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DOI: 10.1016/j.foodhyd.2019.105352

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Document Version Peer reviewed version

Citation for published version (Harvard):

Smaniotto, F, Prosapio, V, Zafeiri, I & Spyropoulos, F 2020, 'Freeze drying and rehydration of alginate fluid gels', Food Hydrocolloids, vol. 99, 105352. https://doi.org/10.1016/j.foodhyd.2019.105352

Link to publication on Research at Birmingham portal

#### Publisher Rights Statement:

Smaniotto, F. et al (2019) Freeze drying and rehydration of alginate fluid gels, Food Hydrocolloids, article no. 105352, https://doi.org/10.1016/j.foodhyd.2019.105352

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# **1** Freeze drying and rehydration of alginate fluid gels

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# 6 Abstract

7 The aim of this work was to study the effect of freeze drying (FD) and rehydration on 8 alginate fluid gels (AFG). FD was employed as a widely used drying method in order to 9 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 10 11 restored after drying and rehydration processes. First, it was investigated the influence of 12 the material formulation (alginate (ALG) and calcium chloride (CaCl<sub>2</sub>) concentrations) on the 13 particle size distribution (PSD) and the rheological properties. The used ALG/CaCl<sub>2</sub> ratio 14 demonstrated to be responsible for forming nanoparticles or microparticles. The impact of 15 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 16 17 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<sub>2</sub> in 18 19 quantities leading to nanoparticles formation. Preliminary encapsulation experiments were 20 also carried out to highlight the impact of this process on the release behaviour of the 21 loaded active from AFG. Results obtained from in vitro release studies and their model 22 fitting showed that the active release behaviour from AFG was not affected by freeze drying 23 when AFG was formed of nanoparticles. On the contrary, the active release behaviour was 24 affected by freeze drying when AFG was formed by microparticles.

25 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., 2014; Le Révérend, et al., 2010), texturing (Fernández Farrés, et al., 2014), satiety enhancers 40 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 provided. 48

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<sub>w</sub>) 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 poor recovery of the initial properties (Vega-Gálvez, et al., 2015). Among the most common 58 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 (Karam, et al., 2016; Prosapio & Norton, 2017a). Specifically, for the dehydration of 61 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.

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

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

94

95

# 2. Materials and methods

**96** 2.1 Materials

Sodium alginate (ALG), and nicotinamide (NIC, ≥98%, HPLC) were purchased from
Sigma-Aldrich<sup>®</sup> (Sigma–Aldrich Company Ltd., Dorset, UK). Calcium chloride (CaCl<sub>2</sub>,
anhydrous, 93%) was purchased from Alfa Aesar<sup>™</sup> (USA). All materials were used without
further purification. Milli-Q water was made using an Elix<sup>®</sup> 5 distillation apparatus (Millipore<sup>®</sup>,
USA) and was used for all water-based preparations.

102

**103** 2.2 Gel preparation

**104** 2.2.1 Blank fluid gels

Firstly, an alginate solution was prepared by dissolving different amounts of ALG (1%, 105 106 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<sub>2</sub> 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 16 pins fixed on the internal wall. 1000 rpm shaft speed was used for AFG production. The 113 114 alginate solution was pumped using a flowrate of 33 mL/min with a peristaltic pump 115 (Masterflex L/S Peristaltic, DE), while the CaCl<sub>2</sub> 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

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

126

127 2.3 Rheological properties

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

132

133 2.4 Particle Size Distribution

134 2.4.1 Mastersizer

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

140 2.4.2 Zetasizer

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

145 2.5 Optical microscopy

Optical microscope (DM 2500 LED, Leica<sup>®</sup>, CH) was used to observe and record alginate microparticles. Samples were, firstly, diluted with distilled water using a water to sample ratio of 10:1 and they were mixed using a vortex mixer (VM20, Rigal Bennet<sup>®</sup>, UK) for 20 seconds. DIC settings were employed to increase the contrast, allowing the average dimensions of particles to be measured. Images were captured using a CCD camera (DFC450 151 C, Leica<sup>®</sup>, CH) coupled to the microscope. Image analysis software IPP (LAS 4.8.0, Leica<sup>®</sup>, CH)

152 was used to evaluate the dimensions of the microparticles through the images.

153

# **154** 2.6 Freeze drying

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

$$NMC = \frac{(Md - Ms)}{(Mo - Ms)} \tag{1}$$

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

168

169 2.8 Water activity analysis

Water activity (a<sub>w</sub>) of dried samples was measured using an AquaLab<sup>®</sup> dew point water activity meter (model 4TE, Decagon Devices Inc., Pullman, WA, USA). The temperature controlled sample chamber was set to 25 °C. Analyses were carried out in triplicate.

**173** 2.9 Rehydration of samples

After dehydration, amounts of distilled water were placed in each sample in order to obtain the same sample weight before FD. Samples were then transferred in a shacking incubator (Incu-Shake MIDI, SciQuip, UK), in order to obtain a homogeneous rehydration of 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 (approximatively 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 temperature (thermostated at 21.5 °C), under stirring at 150 rpm. At regular intervals, 185 186 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 wavelength (NIC maximum of absorbance) and was set for concentrations between 0.08 and 188 45 µg/mL and was used to correlate the absorbance value to the actual concentration of NIC 189 190 into the release medium. Each analysis was carried out in triplicate.

#### **191** 2.11 Data fitting

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

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

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

### 201 3 Results and discussion

In the first part of the experimentation, the amounts of alginate (ALG) and CaCl<sub>2</sub> were varied to identify the minimum ratio between CaCl<sub>2</sub> and ALG required to obtain microparticles formation. Both formulations (forming and non-forming particles) were used as matrices for the encapsulation of nicotinamide. In vitro release behaviour of NIC from these materials was then studied. Freeze-drying experiments on AFG were carried out to investigate the effect of this method on fluid gel properties (particle size and viscosity) and to identify the conditions needed to assure the complete removal of free water (NMC < 0.1 and  $a_w < 0.6$ ) (Brown, et al.,

209 2010; Ratti, 2001; Stevenson, et al., 2015). The effect of CaCl<sub>2</sub> 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/CaCl<sub>2</sub> ratio

The amount of CaCl<sub>2</sub> needed to obtain the complete gelation of AFG was investigated by producing several samples at fixed ALG concentration and changing the CaCl<sub>2</sub> one; the final properties of the obtained materials in terms of PSD were then assessed. Samples compositions used in this part of the work alongside their acronyms are reported in Table 1.

**219** 3.1.1 Particle size distribution

Particle dimensions were evaluated using the Mastersizer and Zetasizer as reported in
 section 2.4 (Particle Size Distribution). PSD curves obtained using the Mastersizer and the
 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  $\mu$ m, while AFG\_2%\_0.15% and AFG\_2%\_0.25% were in the range between 0.02  $\mu$ m 225 and 0.2  $\mu$ m. 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 CaCl<sub>2</sub> and ALG necessary to ensure 231 particle formation. Samples compositions used are reported in Table 2.

Microparticles formation was observed for samples AFG\_1%\_0.25% and AFG\_3%\_0.45%, while nanoparticles were detected for the samples AFG\_1%\_0.15% and AFG\_3%\_0.35%. These results showed that microparticles formation was achieved only when a critical ratio between ALG and CaCl<sub>2</sub> was used during sample preparation. This ratio can be estimated to be around 0.155 of CaCl<sub>2</sub> to ALG (w/w) and from now on this ratio will be called Critical Ratio for Microparticles Formation (CRMF). This value was estimated considering the used percentages of CaCl<sub>2</sub> and ALG in samples that consisted of microparticles. However, even

239 in samples produced with a CaCl<sub>2</sub>/ALG ratio lower than the CRMF, PSD curves showed the presence of nanoparticles in the range of 100 nm and due to these very small dimensions they 240 241 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 morphology. Additionally, the authors observed the formation of an alginate secondary 243 structure in the presence of Ca<sup>2+</sup> ions, but only when a Ca<sup>2+</sup> concentration higher than a critical 244 245 concentration was used. Nano-domains dimensions of AFG produced using a CaCl<sub>2</sub>/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 CaCl<sub>2</sub>/ALG ratio lower than a critical value was used. When a CaCl<sub>2</sub>/ALG ratio lower than 249 the CRMF was used for the production of AFG, some cross-linking points were generated; however, it appears that the Ca<sup>2+</sup> concentration was not enough to crosslink all the  $\alpha$ -L-250 251 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 CaCl<sub>2</sub>/ALG ratio higher than the CRMF were able 255 to fully form microparticles. The folding of ALG polymer chains on themselves is a process induced by the applied shear regime. Ca<sup>2+</sup> ions can "lock" ALG chains in position only when a 256 257 concentration high enough to achieve a full crosslinking is used. After the removal of the shear regime, due to the full crosslinking of ALG, polymer chains were unable to unfold and interact 258 259 with other ALG chains, but stable microparticles could still be formed. Even when a CaCl<sub>2</sub>/ALG 260 ratio lower than the CRMF was used, ALG folded on themselves, but due to the lack of a complete crosslinking, they were able to unfold and interact with each other, obtain an 261 262 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 CaCl<sub>2</sub>/ALG ratio lower (0.25% CaCl<sub>2</sub> concentration) and higher (0.35% 269 CaCl<sub>2</sub> 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 chosen with a view to understand how the release behaviour of the incorporated active 271 272 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 CaCl<sub>2</sub> 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. Comparing these two curves with the one of NIC water solution, it can be observed that both 284 285 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 CaCl<sub>2</sub>. 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 CaCl<sub>2</sub> 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 formulation and on its release behaviour. 293

294

# **295** 3.2 Freeze drying

Freeze drying experiments were carried out to investigate the effect of processing time and CaCl<sub>2</sub> concentration, used during material production step, on samples moisture content and water activity. Rheological properties and particle size distributions were studied on both non freeze-dried (AFG) and freeze-dried and rehydrated (FD-AFG) fluid gels

formulations. ALG concentration was fixed at 2% w/w and three different fluid gels were
 prepared using CaCl<sub>2</sub> concentration of 0.15%, 0.25% and 0.35.

302

**303** 3.2.1 Moisture content and water activity

Normalised moisture content (NMC) and water activity (a<sub>w</sub>) were measured for 48 h at different time intervals during the freeze drying experiments. The values obtained 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  $h^{-1}$ , whereas for the alginate solution the process was faster (approx. 0.05 h<sup>-1</sup>), likely due to the 310 311 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 sublimation. After 30 h, a zero drying rate with no substantial changes to moisture content 314 315 was recorded, i.e. all free water was removed from the samples. The experimental values measured for a<sub>w</sub> of the materials during drying, reported in Figure 3b, showed a slightly 316 317 different behaviour in the first stage. In fact, water activity remained nearly unchanged for 6h in the case of the alginate solution and 18 h in the case of AFG and then gradually decreased 318 319 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<sub>w</sub> 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 CaCl<sub>2</sub> added), the NMC decreased more rapidly if compared to materials in which some 325 326 amount of CaCl<sub>2</sub> was used. A similar trend can be identified from the water activity (a<sub>w</sub>) curves; 327 especially at 18 hours of drying time big differences between samples can be seen. The slower drying kinetics of AFG, when compared to an ALG solution in which no CaCl<sub>2</sub> was added, can 328 329 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.

Additionally, comparing the a<sub>w</sub> and NMC curves obtained at different CaCl<sub>2</sub> concentrations for
AFG samples (Figure 3a and 3b), it can be seen that there is negligible difference among them,
suggesting that once the network is formed, this parameter does not have substantial effect
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, et al., 2019; Maldonado, et al., 2010; Ratti, 2008). Rehydrated samples were characterised in 343 344 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

The rheological behaviour of rehydrated fluid gel samples having different CaCl<sub>2</sub> content was compared to that of systems prior to FD. Samples were rehydrated for 1 hour before recording viscosity measurements. Viscosity curves for the different formulations are 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<sub>2</sub> 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<sub>2</sub>/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<sub>2</sub>/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<sub>2</sub>/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.

In order to identify the time needed for AFG produced using a CaCl<sub>2</sub>/ALG ratio lower than the CRMF to recover the viscosity they had before FD, an additional rehydration experiment was carried out on AFG\_2%\_0.25% using a rehydration time of 5 min and 10 min in the shacking incubator before performing the rheological test. A comparison among the 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<sub>2</sub>/ALG ratio lower than the CRMF are able to fully recover the rheological properties 391 they had before FD in a short time.

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### **393** 3.2.3 Particle Size distribution

The PSD of rehydrated samples of AFG\_2%\_0.15%, AFG\_2%\_0.25% and AFG\_2%\_0.35% were compared to those of the samples before FD to identify if some particle aggregation was induced by the drying process. Samples were rehydrated for 1 hour before performing particle size measurements using the Mastersizer as described in section 2.4.1. PSD curves are displayed in Figure 6.

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

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403 3.2.4 Release behaviour of nicotinamide-loaded fluid gels

After NFG production, a fraction of both NFG\_025 and NFG-035 was freeze-dried, as described in section 2.6 (Freeze drying), until constant weight (48 h). Freeze dried samples were then rehydrated for 1 hour and release of the active over time was studied, as described in section 2.10 (In vitro release), to highlight the influence of FD on the release behaviour of NIC. The NIC release profiles from FG before drying and following drying and rehydration are depicted in Figure 7 for two different concentrations of the active:

As can be seen in Figure 7a-b, both FD and rehydration processes do not seem to affect the release behaviour of NIC from AFG; in fact, release curves are almost overlapping. In order to better understand the release behaviour of NIC a model fitting analysis was conducted: data obtained from in vitro release studies of NFG formulations were used to fit a mathematical model as described in section 2.11 Data fitting and the obtained parameters 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<sub>2</sub>/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<sub>2</sub>/ALG ratio higher 437 than the CRMF cannot be completely rehydrated. This is not true for AFG made using a 438 CaCl<sub>2</sub>/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.

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

## 447 4. Conclusions

In this work, the suitability of freeze drying as preservation method for alginate fluid gels was investigated. The effect of particle dimensions on drying kinetics and rehydration behaviour of these materials was studied. It was demonstrated that the drying and rehydration processes do not affect the particle size distribution. However, the rheological properties of AFG can be completely recovered only when materials where made using a CaCl<sub>2</sub>/ALG ratio that allowed the formation of nanoparticles. Preliminary encapsulation experiments of NIC 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 nanoparticles. This showed to be the most suitable system to deliver the active compound. 456 457 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 microparticles. This is probably due to the fact that microparticles-based AFG cannot be fully 459 460 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 actives. These results can be relevant from both a scientific and industrial point of view, since 462 they lead to a better understanding of the AFG behaviour and suggest that FD can be applied 463 464 to extend their shelf life, without any compromise in the material properties.

465

# 466 Acknowledgement

467 This research was funded by the Engineering and Physical Sciences Research Council

468 [grant number EP/K030957/1], the EPSRC Centre for Innovative Manufacturing in Food.

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





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



Figure 3- Drying kinetics of fluid gels at 2% ALG (w/w) and different CaCl<sub>2</sub> concentrations: (a) Normalised
 Moisture Content; (b) water activity.







Figure 4: Shear viscosity curves of blank AFG before freeze drying and after drying/rehydration: (a) 0.15% CaCl<sub>2</sub>;
(b) 0.25% CaCl<sub>2</sub>; (c) 0.35% CaCl<sub>2</sub>.



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





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



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

Table 1-ALG and  $CaCl_2$  concentrations used for sample preparations

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

Table 2- ALG and  $CaCl_2$  concentrations used for sample preparations

Sample	Alginate concentration (w/w)	CaCl <sub>2</sub> 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%

659 Table 3 - k, n,  $R^2$ , parameters obtained from eq. 2 data fitting

		NFG-025 after		NFG-0.35 after		
	NFG-0.25	rehydration	NFG-0.35	rehydration	<b>NIC Solution</b>	NIC&ALG
	0.9526	0.8835	0.9187	0.3911	1.3440	0.9814
k	(± 0.0835)	(± 0.0518)	(± 0.0785)	(± 0.0504)	(± 0.1925)	(± 0.0488)
	0.5826	0.5118	0.5578	0.5600	0.5851	0.5464
n	(± 0.0654)	(± 0.0413)	(± 0.0625)	(± 0.0396)	(± 0.0887)	(± 0.0361)
R <sup>2</sup>	0.995	0.997	0.995	0.998	0.994	0.998