



HAL
open science

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

Cheng Zhang, Romain A Grossier, Nadine Candoni, Stéphane Veesler

► To cite this version:

Cheng Zhang, Romain A Grossier, Nadine Candoni, Stéphane Veesler. Preparation of alginate hydrogel microparticles by gelation introducing cross-linkers using droplet-based microfluidics: a review of methods. *Biomaterials Research*, 2021, 25 (1), pp.41. 10.1186/s40824-021-00243-5 . hal-02938090v5

HAL Id: hal-02938090

<https://hal.science/hal-02938090v5>

Submitted on 15 Nov 2021 (v5), last revised 24 Nov 2021 (v6)

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

REVIEW

Open Access

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

* Correspondence: veessler@cinam.univ-mrs.fr
CNRS, Aix-Marseille Université, CINaM (Centre Interdisciplinaire de Nanosciences de Marseille), Campus de Luminy, Case 913, F-13288 Marseille Cedex 09, France



© The Author(s). 2021 **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

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 β -D-mannuronate (M) and α -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 rates
 103 and viscosities of fluids, etc. [41, 46]. Furthermore,
 104 monodisperse droplets can be generated without using
 105 surfactant [28, 48, 59], which is impossible with
 106 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 μ 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.

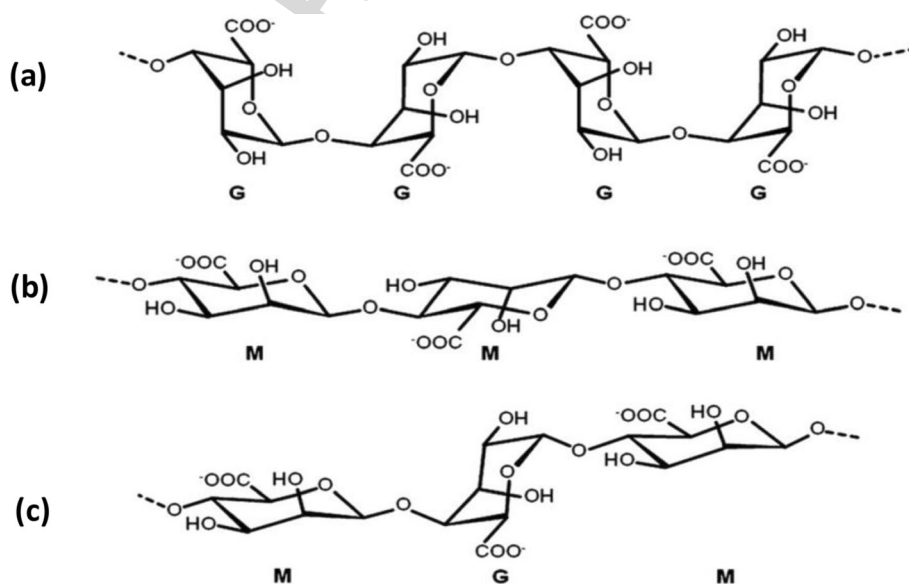


Fig. 1 Chemical structures of a G-block, b M-block and c alternating G and M-blocks in alginate. Figure reprinted with permission from Reference [25]

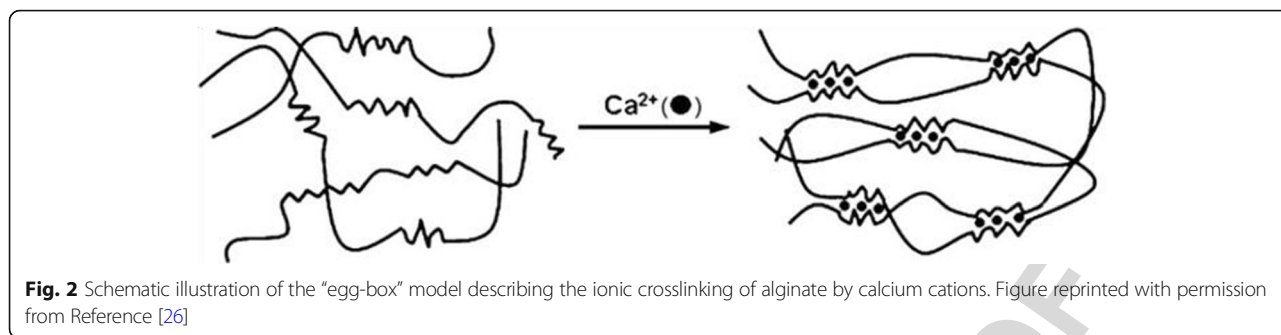


Fig. 2 Schematic illustration of the “egg-box” model describing the ionic crosslinking of alginate by calcium cations. Figure reprinted with permission from Reference [26]

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 (ρ_C), dynamic
 129 viscosity (μ_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 γ_{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 μ_C of the
 continuous fluid, and γ_{CD} of the interface between
 the continuous and the dispersed fluid:

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

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 θ ($0^\circ < \theta \leq 180^\circ$) at the

150
151
152
153
154
155
156

F3

169

170

171

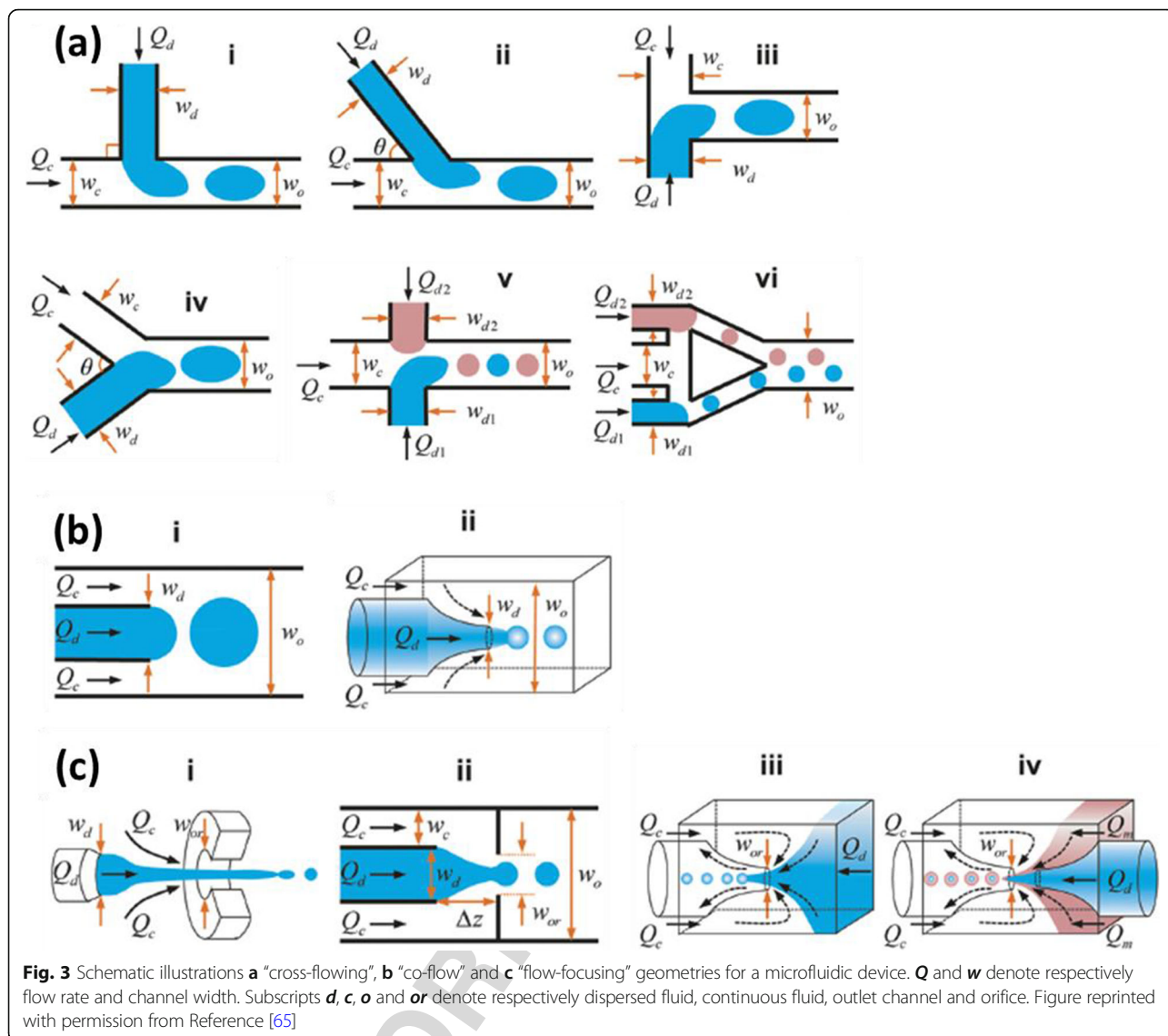


Fig. 3 Schematic illustrations **a** “cross-flowing”, **b** “co-flow” and **c** “flow-focusing” geometries for a microfluidic device. **Q** and **w** denote respectively flow rate and channel width. Subscripts **d, c, o** and **or** denote respectively dispersed fluid, continuous fluid, outlet channel 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 θ (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**

243 A first 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

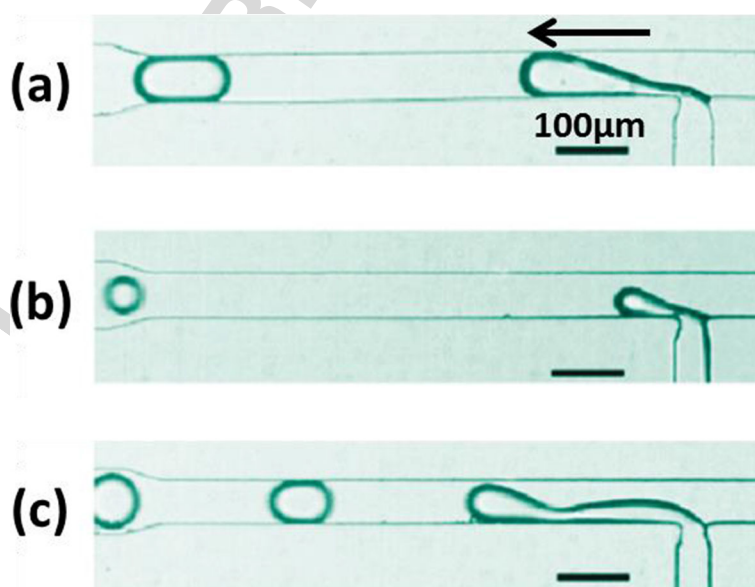


Fig. 4 Three break-off mechanisms of droplet generation with a cross-flowing geometry: **a** squeezing, **b** dripping and **c** jetting. The arrow indicates the droplet flow direction. Figure reprinted with permission from Reference [57]. Copyright 2010 American Chemical Society

f4.1
f4.2
f4.3

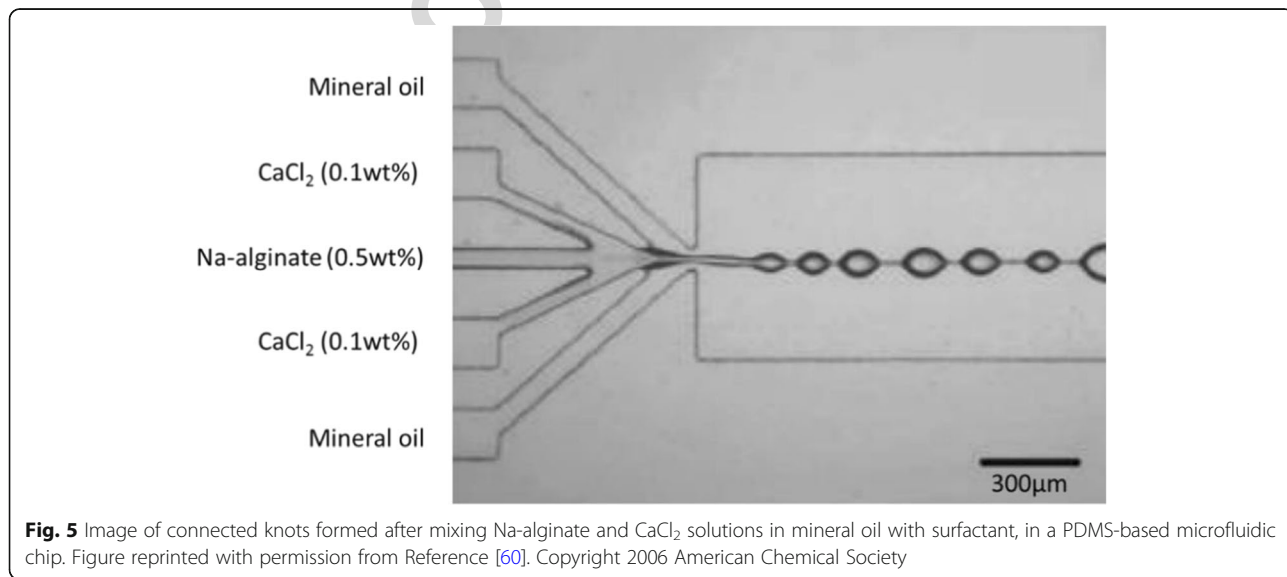
t1.1 **Table 1** Internal gelation with water-soluble cross-linkers: mixing cross-linkers with Na-alginate before droplet generation

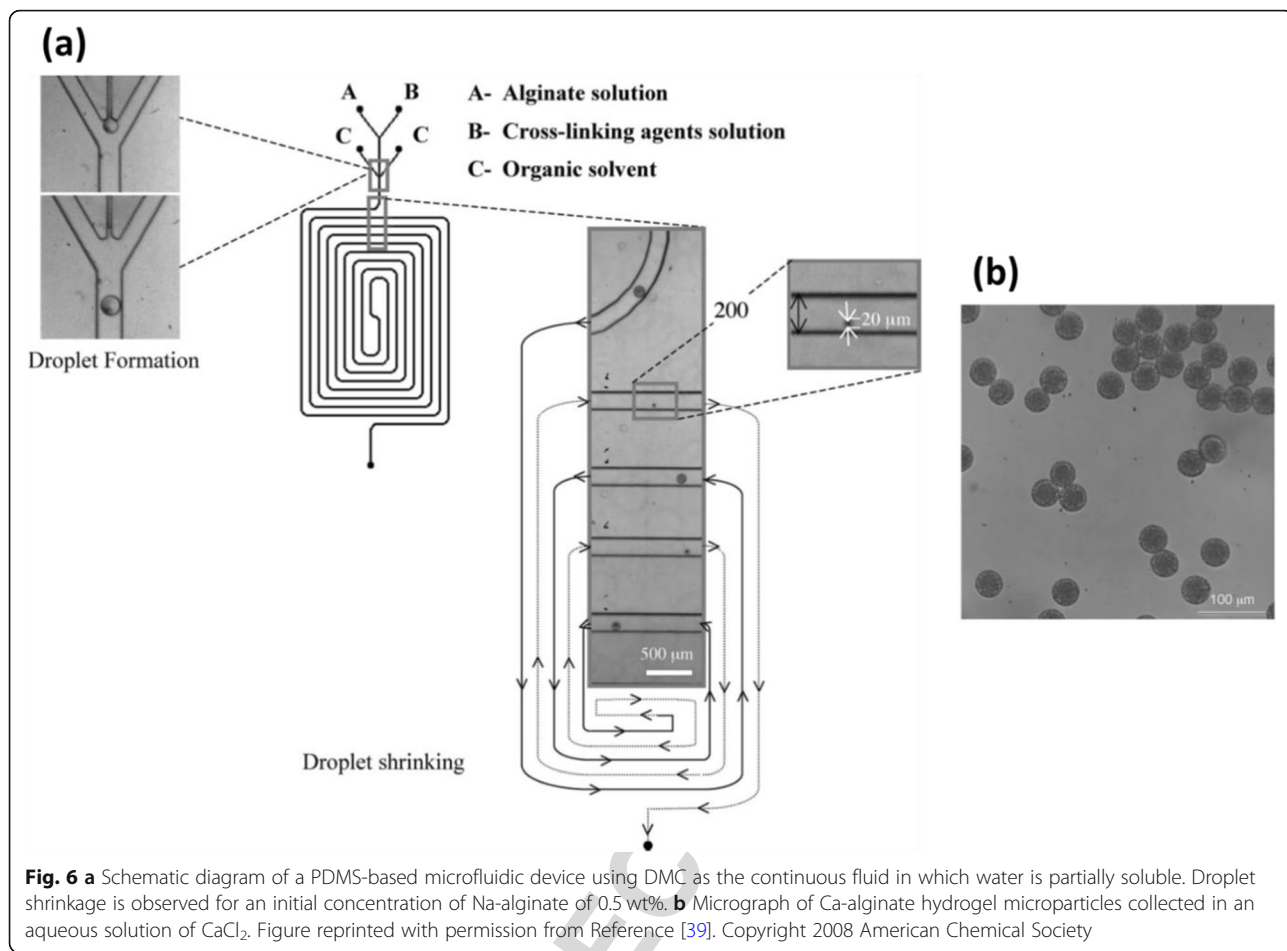
t1.2	References		Trivedi et al., 2009 [48]	Zhang et al., 2006 [60]	Rondeau and Cooper-White, 2008 [39]	Present review	Present review
t1.3	Droplet generation	Concentration of Na-alginate	1 wt%	0.5 wt%	0.5 wt%	0.06 wt%	0.006 wt%
t1.4		Continuous fluid	Silicone oil	Mineral oil	DMC ^a	DMC ^a	DMC ^a
t1.5		Use of surfactant	NO	Span 80	Not mentioned	NO	NO
t1.6		Geometry	Flow-focusing	Flow-focusing	Flow-focusing	Cross-flowing	Cross-flowing
t1.7		Microfluidic material	Fluoropolymer tubing and junctions	PDMS chip	PDMS chip	Fluoropolymer tubing and junctions	Fluoropolymer tubing and junctions
t1.9	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%)
t1.10		Geometry	Flow-focusing	Cross-flowing	Flow-focusing	–	Cross-flowing
t1.11		Mixing	Before droplet generation	During droplet generation	Before droplet generation	Off-line, before droplet generation	During droplet generation

t1.16 ^aContinuous 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%), CaCl₂ 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 CaCl₂ 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 CaCl₂ 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 CaCl₂ (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/CaCl₂ 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



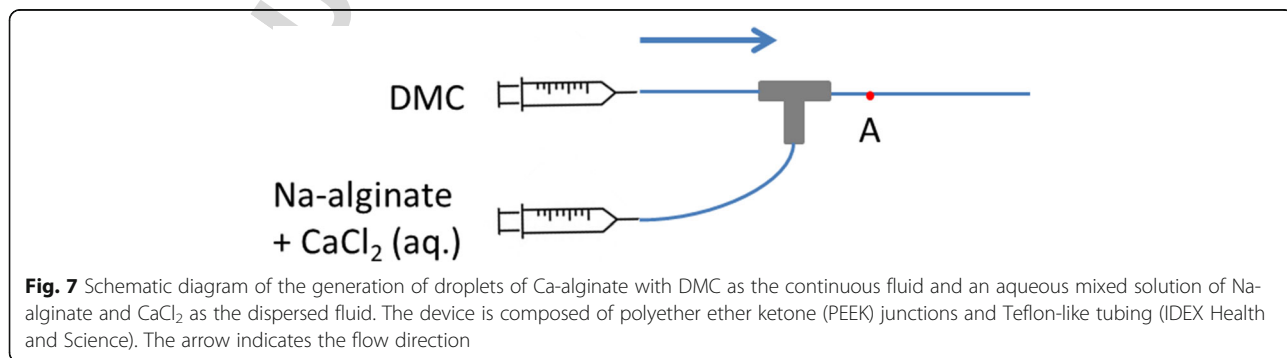


f6.1
 f6.2
 f6.3
 f6.4

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 CaCl₂ (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 CaCl₂ 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 CaCl₂ 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 CaCl₂ (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 BaCl₂ and CaCl₂ 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 CaCl₂ (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 CaCl₂. Then octyl alcohol oil (no mention of surfac- 366
 367 tant) was injected at a second cross-junction. Droplets of 367
 368 Na-alginate/CaCl₂ were generated by flow-focusing. In 368
 369 the “synthesizing channel” (Fig. 9a), within each droplet, 369

F9

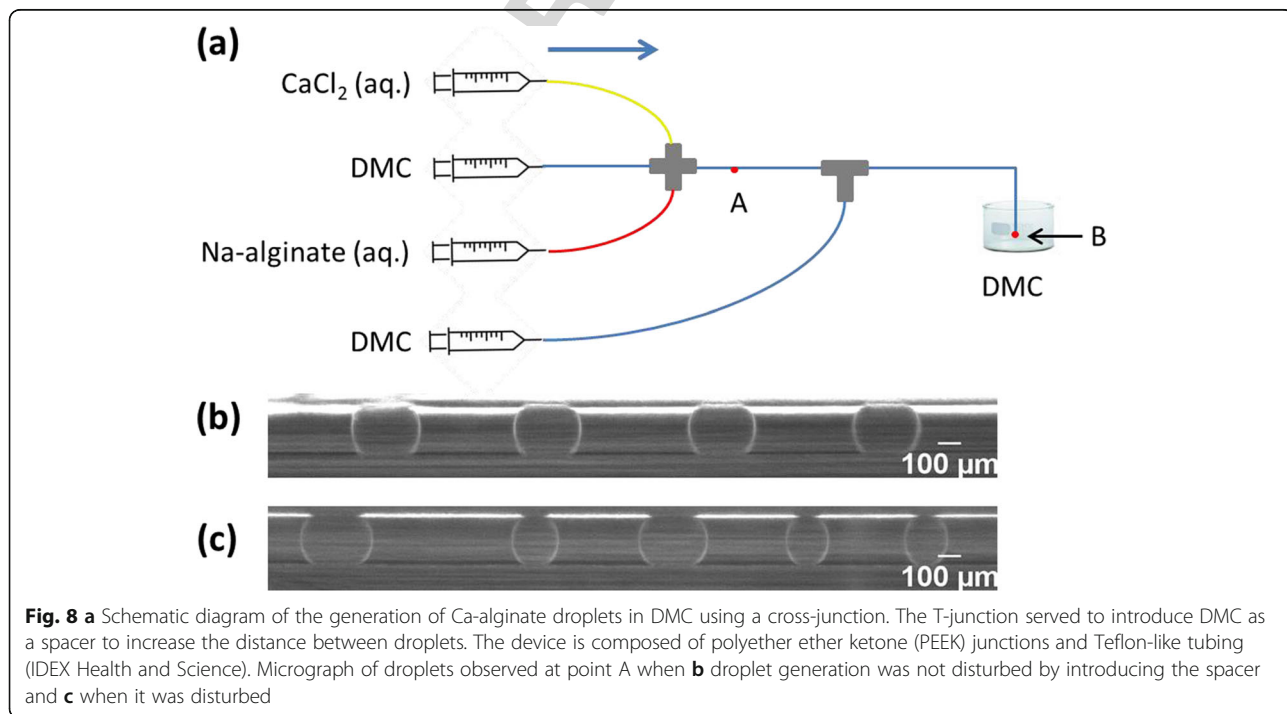


Fig. 8 a Schematic diagram of the generation of Ca-alginate droplets in DMC using a cross-junction. The T-junction served to introduce DMC as a spacer to increase the distance between droplets. The device is composed of polyether ether ketone (PEEK) junctions and Teflon-like tubing (IDEX Health and Science).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

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

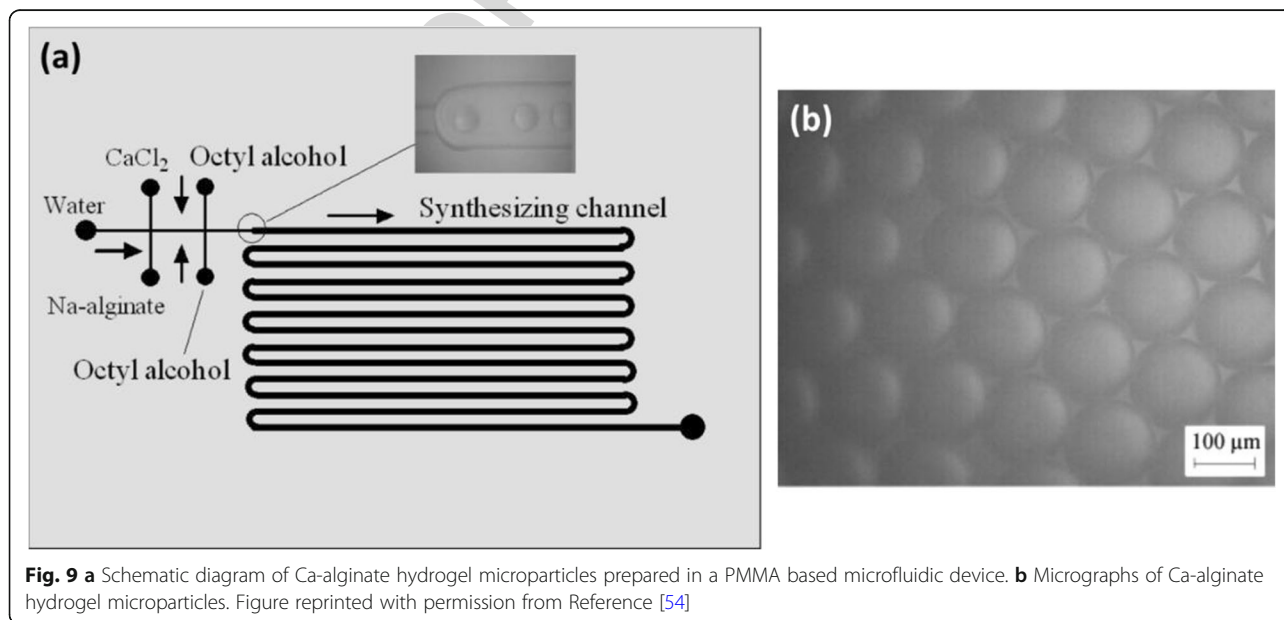
t2.2	Reference	Xu et al., 2008 [54]	Liu et al., 2006 [28]	Trivedi et al., 2010 [47]
t2.3	Droplet generation			
	Concentration of Na-alginate	2 wt%	2 wt%	1 wt%
t2.4	Continuous fluid	octyl alcohol oil	soybean oil	silicone oil
t2.5	Use of surfactant	Not mentioned	NO	NO
t2.6	Geometry	Flow-focusing	Flow-focusing	Flow-focusing
t2.7	Microfluidic material	PMMA chip	PDMS chip	Fluoropolymer tubing and junctions
t2.8	Internal gelation by mixing Na-alginate and water-soluble cross-linker			
t2.9		Cross-linkers	CaCl ₂ (2 wt%)	BaCl ₂ (50 mM)
t2.10		Geometry	Cross-flowing	Expansion chamber
t2.11			Expansion chamber	Cross-flowing
t2.12		Mixing	Coalescence of flows	Coalescence of droplets with flow

370 mixing Na-alginate and CaCl₂ 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.

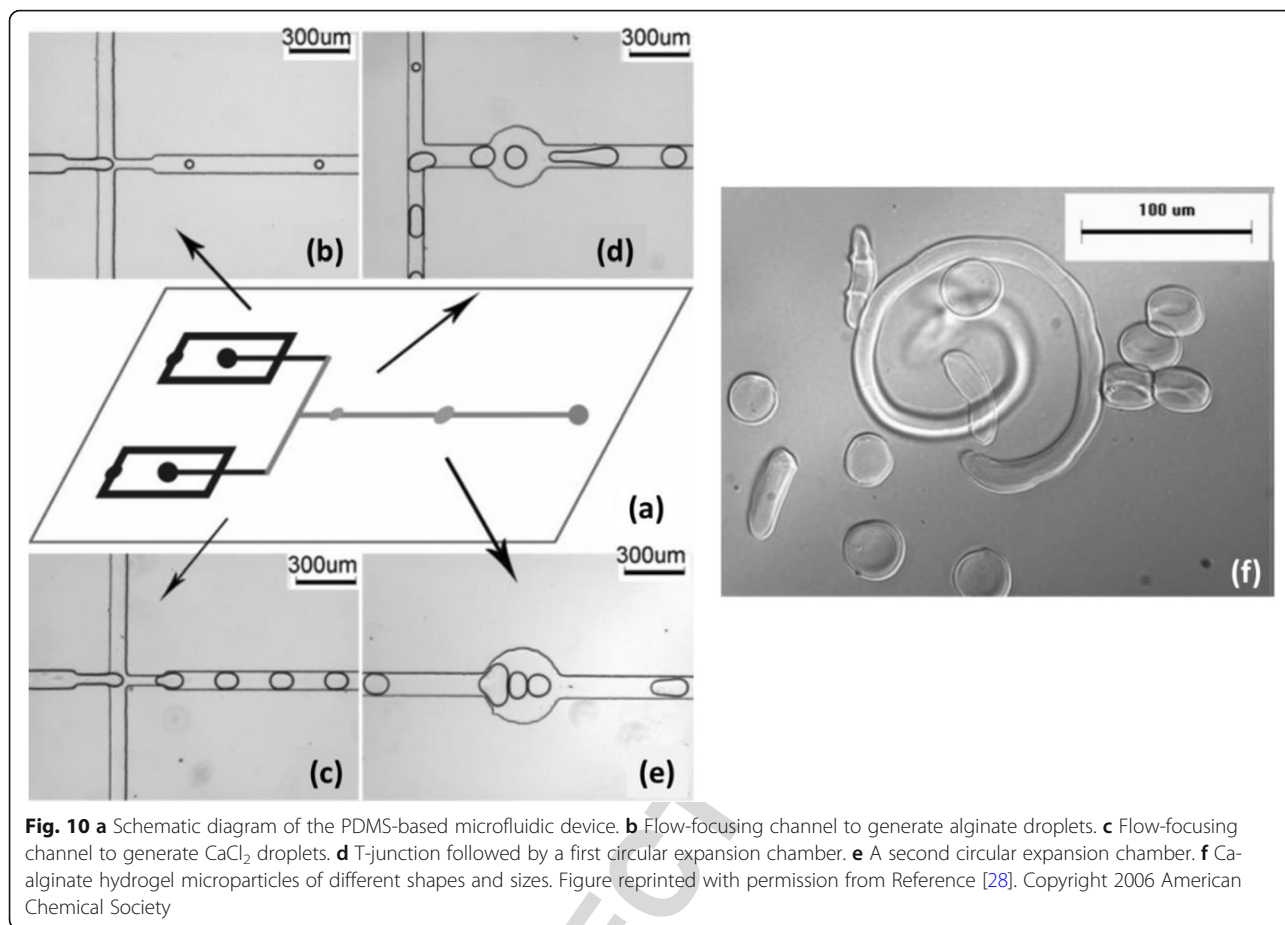
378 Another strategy to delay gelation was carried out by
 379 Liu et al. [28] involving coalescence of Na-alginate drop-
 380 lets with CaCl₂ droplets generated separately. First, on a
 F10381 microfluidic chip (Fig. 10a), Na-alginate (2 wt%) droplets
 382 (Fig. 10b) and CaCl₂ (2 wt%) droplets (Fig. 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 BaCl₂ (50 mM) was injected 403
 downstream by a T-junction. With the help of dye, obser- 404
 vations at the T-junction indicated that BaCl₂ 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 CaCl₂ 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 BaCl₂ 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 γ_{CD} soybean oil, independent droplets
 412 of BaCl₂ 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 (CaCO₃)
 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 CaCO₃ (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/CaCO₃ 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 CaCO₃, 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 CaCO₃. More-
 over, a higher concentration of CaCO₃ 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 CaCO₃ nanoparticles (35 mM) was introduced by
 two side channels (Fig. 12b). This geometry was used to

F11

F12

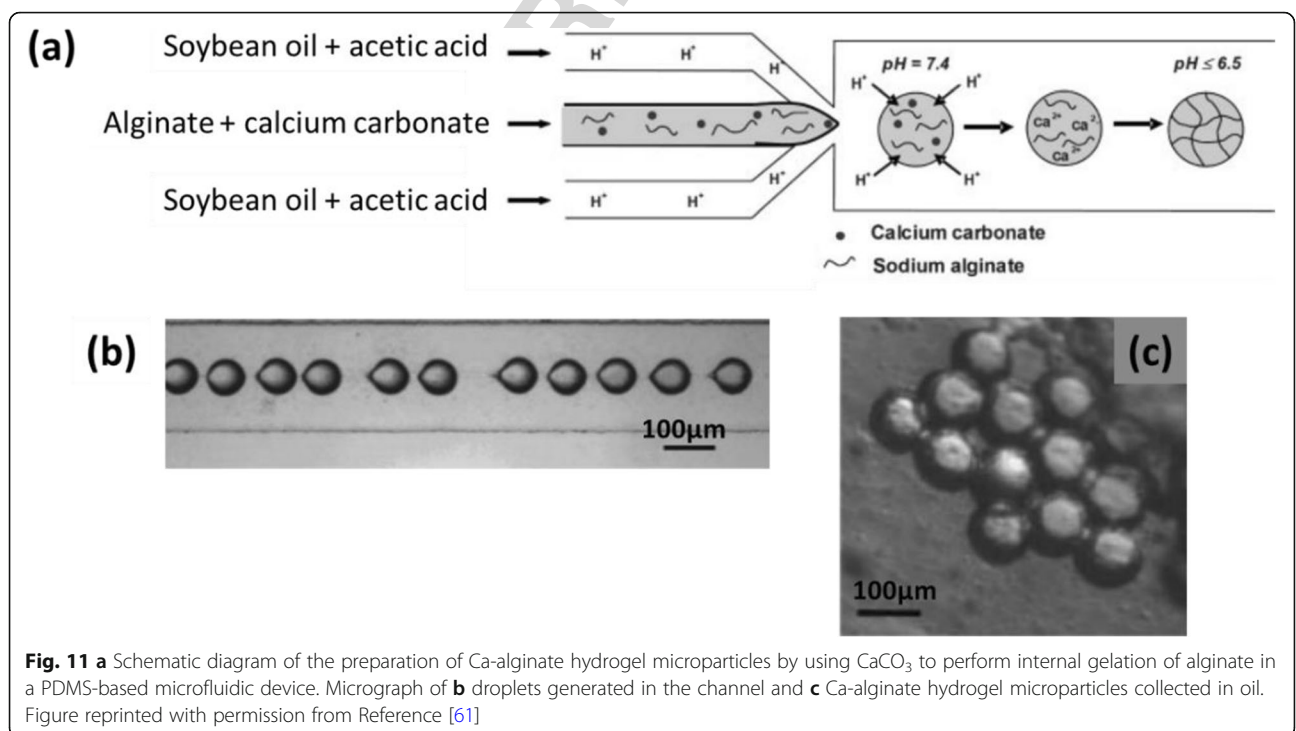
t3.1 **Table 3** Internal gelation with water-insoluble or weakly soluble cross-linkers which are pH sensitive

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	Droplet generation	Concentration of Na-alginate	2 wt%	1,5 wt%	2 w/v%	2 wt%
t3.4		Continuous fluid	Soybean oil	Fluorocarbon oil	Mineral oil	Fluorinated carbon oil
t3.5		Use of surfactant	Span 80	Fluorinated surfactant	Span 80	Biocompatible surfactant
t3.6		Geometry	Flow-focusing	Flow-focusing	Flow-focusing	Flow-focusing
t3.7		Microfluidic material	PDMS chip	PDMS chip	PDMS chip	PDMS chip
t3.8						
t3.9	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)
t3.10		Mixing	Off-line, before droplet generation	Before droplet generation	Off-line, before droplet generation	Off-line, before droplet generation
t3.11		Gelation by acid addition	In continuous fluid	In continuous fluid	In collecting fluid	In continuous fluid
t3.12						
t3.13						
t3.14						

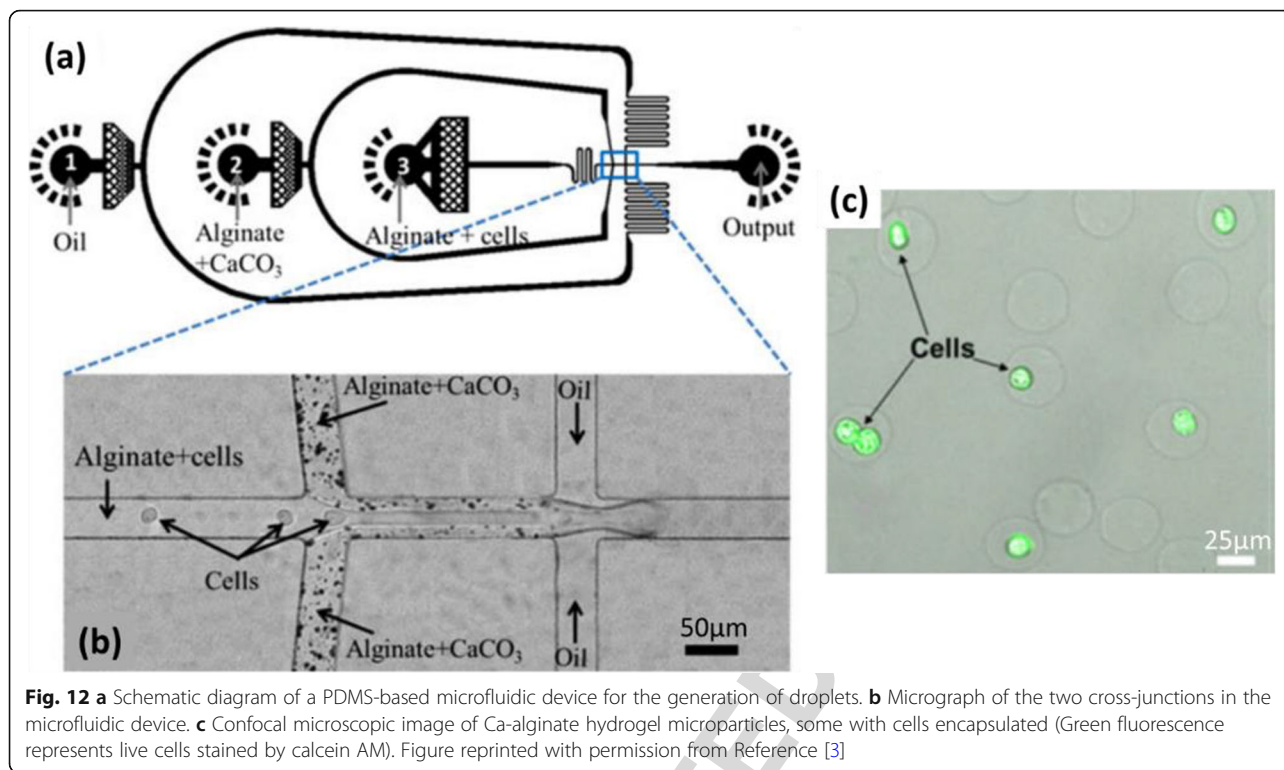
453 create a coaxial stream while avoiding direct mechanical
 454 contact between cells and the potentially damaging
 455 CaCO₃ particles. At a second T-junction, fluorocarbon
 456 oil with surfactant (fluorinated surfactant, 1 wt%) was
 457 injected. Droplets of Na-alginate/cells/CaCO₃ were then
 458 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 CaCO₃ and
 464 Na-alginate was not homogeneous, which can be seen

from Fig. 12b. Thus, the varying amounts of CaCO₃ 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 CaCO₃ 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



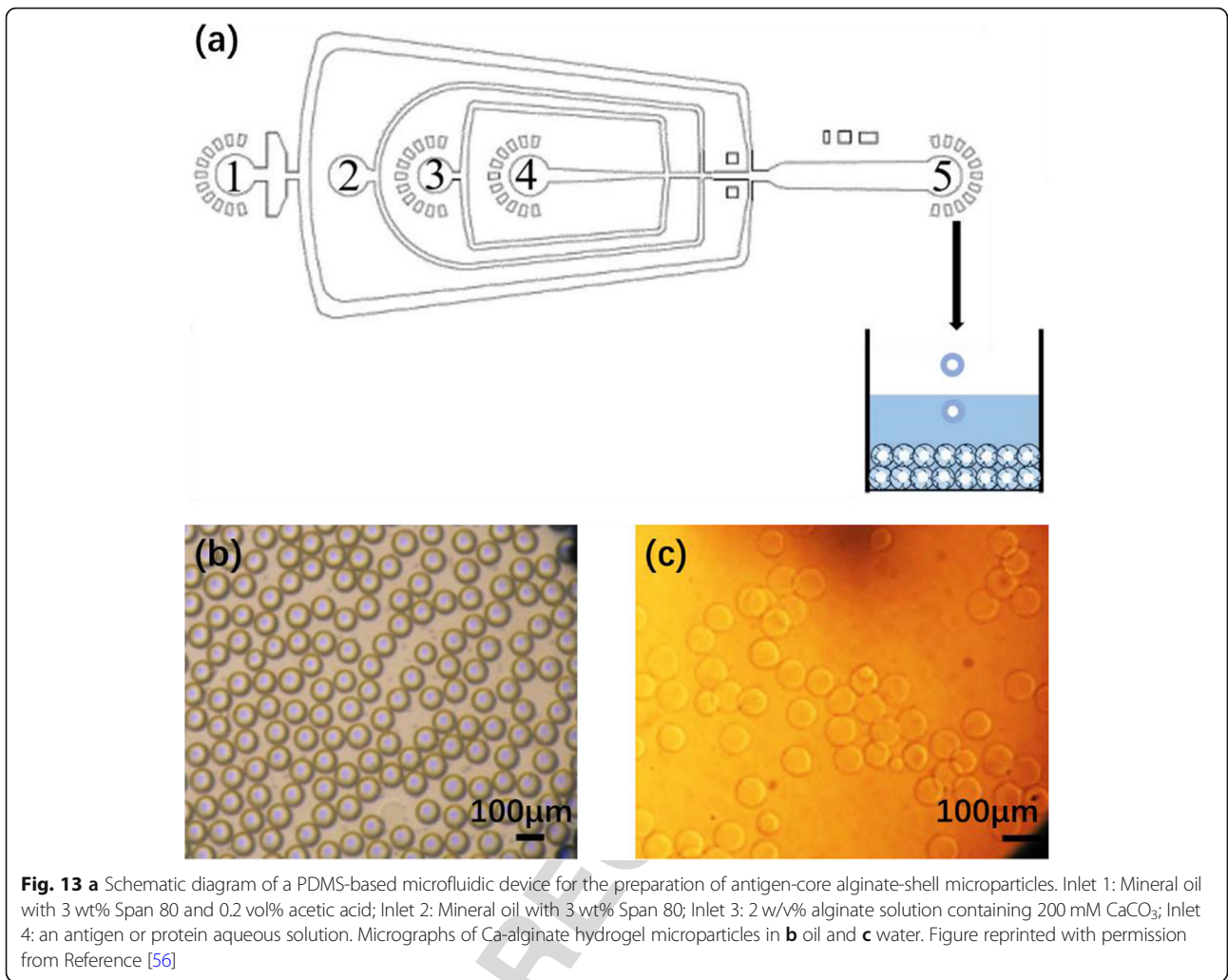
f12.1 **Fig. 12 a** Schematic diagram of a PDMS-based microfluidic device for the generation of droplets. **b** Micrograph of the two cross-junctions in the
 f12.2 microfluidic device. **c** Confocal microscopic image of Ca-alginate hydrogel microparticles, some with cells encapsulated (Green fluorescence
 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_3 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_3 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_2 (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_2 . As
 495 mentioned above, however, since CaCO_3 is not soluble in
 496 water, a high concentration of CaCO_3 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_3 particles are well dis-
 500 persed.

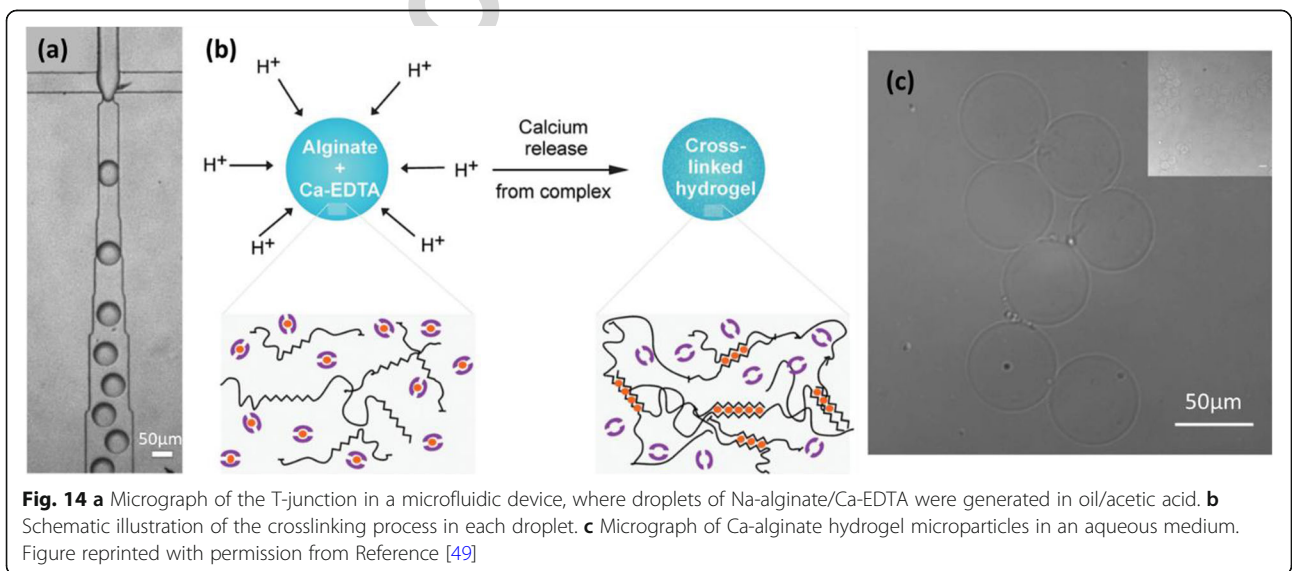
501 In order to obtain a homogeneous internal structure
 502 of hydrogel microparticles, Utech et al. used a slightly
 503 water-soluble calcium-ethylenediaminetetraacetic acid
 504 (Ca-EDTA) complex as the cross-linker [49]. An aque-
 505 ous solution of Na-alginate (2 wt%) mixed with Ca-

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 micropar-
 ticles 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



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 (Ca (CH₃COO)₂) or CaCl₂ as cross-linkers, as summarized in Table 4.

Cross-linkers can be contained in the continuous fluid, as described in Zhang et al. [61] Ca (CH₃COO)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/Ca (CH₃COO)₂, with surfactant (Span 80, 3 wt%). Ca (CH₃COO)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 Ca (CH₃COO)₂ in soybean oil caused clogging in the microchannel [61]. Thus, it is difficult to vary the rate of gelation of microparticles.

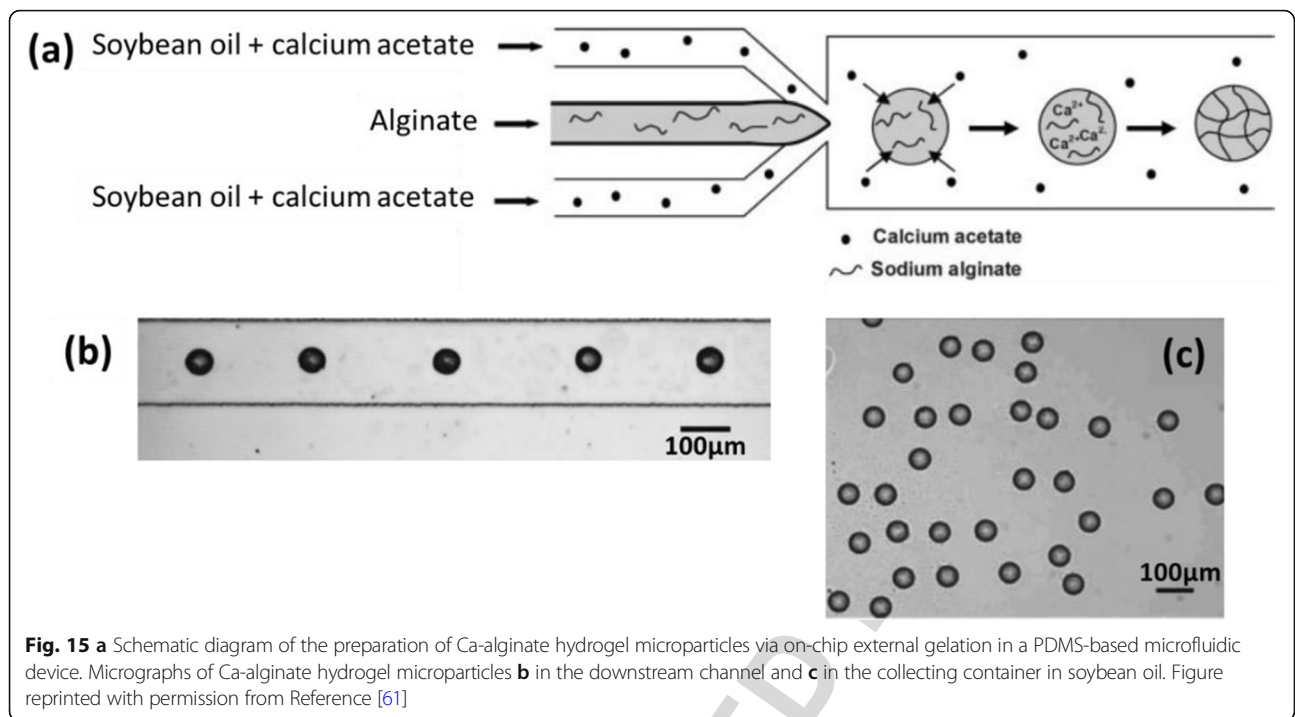
One way to limit channel clogging is to make the cross-linkers diffuse slowly in Na-alginate droplets. Thus, Liu et al. used emulsion fluids to introduce cross-linker s[29]. A glass-based microfluidic device was used, with channels modified so as to be hydrophobic. Droplets of Na-alginate (3 wt%) were first generated in corn oil at the first flow-focusing channel (Fig. 16a). The emulsion of CaCl₂, containing CaCl₂ droplets in corn oil (with surfactant SY-Glyster CRS-75), was injected downstream of the cross-junction. The contact between CaCl₂ 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

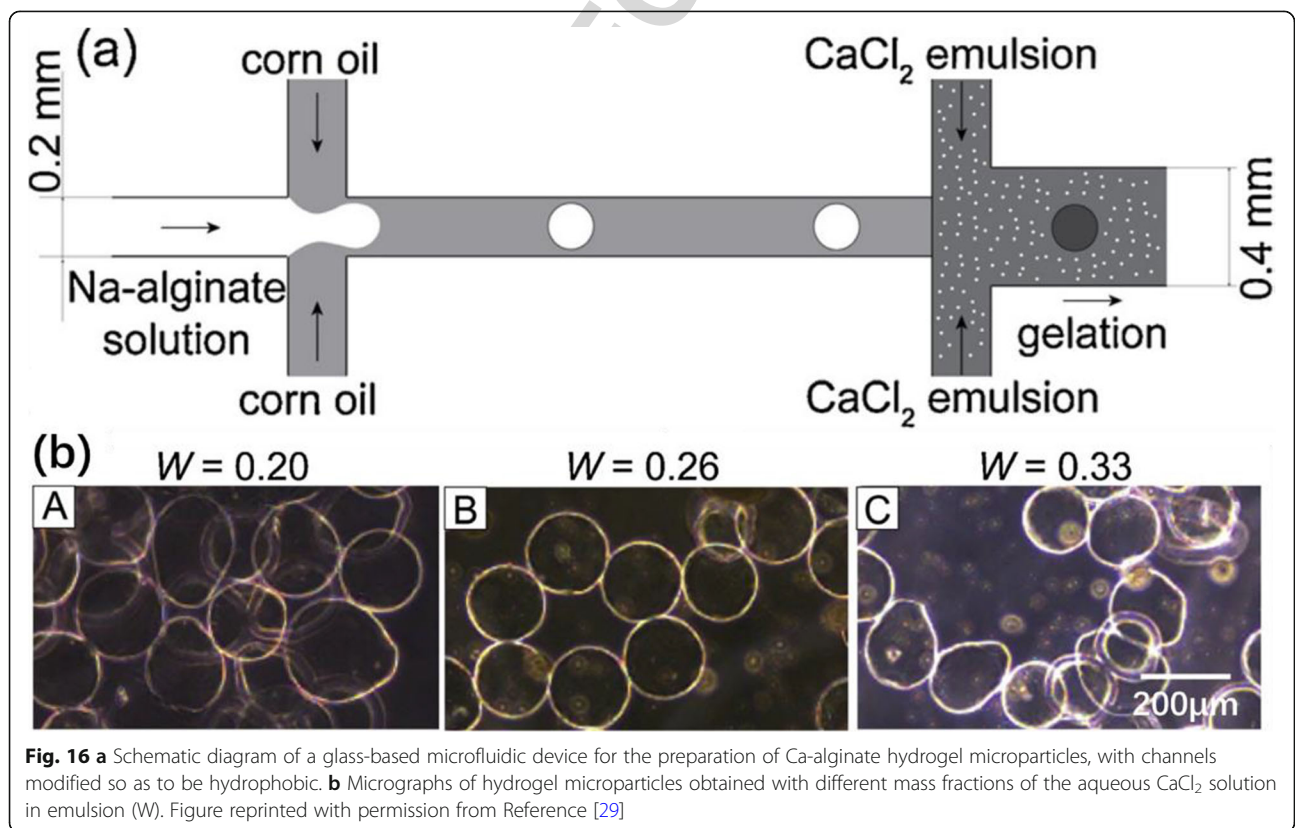
F16

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	Droplet generation	Concentration of Na-alginate	2 wt%	3 wt%	0.025–0.15 wt%	1 w/v%
t4.4		Continuous fluid	Soybean oil	Corn oil	Methyl acetate	1-undecanol
t4.5		Use of surfactant	Span 80	SY-Glyster CRS-75	Not mentioned	Abil Em 90
t4.6		Geometry	Flow-focusing	Flow-focusing	Flow-focusing	Flow-focusing
t4.7		Microfluidic material	PDMS chip	Glass chip	PDMS chip	PDMS chip
t4.8		Cross-linkers	Ca (CH ₃ 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
t4.9	External gelation by introducing Na-alginate and cross-linkers on chip before collection	Geometry	–	Flow-focusing	Cross-flowing	Flow-focusing
t4.10		Mixing	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 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 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. 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 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 CaCl_2 fluid,

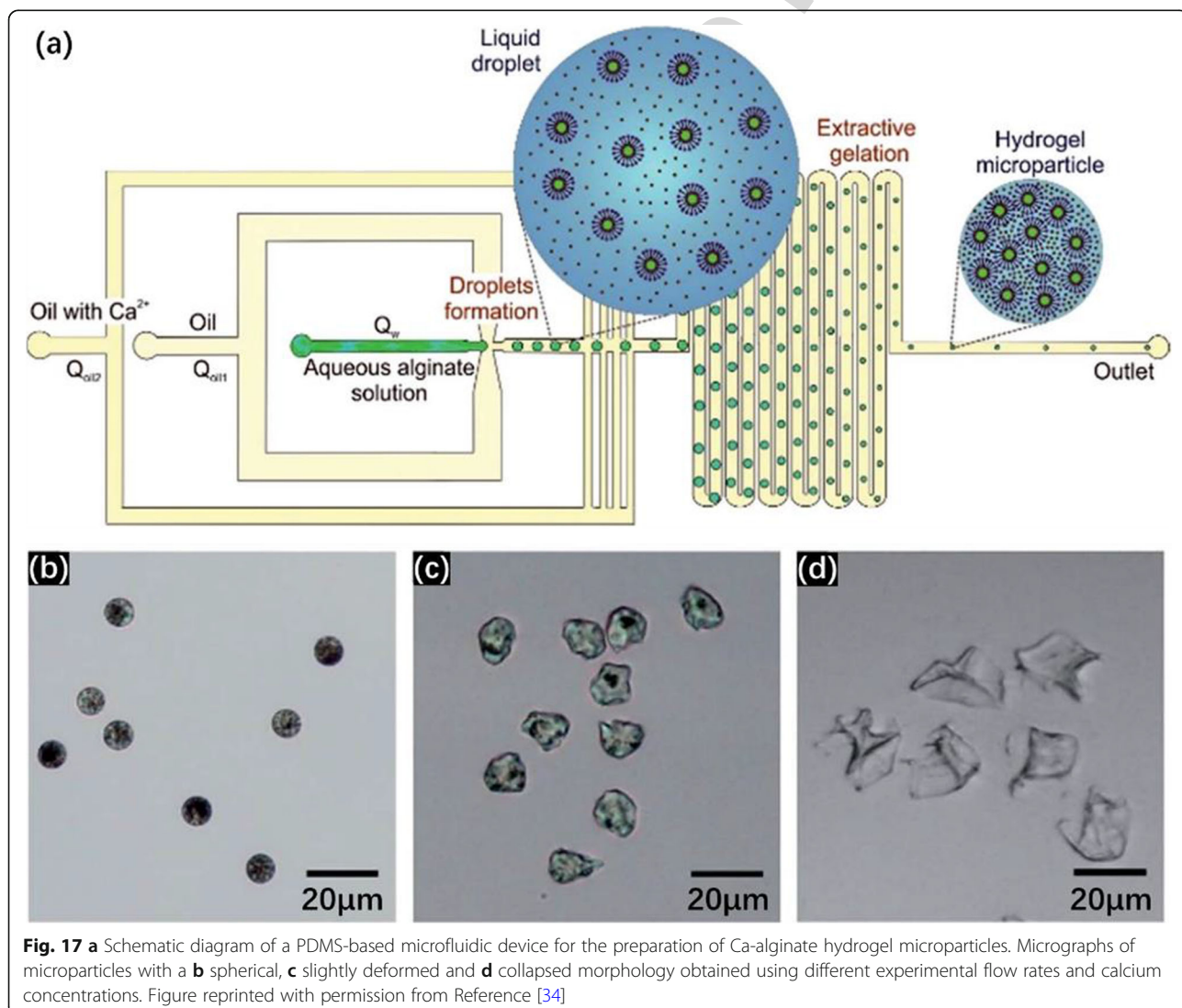


Fig. 17 a Schematic diagram of a PDMS-based microfluidic device for the preparation of Ca-alginate hydrogel microparticles. Micrographs of microparticles with a **b** spherical, **c** slightly deformed and **d** collapsed morphology obtained using different experimental flow rates and calcium concentrations. 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. CaCl₂ (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 CaCl₂, 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

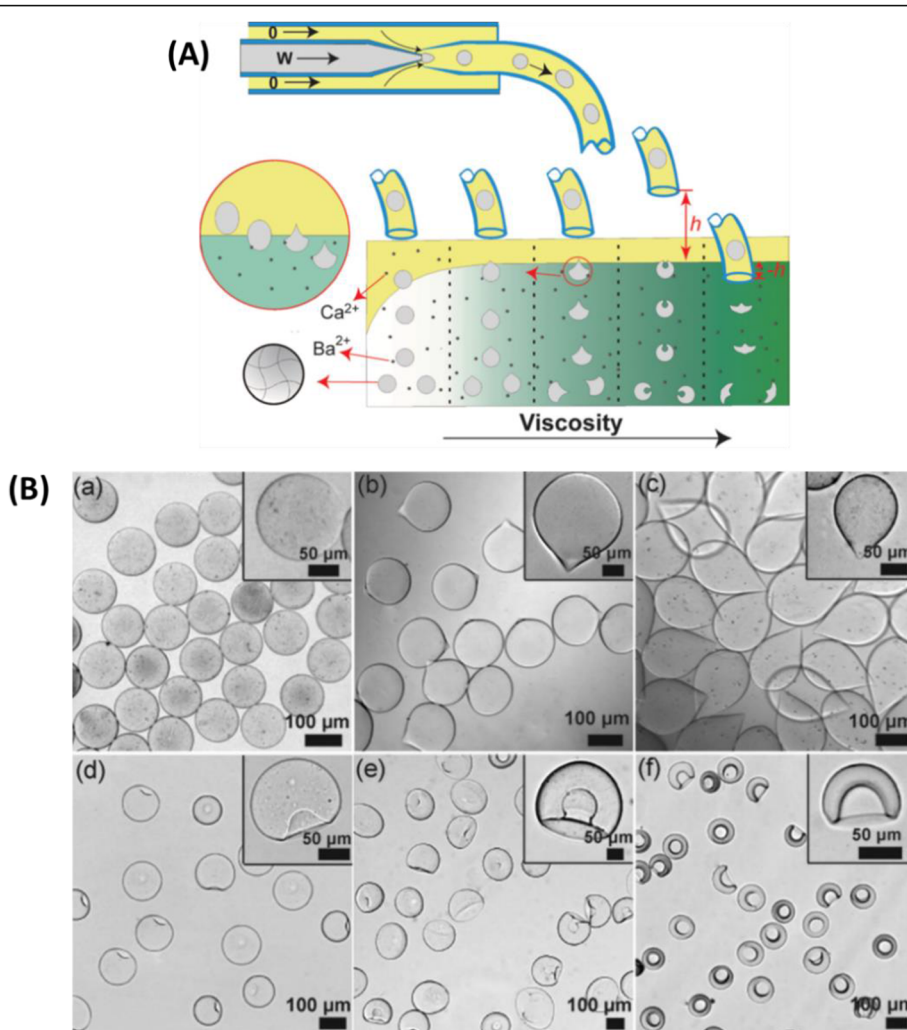
663 Other strategies of external gelation introduce cross-
664 linkers “off-chip”, i.e., during droplet collection. Ca
665 (CH₃COO)₂ or CaCl₂ are used as cross-linkers and the
666 collection bath procedure depends on the strategy, as
667 summarized in Table 5. 668

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 CaCl₂ (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 γ_{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 γ_{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	Droplet generation	Na-alginate	1.5 wt%	0.006–1 wt%	0.006–1 wt%
t5.4		Continuous fluid	N-decanol	DMC ^a	DMC ^a
t5.5		Use of surfactant	Span 80	NO	NO
t5.6		Geometry	Flow-focusing	Cross-flowing	Cross-flowing
t5.7		Microfluidic material	Glass chip	Fluoropolymer tubing and junctions	Fluoropolymer tubing and junctions
t5.8	External gelation by introducing Na-alginate and cross-linkers off-chip during collection	Number of phases or steps	2 phases - 1 collection bath	1 phase - 1 collection bath	2 phases - 2 successive collection baths
t5.9		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
t5.10			Phase 2 Ba (CH ₃ COO) ₂ (15 wt%) in water and glycerol		Step 2 CaCl ₂ (0.5–10 wt%) in water
t5.11					
t5.12					
t5.13		Surfactant	Span 80	NO	NO
t5.14					
t5.15					
t5.16					

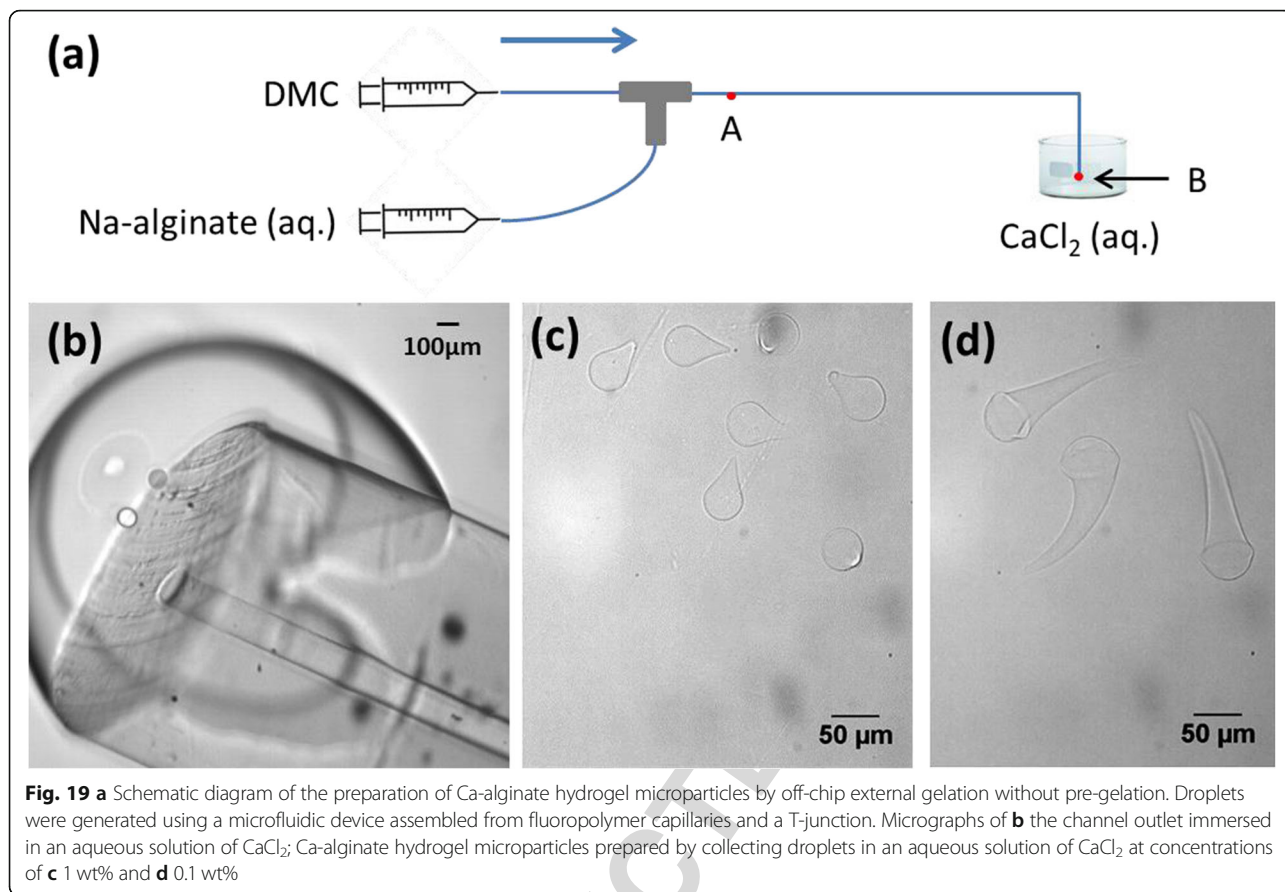
^aContinuous and dispersed fluids partially miscible



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
 F19 701 aqueous solution containing 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 μm . 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 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 ets passed through the interface and entered the 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 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 CaCl₂; Ca-alginate hydrogel microparticles prepared by collecting droplets in an aqueous solution of CaCl₂ 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 CaCl₂ (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 CaCl₂ 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.

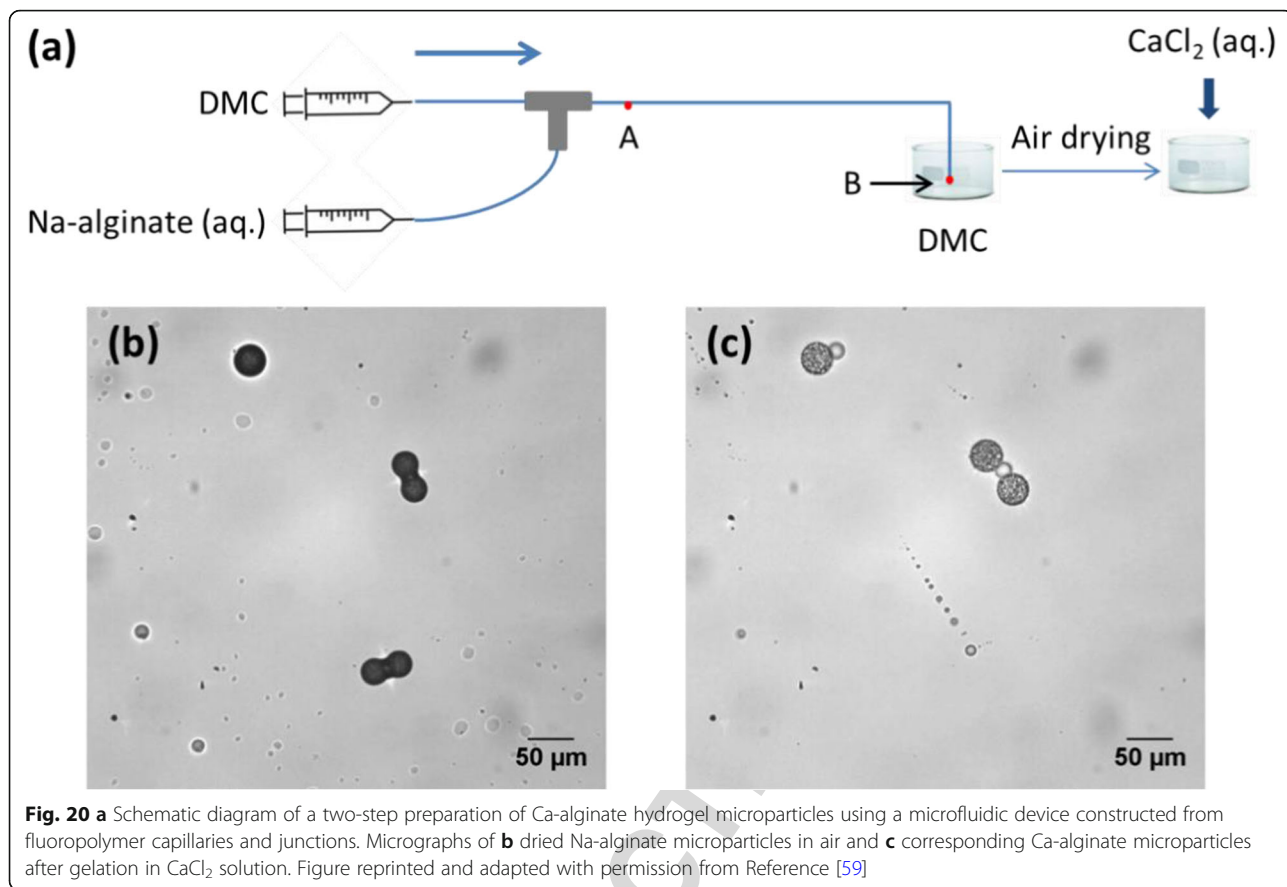
746 In conclusion, external gelation of alginate can be per-
 747 formed both on-chip and off-chip. For on-chip external
 748 gelation (Table 4), cross-linkers can be added to the con-
 749 tinuous fluid, i.e., the oil. However, only limited concen-
 750 trations can be used, since most are slightly 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

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

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

Properties of alginate hydrogel microparticles 773

After preparation, alginate hydrogel microparticles 774
 should be characterized to obtain better knowledge of 775
 their properties, which will determine their further appli- 776
 cations. This section discusses characterization ap- 777
 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 ^a [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 ^a [34]
t6.15	6–10 μm		Sugaya et al. 2011 ^a [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 ^a [59]

t6.18 ^aPartially 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].

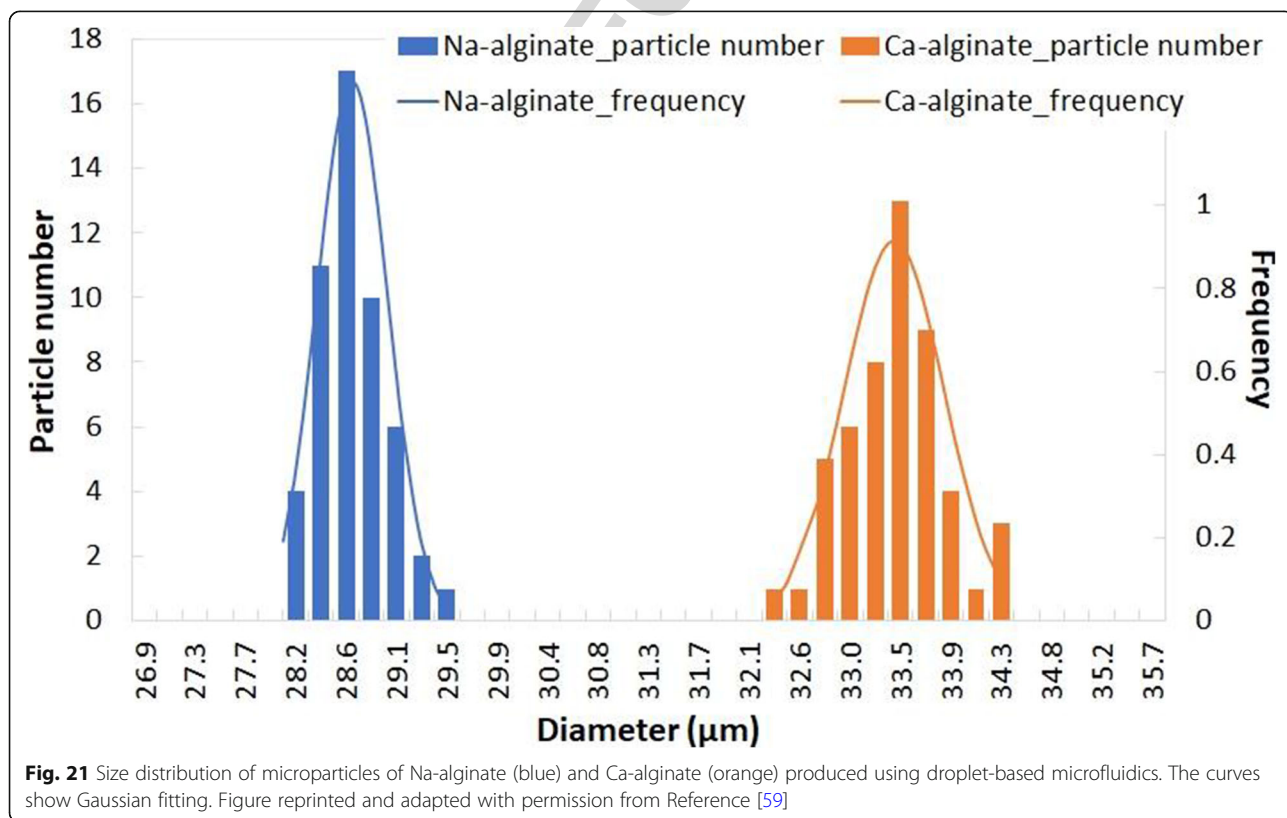
786 Droplet-based microfluidics allows monodisperse mi-
 787 croparticles to be produced with accurate control of size
T6 788 and size distribution. Table 6 shows the average size
 789 attained under droplet-based microfluidics using differ-
 790 ent gelation methods.

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 μm remains challenging. 815

Droplets of this size, below 10 μm , 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 γ_{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].

845 With droplet-based microfluidics, the spherical drop- 895
846 lets initially generated can be transformed into spherical 896
847 alginate hydrogel microparticles after gelation. Non- 897
848 spherical microparticles can also be obtained. For ex- 898
849 ample, as presented previously, Liu et al. first generated 899
850 droplets of Na-alginate and CaCl_2 separately [28]. Then 900
851 the droplets were fused in a specifically designed micro- 901
852 fluidic device, leading to gelation. By varying the channel 902
853 geometry and controlling the flow rates of fluids, Ca- 903
854 alginate microparticles of different shapes were obtained 904
855 (Fig. 10f). A different method was presented by Hu et al. 905
856 [20] Na-alginate droplets were first generated and then 906
857 collected in a two-phase gelation bath. Spherical droplets 907
858 were deformed via interfacial energy derived from sur- 908
859 factant and viscous force. Thus, different shapes were 909
860 produced (Fig. 18B) by controlling the surfactant used 910
861 and the viscosity of the gelation bath.

862 Concentration

863 After preparation, the concentration of alginate in the 911
864 microparticles can be calculated approximately. For in- 912
865 stance, Zhang et al. used partially miscible fluids [59]. 913
866 An aqueous solution of Na-alginate was prepared with a 914
867 known concentration. After droplet generation, droplet 915
868 shrinkage occurred during passage through the channel 916
869 due to water diffusion into the continuous fluid. Drop- 917
870 lets were hence transformed into microparticles. As the 918
871 diffusion of Na-alginate into the continuous fluid is neg- 919
872 ligible [39], the quantity of Na-alginate is constant. It 920
873 can be calculated by multiplying the droplet volume and 921
874 initial concentration. Finally, by measuring microparticle 922
875 size, the concentration of Na-alginate can be calculated. 923
876 The final concentration of Na-alginate varies from 20 to 924
877 100 wt%, depending on the initial concentration and 925
878 diameter of the droplets generated [59]. Furthermore, as 926
879 presented in Utech et al., the homogeneity of composi- 927
880 tion of microparticles can be determined with the help 928
881 of fluorescence technology [49].

882 Stability

883 In most cases, surfactant is added in the continuous fluid 929
884 [48, 49, 61] to lower interfacial energy between the 930

931 continuous and the dispersed fluids γ_{CD} . Note that for 932
932 each of the above studies, use or non-use of surfactant is 933
933 mentioned when indicated by the authors (Tables 1, 2, 934
934 3, 4, 5). Surfactant facilitates the creation of a new inter- 935
935 face, and thus the formation of droplets. It also stabilizes 936
936 the formed droplets by preventing their coalescence 937
937 [41]. Before the application of microparticles, the surfac- 938
938 tant should be dissolved [3], except for biocompatible 939
939 surfactant [49], although protocols for removing surfac- 940
940 tant are rarely reported in the literature. To remove the 941
941 oil used during the preparation, microparticles should be 942
942 washed several times with an aqueous solution, followed 943
943 by centrifugation [61]. 944

945 However, despite its advantages, the use of surfactant 945
946 may be undesirable. Surfactant has been shown to im- 946
947 pact the surface properties of microparticles, such as 947
948 morphology [45] and surface hydrophobicity [22]. Addi- 948
949 tionally, if rinsing is insufficient, the traces of surfactant 949
949 on microparticles can damage the devices during appli- 950
950 cation. In this situation, microparticles should be pre- 951
951 pared without surfactant, which is possible with droplet- 952
952 based microfluidics. In the microchannel, the coales- 953
953 cence of droplets can be avoided by enlarging the dis- 954
954 tance between droplets, which can be achieved by 955
955 regulating flow rates. Furthermore, gelation, either on- 956
956 chip or off-chip, solidifies droplets and thus helps to 957
957 avoid coalescence as well. Another strategy consists of 958
958 using partially miscible fluids. This means that the drop- 959
959 lets shrink and become more and more condensed dur- 960
960 ing passage through the channel. At the outlet, either 961
961 gelation [39] or a final shrinkage [59] can help avoid 962
962 coalescence. 963

964 Moreover, for their stability, alginate hydrogel micro- 964
965 particles should be insoluble in water. This can be 965
966 achieved by adopting proper gelation methods using a 966
966 sufficient quantity of cross-linkers for effective gelation. 967

921 Mechanical properties

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

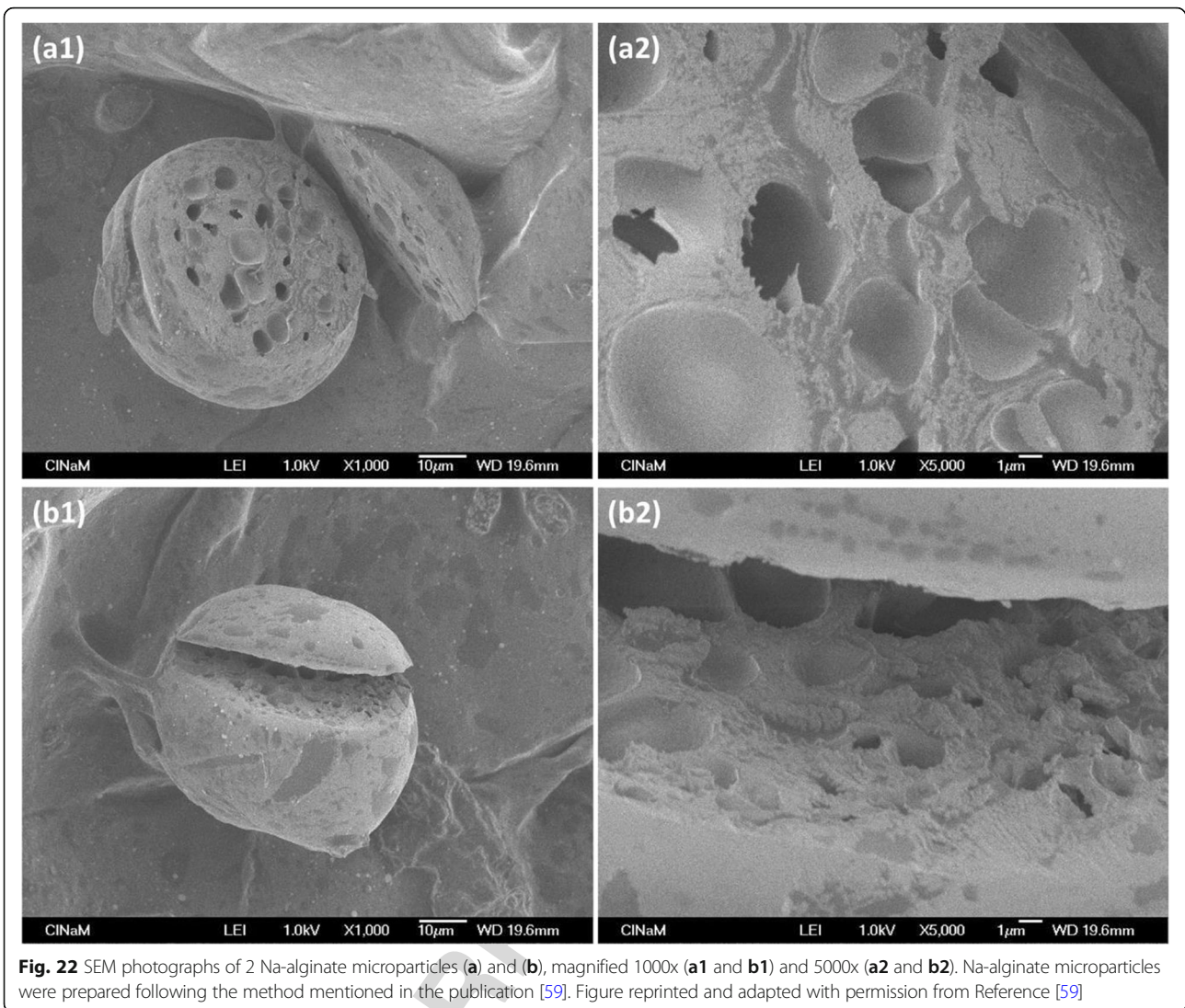
Lastly, we wish to stress the ease of constructing a 994
microfluidic device with Fluoropolymer tubing and junc- 995
tions compared to a chip, whatever its composition: 996
PDMS, PMMA or glass. A microfluidic device's geom- 997
etry can be tuned flexibly, whereas in a chip, the geom- 998
etry of channels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

- 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
1024 needs to be produced. However, the throughput of
1025 microparticles fabricated by microfluidics is still
1026 limited. Exploring ways to scale up microparticle
1027 production would therefore be a welcome
1028 contribution.
- 1029 3. Currently, most publications concern proof-of-
1030 concepts or preliminary demonstrations. Little work
1031 is available on microparticles in real biological or
1032 biomedical applications [53]. Apart from biological
1033 barriers [35], the physiological environment is quite
1034 complex in terms of composition and rheological
1035 characteristics, which makes it extremely unlikely
1036 that microparticles can provide the functions re-
1037 quired. The feasibility of using alginate hydrogel mi-
1038 croparticles in real-life applications needs to be
1039 assessed.



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

Acknowledgements 1061
 We thank Marjorie Sweetko for English revision. 1062

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

Funding 1068
 We are grateful to ANR CUMBA (ANR-15-CE19-0017-03) for its financial 1069
 support. 1070

Availability of data and materials 1071
 Not applicable. 1072

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.

1080 **Received: 23 August 2021 Accepted: 6 November 2021**

1081

1082 **References**

- 1083 1. Agüero L, Zaldivar-Silva D, Peña L, Dias ML. Alginate microparticles as oral
1084 colon drug delivery device: a review. *Carbohydr Polym.* 2017;168:32–43.
1085 <https://doi.org/10.1016/j.carbpol.2017.03.033>.
- 1086 2. Ahmed EM. Hydrogel: preparation, characterization, and applications: a
1087 review. *J Adv Res.* 2015;6(2):105–21. <https://doi.org/10.1016/j.jare.2013.07.006>.
- 1088
- 1089 3. Akbari S, Pirbodaghi T. Microfluidic encapsulation of cells in alginate
1090 particles via an improved internal gelation approach. *Microfluid Nanofluid.*
1091 2014;16(4):773–7. <https://doi.org/10.1007/s10404-013-1264-z>.
- 1092 4. Barbieri M, Cellini F, Cacciotti I, Peterson SD, Porfiri M. In situ temperature
1093 sensing with fluorescent chitosan-coated PNIPAAm/alginate beads. *J Mater*
1094 *Sci.* 2017;52(20):12506–12. <https://doi.org/10.1007/s10853-017-1345-6>.
- 1095 5. Baroud CN, Gallaire F, Danga R. Dynamics of microfluidic droplets. *Lab*
1096 *Chip.* 2010;10(16):2032–45. <https://doi.org/10.1039/c001191f>.
- 1097 6. Caccavo D, Cascone S, Lamberti G, Barba AA. Hydrogels: experimental
1098 characterization and mathematical modelling of their mechanical and
1099 diffusive behaviour. *Chem Soc Rev.* 2018;47(7):2357–73. <https://doi.org/10.1039/C7CS00638A>.
- 1100
- 1101 7. Carin M, Barthès-Biesel D, Edwards-Lévy F, Postel C, Andrei DC. Compression
1102 of biocompatible liquid-filled HSA-alginate capsules: determination of the
1103 membrane mechanical properties. *Biotechnol Bioeng.* 2003;82(2):207–12.
1104 <https://doi.org/10.1002/bit.10559>.
- 1105 8. Chan LW, Lee HY, Heng PWS. Production of alginate microspheres by
1106 internal gelation using an emulsification method. *Int J Pharm.* 2002;242(1–2):
1107 259–62. [https://doi.org/10.1016/S0378-5173\(02\)00170-9](https://doi.org/10.1016/S0378-5173(02)00170-9).
- 1108 9. Christopher GF, Anna SL. Microfluidic methods for generating continuous
1109 droplet streams. *J Phys D Appl Phys.* 2007;40(19):R319–36. <https://doi.org/10.1088/0022-3727/40/19/R01>.
- 1110
- 1111 10. De Menech M, Garstecki P, Jousse F, Stone HA. Transition from squeezing to
1112 dripping in a microfluidic T-shaped junction. *J Fluid Mech.* 2008;595:141–61.
- 1113 11. Draget KL. 29 - alginates. In: Phillips GO, Williams PA, editors. *Handbook of*
1114 *hydrocolloids*. 2nd ed: Woodhead Publishing; 2009. p. 807–28.
- 1115 12. Enck K, Rajan SP, Aleman J, Castagno S, Long E, Khalil F, et al. Design of an
1116 adhesive film-based microfluidic device for alginate hydrogel-based cell
1117 encapsulation. *Ann Biomed Eng.* 2020;48(3):1103–11. <https://doi.org/10.1007/s10439-020-02453-9>.
- 1118
- 1119 13. Freiberg S, Zhu XX. Polymer microspheres for controlled drug release. *Int J*
1120 *Pharm.* 2004;282(1–2):1–18. <https://doi.org/10.1016/j.ijpharm.2004.04.013>.
- 1121 14. Fundueanu G, Nastruzzi C, Carpov A, Desbrieres J, Rinaudo M. Physico-
1122 chemical characterization of Ca-alginate microparticles produced with
1123 different methods. *Biomaterials.* 1999;20(15):1427–35. [https://doi.org/10.1016/S0142-9612\(99\)00050-2](https://doi.org/10.1016/S0142-9612(99)00050-2).
- 1124
- 1125 15. Gacosa P. Alginates. *Carbohydr Polym.* 1988;8(3):161–82. [https://doi.org/10.1016/0144-8617\(88\)90001-X](https://doi.org/10.1016/0144-8617(88)90001-X).
- 1126
- 1127 16. Grant GT, Morris ER, Rees DA, Smith PJC, Thom D. Biological interactions
1128 between polysaccharides and divalent cations: the egg-box model. *FEBS*
1129 *Lett.* 1973;32(1):195–8. [https://doi.org/10.1016/0014-5793\(73\)80770-7](https://doi.org/10.1016/0014-5793(73)80770-7).
- 1130 17. Guevorkian K, Maître JL. Chapter 10 - micropipette aspiration: a unique tool
1131 for exploring cell and tissue mechanics in vivo. In: Lecuit T, editor. *Methods*
1132 *in cell biology*: Academic Press; 2017. p. 187–201.
- 1133 18. Haghgooei R, Toner M, Doyle PS. Squishy non-spherical hydrogel
1134 microparticles. *Macromol Rapid Commun.* 2010;31(2):128–34. <https://doi.org/10.1002/marc.200900302>.
- 1135
- 1136 19. Hoffman AS. Hydrogels for biomedical applications. *Adv Drug Deliv Rev.*
1137 2012;64:18–23. <https://doi.org/10.1016/j.addr.2012.09.010>.
20. Hu Y, Wang Q, Wang J, Zhu J, Wang H, Yang Y. Shape controllable microgel
1138 particles prepared by microfluidic combining external ionic crosslinking.
1139 *Biomicrofluidics.* 2012;6:026502. <https://doi.org/10.1063/1.4720396>.
1140
21. Joye IJ, McClements DJ. Biopolymer-based nanoparticles and microparticles:
1141 fabrication, characterization, and application. *Curr Opin Colloid Interface Sci.*
1142 2014;19(5):417–27. <https://doi.org/10.1016/j.cocis.2014.07.002>.
1143
22. Kidane A, Guimond P, Rob Ju T-C, Sanchez M, Gibson J, North A, et al. 1144
1145 Effects of cellulose derivatives and poly (ethylene oxide)–poly (propylene
1146 oxide) tri-block copolymers (Pluronic®surfactants) on the properties of
1147 alginate based microspheres and their interactions with phagocytic cells. *J*
1148 *Control Release.* 2002;85(1–3):181–9. [https://doi.org/10.1016/S0168-3659\(02\)00281-X](https://doi.org/10.1016/S0168-3659(02)00281-X).
1149
23. Kleinberger RM, Burke NaD, Dalnoki-Veress K, Stöver HDH. Systematic study
1150 of alginate-based microcapsules by micropipette aspiration and confocal
1151 fluorescence microscopy. *Mater Sci Eng C.* 2013;33(7):4295–304. <https://doi.org/10.1016/j.msec.2013.06.033>.
1152
24. Lee B-B, Ravindra P, Chan E-S. Size and shape of calcium alginate beads
1153 produced by extrusion dripping. *Chem Eng Technol.* 2013;36:1627–42.
1154 <https://doi.org/10.1002/ceat.201300230>.
1155
25. Lee KY, Mooney DJ. Alginate: properties and biomedical applications. *Prog Polym*
1156 *Sci.* 2012;37(1):106–26. <https://doi.org/10.1016/j.progpolymsci.2011.06.003>.
1157
26. Lee KY, Yuk SH. Polymeric protein delivery systems. *Prog Polym Sci.* 2007;
1158 32(7):669–97. <https://doi.org/10.1016/j.progpolymsci.2007.04.001>.
1159
27. Li W, Zhang L, Ge X, Xu B, Zhang W, Qu L, et al. Microfluidic fabrication of
1160 microparticles for biomedical applications. *Chem Soc Rev.* 2018;47(15):5646–
1161 83. <https://doi.org/10.1039/C7CS00263G>.
1162
28. Liu K, Ding H-J, Liu J, Chen Y, Zhao X-Z. Shape-controlled production of
1163 biodegradable calcium alginate gel microparticles using a novel microfluidic
1164 device. *Langmuir.* 2006;22(22):9453–7. <https://doi.org/10.1021/la061729+>.
1165
29. Liu Y, Tottori N, Nisisako T. Microfluidic synthesis of highly spherical calcium
1166 alginate hydrogels based on external gelation using an emulsion reactant.
1167 *Sensors Actuators B Chem.* 2019;283:802–9. <https://doi.org/10.1016/j.snb.2018.12.101>.
1168
30. Majtra J, Shukla V. Cross-linking in hydrogels - a review. *Am J Polym Sci.*
1169 2014;4:25–31.
1170
31. Markert CD, Guo X, Skardal A, Wang Z, Bharadwaj S, Zhang Y, et al. 1171
1172 Characterizing the micro-scale elastic modulus of hydrogels for use in
1173 regenerative medicine. *J Mech Behav Biomed Mater.* 2013;27:115–27.
1174 <https://doi.org/10.1016/j.jmbm.2013.07.008>.
1175
32. Merkel TJ, Jones SW, Herlihy KP, Kersey FR, Shields AR, Napier M, et al. 1176
1177 Using mechanobiological mimicry of red blood cells to extend circulation times of
1178 hydrogel microparticles. *Proc Natl Acad Sci.* 2011;108(2):586–91. <https://doi.org/10.1073/pnas.1010013108>.
1179
33. Mørch YA, Donati I, Strand BL, Skjåk-Bræk G. Effect of Ca²⁺, Ba²⁺, and Sr²⁺
1180 on alginate microbeads. *Biomacromolecules.* 2006;7(5):1471–80. <https://doi.org/10.1021/bm060010d>.
1181
34. Pittermannová A, Ruberová Z, Zadražil A, Bremond N, Bibette J, Štěpánek F. 1182
1183 Microfluidic fabrication of composite hydrogel microparticles in the size
1184 range of blood cells. *RSC Adv.* 2016;6(105):103532–40. <https://doi.org/10.1039/C6RA23003B>.
1185
35. Poon W, Kingston BR, Ouyang B, Ngo W, Chan WCW. A framework for
1186 designing delivery systems. *Nat Nanotechnol.* 2020;15(10):819–29. <https://doi.org/10.1038/s41565-020-0759-5>.
1187
36. Qiu C, Chen M, Yan H, Wu H. Generation of uniformly sized alginate
1188 microparticles for cell encapsulation by using a soft-lithography approach.
1189 *Adv Mater.* 2007;19(12):1603–7. <https://doi.org/10.1002/adma.200602574>.
1190
37. Ren K, Zhou J, Wu H. Materials for microfluidic chip fabrication. *Acc Chem*
1191 *Res.* 2013;46(11):2396–406. <https://doi.org/10.1021/ar300314s>.
1192
38. Roh YH, Lee HJ, Bong KW. Microfluidic fabrication of encoded hydrogel
1193 microparticles for application in multiplex immunoassay. *BioChip J.* 2019;
1194 13(1):64–81. <https://doi.org/10.1007/s13206-019-3104-z>.
1195
39. Rondeau E, Cooper-White JJ. Biopolymer microparticle and nanoparticle
1196 formation within a microfluidic device. *Langmuir.* 2008;24(13):6937–45.
1197 <https://doi.org/10.1021/la703339u>.
1198
40. Santa-Maria M, Scher H, Jeoh T. Microencapsulation of bioactives in cross-
1199 linked alginate matrices by spray drying. *J Microencapsul.* 2012;29(3):286–95.
1200 <https://doi.org/10.3109/02652048.2011.651494>.
1201
41. Seemann R, Brinkmann M, Pfohl T, Herminghaus S. Droplet based
1202 microfluidics. *Rep Prog Phys.* 2011;75(1):016601. <https://doi.org/10.1088/0034-4885/75/1/016601>.
1203
1204
1205
1206
1207

- 1208 42. Shikha S, Zheng X, Zhang Y. Upconversion nanoparticles-encoded hydrogel
1209 microbeads-based multiplexed protein detection. *Nano-Micro Lett.* 2018;
1210 10(2):31. <https://doi.org/10.1007/s40820-017-0184-y>.
- 1211 43. Stephenson R, Stuart J. Mutual binary solubilities: water-alcohols and water-esters. *J*
1212 *Chem Eng Data.* 1986;31(1):56–70. <https://doi.org/10.1021/je00043a019>.
- 1213 44. Sugaya S, Yamada M, Seki M. Production of extremely-small hydrogel
1214 microspheres by utilizing water-droplet dissolution in a polar solvent. In:
1215 Landers J, editor. 15th International Conference on Miniaturized Systems for
1216 Chemistry and Life Sciences. Seattle: Chemical and Biological Microsystems
1217 Society (CBMS); 2011. p. 18–20.
- 1218 45. Sundberg DC, Casassa AP, Pantazopoulos J, Muscato MR, Kronberg B, Berg J.
1219 Morphology development of polymeric microparticles in aqueous
1220 dispersions. I. Thermodynamic considerations. *J Appl Polym Sci.* 1990;41(78):
1221 1425–42. <https://doi.org/10.1002/app.1990.070410706>.
- 1222 46. Teh SY, Lin R, Hung LH, Lee AP. Droplet microfluidics. *Lab Chip.* 2008;8(2):
1223 198–220. <https://doi.org/10.1039/b715524g>.
- 1224 47. Trivedi V, Doshi A, Kurup GK, Ereifej E, Vandevord PJ, Basu AS. A modular
1225 approach for the generation, storage, mixing, and detection of droplet
1226 libraries for high throughput screening. *Lab Chip.* 2010;10(18):2433–42.
1227 <https://doi.org/10.1039/c004768f>.
- 1228 48. Trivedi V, Ereifej ES, Doshi A, Sehgal P, Vandevord PJ, Basu AS. Microfluidic
1229 encapsulation of cells in alginate capsules for high throughput screening,
1230 Proceedings of the 31st annual international conference of the IEEE
1231 engineering in medicine and biology society: engineering the future of
1232 biomedicine, EMBC 2009; 2009. p. 7037–40.
- 1233 49. Utech S, Prodanovic R, Mao AS, Ostafe R, Mooney DJ, Weitz DA. Microfluidic
1234 generation of monodisperse, structurally homogeneous alginate microgels
1235 for cell encapsulation and 3D cell culture. *Adv Healthc Mater.* 2015;4(11):
1236 1628–33. <https://doi.org/10.1002/adhm.201500021>.
- 1237 50. Uyen NTT, Hamid ZaA, Tram NXT, Ahmad N. Fabrication of alginate
1238 microspheres for drug delivery: a review. *Int J Biol Macromol.* 2020;153:
1239 1035–46. <https://doi.org/10.1016/j.ijbiomac.2019.10.233>.
- 1240 51. Velings NM, Mestdagh MM. Physico-chemical properties of alginate gel
1241 beads. *Polym Gels Netw.* 1995;3(3):311–30. [https://doi.org/10.1016/0966-7822\(94\)00043-7](https://doi.org/10.1016/0966-7822(94)00043-7).
- 1242
- 1243 52. Wang CX, Cowen C, Zhang Z, Thomas CR. High-speed compression of
1244 single alginate microspheres. *Chem Eng Sci.* 2005;60(23):6649–57. <https://doi.org/10.1016/j.ces.2005.05.052>.
- 1245
- 1246 53. Wang W, Zhou C. A journey of nanomotors for targeted cancer therapy:
1247 principles, challenges, and a critical review of the state-of-the-art. *Adv*
1248 *Healthc Mater.* 2020;10:e2001236.
- 1249 54. Xu JH, Li SW, Tan J, Luo GS. Controllable preparation of monodispersed
1250 calcium alginate microbeads in a novel microfluidic system. *Chem Eng*
1251 *Technol.* 2008;31(8):1223–6. <https://doi.org/10.1002/ceat.200800027>.
- 1252 55. Xu Q, Hashimoto M, Dang TT, Hoare T, Kohane DS, Whitesides GM, et al.
1253 Preparation of monodisperse biodegradable polymer microparticles using a
1254 microfluidic flow-focusing device for controlled drug delivery. *Small.* 2009;
1255 5(13):1575–81. <https://doi.org/10.1002/smll.200801855>.
- 1256 56. Yu L, Sun Q, Hui Y, Seth A, Petrovsky N, Zhao C-X. Microfluidic formation of
1257 core-shell alginate microparticles for protein encapsulation and controlled
1258 release. *J Colloid Interface Sci.* 2019;539:497–503. <https://doi.org/10.1016/j.jcis.2018.12.075>.
- 1259
- 1260 57. Zagnoni M, Anderson J, Cooper JM. Hysteresis in multiphase microfluidics at
1261 a T-junction. *Langmuir.* 2010;26(12):9416–22. <https://doi.org/10.1021/la1004243>.
- 1262
- 1263 58. Zhang C. Development of a microfluidic method for the preparation of
1264 mimetic microparticles of red blood cells with controllable size and
1265 mechanical properties: Université d'Aix-Marseille; 2020.
- 1266 59. Zhang C, Grossier R, Lacaria L, Rico F, Candoni N, Veessler S. A microfluidic
1267 method generating monodispersed microparticles with controllable sizes
1268 and mechanical properties. *Chem Eng Sci.* 2020;211:115322. <https://doi.org/10.1016/j.ces.2019.115322>.
- 1269
- 1270 60. Zhang H, Tumarkin E, Peerani R, Nie Z, Sullan RMA, Walker GC, et al.
1271 Microfluidic production of biopolymer microcapsules with controlled
1272 morphology. *J Am Chem Soc.* 2006;128(37):12205–10. <https://doi.org/10.1021/ja0635682>.
- 1273
- 1274 61. Zhang H, Tumarkin E, Sullan RMA, Walker GC, Kumacheva E. Exploring
1275 microfluidic routes to microgels of biological polymers. *Macromol Rapid*
1276 *Commun.* 2007;28(5):527–38. <https://doi.org/10.1002/marc.200600776>.
62. Zhang J, Shikha S, Mei Q, Liu J, Zhang Y. Fluorescent microbeads for point-
of-care testing: a review. *Microchim Acta.* 2019;186(6):361. <https://doi.org/10.1007/s00604-019-3449-y>. 1277
63. Zhang S, Guivier-Curien C, Veessler S, Candoni N. Prediction of sizes and
frequencies of nanoliter-sized droplets in cylindrical T-junction microfluidics. 1278
- Chem Eng Sci.* 2015;138:128–39. <https://doi.org/10.1016/j.ces.2015.07.046>. 1279
64. Zhao X, Cui Y, He Y, Wang S, Wang J. Synthesis of multi-mode quantum
dots encoded molecularly imprinted polymers microspheres and
application in quantitative detection for dopamine. *Sensors Actuators B*
Chem. 2020;304:127265. <https://doi.org/10.1016/j.snb.2019.127265>. 1280
65. Zhu P, Wang L. Passive and active droplet generation with microfluidics: a
review. *Lab Chip.* 2017;17(1):34–75. <https://doi.org/10.1039/C6LC01018K>. 1281
- 1282
- 1283
- 1284
- 1285
- 1286
- 1287
- 1288

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1289

1290

1291

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions



Author Query Form

Journal: Biomaterials Research

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

Authors: Cheng Zhang, Romain Grossier, Nadine Candoni, Stéphane Veessler

Article: 243

Dear Authors,

During production of your paper, the following queries arose. Please respond to these by annotating your proofs with the necessary changes/additions. If you intend to annotate your proof electronically, please refer to the E-annotation guidelines. We recommend that you provide additional clarification of answers to queries by entering your answers on the query sheet, in addition to the text mark-up.

Query No.	Query	Remark
Q1	Please check if the affiliation is presented correctly.	
Q2	Citation details for references [11, 17, 58] are incomplete. Please supply the "publisher location" of these references. Otherwise, kindly advise us on how to proceed.	