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Smaniotto, Fabio; Prosapio, Valentina; Zafeiri, Ioanna; Spyropoulos, Fotios

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Freeze drying and rehydration of alginate fluid gels

F. Smaniotto, V. Prosapio*, I. Zafeiri, F. Spyropoulos

School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT,
United Kingdom

[*v.prosapio@bham.ac.uk](mailto:v.prosapio@bham.ac.uk)

Abstract

The aim of this work was to study the effect of freeze drying (FD) and rehydration on alginate fluid gels (AFG). FD was employed as a widely used drying method in order to evaluate whether its application causes any change in the material characteristics crucial for AFG behaviour. Rehydration studies were performed to assess if AFG properties could be restored after drying and rehydration processes. First, it was investigated the influence of the material formulation (alginate (ALG) and calcium chloride (CaCl_2) concentrations) on the particle size distribution (PSD) and the rheological properties. The used ALG/ CaCl_2 ratio demonstrated to be responsible for forming nanoparticles or microparticles. The impact of fluid gel particle dimensions on drying and rehydration processes was also investigated. Overall, particle dimensions do not have a significant effect on drying kinetics. Analyses on rehydrated samples showed that the PSD is not affected by the processing, whereas the same viscosity was completely recovered only for samples formed by ALG and CaCl_2 in quantities leading to nanoparticles formation. Preliminary encapsulation experiments were also carried out to highlight the impact of this process on the release behaviour of the loaded active from AFG. Results obtained from in vitro release studies and their model fitting showed that the active release behaviour from AFG was not affected by freeze drying when AFG was formed of nanoparticles. On the contrary, the active release behaviour was affected by freeze drying when AFG was formed by microparticles.

Keywords: Fluid gel; freeze-drying; rehydration; rheological properties; encapsulation

26 1. Introduction

27 Fluid gels are suspensions of gel particles in a non-gelled continuous medium and are
28 formed by applying shear forces to a polymeric solution undergoing a sol-gel transition
29 (Garrec & Norton, 2012). They can be prepared using both biological (polysaccharides and
30 proteins) and synthetic polymers (Norton, et al., 1999), and can be produced using typical
31 shear devices, such as a jacketed pin-stirrer, which allows a continuous process. Fluid gels can
32 be temperature and/or ionically set, depending on the hydrocolloid used and to its gelation
33 mechanism (García, et al., 2018a; Wang, et al., 2016). The particle size can be controlled by
34 varying the polymer concentration and the shear rate (García, et al., 2018b; Norton, et al.,
35 1999).

36 Fluid gels show unique characteristics: at low deformations their flow behaviour tends to
37 resemble that of quiescent gels, whereas at high deformations exhibits yield and flow
38 characteristics like a viscoelastic fluid (Norton, et al., 2015). Due to this rheological
39 performance, fluid gels find applications in the food industry as fat replacers (Chung, et al.,
40 2014; Le Révérend, et al., 2010), texturing (Fernández Farrés, et al., 2014), satiety enhancers
41 (Norton, et al., 2006) and emulsion stabiliser (García, et al., 2014). A novel application involves
42 their use for encapsulation of bioactive molecules to protect them from the surrounding
43 environment, to control their release in a specific medium and/or to mask their flavour
44 (Mahdi, et al., 2016; Torres, et al., 2016). The use of fluid gels as encapsulating material has
45 the following advantages compared to other carriers (such as microcapsules, liposomes, etc.):
46 organic solvents are not needed, the process can be run in continuous and can be easily
47 scaled-up, mild operating conditions can be employed and good control over particle size is
48 provided.

49 Being formed by a large amount of water, the shelf-life of fluid gels is relatively short and
50 microbial spoilage can easily occur. This limitation can be overcome by drying the material, as
51 water removal prevents bacteria proliferation. A dried product can be considered as safe if it
52 is characterised by water activity (a_w) lower than 0.6 (de Bruijn, et al., 2016) and normalised
53 moisture content (NMC) lower than 0.1 (Brown, et al., 2010; Prosapio & Norton, 2018). In
54 addition, the lower volume/weight of the product allows packaging, transport and storage
55 costs to be reduced (Brown, et al., 2008; Prosapio, et al., 2017b). On the other hand, drying
56 of food products has been reported to cause some damage to the product microstructure

57 according to the method and conditions employed, leading to low rehydration capacity and
58 poor recovery of the initial properties (Vega-Gálvez, et al., 2015). Among the most common
59 drying methods, freeze-drying (FD) showed the best performance in terms of water
60 desorption, retention of the product characteristics and rehydration of the dried material
61 (Karam, et al., 2016; Prosapio & Norton, 2017a). Specifically, for the dehydration of
62 microstructures/formulations containing encapsulated matters, FD can be a suitable method
63 to prevent the degradation of thermosensitive compounds, as it involves the freezing of the
64 product, followed by ice sublimation under vacuum conditions (Barbosa, et al., 2015; Sanchez,
65 et al., 2013).

66 The drying of quiescent gels has been extensively investigated using different materials
67 (gellan gum (Bonifacio, et al., 2017; Cassanelli, et al., 2018), starch (De Marco & Reverchon,
68 2017; Franco, et al., 2018), cellulose (Jin, et al., 2004; Long, et al., 2018), agarose (Mao, et al.,
69 2017), silica (Smirnova, et al., 2003), chitosan (Cardea, et al., 2010), etc.) and techniques (air-
70 drying, freeze-drying, supercritical carbon dioxide drying, microwave drying (Cassanelli, et al.,
71 2019; Hu, et al., 2013)). Tiwari et al. (Tiwari, et al., 2015) analysed the structure of freeze-
72 dried gellan gum and agar gels; they observed for both materials that mechanically stable
73 cellular solids were produced. Cassanelli et al. (Cassanelli, et al., 2019) studied the drying of
74 gellan gum gels using oven drying, freeze drying and supercritical carbon dioxide drying; they
75 observed that the gel microstructure was better preserved when FD was employed, leading
76 to a significantly higher rehydration capacity compared to the other methods. Hu et al. (Hu,
77 et al., 2013) employed air drying, microwave vacuum drying and freeze drying to dry hairtail
78 fish meat gels and stated that FD samples showed better quality attributes in terms of
79 moisture content, water absorption index, protein degradation and sensory acceptance than
80 air and microwave-dried ones.

81 Despite the potentiality in providing long/safe storage and protection of the actives, the
82 drying of fluid gels has not been studied so far. In order to fill the gap in the current literature,
83 this work investigates the freeze drying of alginate fluid gels (AFG). Alginate is a hydrocolloid
84 that undergoes gelation in the presence of multivalent cations, usually Ca^{2+} (McHugh & Pap,
85 1987). AFG were prepared in a pin-stirrer and thereafter freeze-dried. Rheological
86 measurements were carried out on both unprocessed and processed fluid gels to verify if the
87 material properties (particle size and viscosity) can be completely recovered after drying and

88 rehydration. In addition to the preservation of rheological behaviour upon rehydration, the
89 capacity of the fluid gels to modulate the release of a model active (present within the fluid
90 gel microstructure) prior and following FD was investigated. Preliminary encapsulation
91 experiments were also performed, using Nicotinamide as model active, to investigate the
92 release behaviour before and after drying/rehydration, and identify possible changes due to
93 the processing.

94

95 2. Materials and methods

96 2.1 Materials

97 Sodium alginate (ALG), and nicotinamide (NIC, $\geq 98\%$, HPLC) were purchased from
98 Sigma-Aldrich[®] (Sigma–Aldrich Company Ltd., Dorset, UK). Calcium chloride (CaCl_2 ,
99 anhydrous, 93%) was purchased from Alfa Aesar[™] (USA). All materials were used without
100 further purification. Milli-Q water was made using an Elix[®] 5 distillation apparatus (Millipore[®],
101 USA) and was used for all water-based preparations.

102

103 2.2 Gel preparation

104 2.2.1 Blank fluid gels

105 Firstly, an alginate solution was prepared by dissolving different amounts of ALG (1%,
106 2%, 3% (w/w), calculated on the final weight of the material) in distilled water at 95°C for 45
107 min under stirring to ensure complete powder dissolution, as reported by Farrés et al.
108 (Fernández Farrés, et al., 2013). Thereafter, the solution was cooled at room temperature
109 (R.T.). Secondly, another solution was prepared by dissolving CaCl_2 at a different
110 concentration for each experiment (0.15%, 0.25%, 0.35% (w/w), calculated on the final weight
111 of the material) in water at R.T.. Alginate Fluid Gels (AFG) were prepared using a pin-stirrer
112 vessel (Het Stempel, HL) having a volume of 150 mL, 16 pins placed on the rotating shaft and
113 16 pins fixed on the internal wall. 1000 rpm shaft speed was used for AFG production. The
114 alginate solution was pumped using a flowrate of 33 mL/min with a peristaltic pump
115 (Masterflex L/S Peristaltic, DE), while the CaCl_2 solution was injected using a flowrate of 4.02
116 mL/min with a syringe pump (Cole-Parmer Single-syringe, US) through a stainless steel needle
117 of 1.25 mm internal diameter.

118

119 2.2.2 Nicotinamide fluid gels

120 Firstly, a solution of 2% (w/w) was prepared by dissolving ALG in distilled water at 95
121 °C for 45 min under stirring to ensure complete powder dissolution. Then, the solution was
122 cooled at R.T.. Secondly, three solutions at 0.10% (w/w) were prepared by dissolving NIC in
123 distilled water at R.T.. To these solutions different amounts of CaCl₂ (0.15%, 0.25%, 0.35%
124 (w/w), calculated on the final weight) were dissolved at R.T.. Nicotinamide Fluid Gels (NFG)
125 were then prepared using a pin-stirrer vessel as described above (2.2.1 Blank fluid gels).

126

127 2.3 Rheological properties

128 The rheological properties of fluid gels were determined by shear viscosity tests using
129 a rotational rheometer (Kinexus™, Malvern®, UK) equipped with a 40 mm diameter sand
130 blasted plate geometry. The analyses were carried out at 25 °C using a shear rate ramp of 31
131 points between 0.01 and 100 s⁻¹. Measurements were performed in triplicate.

132

133 2.4 Particle Size Distribution

134 2.4.1 Mastersizer

135 The particle size distribution (PSD) of fluid gels was evaluated using a Mastersizer-
136 2000 (Malvern®, UK). Few drops of sample were placed into the mixing chamber and stirred
137 for 10 min at 1300 rpm before performing the analysis to disrupt any possible macro-
138 aggregation. PSD was evaluated as numerical particle sizes percentage. Measurements were
139 performed in triplicate.

140 2.4.2 Zetasizer

141 The PSD of fluid gels was also evaluated by using Zetasizer (Malvern Instruments Ltd.,
142 UK). Samples were diluted with water before analysis to yield a suitable scattering intensity
143 and measured at 25 °C. PSD curves were evaluated as numerical particle sizes percentage. All
144 measurements were performed at 25 °C in triplicate.

145 2.5 Optical microscopy

146 Optical microscope (DM 2500 LED, Leica®, CH) was used to observe and record
147 alginate microparticles. Samples were, firstly, diluted with distilled water using a water to
148 sample ratio of 10:1 and they were mixed using a vortex mixer (VM20, Rigal Bennet®, UK) for
149 20 seconds. DIC settings were employed to increase the contrast, allowing the average
150 dimensions of particles to be measured. Images were captured using a CCD camera (DFC450

151 C, Leica[®], CH) coupled to the microscope. Image analysis software IPP (LAS 4.8.0, Leica[®], CH)
152 was used to evaluate the dimensions of the microparticles through the images.

153

154 2.6 Freeze drying

155 Fluid gels were frozen at -20 °C overnight and then lyophilised using a bench top
156 Freeze Dryer (SCANVAC Coolsafe™, model 110-4, DK), condenser temperature -110 °C,
157 pressure 10 Pa, condition that is defined by the equipment. The processing time was varied
158 from 2 to 48 h to investigate the drying kinetics. Experiments were performed in triplicate for
159 each condition investigated.

160

161 2.7 Moisture content analysis

162 Moisture content analyses were carried out measuring the sample weight before and
163 after drying. Moisture content was expressed as NMC (Normalised Moisture Content) and
164 calculated through the following equation (1):

$$NMC = \frac{(M_d - M_s)}{(M_o - M_s)} \quad (1)$$

165 where M_s is the solid sample mass, M_d the sample mass after drying and M_o the pre-dried
166 sample. The drying kinetics was determined plotting NMC as a function of the drying time.
167 Analyses were carried out in triplicate.

168

169 2.8 Water activity analysis

170 Water activity (a_w) of dried samples was measured using an AquaLab[®] dew point
171 water activity meter (model 4TE, Decagon Devices Inc., Pullman, WA, USA). The temperature
172 controlled sample chamber was set to 25 °C. Analyses were carried out in triplicate.

173 2.9 Rehydration of samples

174 After dehydration, amounts of distilled water were placed in each sample in order to
175 obtain the same sample weight before FD. Samples were then transferred in a shaking
176 incubator (Incu-Shake MIDI, SciQuip, UK), in order to obtain a homogeneous rehydration of
177 the material, for different times at 400 rpm and 20 °C.

178

179 2.10 In vitro release

180 In vitro release studies from NFG were performed using a UV/vis spectrophotometer
181 (Orion Aquamate, Thermo Scientific, UK) to determine the concentration of NIC in the
182 medium over time. A weighted amount of NFG (approximately 2.5 g) was enclosed in a
183 dialysis sack (Sigma–Aldrich Company Ltd., Dorset, UK, dialysis tubing cellulose membrane,
184 width 43 mm, M.W. cut-off of 14000 Da) and placed in 500 mL of distilled water at room
185 temperature (thermostated at 21.5 °C), under stirring at 150 rpm. At regular intervals,
186 aliquots of 2 mL were withdrawn, measured using the spectrophotometer at 214 nm
187 wavelength and then poured again into the medium. A calibration curve was made at 214 nm
188 wavelength (NIC maximum of absorbance) and was set for concentrations between 0.08 and
189 45 µg/mL and was used to correlate the absorbance value to the actual concentration of NIC
190 into the release medium. Each analysis was carried out in triplicate.

191 2.11 Data fitting

192 Data obtained from NIC in vitro release studies from NFG were model fitted using
193 equation (2):

194
$$\frac{M_t}{M_\infty} = kt^n \quad (2)$$

195 where M_t and M_∞ are respectively the cumulative amounts of drug released at time t and
196 at time when the release plateau was reached, k is the kinetic constant and n is an exponent
197 characterizing the diffusional mechanism. The use of Eq. (2) is validated by several authors
198 and it is generally used to understand the release mechanism of drugs and bioactives from
199 formulations from release data obtained in in vitro release experiments (Korsmeyer, et al.,
200 1983; Rinaki, et al., 2003; Siepmann, et al., 2002).

201 3 Results and discussion

202 In the first part of the experimentation, the amounts of alginate (ALG) and CaCl_2 were
203 varied to identify the minimum ratio between CaCl_2 and ALG required to obtain microparticles
204 formation. Both formulations (forming and non-forming particles) were used as matrices for
205 the encapsulation of nicotinamide. In vitro release behaviour of NIC from these materials was
206 then studied. Freeze-drying experiments on AFG were carried out to investigate the effect of
207 this method on fluid gel properties (particle size and viscosity) and to identify the conditions
208 needed to assure the complete removal of free water ($\text{NMC} < 0.1$ and $a_w < 0.6$) (Brown, et al.,

209 2010; Ratti, 2001; Stevenson, et al., 2015). The effect of CaCl₂ concentration in AFG on the
210 freeze drying kinetics was also investigated. Afterwards, a second set of release experiments
211 was performed on NFG to study the effect of the freeze drying process and rehydration on
212 the release behaviour of NIC from AFG.

213

214 3.1 Effect of ALG/CaCl₂ ratio

215 The amount of CaCl₂ needed to obtain the complete gelation of AFG was investigated
216 by producing several samples at fixed ALG concentration and changing the CaCl₂ one; the final
217 properties of the obtained materials in terms of PSD were then assessed. Samples
218 compositions used in this part of the work alongside their acronyms are reported in Table 1.

219 3.1.1 Particle size distribution

220 Particle dimensions were evaluated using the Mastersizer and Zetasizer as reported in
221 section 2.4 (Particle Size Distribution). PSD curves obtained using the Mastersizer and the
222 Zetasizer are reported in Figure 1.

223 Mastersizer and Zetasizer curves of AFG_2%_0.35% displayed a PSD in the range of
224 0.5 to 5 µm, while AFG_2%_0.15% and AFG_2%_0.25% were in the range between 0.02 µm
225 and 0.2 µm. The particle formation behaviour of samples was also studied using light
226 microscopy (as shown in Figure 1b).

227 Due to the small dimensions, particles in samples AFG_2%_0.25% and AFG_2%_0.15%
228 could not be recorded/visualised by optical microscopy analysis, while it can be noticed the
229 presence of microparticles for AFG_2%_0.35% (Figure 1b). Samples were also produced using
230 different percentages of ALG to identify the ratio between CaCl₂ and ALG necessary to ensure
231 particle formation. Samples compositions used are reported in Table 2.

232 Microparticles formation was observed for samples AFG_1%_0.25% and
233 AFG_3%_0.45%, while nanoparticles were detected for the samples AFG_1%_0.15% and
234 AFG_3%_0.35%. These results showed that microparticles formation was achieved only when
235 a critical ratio between ALG and CaCl₂ was used during sample preparation. This ratio can be
236 estimated to be around 0.155 of CaCl₂ to ALG (w/w) and from now on this ratio will be called
237 Critical Ratio for Microparticles Formation (CRMF). This value was estimated considering the
238 used percentages of CaCl₂ and ALG in samples that consisted of microparticles. However, even

239 in samples produced with a CaCl₂/ALG ratio lower than the CRMF, PSD curves showed the
240 presence of nanoparticles in the range of 100 nm and due to these very small dimensions they
241 could not be visualised using optical microscopy.. In accordance with results reported by
242 Badita et al., these nano-domains can be attributed to the alginate polymer chains
243 morphology. Additionally, the authors observed the formation of an alginate secondary
244 structure in the presence of Ca²⁺ ions, but only when a Ca²⁺ concentration higher than a critical
245 concentration was used. Nano-domains dimensions of AFG produced using a CaCl₂/ALG ratio
246 lower than the CRMF were in accordance with the values obtained by Badita et al. (Badita, et
247 al., 2016). In addition, He et al. (He, et al., 2016) reported the formation of “nuclei” of gelation
248 when CaCl₂/ALG ratio lower than a critical value was used. When a CaCl₂/ALG ratio lower than
249 the CRMF was used for the production of AFG, some cross-linking points were generated;
250 however, it appears that the Ca²⁺ concentration was not enough to crosslink all the α-L-
251 guluronate (G) monomers of ALG that are responsible for the formation of the egg-box model
252 (Braccini & Pérez, 2001). The non-crosslinked residues of polymer chains were able to interact
253 with other non-crosslinked chains, explaining the formation of an extended network all over
254 the whole material. Samples produced with a CaCl₂/ALG ratio higher than the CRMF were able
255 to fully form microparticles. The folding of ALG polymer chains on themselves is a process
256 induced by the applied shear regime. Ca²⁺ ions can “lock” ALG chains in position only when a
257 concentration high enough to achieve a full crosslinking is used. After the removal of the shear
258 regime, due to the full crosslinking of ALG, polymer chains were unable to unfold and interact
259 with other ALG chains, but stable microparticles could still be formed. Even when a CaCl₂/ALG
260 ratio lower than the CRMF was used, ALG folded on themselves, but due to the lack of a
261 complete crosslinking, they were able to unfold and interact with each other, obtain an
262 extended matrix, after the removal of the shear regime. In that case, only “pre-particles”
263 within an ALG matrix could be identified by PSD analyses. However, due to their small
264 dimensions they could not be visualised using the optical microscope.

265

266 3.1.2 Release behaviour

267 Nicotinamide loaded alginate fluid gels NFG-025 and NFG-035 were produced
268 respectively using a CaCl₂/ALG ratio lower (0.25% CaCl₂ concentration) and higher (0.35%
269 CaCl₂ concentration) than the CRMF; they were produced in the presence of NIC (0.10% w/w),

270 as described in the section 2.2.2 (Nicotinamide fluid gels). These two formulations were
271 chosen with a view to understand how the release behaviour of the incorporated active
272 compound is affected by the formed particle sizes. NIC was chosen as a model active because
273 of its small dimension and its low molecular weight (122.13 g/mol) to avoid its physical
274 entrapment into the gel-network, which would limit the diffusion into the release medium.
275 Additionally, NIC is a highly hydrophilic molecule and displays a very high water solubility
276 (>500 mg/mL at 25 °C), which is a suitable characteristic for the production of water-based
277 fluid gels (Budavari, 1996). Additionally, the release profiles of these materials were
278 compared to that of a NIC water solution and a NIC and ALG (2% w/w) water solution, both
279 containing 0.10% w/w of the active (National Center for Biotechnology Information. PubChem
280 Database. Nicotinamide), in order to fully understand if and how the CaCl₂ presence affects
281 release behaviour (Figure 2).

282 As depicted from Figure 2, the formation of microparticles did not prevent the
283 diffusion of NIC out of AFG. In fact, NFG-025 and NFG-035 presented the same release profile.
284 Comparing these two curves with the one of NIC water solution, it can be observed that both
285 AFG formulations were able to slightly slow down the release of NIC. This delay is obvious
286 even when comparing the NIC&ALG solution curve with the NIC water solution one; however,
287 the delay effect is enhanced in the presence of CaCl₂. It is possible to conclude that AFG are
288 able to slow down the diffusion of the loaded NIC and this effect is due to a combination of
289 the ALG and CaCl₂ presence. The delay ability can be attributed to the increase of viscosity of
290 the solutions as suggested by Secouard et al. (Secouard, et al., 2003), in a release experiments
291 of limonene from three different polysaccharide based water solutions and gel systems. They
292 suggested that materials viscosities contribute significantly in limonene retention with the
293 formulation and on its release behaviour.

294

295 3.2 Freeze drying

296 Freeze drying experiments were carried out to investigate the effect of processing
297 time and CaCl₂ concentration, used during material production step, on samples moisture
298 content and water activity. Rheological properties and particle size distributions were studied
299 on both non freeze-dried (AFG) and freeze-dried and rehydrated (FD-AFG) fluid gels

300 formulations. ALG concentration was fixed at 2% w/w and three different fluid gels were
301 prepared using CaCl₂ concentration of 0.15%, 0.25% and 0.35.

302

303 3.2.1 Moisture content and water activity

304 Normalised moisture content (NMC) and water activity (a_w) were measured for 48 h
305 at different time intervals during the freeze drying experiments. The values obtained
306 alongside the corresponding standard deviations are shown in Figure 3.

307 From Figure 3a, it is possible to observe that the first stage of drying (about 18 h) was
308 quite fast and characterised by a constant rate. Specifically, for AFG at this stage sublimation
309 of the ice formed from inter-particle water took place, with a drying rate of about 0.02 h⁻¹,
310 whereas for the alginate solution the process was faster (approx. 0.05 h⁻¹), likely due to the
311 absence of aggregates that act as a resistance to heat and mass transfer. As a result, AFG at
312 18h drying still showed a NMC around 0.4, while the solution was already dried. Thereafter,
313 for fluid gels a falling rate was observed up to 30 h, related to the intra-particle ice
314 sublimation. After 30 h, a zero drying rate with no substantial changes to moisture content
315 was recorded, i.e. all free water was removed from the samples. The experimental values
316 measured for a_w of the materials during drying, reported in Figure 3b, showed a slightly
317 different behaviour in the first stage. In fact, water activity remained nearly unchanged for 6h
318 in the case of the alginate solution and 18 h in the case of AFG and then gradually decreased
319 until achieving a constant value at 48 h. From these diagrams, it can be concluded that at least
320 48 h drying is needed for AFG to lower both a_w and NMC under the threshold limits (0.6 and
321 0.1, respectively (Brown, et al., 2010; de Bruijn, et al., 2016)) and, therefore, prevent microbial
322 growth.

323 The effect of fluid gel formation on alginate drying behaviour can be appreciated in
324 the first hours of drying (until 18 hours). In fact, for the sample not formed by a fluid gel (no
325 CaCl₂ added), the NMC decreased more rapidly if compared to materials in which some
326 amount of CaCl₂ was used. A similar trend can be identified from the water activity (a_w) curves;
327 especially at 18 hours of drying time big differences between samples can be seen. The slower
328 drying kinetics of AFG, when compared to an ALG solution in which no CaCl₂ was added, can
329 be due to the formation of a gel network in which water is entrapped between alginate
330 polymer chains leading to more time being required to remove water from that network.

331 Additionally, comparing the a_w and NMC curves obtained at different CaCl_2 concentrations for
332 AFG samples (Figure 3a and 3b), it can be seen that there is negligible difference among them,
333 suggesting that once the network is formed, this parameter does not have substantial effect
334 on the gel drying.

335

336 3.2.2 Rehydration performance

337 FD samples were rehydrated, following the procedure reported in section 2.9, to
338 assess the impact of the freezing/drying on the material properties of fluid gels. Rehydration
339 is one of the key parameters that quantify the quality of a dried product as it describes its
340 ability to reacquire the initial amount of water within its structure. Rehydration is a complex
341 phenomenon, in which different mechanisms take place: water absorption into the dried
342 product, diffusion through the porous network and swelling of the structure (Lopez-Quiroga,
343 et al., 2019; Maldonado, et al., 2010; Ratti, 2008). Rehydrated samples were characterised in
344 terms of viscosity, PSD and release behaviour and compared with unprocessed (non-freeze
345 dried) fluid gels.

346

347 3.2.3 Recovery of rheological properties

348 The rheological behaviour of rehydrated fluid gel samples having different CaCl_2
349 content was compared to that of systems prior to FD. Samples were rehydrated for 1 hour
350 before recording viscosity measurements. Viscosity curves for the different formulations are
351 presented in Figure 4.

352 It is possible to notice that for AFG_2%_0.15% and AFG_2%_0.25%, viscosity curves
353 before FD and after FD/rehydration are almost overlapping. It can be stated that freeze-dried
354 materials with a CaCl_2 concentration of 0.15% or 0.25% were able to fully recover the
355 rheological behaviour they had before freeze-drying. AFG_2%_0.15% and AFG_2%_0.25%
356 were produced using a 2% ALG concentration and, as shown in Figure 4, they were not able
357 to produce microparticles. The comparison between the viscosity curves of AFG_2%_0.35%
358 in the fresh and dried/rehydrated form showed more significant changes (Figure 6c). More
359 specifically, an overall decrease in the shear viscosity can be observed and, in particular, the
360 difference with the untreated material becomes more evident as shear rate increases. This
361 behaviour can be due to the inability of AFG_2%_0.35% to fully reabsorb the added water or

362 by its delay in doing that. Additional rehydration experiments were carried out on freeze-
363 dried AFG_2%_0.35%, increasing the rehydration time up to 48h, to assess if they were able
364 to recover the rheological properties they had before freeze drying by prolonging the time of
365 the rehydration step; however, the viscosity profile was not restored even after 2 days of
366 rehydration in the shaker incubator. This behaviour suggests that AFG produced using a
367 CaCl_2/ALG ratio higher than the CRPF, i.e. when microparticles formation is achieved, cannot
368 recover the rheological properties they had before FD. This set of experiments showed that
369 the rehydration of AFG, and the complete recovery of the rheological properties they had
370 before the FD, is achievable only when materials were prepared using a CaCl_2/ALG ratio lower
371 than CRPF. Below that ratio, polymer chains are not fully cross-linked leaving some empty
372 zones between them in the alginate gel formed. Because of the presence of less cross-linking
373 junctions, polymer chains present a higher mobility than polymer chains of fully cross-linked
374 materials (when a CaCl_2/ALG ratio higher than the CRMF was used). The rehydrated
375 AFG_2%_0.35% samples were not visually homogeneous and regions having higher particle
376 concentration and regions at higher water concentration could be identified. This higher
377 mobility may explain why viscosity profiles before and after FD completely match solely for
378 samples not completely cross-linked. In fact, the more mobile polymer chains are less rigid
379 and they can move more and faster to fit the water molecules between them. In contrast,
380 polymer chains having a lower mobility due to the presence of more cross-linking points, take
381 more time to fit all water molecules or just a fraction of the removed water molecules can be
382 reabsorbed.

383 In order to identify the time needed for AFG produced using a CaCl_2/ALG ratio lower
384 than the CRMF to recover the viscosity they had before FD, an additional rehydration
385 experiment was carried out on AFG_2%_0.25% using a rehydration time of 5 min and 10 min
386 in the shaking incubator before performing the rheological test. A comparison among the
387 resulting viscosity curves is reported in Figure 5.

388 From Figure 5 becomes evident that the complete recovery of viscosity was achieved
389 after approximately 10 min of rehydration time. It can be concluded that AFG produced using
390 a CaCl_2/ALG ratio lower than the CRMF are able to fully recover the rheological properties
391 they had before FD in a short time.

392

393 3.2.3 Particle Size distribution

394 The PSD of rehydrated samples of AFG_2%_0.15%, AFG_2%_0.25% and
395 AFG_2%_0.35% were compared to those of the samples before FD to identify if some particle
396 aggregation was induced by the drying process. Samples were rehydrated for 1 hour before
397 performing particle size measurements using the Mastersizer as described in section 2.4.1.
398 PSD curves are displayed in Figure 6.

399 The PSD curves after rehydration were perfectly overlapping with the curves obtained
400 from materials before FD. It is possible to conclude that AFG do not form aggregates during
401 the freeze-drying process and that they can retain their initial sizes.

402

403 3.2.4 Release behaviour of nicotinamide-loaded fluid gels

404 After NFG production, a fraction of both NFG_025 and NFG-035 was freeze-dried, as
405 described in section 2.6 (Freeze drying), until constant weight (48 h). Freeze dried samples
406 were then rehydrated for 1 hour and release of the active over time was studied, as described
407 in section 2.10 (In vitro release), to highlight the influence of FD on the release behaviour of
408 NIC. The NIC release profiles from FG before drying and following drying and rehydration are
409 depicted in Figure 7 for two different concentrations of the active:

410 As can be seen in Figure 7a-b, both FD and rehydration processes do not seem to affect
411 the release behaviour of NIC from AFG; in fact, release curves are almost overlapping. In order
412 to better understand the release behaviour of NIC a model fitting analysis was conducted:
413 data obtained from in vitro release studies of NFG formulations were used to fit a
414 mathematical model as described in section 2.11 Data fitting and the obtained parameters
415 are reported in Table 3:

416 As described by Rinaki et al. (Rinaki, et al., 2003), the n parameter obtained from data
417 fitting of eq. 2 describes the mechanism of drug release. As can be noticed from Table 3, this
418 value is almost constant for all in vitro experiments and it is in the range between 0.51-0.59.
419 As reported by the same authors and by Korsmeyer et al. (Korsmeyer, et al., 1983; Rinaki, et
420 al., 2003), n values in the range 0.4-0.65 are related to pure diffusion mechanism (anomalous
421 non-Fickian diffusion) of drug. In NFG the release of NIC can be related to its diffusion through
422 the ALG matrix; this is very similar to the diffusion of NIC from a water solution or from a
423 water and alginate solution. This supports the theory that no drug-matrices interactions were

424 formed between NIC and AFG. In a previous reported study, the interaction between
425 tryptophan and AFG were recorded (Smaniotto, et al., 2019). However, in that case the
426 amount of drug detected during in vitro release studies was not equal to the overall amount
427 of drug introduced in AFG and the amount released was also affected by storage time,
428 suggesting a possible interaction between tryptophan and alginate polymer chains, as
429 reported in literature by Yang et al. (Yang, et al., 2015). As described by Korsmeyer et al.
430 (Korsmeyer, et al., 1983), the kinetic constant k is characteristic of each drug-matrix system.
431 As can be seen in Table 3, k values of NFG-0.35 are substantially different between the
432 “untreated” material and the one after the drying and rehydration processes. As reported
433 above (see section 3.2.2 Recovery of rheological properties), AFG made using a CaCl_2/ALG
434 ratio higher than the CRMF, as in the case of NFG-0.35, were not able to be completely
435 rehydrated and their rheological properties were affected by the drying and rehydration
436 steps. Changes in k values of NFG-0.35 confirm that AFG made using a CaCl_2/ALG ratio higher
437 than the CRMF cannot be completely rehydrated. This is not true for AFG made using a
438 CaCl_2/ALG ratio lower than the CRMF, as the k values of NFG-0.25 is very similar to the one of
439 the same sample submitted to freeze drying and rehydration processes, confirming that a
440 complete rehydration of the matrix can be achieved, as showed in section 3.2.2 Recovery of
441 rheological properties.

442 It is possible to conclude that AFG are able to slow down the diffusion of the loaded NIC
443 even after FD. Additionally, freeze drying is a suitable technique for preserving AFG by
444 decreasing their moisture content and, consequently, their water activity, preventing the
445 bacterial growth. Rehydration can restore the release behaviour they had before freeze
446 drying.

447 4. Conclusions

448 In this work, the suitability of freeze drying as preservation method for alginate fluid gels was
449 investigated. The effect of particle dimensions on drying kinetics and rehydration behaviour
450 of these materials was studied. It was demonstrated that the drying and rehydration
451 processes do not affect the particle size distribution. However, the rheological properties of
452 AFG can be completely recovered only when materials were made using a CaCl_2/ALG ratio
453 that allowed the formation of nanoparticles. Preliminary encapsulation experiments of NIC
454 in AFG were also carried out, showing that the release behaviour of this active from AFG was

455 not modified by the drying/rehydration processes when AFG were composed of
456 nanoparticles. This showed to be the most suitable system to deliver the active compound.
457 On the other hand, data fitting models of in vitro release experiments showed some changes
458 in the release behaviour of NIC after freeze drying and rehydration of AFG made of
459 microparticles. This is probably due to the fact that microparticles-based AFG cannot be fully
460 rehydrated. More experiments should be conducted in the future, loading other types of
461 actives in AFG to investigate their usage as materials for the controlled release overtime of
462 actives. These results can be relevant from both a scientific and industrial point of view, since
463 they lead to a better understanding of the AFG behaviour and suggest that FD can be applied
464 to extend their shelf life, without any compromise in the material properties.

465

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469

470

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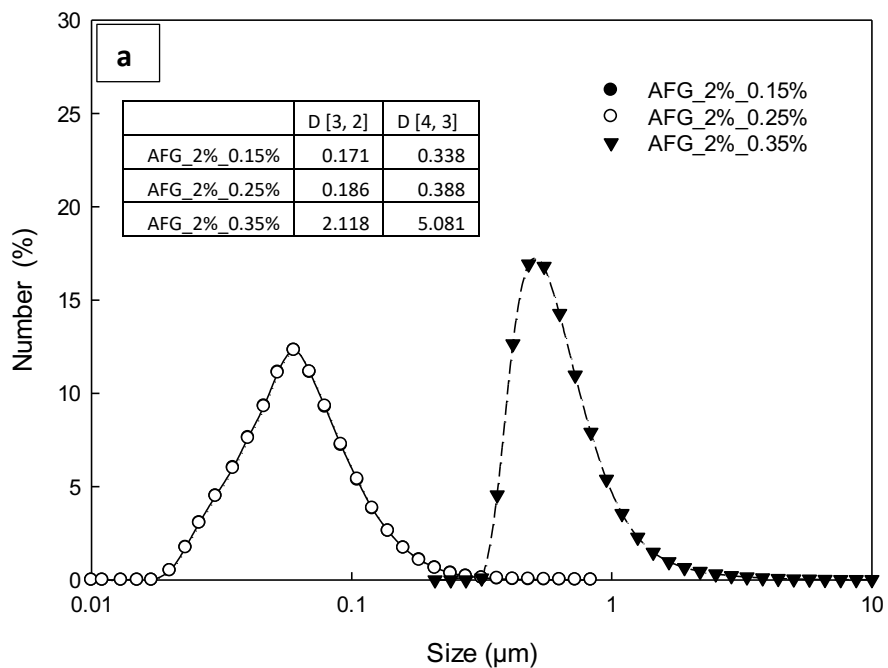
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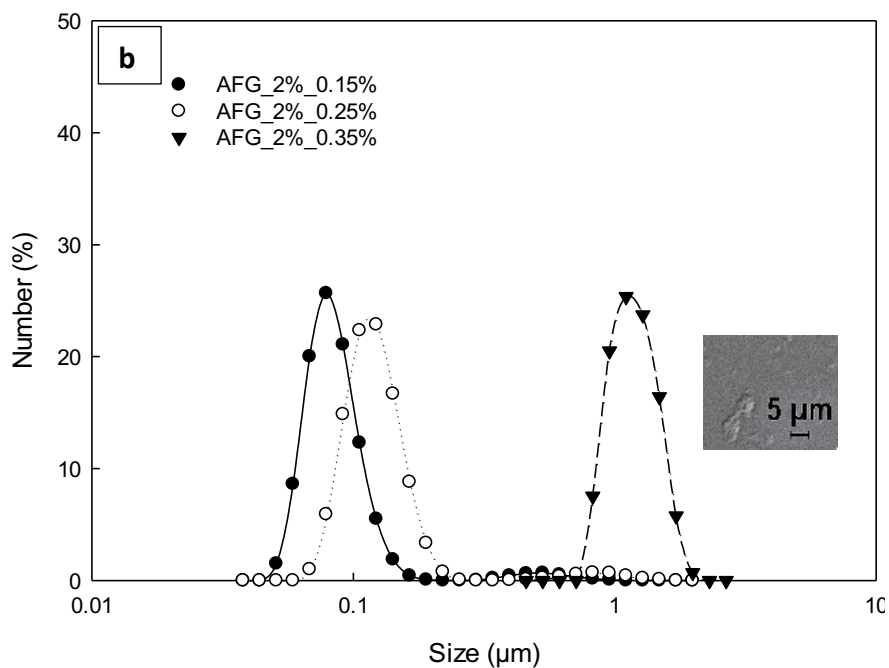
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Figure 1- PSD curves of AFG produced by using 2% ALG (w/w): (a) Mastersizer, (b) Zetasizer.

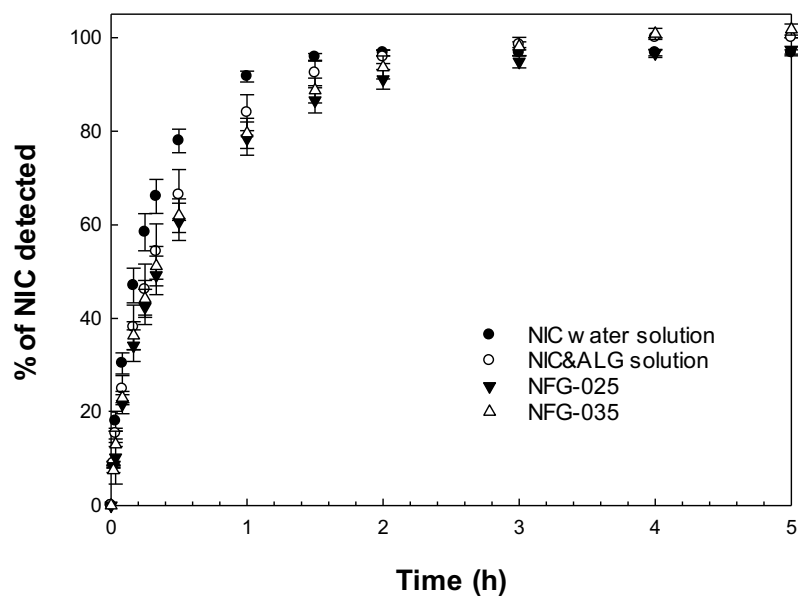
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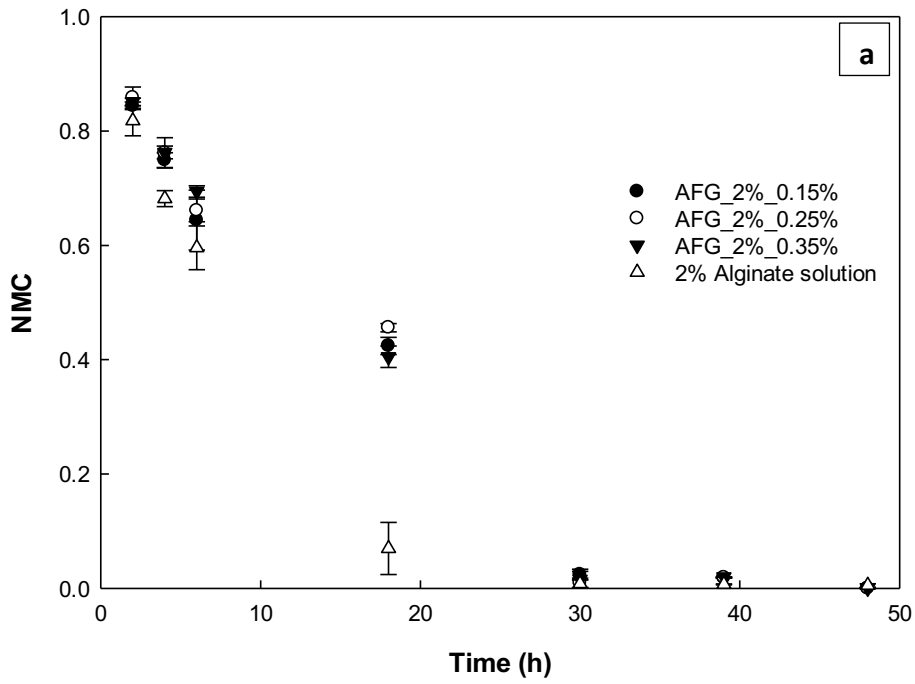


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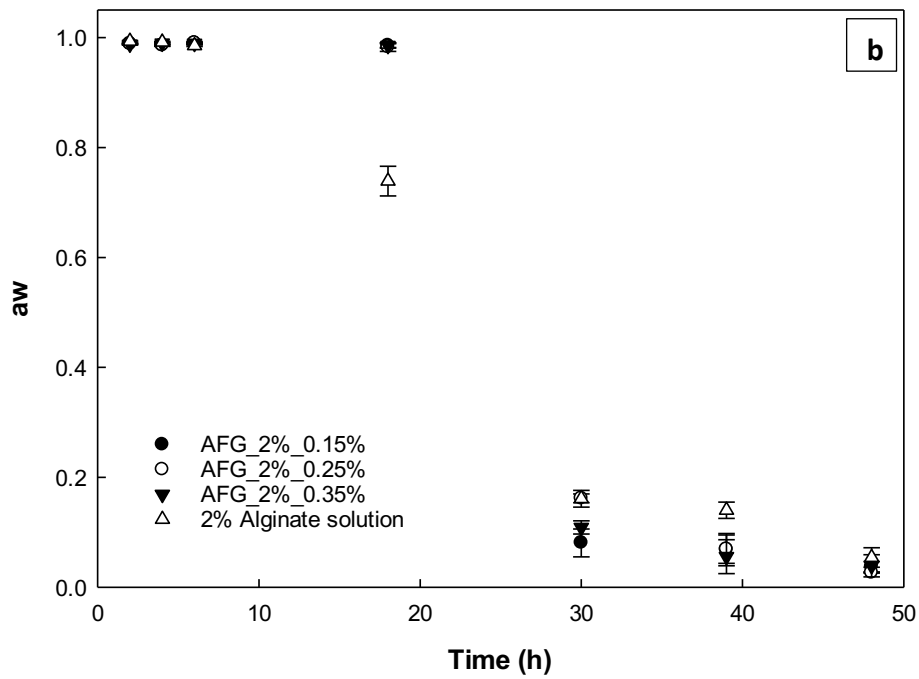
623 Figure 2- In vitro release profile of nicotinamide (National Center for Biotechnology Information. PubChem
624 Database. Nicotinamide) from NIC in aqueous medium, NIC&ALG solution, and NIC-loaded alginate fluid gels
625 produced using different concentrations of CaCl_2 (NFG-025 and NFG-035).

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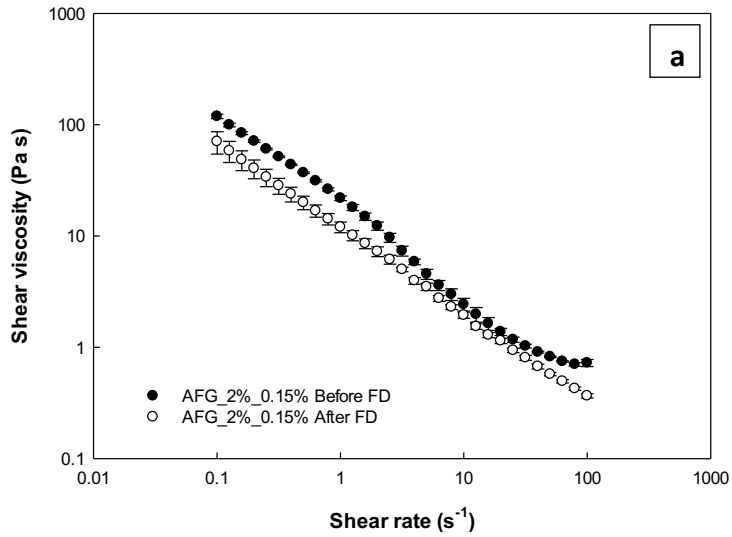
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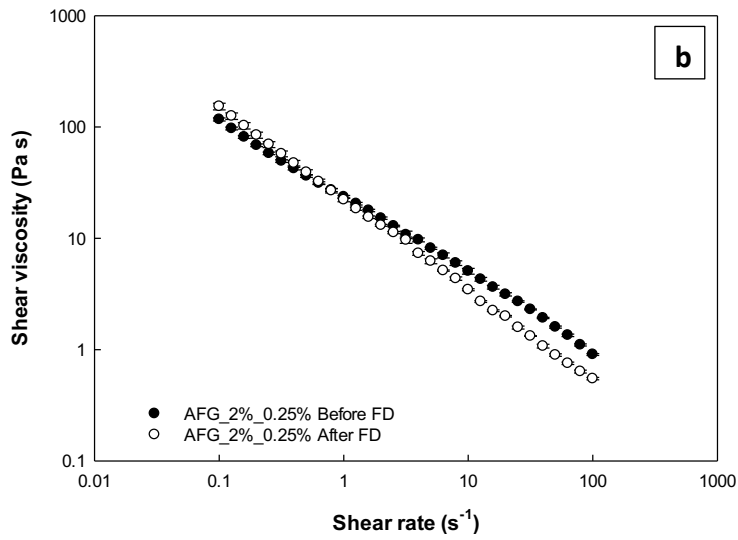
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630 *Figure 3- Drying kinetics of fluid gels at 2% ALG (w/w) and different CaCl₂ concentrations: (a) Normalised*
 631 *Moisture Content; (b) water activity.*

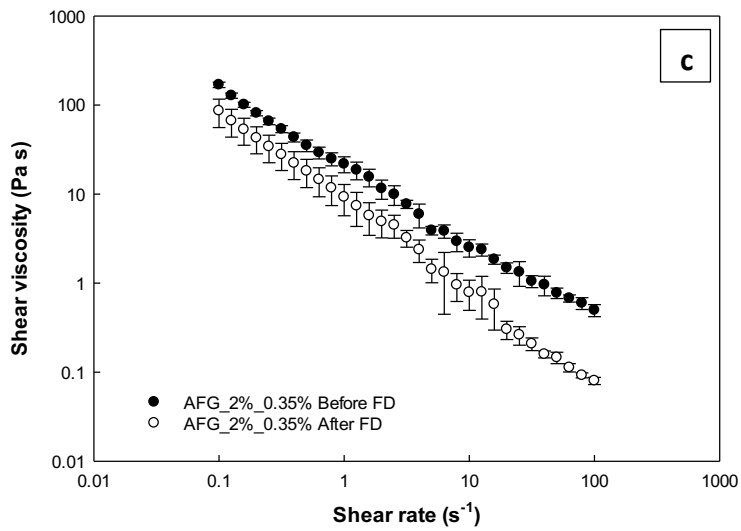
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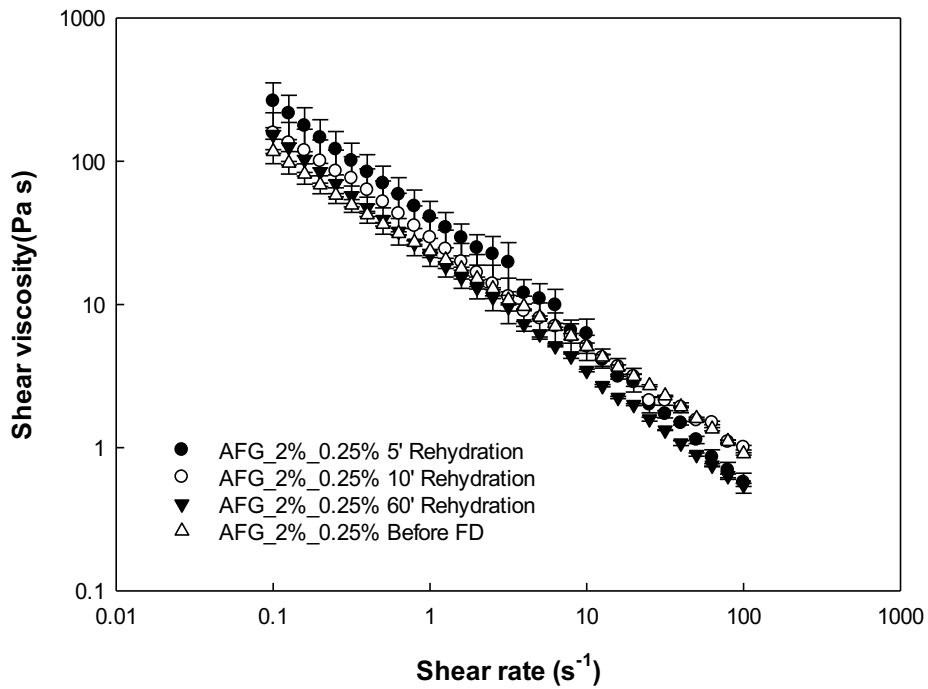
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636 *Figure 4: Shear viscosity curves of blank AFG before freeze drying and after drying/rehydration: (a) 0.15% CaCl₂;*
 637 *(b) 0.25% CaCl₂; (c) 0.35% CaCl₂.*

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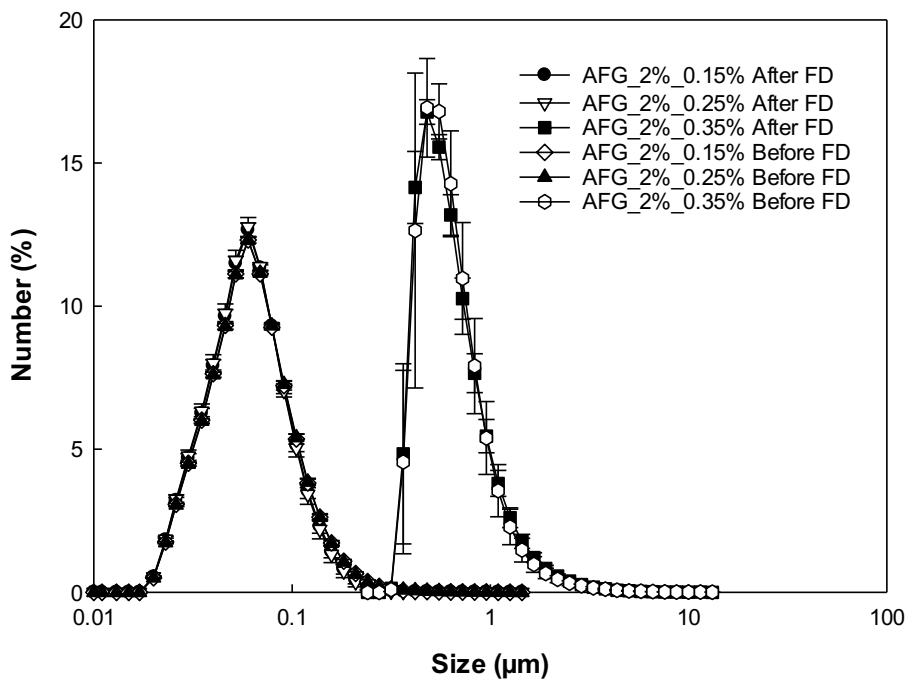
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Figure 5- Shear viscosity curves of AFG-025 as a function of the rehydration time

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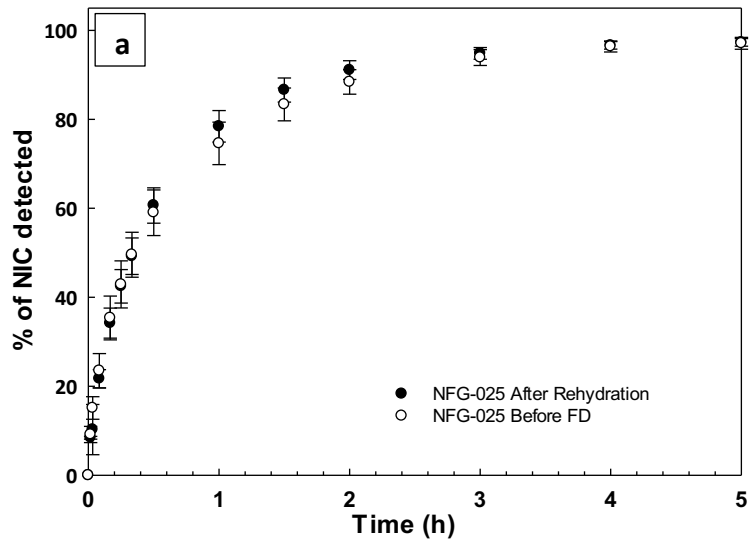
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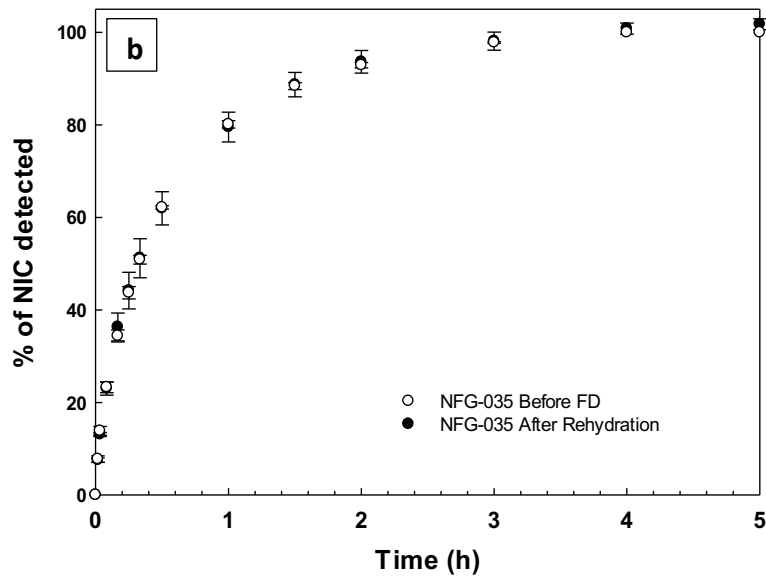
Figure 6- PSD curves of blank (no active) AFG before freeze drying and after drying and rehydration

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649 Figure 7- In vitro release profiles of NIC from forming nanoparticles AFG (a) and forming microparticles AFG (b)
 650 before freeze drying and after drying and rehydration.

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Table 1-ALG and CaCl₂ concentrations used for sample preparations

Sample	Alginate concentration (w/w)	CaCl ₂ concentration (w/w)
AFG_2%_0.15%	2%	0.15%
AFG_2%_0.25%	2%	0.25%
AFG_2%_0.35%	2%	0.35%

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Table 2- ALG and CaCl₂ concentrations used for sample preparations

Sample	Alginate concentration (w/w)	CaCl ₂ concentration (w/w)
AFG_1%_0.15%	1%	0.15%
AFG_1%_0.25%	1%	0.25%
AFG_3%_0.35%	3%	0.35%
AFG_3%_0.45%	3%	0.45%

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Table 3 – k, n, R², parameters obtained from eq. 2 data fitting

	NFG-0.25	NFG-0.25 after rehydration	NFG-0.35	NFG-0.35 after rehydration	NIC Solution	NIC&ALG
k	0.9526 (± 0.0835)	0.8835 (± 0.0518)	0.9187 (± 0.0785)	0.3911 (± 0.0504)	1.3440 (± 0.1925)	0.9814 (± 0.0488)
n	0.5826 (± 0.0654)	0.5118 (± 0.0413)	0.5578 (± 0.0625)	0.5600 (± 0.0396)	0.5851 (± 0.0887)	0.5464 (± 0.0361)
R²	0.995	0.997	0.995	0.998	0.994	0.998

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