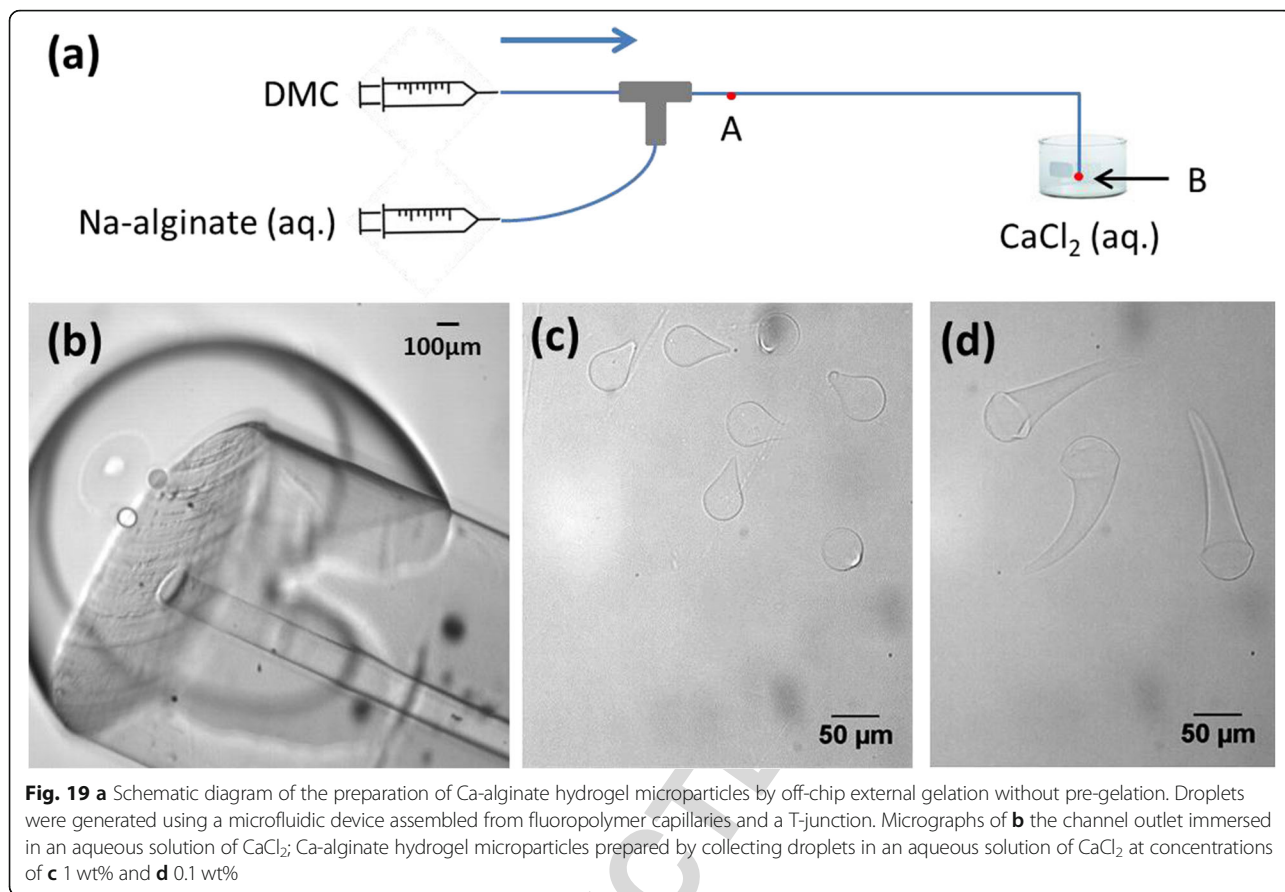




f18.1 **Fig. 18 A** Schematic diagram of the preparation of Ca-alginate hydrogel microparticles using a microfluidic device constructed with glass capillaries,  
 f18.2 and off-chip gelation in a two-phase gelation bath. **B** Micrographs of Ca-alginate hydrogel microparticles of different shapes prepared under different  
 f18.3 experimental conditions. Figure reprinted with permission from Reference [20]  
 f18.4

697 sufficient gelation. However, in practice, when and how  
 698 to replenish them remains an issue.  
 699 To avoid the problem of replenishing the bath with  
 700 the two cations, we collected Na-alginate droplets in an  
 F19701 aqueous solution containing  $\text{CaCl}_2$  (Fig. 19a) without  
 702 pre-gelation. Na-alginate (0.006–1 wt%) droplets were  
 703 first generated in DMC in a T-junction and Teflon-like  
 704 capillaries (IDEX Health and Science), without using  
 705 surfactant [59]. Because water is slightly soluble in  
 706 DMC, 3 wt%, water diffused gradually from droplets into  
 707 DMC, causing the droplets to shrink as they passed  
 708 through the channel (point A to B in Fig. 19a). Thus,  
 709 droplet size reduced to below 100  $\mu\text{m}$ . Furthermore,  
 710 since alginate dissolution in the continuous fluid is neg-  
 711 ligible [39], with the loss of water, the alginate concen-  
 712 tration in droplets increased. Then, the channel outlet  
 713 (point B in Fig. 19a) was immersed in an aqueous

714 solution of  $\text{CaCl}_2$  (0.1–1 wt%). An interface was created  
 715 at the channel outlet (Fig. 19b) because of the non-total  
 716 miscibility between DMC and water. Na-alginate droplet-  
 717 s passed through the interface and entered the  $\text{CaCl}_2$   
 718 solution, leading to off-chip external gelation. After gelation,  
 719 Ca-alginate hydrogel microparticles were droplet-  
 720 shaped (Fig. 19c) and tadpole-shaped (Fig. 19d), as in  
 721 Fig. 18B, b-c. The shape of the microparticles varied  
 722 with the flow rates, the concentration of Na-alginate and  
 723 that of  $\text{CaCl}_2$ . It is likely that the deformation mechan-  
 724 ism involved the forces applied to droplets at the inter-  
 725 face, as explained by Hu et al. [20].  
 726 To improve the spherical shape of microparticles, we  
 727 collected droplets in a bath of the continuous fluid, i.e.,  
 728 DMC. Hence, the droplets continued to shrink and were  
 729 finally transformed into spherical condensed Na-alginate  
 730 microparticles, not yet gelled. For the gelation of the



f19.1 **Fig. 19 a** Schematic diagram of the preparation of Ca-alginate hydrogel microparticles by off-chip external gelation without pre-gelation. Droplets  
 f19.2 were generated using a microfluidic device assembled from fluoropolymer capillaries and a T-junction. Micrographs of **b** the channel outlet immersed  
 f19.3 in an aqueous solution of  $\text{CaCl}_2$ ; Ca-alginate hydrogel microparticles prepared by collecting droplets in an aqueous solution of  $\text{CaCl}_2$  at concentrations  
 f19.4 of **c** 1 wt% and **d** 0.1 wt%  
 f19.5

731 microparticles, the bath of DMC was first evaporated.  
 732 Then, an aqueous solution of  $\text{CaCl}_2$  (0.5–10 wt%) was  
 733 added to the dried Na-alginate microparticles, inducing  
 734 off-chip external gelation. Observations showed that this  
 735 process was accompanied by the swelling of the micro-  
 F20 736 particles without deformation (Fig. 20b-c). In the end,  
 737 spherical Ca-alginate hydrogel microparticles were ob-  
 738 tained. They were insoluble in water, indicating efficient  
 739 gelation. Moreover, the concentration of  $\text{CaCl}_2$  had no  
 740 significant effect on the size of the Ca-alginate micropar-  
 741 ticles. Since no surfactant is used in this method, no  
 742 surfactant-removing step is needed, which simplifies the  
 743 process. However, the quantity of microparticles pro-  
 744 duced is limited by the need to avoid droplet  
 745 coalescence.

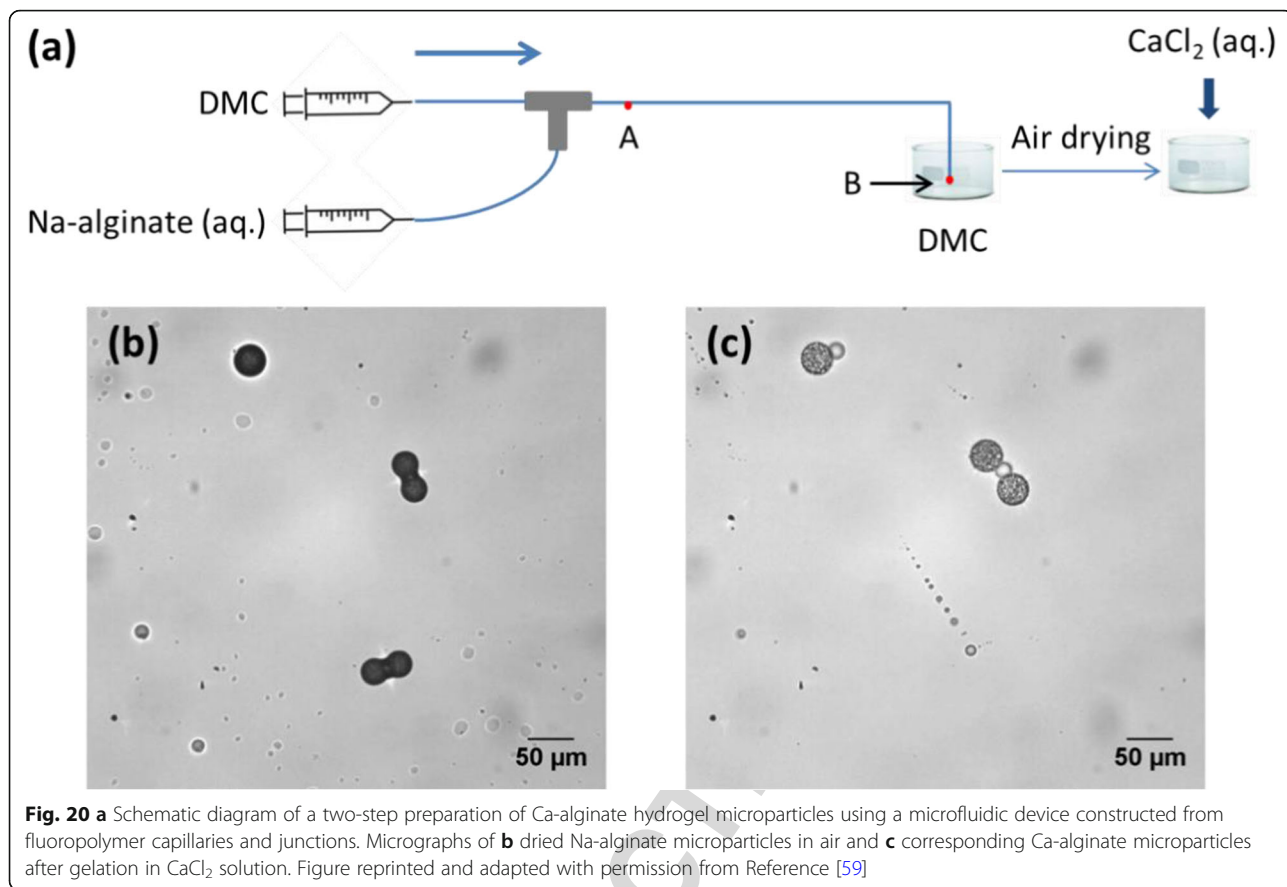
746 In conclusion, external gelation of alginate can be per-  
 747 formed both on-chip and off-chip. For on-chip external  
 748 gelation (Table 4), cross-linkers can be added to the con-  
 749 tinuous fluid, i.e., the oil. However, only limited concen-  
 750 trations can be used, since most are slightly soluble in  
 751 oil. Therefore, introducing cross-linkers in emulsion  
 752 form increases the quantity of alginate available for gel-  
 753 ation. However, the particles are large and deformed. On  
 754 the other hand, if partially miscible fluids are used, an  
 755 aqueous solution of cross-linkers can be injected after

756 droplet shrinkage. Particle size is reduced but the gel- 756  
 757 ation is too rapid. Things can be improved by dissolving 757  
 758 cross-linkers in an oil-based emulsion, introduced in 758  
 759 small quantities but repeatedly. 759

760 For off-chip external gelation (Table 5), cross-linkers 760  
 761 are introduced into the collection bath. A two-phase col- 761  
 762 lection bath permits pre-gelation of Na-alginate droplets 762  
 763 before gelation. However, the particles are large. Droplet 763  
 764 size can be reduced to below the channel diameter by 764  
 765 using partially miscible fluids for droplet generation, and 765  
 766 the droplets can then be collected directly in the dis- 766  
 767 persed phase containing the cross-linker. However, the 767  
 768 microparticles are deformed. To further reduce particle 768  
 769 size and improve gelation, our solution is to perform 769  
 770 two-step collection. Thus, off-chip external gelation can 770  
 771 be used to produce shape-controlled and size-controlled 771  
 772 microparticles. 772

773 **Properties of alginate hydrogel microparticles** 773

774 After preparation, alginate hydrogel microparticles 774  
 775 should be characterized to obtain better knowledge of 775  
 776 their properties, which will determine their further appli- 776  
 777 cations. This section discusses characterization ap- 777  
 778 proaches and factors influencing particle properties. 778



f20.1  
f20.2  
f20.3  
f20.4

t6.1 **Table 6** Average size of alginate microparticles prepared using droplet-based microfluidics with different gelation methods

t6.2	Average size of microparticles	Gelation method	Reference
t6.3	1–50 μm	Internal gelation with water-soluble cross-linkers mixed with Na-alginate before droplet generation	Rondeau and Cooper-White 2008 <sup>a</sup> [39]
t6.4	10–300 nm		
t6.5	50–300 μm	Internal gelation with water-soluble cross-linkers mixed with Na-alginate after droplet generation	Xu et al. 2008 [54]
t6.6	20–50 μm		Liu et al. 2006 [28]
t6.7	22–42 μm		Trivedi et al. 2009 [48]
t6.8	60–100 μm	Internal gelation with water-insoluble cross-linkers mixed with Na-alginate after droplet generation	Zhang et al. 2007 [61]
t6.9	26 μm		Akbari and Pirbodaghi 2014 [3]
t6.10	50–100 μm		Yu et al. 2019 [56]
t6.11	10–50 μm	Internal gelation with slightly water-soluble cross-linkers mixed with Na-alginate after droplet generation	Utech et al. 2015 [49]
t6.12	50–70 μm	External gelation with on-chip introduction of cross-linkers	Zhang et al. 2007 [61]
t6.13	147–176 μm		Liu et al. 2019 [29]
t6.14	5–10 μm		Pittermannová et al. 2016 <sup>a</sup> [34]
t6.15	6–10 μm		Sugaya et al. 2011 <sup>a</sup> [44]
t6.16	100–200 μm	External gelation with off-chip introduction of cross-linkers	Hu et al. 2012 [20]
t6.17	7–40 μm		Zhang et al. 2020 <sup>a</sup> [59]

t6.18 <sup>a</sup>Partially miscible fluids were used

779 **Size**

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

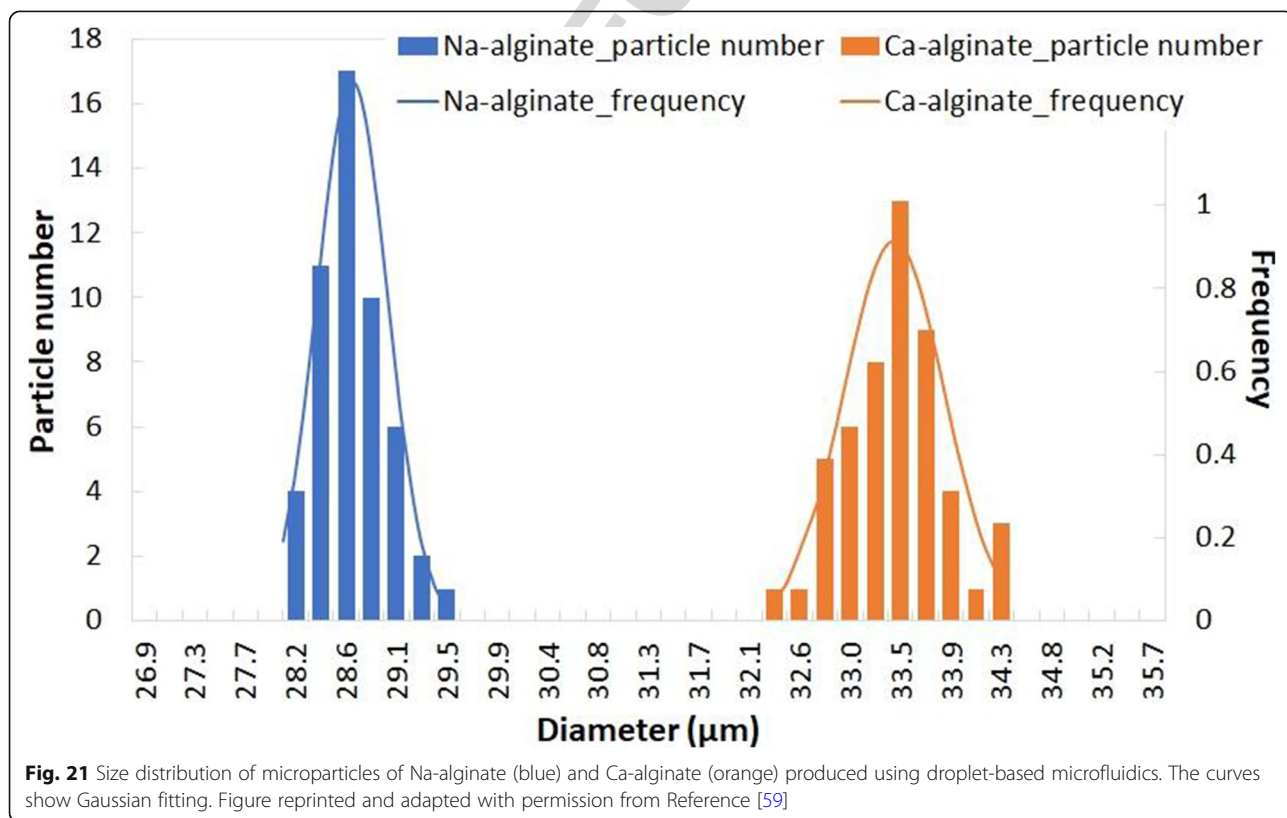
786 Droplet-based microfluidics allows monodisperse mi-  
 787 croparticles to be produced with accurate control of size  
**T6** 788 and size distribution. Table 6 shows the average size  
 789 attained under droplet-based microfluidics using differ-  
 790 ent gelation methods.

**F21** 791 Figure 21 shows an example of the narrow size distribu-  
 792 tion of Na-alginate and Ca-alginate microparticles pro-  
 793 duced by droplet-based microfluidics [59] (part III.2.2.),  
 794 indicating the monodispersity of the particle size. This is  
 795 an advantage compared to conventional emulsification,  
 796 which yields a broader size distribution [55].

797 With droplet-based microfluidics, the size of alginate  
 798 hydrogel microparticles is influenced by several factors  
 799 linked to the fluids used to generate them. When immis-  
 800 sible fluids are used for the dispersed and the continu-  
 801 ous fluids, the size of alginate hydrogel microparticles is  
 802 completely dependent on the size of the droplets first  
 803 generated. Droplet size is influenced by channel size,  
 804 and smaller droplets can be generated by using narrower  
 805 channels. Other important factors are flow rates, alginate

concentration [39, 59], and fluid viscosities [41, 46]. 806  
 However, reducing channel size increases hydraulic re- 807  
 sistance, as well as the pressure required to generate 808  
 droplets. Moreover, it should be noted that in most 809  
 cases, the Na-alginate solution used is relatively viscous. 810  
 Therefore, high pressure may cause leakage or even de- 811  
 struction of the microfluidic device [3, 49]. Thus, even 812  
 when channel diameter is decreased and/or the flow rate 813  
 of the continuous fluid is increased, producing droplets 814  
 of a diameter below 10  $\mu\text{m}$  remains challenging. 815

Droplets of this size, below 10  $\mu\text{m}$ , can be obtained 816  
 without applying high pressure (Table 6), by using parti- 817  
 ally miscible fluids with low solubility in each other 818  
 [39, 44, 59]. The dispersed fluid is an aqueous solution 819  
 containing Na-alginate; the continuous fluid is an or- 820  
 ganic solvent that is partially miscible with water and in 821  
 which water has low solubility. The partial miscibility 822  
 between the continuous and the dispersed fluids should 823  
 be slight enough so that interfacial energy  $\gamma_{CD}$  still al- 824  
 lows the generation of droplets. The low solubility of 825  
 water in the continuous fluid allows water diffusion from 826  
 droplets into it, causing the droplets to shrink. As a re- 827  
 sult, the initially obtained diluted large droplets are 828  
 transformed into concentrated small droplets or micro- 829  
 particles. Thus, their size is no longer dependent on the 830  
 size of droplets initially generated but varies with the 831  
 interaction between water and the continuous fluid. 832



f21.1 **Fig. 21** Size distribution of microparticles of Na-alginate (blue) and Ca-alginate (orange) produced using droplet-based microfluidics. The curves  
 f21.2 show Gaussian fitting. Figure reprinted and adapted with permission from Reference [59]  
 f21.3



### 833 Shape

834 The shape of microparticles is another important prop- 885  
835 erty. A specific shape is sometimes needed; for example, 886  
836 red blood cell-mimicking microparticles are often re- 887  
837 quired in a biconcave shape [32]. In drug delivery, the 888  
838 shape of microparticles has an impact on the drug- 889  
839 release profile [13]. The overall shape of microparticles 890  
840 can be observed by using optical microscopy. Confocal 891  
841 microscopy of fluorescent samples can be used to form a 892  
842 spatial 3D image [21]. Better resolution can be obtained 893  
843 by using atomic force microscopy (AFM) or scanning 894  
844 electron microscopy (SEM) [59]. 895

845 With droplet-based microfluidics, the spherical drop- 896  
846 lets initially generated can be transformed into spherical 897  
847 alginate hydrogel microparticles after gelation. Non- 898  
848 spherical microparticles can also be obtained. For ex- 899  
849 ample, as presented previously, Liu et al. first generated 900  
850 droplets of Na-alginate and  $\text{CaCl}_2$  separately [28]. Then 901  
851 the droplets were fused in a specifically designed micro- 902  
852 fluidic device, leading to gelation. By varying the channel 903  
853 geometry and controlling the flow rates of fluids, Ca- 904  
854 alginate microparticles of different shapes were obtained 905  
855 (Fig. 10f). A different method was presented by Hu et al. 906  
856 [20] Na-alginate droplets were first generated and then 907  
857 collected in a two-phase gelation bath. Spherical droplets 908  
858 were deformed via interfacial energy derived from sur- 909  
859 factant and viscous force. Thus, different shapes were 910  
860 produced (Fig. 18B) by controlling the surfactant used 911  
861 and the viscosity of the gelation bath. 912

### 862 Concentration

863 After preparation, the concentration of alginate in the 913  
864 microparticles can be calculated approximately. For in- 914  
865 stance, Zhang et al. used partially miscible fluids [59]. 915  
866 An aqueous solution of Na-alginate was prepared with a 916  
867 known concentration. After droplet generation, droplet 917  
868 shrinkage occurred during passage through the channel 918  
869 due to water diffusion into the continuous fluid. Drop- 919  
870 lets were hence transformed into microparticles. As the 920  
871 diffusion of Na-alginate into the continuous fluid is neg- 921  
872 ligible [39], the quantity of Na-alginate is constant. It 922  
873 can be calculated by multiplying the droplet volume and 923  
874 initial concentration. Finally, by measuring microparticle 924  
875 size, the concentration of Na-alginate can be calculated. 925  
876 The final concentration of Na-alginate varies from 20 to 926  
877 100 wt%, depending on the initial concentration and 927  
878 diameter of the droplets generated [59]. Furthermore, as 928  
879 presented in Utech et al., the homogeneity of composi- 929  
880 tion of microparticles can be determined with the help 930  
881 of fluorescence technology [49]. 931

### 882 Stability

883 In most cases, surfactant is added in the continuous fluid 932  
884 [48, 49, 61] to lower interfacial energy between the 933

continuous and the dispersed fluids  $\gamma_{\text{CD}}$ . Note that for 885  
each of the above studies, use or non-use of surfactant is 886  
mentioned when indicated by the authors (Tables 1, 2, 887  
3, 4, 5). Surfactant facilitates the creation of a new inter- 888  
face, and thus the formation of droplets. It also stabilizes 889  
the formed droplets by preventing their coalescence 890  
[41]. Before the application of microparticles, the surfac- 891  
tant should be dissolved [3], except for biocompatible 892  
surfactant [49], although protocols for removing surfac- 893  
tant are rarely reported in the literature. To remove the 894  
oil used during the preparation, microparticles should be 895  
washed several times with an aqueous solution, followed 896  
by centrifugation [61]. 897

However, despite its advantages, the use of surfactant 898  
may be undesirable. Surfactant has been shown to im- 899  
pact the surface properties of microparticles, such as 900  
morphology [45] and surface hydrophobicity [22]. Addi- 901  
tionally, if rinsing is insufficient, the traces of surfactant 902  
on microparticles can damage the devices during appli- 903  
cation. In this situation, microparticles should be pre- 904  
pared without surfactant, which is possible with droplet- 905  
based microfluidics. In the microchannel, the coales- 906  
cence of droplets can be avoided by enlarging the dis- 907  
tance between droplets, which can be achieved by 908  
regulating flow rates. Furthermore, gelation, either on- 909  
chip or off-chip, solidifies droplets and thus helps to 910  
avoid coalescence as well. Another strategy consists of 911  
using partially miscible fluids. This means that the drop- 912  
lets shrink and become more and more condensed dur- 913  
ing passage through the channel. At the outlet, either 914  
gelation [39] or a final shrinkage [59] can help avoid 915  
coalescence. 916

Moreover, for their stability, alginate hydrogel micro- 917  
particles should be insoluble in water. This can be 918  
achieved by adopting proper gelation methods using a 919  
sufficient quantity of cross-linkers for effective gelation. 920

### 921 Mechanical properties

922 Mechanical properties of alginate hydrogel microparti- 922  
cles are usually characterized by measuring the 923  
Young's modulus, which varies with several factors. 924  
According to the type of bond between alginate and 925  
cross-linkers, covalent crosslinking results in a higher 926  
Young's modulus than ionic crosslinking in microparti- 927  
cles [6]. For ionic crosslinking, different cations 928  
present different affinities, i.e. different forces with al- 929  
ginate, thus different Young's moduli [33]. In 930  
addition, the Young's modulus increases with the con- 931  
centration of alginate [31]. To measure the Young's 932  
modulus of a microparticle, it needs to be deformed 933  
under a known force, which can be either compressive 934  
or tensile [17]. The techniques used in the literature 935  
include micropipette aspiration [23], compression 936  
[7, 52] or Atomic Force Microscopy (AFM) [59, 61]. 937



### 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  $\mu\text{m}$ ). 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

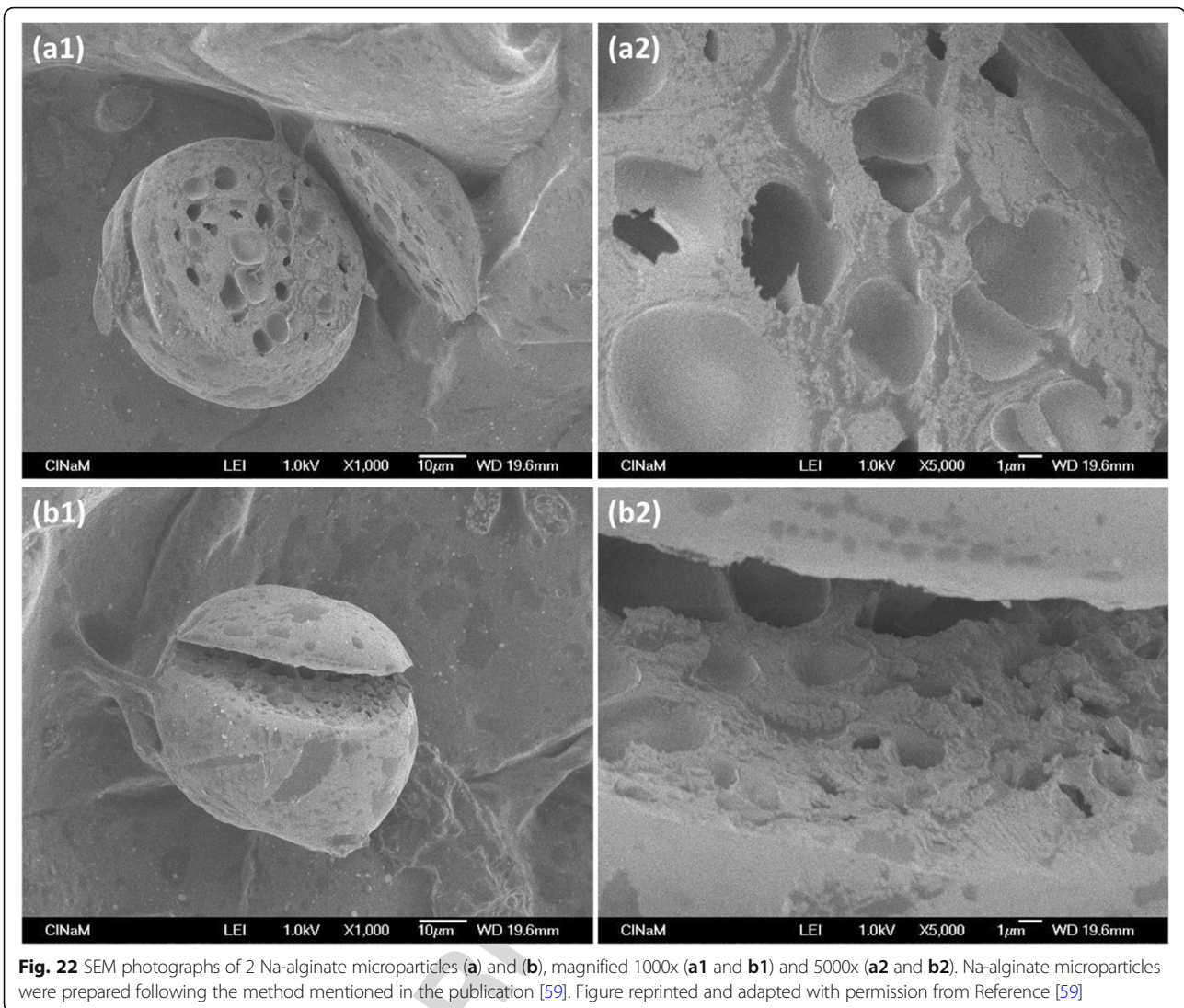
Lastly, we wish to stress the ease of constructing a 994  
microfluidic device with Fluoropolymer tubing and junc- 995  
tions compared to a chip, whatever its composition: 996  
PDMS, PMMA or glass. A microfluidic device's geom- 997  
etry can be tuned flexibly, whereas in a chip, the geom- 998  
etry 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.

- 1011 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
- 1023 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
- 1029 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



f22.1 **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  
 f22.2 were prepared following the method mentioned in the publication [59]. Figure reprinted and adapted with permission from Reference [59]  
 f22.3

1040 4. The relation between degree of gelation and  
 1041 mechanical properties is rarely discussed in the  
 1042 literature. In fact, the degree of gelation itself is  
 1043 barely mentioned although, from a microscopic  
 1044 point of view, it influences mechanical properties.  
 1045 Thus, we believe it is worth assessing degree of  
 1046 gelation by measuring the concentration of cross-  
 1047 linkers inside the final hydrogel microparticles. Sub-  
 1048 sequently, the relation between degree of gelation  
 1049 and mechanical properties should also be addressed.  
 1050 5. In practical applications, the surface of  
 1051 microparticles usually needs to be modified. For  
 1052 example, one way to detect antigens is to graft  
 1053 antibodies onto the surface of microparticles [38].  
 1054 We note that the surface modification is always an  
 1055 extra step after the preparation of microparticles,  
 1056 thus complicating the process. The feasibility of an  
 1057 “all-in-one” process should be studied: can all the

steps be performed within one single microfluidic 1058  
 device, combining microparticle production and 1059  
 surface modification? 1060

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**Authors’ contributions** 1063  
 CZ, RG, NC and SV searched references and collected information. CZ and 1064  
 NC established the structure of whole manuscript. All authors wrote and 1065  
 reviewed the manuscript. All authors read and approved the final 1066  
 manuscript. 1067

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

1075 Not applicable.

1076 **Consent for publication**

1077 Not applicable.

1078 **Competing interests**

1079 The authors declare no conflict of interest.

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1081

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