

1 Development of an immobilization system for in situ micronutrients release

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3 *Philippe E. Ramos<sup>a\*</sup>*

4 *Miguel A. Cerqueira<sup>b</sup>*

5 *Michael T. Cook<sup>cd</sup>*

6 *Ana I. Bourbon<sup>a</sup>*

7 *Vitaliy V. Khutoryanskiy<sup>d</sup>*

8 *Dimitris Charalampoulos<sup>e</sup>*

9 *José A. Teixeira<sup>a</sup>*

10 *António A. Vicente<sup>a</sup>*

11

12 *<sup>a</sup>CEB - Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-*  
13 *057 Braga, Portugal*

14 *<sup>b</sup>INL - International Iberian Nanotechnology Laboratory, Av. Mestre José Veiga s/n, 4715-*  
15 *330 Braga Portugal*

16 *<sup>c</sup> School of Pharmacy, University of Hertfordshire, Hatfield, AL10 9AB, UK*

17 *<sup>d</sup> School of Pharmacy, University of Reading, Reading, RG6 6AD, UK*

18 *<sup>e</sup> Food and Nutritional Sciences, University of Reading, Reading, RG6 6AD, UK*

19 *\*Corresponding author. Tel.: +351253601962, fax: +351 253 678 986*

20 *e-mail address: phramos@ceb.uminho.pt (P.E. Ramos).*

21

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28

29 **Abstract**

30

31 An immobilization system constituted by coated microcapsules was developed aiming at  
32 immobilizing probiotic bacteria capable of producing folate and release it in a sustained  
33 manner into the intestine. Despite no probiotic folate-producers have been immobilized so  
34 far, the system has been developed with this goal and this work reports its stability and ability  
35 to release folate under gastro-intestinal conditions.

36 Microcapsules were made of alginate with three consecutive coatings of poly-L-lysine,  
37 sodium alginate and chitosan. Turbidity experiments showed a strong electrostatic interaction  
38 between these polymers. Fourier transform infrared spectroscopy (FTIR) and confocal  
39 analysis showed the stability of the coating materials when applied on the microcapsules,  
40 even after they were immersed in solutions simulating conditions in the stomach and small  
41 intestine (i.e. pH 2, 60 min and pH 7.2, 120 min, respectively). Coated microcapsules have  
42 an average diameter size ranged from 20 and 40  $\mu\text{m}$ , and swelled upon exposure to a neutral  
43 medium, without dissolution as showed by microscopy analyses. Release experiments proved  
44 the ability of the coated microcapsules to release folic acid, at different rates, depending on  
45 the applied coating. Release experiments showed that the first coating ( $\epsilon$ -PLL) is  
46 characterized by Fickian diffusion as the main release mechanism of folic acid. Fickian rate  
47 constant ( $k_F$ ) decreased with the number of consequent coatings, reflecting the decrease of  
48 predominance of Fick's behavior. Results showed that the developed coated microcapsules  
49 have suitable characteristics for encapsulation of folic acid aiming *in situ* release in the  
50 intestine.

51

## 52 1. Introduction

53

54 Folate is a vitamin that occurs in a large number of forms, being all derived from folic acid  
55 (pteroylglutamic acid). However there are different forms of folate, being the most known  
56 folic acid and 5-methyltetrahydrofolic acid (Belz & Nau, 1998). Folate is part of some  
57 important metabolic pathways, such as methyl group biogenesis and synthesis of nucleotides,  
58 vitamins and amino acids (Jacob, 2000). According to the World Health Organization, folate  
59 deficiency is often associated with megaloblastic anemia, risk of low birthweight and  
60 placental abruption, risk of delivering preterm or small-for-gestational-age infants, risk of  
61 neural tube defects (NTD), depression or even dementia (de Benoist, 2008). The increased

62 cancer risk is also reported in other studies (Stolzenberg-solomon *et al.*, 2006). Folate  
63 fortification in food to maintain recommended daily intake (350 µg for adults and 600 µg for  
64 pregnant women) has been used using the synthetic form of B9 vitamin, folic acid (European  
65 Food Safety Authority, 2014). However, folic acid has low bioavailability after food  
66 processing, storage and consumption, due to inefficient absorption (de Meer *et al.*, 2005).  
67 More than that, folic acid has the capacity to mask, in an initial phase, vitamin B12 deficiency  
68 (Bailey & Ayling, 2009; Morris & Tangney, 2007), while also changing the activity of the  
69 hepatic dihydrofolate reductase enzyme (Bailey & Ayling, 2009) and promoting cancer  
70 (Baggott *et al.*, 2012; Ulrich & Potter, 2006). Considering all these reasons, food fortification  
71 by a natural folate form is highly recommended.

72 Some probiotic bacteria, such as *Lactococcus lactis cremoris*, *Streptococcus thermophilus*,  
73 *Bifidobacterium lactis*, *B. breve*, *B. infantis* and *B. animalis* are capable of producing large  
74 amounts of folate (Crittenden *et al.*, 2003; Sybesma *et al.*, 2003). However, several factors  
75 limit probiotics' action in the human body, such as very weak resistance to gastro-intestinal  
76 conditions (Gueimonde & Salminen, 2006) and low residence time in the intestine (Gardiner  
77 *et al.*, 2004; Klingberg & Budde, 2006). A possible solution to these problems mentioned  
78 above could be the encapsulation of folate-producers probiotics. Microcapsules are able to  
79 protect probiotics against high oxygen levels (Sunohara *et al.* 1995), food products (Tripathi  
80 & Giri 2014), freezing (Azizi *et al.* 2010; Sousa *et al.* 2013), and during the passage through  
81 the gastrointestinal tract (Sun & Griffiths 2000). Other limitation could be the direct contact  
82 of these bacteria with human gut, following reports mentioning concerns about the probiotics  
83 utilization in humans, such as: possible passage from the digestive tract to extra-intestinal  
84 sites, leading to infections (Butel, 2014), and an excessive immune stimulation by a direct  
85 contact of probiotics with the gut, that creates continuous immunological responses by  
86 human organism (Marteau & Shanahan, 2003). However, a full and continuous encapsulation  
87 during the passage through the gastrointestinal system can provide other advantages such as  
88 the prevention of the interfacial activation, stimulation of production and excretion of  
89 secondary metabolites (Nazzaro *et al.* 2012).

90 Alginate is the most applied material in microcapsules formation due to its low price, ease of  
91 gelation and biocompatibility (Chen *et al.*, 2012; Klein *et al.*, 1983; Quong *et al.*, 1998;  
92 Smidsrd & Skjak-Brae, 1990; Tanaka & Matsumura, 1983). Alginate is a polysaccharide

93 extracted from brown algae and is composed of randomly 1–4 linked  $\beta$ -D-mannuronic acid  
94 and  $\alpha$ -L-guluronic acid, M blocks and G blocks, respectively (Smidsrd & Skjak-Brae, 1990).  
95 The ratio between these two blocks (M/G ratio) leads to alginates with different  
96 characteristics when crosslinked with calcium, as only G blocks bind to calcium. This has a  
97 direct influence on the encapsulation efficiency, swelling and release kinetics. In  
98 microcapsule formation, alginates with a high M/G ratio will be more permeable and will  
99 have a faster release of encapsulated compounds (Khanna *et al.*, 2010). Alginates with a  
100 lower M/G ratio will form stronger structures, which are less permeable and more viscous  
101 due to the greater affinity of the G blocks to calcium ions, compared to M blocks (Sarmiento  
102 *et al.*, 2007). Divalent calcium ( $\text{Ca}^{2+}$ ) is the most commonly used ion to create alginate-based  
103 microcapsules, although other ions can also be used (Tam *et al.*, 2011). There are different  
104 techniques for microencapsulation of probiotics, such as extrusion, spray drying,  
105 emulsification and coacervation (Rathore *et al.*, 2013) but the production of microcapsules  
106 smaller than 100  $\mu\text{m}$  is less common in the literature. Microcapsules smaller than 100  $\mu\text{m}$   
107 are important as they will not alter food texture (Adhikari *et al.*, 2003) and thus their sensorial  
108 aspects. Nevertheless, depending on their hardness there are even works that refer  
109 microcapsules' sizes of 40  $\mu\text{m}$  that change food texture (Engelen *et al.*, 2005). Sheu &  
110 Marshall (1993) produced sodium alginate microcapsules smaller than 100  $\mu\text{m}$ , by emulsion  
111 technique, as support to the alginate ionotropic gelation. Considering the small diameters of  
112 microcapsules below 100  $\mu\text{m}$ , and that small capsules (below 200  $\mu\text{m}$ ) are less efficient for  
113 probiotics protection (Heidebach *et al.*, 2012), added barriers to protect microencapsulated  
114 probiotics have been developed, namely by using layer-by-layer (LbL) assembly.  
115 Electrostatic LbL coating involves the assembly of materials of opposite charge e.g. through  
116 exposure to alternating solutions of cationic and anionic polymers (Cook *et al.*, 2013;  
117 Krasaekoopt *et al.*, 2006).  
118 Different polysaccharides and proteins have been used to form LbL coatings (Tang *et al.*,  
119 2006; Yan *et al.*, 2014). Alginate|poly-L-lysine|alginate is a well-known combination of  
120 coatings which have been used on alginate based microcapsules. The interactions between  
121 these two polymers (alginate and poly-L-lysine (PLL)), in most cases leading to a LbL  
122 assembly, are based in the electrostatic interactions between the anionic groups  $\text{COO}^-$  present  
123 in alginate and the cationic groups  $\text{NH}_3^+$  present in PLL (Orive *et al.*, 2006). The electrostatic

124 interactions of PLL are enhanced when the amount of M blocks present in alginate is higher,  
125 in others words, PLL cationic groups have more affinity for M blocks compared to the G  
126 blocks (Thu *et al.*, 1996). Chitosan has been one of the most used materials to improve the  
127 protection of probiotics, e.g. as a coating on alginate microcapsules (Chávarri *et al.*, 2010;  
128 Kamalian *et al.*, 2014). Chitosan and alginate have also demonstrated mucoadhesive  
129 properties that are important to be used to increase the residence time of microcapsules in the  
130 gut (Sarmiento, *et al.*, 2007; Takeuchi *et al.*, 2005).

131 The objective of this work was developing an alginate-based probiotics encapsulation system  
132 smaller than 100  $\mu\text{m}$ , with a rationally designed coating developed through LbL assembly. It  
133 has being developed aiming to host probiotic bacteria while being able to pass through the  
134 gastrointestinal system up to the point at which adhesion to the intestinal mucosa can be  
135 achieved, with the consequent exchange of nutrients and products (probiotic activation and  
136 a continuous encapsulation) in the intestine. This approach will increase the residence time  
137 of probiotics in the intestine and will avoid possible inflammatory responses or infections  
138 provoked by direct contact of probiotic bacteria with the intestinal mucosa. In this work will  
139 only be presented the development and characterization of the system and the results  
140 considering probiotics encapsulation and protection, as adhesion will not be explored. The  
141 developed system will be characterized in terms of size, swelling capacity, folate release  
142 behavior and the chemical interactions between the capsule and the coatings (FTIR analysis).

143

## 144 2. Materials and methods

### 145 2.1. Materials

146 Sodium alginates “Protanal CR 8223” (M/G ratio 65/35; 250 - 350 kDa) and “Protanal  
147 LFR5/60” (M/G ratio 30/70; 20 - 60 kDa) were purchased from FMC Biopolymer (Belgium).  
148  $\epsilon$ -poly-L-lysine ( $\epsilon$ -PLL) was purchased from Handary (Belgium – Molecular Weight - 30  
149 kDa). Chitosan was obtained from Golden-Shell Biochemical Co. Ltd. (China - molecular  
150 weight - 5-10 kDa) with a degree of deacetylation of 95 %. Corn oil, Tween 80 (Panreac,  
151 Germany), fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RITC), 1-ethyl-3-  
152 (-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N,N-dimethylformamide and  
153 folic acid were purchased from Sigma (USA).

## 154 2.2. Turbidity measurements

155 All polymer solutions (0.1 % w/v) were prepared in deionized water, except chitosan which  
156 was dissolved in a 1 % (v/v) lactic acid solution. A mixture of 5 mL of each solution was  
157 used, testing some combinations of biopolymers to be used as coating materials (sodium  
158 alginate CR 8223 with  $\epsilon$ -PLL;  $\epsilon$ -PLL with sodium alginate LFR5/60; sodium alginate  
159 LFR5/60 with chitosan). The pH of each mixture was adjusted with 0.2 mol.L<sup>-1</sup> hydrogen  
160 chloride or 0.2 mol.L<sup>-1</sup> sodium hydroxide solutions, in the range of 2 to 8. In order to  
161 standardize the compounds concentration in all samples after pH adjustment, sodium chloride  
162 (0.2 mol.L<sup>-1</sup>) was added to obtain the same final volume in all experiments. Turbidity  
163 measurements were performed on a spectrophotometer at 400 nm (Jasco V560, Jasco  
164 Corporation, Japan).

## 165 2.3. Microcapsules production

166 Microcapsules were produced according to the method described by Sheu & Marshall (1993).  
167 These first tests were performed without bacteria to facilitate coated microcapsules  
168 characterization. The microcapsules were produced by dropwise addition of 20 mL sodium  
169 alginate (CR 8220), with a concentration of 1.5 %, into a 100 mL solution of vegetable oil  
170 with a concentration of 0.2 % of Tween 80. The mixture was then stirred for 10 min at  
171 200 rpm. After this, a solution of 200 mL of calcium chloride (0.05 M) was gently added  
172 during 20 s and the mixture stirred at 200 rpm for 20 min. After hardening, the solution was  
173 passed to a separatory funnel, where it remained for 30 min. After this, the liquid (oil and  
174 water) was gently removed with a pipette. The residual volume containing the microcapsules  
175 was then filtered through a 100  $\mu$ m nylon filter using water to remove the residual oil. After  
176 filtration the microcapsules that passed through the filter (smaller than 100  $\mu$ m) were  
177 centrifuged for 5 min at 600 rpm (Centrifuge Heraeus Megafuge 1.0R) and isolated.

## 178 2.4. Layer-by-layer assembly - Coated microcapsules production

179 After production, the microcapsules were immersed in a 0.01 %  $\epsilon$ -PLL solution (10 mL),  
180 with constant stirring of 200 rpm for 15 min, forming the coated microcapsules with a single  
181 coat. Then, the solution was centrifuged in the manner mentioned before. The next step was  
182 the immersion of the coated microcapsules (alginate| $\epsilon$ -PLL) into a sodium alginate

183 (LFR5/60) solution (0.1 %, 10 mL) for 15 min, followed by centrifugation (600 rpm, 10 min).  
184 The recovered coated microcapsules (double coated) were then immersed into a chitosan  
185 solution (0.01 %, 10 mL). After that, another centrifugation step separated the coated  
186 microcapsules from the chitosan solution.

## 187 2.5. Fourier transform infrared (FTIR) spectroscopy

188 In order to confirm the adhesion of the different coatings, FTIR analyses were carried out  
189 with a Perkin Elmer 16 PC spectrometer (Perkin Elmer, Boston, MA, USA) in the  
190 wavenumber region of 600 - 4000  $\text{cm}^{-1}$  using 16 scans for each sample. The microcapsules  
191 and coated microcapsules were freeze-dried prior to FTIR measurements.

## 192 2.6. Diameter measurement

193 Diameter measurements were performed through microscopy with a 10 $\times$  magnification  
194 (Olympus BX51). Images of the samples were taken and at least 200 capsules were measured  
195 with Image J software.

## 196 2.7. Testing of microcapsules and coated microcapsules in different pH's

197 Microcapsules and coated microcapsules were immersed into a 10 mL solution of potassium  
198 chloride - hydrogen chloride (pH 2) for 1 h, stirred at 100 rpm. Then, the solution was  
199 centrifuged and the microcapsules and coated microcapsules were put into a phosphate buffer  
200 saline (PBS) solution (pH 7.2), for 3 h. Aliquots of 100  $\mu\text{L}$  were taken every 15 min. During  
201 the PBS solution test, that have a duration of 3 h, samples were taken every 45 min. Each  
202 experiment was performed in triplicate. These experiments were performed in microcapsules  
203 and coated microcapsules with one coating ( $\epsilon$ -PLL), two coatings (previous coating and  
204 alginate) and with three coatings (previous coatings and chitosan). All these experiments  
205 were performed independently and in triplicate. For each sample, diameter measurements  
206 were performed.

## 207 2.8. Compounds labeling

208 Chitosan-[RITC] was prepared by mixing 100 mL of 1 % chitosan with 50 mg RITC and  
209 20 mg EDC at 4  $^{\circ}\text{C}$  for 1 day. Alginate-[FITC] was prepared by mixing 100 mL of 1.76 %  
210 alginate with 10 mg FITC and 20 mg EDC at 4  $^{\circ}\text{C}$  for 1 day. The residual free dye was then

211 dialyzed off (molecular weight cut-off 3500 Da; Cell-Sep H1, Membrane Filtration products,  
212 USA) with double distilled water for 2 weeks (Chang *et al.*, 2012). To the FITC-labeled  $\epsilon$ -  
213 PLL, 0.1 % of a  $\epsilon$ -PLL solution was dissolved in 0.2 M NaHCO<sub>3</sub> buffer (pH 9) and 1.0 mg  
214 (0.0026 mmol) of FITC was dissolved in N,N-dimethylformamide. Both solutions were  
215 stirred overnight. The  $\epsilon$ -PLL/FITC solution was purified by dialysis (molecular weight cut-  
216 off 1000 Da; Cell-Sep H1, Membrane Filtration products, USA) and the compound was dried  
217 by freeze-drying for further utilization (Kleinberger *et al.*, 2013).

218

## 219 2.9. Confocal microscopy analyses during the gastrointestinal pH simulation

220 The same media and conditions were used as mentioned in section 2.7 but samples were  
221 taken at 5 min and 60 min in the potassium chloride - hydrogen chloride medium and at 5  
222 min and 3 h in the PBS medium. All samples were analyzed by confocal microscopy (Nikon  
223 A1-R Confocal with Resonant Scanner). Independent experiments were performed where  
224 just the studied coating was labeled.

225

## 226 2.10. Kinetic release profiles

227 The release kinetics of folic acid was studied using an *in vitro* dialysis method. Folic acid  
228 was added to sodium alginate solution used for microcapsules' production, forming a  
229 solution with a concentration of folic acid of 2 %. The process of coated microcapsules'  
230 production was the same as mentioned before. Each production batch (10 mL volume) was  
231 divided in two different experiments. Each experiment had 5 mL of coated microcapsules  
232 that were introduced in a dialysis membrane (molecular weight cut-off 15 kDa, Cellu-Sep  
233 H1, Membrane filtration products, USA), which was subsequently placed into 40 mL of PBS  
234 solution, under magnetic stirring, at 37 °C, with no exposure to light. At appropriate time  
235 intervals, 0.8 mL of supernatant were taken and fresh medium was replaced to keep the initial  
236 volume constant. The amount of folic acid released from the coated microcapsules was  
237 evaluated by measuring the absorbance at 309 nm (Elisa Biotech Synergy HT, Biotek, USA).  
238 Coated microcapsules were produced without folic acid and a release experiment was



239 performed proving that no significant absorbance could be measured from the samples of  
240 that experiment.

241 All the tests were run at least in triplicate.

242

243 Mathematical Modeling

244 Folic acid release profile from the coated microcapsules was evaluated using a kinetic model  
245 that accounts for both Fickian and Case II transport (linear superposition model - LSM)  
246 effects in hydrophilic matrices (Berens & Hopfenberg, 1978b):

$$247 \quad M_t = M_F + M_R \quad (\text{Eq.1})$$

248 where  $M_t$  is the total mass released from the coated microcapsule,  $M_F$  and  $M_R$  are the  
249 contributions of the Fickian and relaxation processes, respectively, at time  $t$ .

250 The Fickian process is described by:

$$251 \quad M_{t,F} = M_{\infty,F} \left[ 1 - \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp(-n^2 k_F t) \right] \quad (\text{Eq.2})$$

252 where

$$253 \quad K_F = \frac{4\pi^2 D}{d^2} \quad (\text{Eq. 3})$$

254  $M_{\infty,F}$  is the compound release at equilibrium,  $D$  is diffusion coefficient and  $d$  is the capsule  
255 diameter.

256 As for polymer relaxation, it is driven by the swelling ability of the polymer and it is therefore  
257 related to the dissipation of stress induced by the entry of the penetrant and can be described  
258 as a distribution of relaxation times, each assuming a first order-type kinetic equation (Berens  
259 & Hopfenberg, 1978a).

260

$$261 \quad M_{t,R} = \sum_i M_{\infty,i} [1 - \exp(-K_i t)] \quad (\text{Eq. 4})$$

262

263 where each  $K_i$  is the respective relaxation rate constant and each  $M_{\infty,i}$  represents the  
264 equilibrium sorption of the  $i^{\text{th}}$  relaxation process.

265

266 Substitution of equations (Eq. 2) and (Eq. 4) into equation (Eq. 1) results in:

$$267 \quad M_{t,F} = M_{\infty,F} \left[ 1 - \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp(-n^2 k_F t) \right] + \sum_i M_{\infty,Ri} [1 - \exp(-KRi, t)] \quad (\text{Eq.5})$$

268 This “general” model can then be used to describe pure Fickian ( $M_{t,F} \neq 0$  and  $i = 0$ );  
269 anomalous ( $M_{t,F}$  and  $i \neq 0$ ) or Case II ( $M_{t,F} = 0$  and  $i \neq 0$ ) transports.

270

### 271 2.11. Statistical analyses

272 Statistical analyses were performed using the analyses of variance (ANOVA) procedure with  
273 SigmaPlot 11.0 software for windows, where a  $p < 0.05$  was considered to be statistically  
274 significant, on the diameter and turbidity measurements.

275 Equation 5 was fitted to data by non-linear regression, using STATISTICA v7.0 (Statsoft.  
276 Inc, USA). The Levenberg-Marquardt algorithm for the least squares function minimization  
277 was applied. Adjusted determination coefficient ( $R^2$ ), squared root mean square error  
278 (RMSE) (i.e., the square root of the sum of the squared residues (SSE) divided by the  
279 regression degrees of freedom) and residuals inspection for randomness and normality were  
280 evaluated to determine regressions quality. Standardized Halved Width (SHW %) (i.e. the  
281 ratio between 95 % Standard Error and the value of the estimate) was assessed to determine  
282 precision of the estimated parameters.

283

284

### 285 3. Results and Discussion

286

287 The design of this system was made with the following criteria: the utilization of alginate CR  
288 8223, that has a 65/35 ratio of the M/G blocks and a high molecular weight (app. 300 kDa)  
289 was meant to form a main core with high permeability due to its high molecular weight and

290 high content of mannuronic residues (responsible for swelling and less affinity by calcium  
291 ions). The use of PLL, as the first coating, is expected to limit the continuous swelling of the  
292 alginate microcapsule that leads to erosion in the media containing monovalent ions and  
293 calcium sequestrants, while maintaining the permeability of the coated microcapsule. The  
294 subsequent coating, alginate LFR5/60, has a 30/60 M/G ratio and a low molecular weight.  
295 PLL has more affinity for M blocks than G blocks, which will promote electrostatic  
296 interactions between the first and the second coating (Thu *et al.*, 1996). This second alginate  
297 coating will also work as a way to guarantee the interaction between PLL and chitosan (to be  
298 used in the third and last coating), maintaining the permeability of the system, and working  
299 as a bioadhesive material in the case of chitosan erosion. Chitosan used as the last coating  
300 has two main objectives: to protect the system (mainly the probiotics) in acidic environments  
301 and be responsible for the adhesion of coated microcapsules to the intestinal epithelium. The  
302 presented system, with the utilization of these specific materials and with that specific order,  
303 was never used before for the development of a coated microcapsule system. The  
304 combination of these materials create a new coated microcapsule system that might have new  
305 functions and applications, such as the controlled release of micronutrients. Some of these  
306 characteristics will be studied and demonstrated in this work, others will be explored in a  
307 future work.

308

### 309 3.1. Turbidity measurements

310 Turbidity tests were performed to demonstrate that the coating materials effectively interact  
311 with each other via their charged groups, which are responsible by the electrostatic  
312 interactions established between them. The materials used to build the different coatings on  
313 the alginate microcapsules were: a) alginate CR 8223, negatively charged material at pH  
314 values above its pKa; b)  $\epsilon$ -PLL, positively charged material at pH values below its isoelectric  
315 point (pI); c) alginate LFR5/60, negatively charged material at pH values above its pKa (An  
316 *et al.*, 2013; Cook *et al.*, 2012); d) chitosan, positively charged material at pH values above  
317 its pKa.

318 The turbidity of these four different solutions was measured at pH values between 2 and 8,  
319 ensuring that the OD was never above 0.08 a.u. This confirms that any turbidity above this  
320 is the result of polyelectrolyte complex formation. The first experiment was performed with

321 alginate CR 8223 and  $\epsilon$ -PLL. The results showed a better interaction between the  
322 biopolymers at pH 5 - 6 (Figure 1), as shown by higher OD values, being those statistically  
323 different for all experiments but not different at pH 4 ( $p < 0.05$ ). The rest of the experiments  
324 are all statistically similar, but their minimal values of OD ( $0.68 \pm 0.05$ ) are considerably  
325 higher than the OD of the pure solutions, which indicates that electrostatic interactions  
326 occurred between these materials in all the pH range tested. These results can be justified  
327 considering that the  $pK_a$  of alginate is 3.3 - 3.7 ( $pK_a$  values of mannuronic acid and guluronic  
328 acid are 3.38 and 3.65, respectively (An *et al.*, 2013)) and that the  $pI$  of  $\epsilon$ -PLL is of  
329 approximately 9 (Yoshida & Nagasawa, 2003). The interactions observable at pH 2 and 3 are  
330 possibly due to a remaining percentage of functional groups charged in alginate at these pH  
331 values (pH at one unity below the  $pK_a$  of alginate, the molecule will still have 10% of the  
332 functional groups charged (Po & Senozan, 2001)).

333

334 Figure 1 – Turbidity measurements for Alginate CR 8223,  $\epsilon$ -PLL, Alginate LFR5/60 and  
335 Chitosan. Different letters represent significantly different values ( $p < 0.05$ ).

336

337 The same experiment was performed with  $\epsilon$ -PLL and alginate LFR5/60 solutions (Figure 1),  
338 which correspond to the first and second coating of the microcapsule, respectively. The  
339 results showed that the strongest interactions between the two materials happen between pH  
340 3 and 8, where no statistically significant differences are observed in that interval ( $p < 0.05$ ).  
341 As mentioned before, the lower OD obtained at pH 2 can be justified by a lower number of  
342 negatively charged functional groups in alginate, at this pH. These results have a similar  
343 behavior when compared with the interactions between alginate CR8223 and  $\epsilon$ -PLL and  
344 similar electrostatic interactions are present in this experiment. It is also important to mention  
345 that the observed OD values are higher, in general, when comparing the interaction of  
346 alginate LFR5/60 and alginate CR 8223 with  $\epsilon$ -PLL. This fact can be justified by the  
347 strongest affinity of the amine groups of  $\epsilon$ -PLL with the glucuronic residues (present in  
348 alginate) that are in a higher percentage in the alginate LFR5/60 (Thu *et al.*, 1996).

349 The turbidity measurements performed with alginate LFR5/60 and chitosan showed that pH,  
350 as before, influenced the results (Figure 1). The  $pK_a$  values of alginate and chitosan are 3.3  
351 – 3.7 and 6.5 (Oliveira *et al.*, 2014), respectively, which indicates that strong interactions are

352 to be expected between the biopolymers at pH values between 3 and 5, being those  
353 statistically different of the rest ( $p < 0.05$ ) (as mentioned above, at pH values near the pKa,  
354 half of the molecule's functional groups are charged). The results obtained between pH 6 and  
355 8 cannot be differentiated from a statistical point of view, which considering that those values  
356 are at the chitosan pKa or above that, means that chitosan is reducing the number of its  
357 functional groups with positive charge, and is starting to precipitate. In the tests at lower pH  
358 (pH 2) the same is happening but with the alginate molecule. Electrostatic forces are present  
359 in the interaction of chitosan with alginate thus creating this significant turbidity (Chávarri  
360 *et al.*, 2010). The interaction of these materials were also demonstrated in other works where  
361 a capsule constituted by alginate and PLL was produced (Constantinidis *et al.*, 2007; Tam *et*  
362 *al.*, 2005b). In other works were also proved the interaction of alginate and chitosan on the  
363 production and coating of a microcapsule (Shi *et al.*, 2007a; Zhao *et al.*, 2007).  
364 Turbidity results show that the materials used are able to interact, being a good indication of  
365 the formation of a LbL structure on the microcapsule. This evaluation is also important to  
366 demonstrate that the developed system is stable for pH values between 2 and 7 (i.e. stomach  
367 and intestine), which gives good perspectives to the utilization of this coated microcapsule  
368 as a gastrointestinal delivery system. After turbidity tests the concentrations of the solutions  
369 were optimized by  $\zeta$  - potential measurements in order to add successfully the different  
370 coatings to the microcapsule. These tests were conducted as described in Carneiro-da-Cunha  
371 *et al.* (2010) and the best solutions achieved were 0.01 % for  $\epsilon$ -PLL, 0.1 % for alginate and  
372 0.01 % for chitosan.

373

### 374 3.2. Size measurement

375

376 Figure 2 shows the diameter of the coated microcapsules through consecutive coating steps,  
377 demonstrating that coated microcapsules produced using this method are smaller than 100  
378  $\mu\text{m}$ , achieving that way the first goal of this work (microcapsules/coated microcapsules to  
379 protect bacteria that do not alter the texture/mouthfeel of the food product they are to be  
380 dispersed in). During sequential coating steps, the coated microcapsules' diameter decreased,  
381 comparing with microcapsules, phenomena that can be explained by the different behavior  
382 of these materials at different pH values. The alginate used in the microcapsules production

383 has the capacity to swell through hydration that increased the microcapsules volume; this  
384 happens after the production in the final washing step with water. This was also reported by  
385 Sriamornsak *et al.* (2007) that tested a great number of alginates types and proved that  
386 alginate-based matrices are susceptible to hydrate at a neutral pH. After generating the main  
387 core, the microcapsules were added to a solution of  $\epsilon$ -PLL, creating the alginate| $\epsilon$ -PLL  
388 coated microcapsule (APM). This deposition decreased the diameter of the coated  
389 microcapsules in comparison with the alginate microcapsules, in a neutral pH, due to the first  
390 coating formation. When APM were put into water the swelling was reduced by the  $\epsilon$ -PLL  
391 layer. This behaviour can be explained by the capacity of this coating to limit the high  
392 hydration capacity, and consequent swelling capacity, of alginate microcapsules (Lawrie *et*  
393 *al.*, 2007). The consequent layers adhesion creating the alginate| $\epsilon$ -PLL|alginate coated  
394 microcapsule (APAM) and alginate| $\epsilon$ -PLL|alginate|chitosan (APACM) did not change the  
395 diameter of the coated microcapsules, being the diameter results for the three coated  
396 microcapsules statistically equal and all different from the microcapsule diameter ( $p<0.05$ ).

397

398

399 Figure 2 – Microcapsule and coated microcapsules diameter through the coatings deposition  
400 (capsules are immersed in water), A; and coated microcapsules' picture by microscope with  
401 a 10x lent (scale bar 150  $\mu\text{m}$ ), B. Different letters represent significantly different values  
402 ( $p<0.05$ ).

403

### 404 3.3. FTIR results

405 FTIR results showed a significant similarity between the microcapsules spectra main peaks  
406 and those of alginate CR 8223 (Figure 3), in both, the characteristic peaks of alginate at 3309  
407  $\text{cm}^{-1}$  (stretching vibrations of hydrogen-bonded OH groups), 1590  $\text{cm}^{-1}$  and 1400  $\text{cm}^{-1}$   
408 (stretching vibrations of the  $\text{COO}^-$  - Figure 3 (B)) are present (Shi *et al.*, 2007; Tam *et al.*,  
409 2005). Just on microcapsules spectrum, two consequent peaks can be found at 2922 and 2852  
410  $\text{cm}^{-1}$  (Figure 3 (A)) and another one at 1743  $\text{cm}^{-1}$  (Figure 3 (C)), both being the consequence  
411 of the presence of oil (Meng *et al.*, 2014; Vlachos *et al.*, 2006).

412

413 Figure 3 - FTIR spectra of alginate powder and alginate microcapsules. A – representation  
414 of 2852 and 2922  $\text{cm}^{-1}$  peaks; B – representation of 1590 and 1400  $\text{cm}^{-1}$  peaks; C –  
415 representation of the 1743  $\text{cm}^{-1}$  peak.

416

417 Considering the spectra presented in Figure 4, it is possible to identify that the oil spectra  
418 keep being present in all samples. The consequent coating adhesion showed that there are no  
419 significant modifications between the alginate microcapsules spectra and the coated  
420 microcapsules spectra. On the alginate microcapsules and APM spectra there are relevant  
421 differences such as the formation of two shoulders on both sides of peak 1590 (Figure 4 (A)),  
422 that correspond to the strong Amide I ( $\sim 1637 \text{ cm}^{-1}$ ) and Amide II ( $\sim 1552 \text{ cm}^{-1}$ ) OD bands of  
423 the  $\epsilon$ -PLL (Tam *et al.*, 2005). The presence of those peaks proves the presence of  $\epsilon$ -PLL in  
424 the APM structure. The presence of alginate LFR5/60 is difficult to prove considering that  
425 its spectra is the same as the one of the initial alginate microcapsule, although it is possible  
426 to notice that the two shoulders presented in the last spectrum (APM spectra) characteristic  
427 of  $\epsilon$ -PLL, disappear in the APAM spectrum which proves that the alginate coating was well  
428 succeed. Some characteristic peaks can be seen in Figure 4 (B) in the APACM spectrum, that  
429 are characteristic of the presence of chitosan, such as the one found at 1567  $\text{cm}^{-1}$ ; this is a  
430 typical peak of the interaction between the negatively charged group  $\text{COO}^-$  of alginate and  
431 the positively charged groups  $\text{NH}_3^+$  of chitosan. This peak is also responsible for the  
432 obstruction of the 1590  $\text{cm}^{-1}$  alginate peak. This behavior can be found in other works dealing  
433 with the coating of alginate with chitosan (Shi *et al.*, 2007b). The 1729  $\text{cm}^{-1}$  peak (Figure 4  
434 (C)) can be justified by the protonation of alginate, due to the contact of chitosan (dissolved  
435 in lactic acid) with alginate (Lawrie *et al.*, 2007). These FTIR results show the successive  
436 adhesion of the different coatings.

437

438 Figure 4 - FTIR spectra of alginate microcapsule, APM, APAM and APACM.

439

#### 440 3.4. Confocal microscopy analyses

441 The LbL assembly on alginate microcapsules was evaluated through confocal microscopy in  
442 order to show the adhesion of the materials to the main core microcapsule structure (Figure

443 5). Alginate CR 8223,  $\epsilon$ -PLL and alginate LFR5/60 were labeled with FITC and chitosan  
444 was labeled with rhodamine. To test the presence of each coating only one coating was  
445 labeled in each experiment, in order to differentiate all the coatings that were labeled with  
446 FITC. Confocal microscopy images showed the existence of the alginate-based  
447 microcapsules (Figure 5a). Figure 5b) shows the coated microcapsules labeled with  $\epsilon$ -PLL,  
448 coated due to the electrostatic forces between  $\text{CO}_2^-$  groups, from alginate, and  $\text{NH}_3^+$  from  $\epsilon$ -  
449 PLL (Bystrick *et al.*, 1990). Figure 5c) shows the adhesion of the second coating, composed  
450 of alginate LFR5/60. Figure 5d) shows the presence of the subsequent chitosan layer on that  
451 of alginate LFR5/60 coated microcapsule, being its adhesion justified by electrostatic forces  
452 between the carboxyl groups of alginate and the amine groups of chitosan ( $\text{NH}_2^+$ ) (Gazori *et*  
453 *al.*, 2009; Sarmiento *et al.*, 2007). With these confocal images it was possible to prove the  
454 sequential adhesion of those different coatings to the main microcapsule structure.

455

456 Figure 5 – Representation of: a) alginate coated microcapsules (alginate labeled with FITC -  
457 scale 250  $\mu\text{m}$ ); b) APM ( $\epsilon$ -PLL labeled with FITC - scale 200  $\mu\text{m}$ ); c) APAM (alginate  
458 labeled with FITC - scale 50  $\mu\text{m}$ ); d) APACM (Chitosan labeled with Rhodamine - scale 50  
459  $\mu\text{m}$ ).

460

### 461 3.5. Evaluation of structures into two different media that simulate gastrointestinal pH

462 Tests were performed to determine the coated microcapsules' diameter in an acidic medium  
463 (potassium chloride - hydrogen chloride at pH 2, 1 h), simulating stomach pH, and in a neutral  
464 pH medium (Phosphate Buffer Solution (PBS) – pH 7.2 – 3 h), simulating intestinal pH.  
465 Alginate microcapsules showed a good resistance to the potassium chloride - hydrogen  
466 chloride solution, as shown in Figure 6. The structures presented an average diameter of  
467  $33.81 \pm 3.05 \mu\text{m}$  in that solution.

468

469 Figure 6 - Microscopy images of the microcapsules after 1 h into the acidic medium (scale -  
470 150  $\mu\text{m}$ ).



471 In PBS the microcapsules had a different behavior. After 5 min they were starting to dissolve,  
472 making the measurement of their size impossible. This is due to exchange between the  
473 sodium ions present in the PBS solution and the calcium ions present in the alginate  
474 microcapsule, together with calcium sequestration by phosphates (Corona-Hernandez *et al.*,  
475 2013). This process generates an increase of repulsion between the COO<sup>-</sup> groups present in  
476 the alginate chains leading to a swelling of the structure (water intake), at an initial stage,  
477 which will later lead to a total collapse of the alginate microcapsule structure. Bajpai *et al.*  
478 (Bajpai & Sharma, 2004) showed that alginate beads had the same behavior into a PBS  
479 solution, increasing their diameter during the first 3 h and leading to a total dissolution of the  
480 materials after that. Similar results were reported by Gao *et al.* (2009).

481 To avoid the coated microcapsules' dissolution in the PBS medium, a coating was added on  
482 the alginate microcapsule. This coating will presumably lead to an increase of the structure's  
483 strength decreasing its swelling and thus avoiding dissolution. Some authors have shown that  
484 the utilization of  $\epsilon$ -PLL as a coating on alginate microcapsules decreased the swelling degree  
485 of the alginate microstructure in a monovalent ions solution such as PBS (Capone *et al.*,  
486 2013; Tam *et al.*, 2011). Figure 7 shows the diameter of the coated microcapsules when  
487 immersed in potassium chloride - hydrogen chloride and PBS solutions. It is clear that a)  
488 there is no destruction of the coated microcapsules in the PBS solution; b) coated  
489 microcapsules' diameter is stable during the contact with the different media and there are  
490 no statistically significant differences between all diameters results in the potassium chloride  
491 - hydrogen chloride solution for all systems ( $p < 0.05$ ) (average diameter of app. 20  $\mu\text{m}$ ) and  
492 the same happens in the PBS solution, also for all systems ( $p < 0.05$ ), being the average  
493 diameter around 40  $\mu\text{m}$ ; and c) there is a swelling degree of approximately 2-fold in diameter  
494 when coated microcapsules go from the potassium chloride - hydrogen chloride solution to  
495 the PBS solution, being that difference statistically significant. The  $\epsilon$ -PLL coating showed  
496 that it was able to protect the structure against the ion exchange process, thus retarding its  
497 swelling.

498

499

500 Figure 7 – Microcapsules and coated microcapsules diameter during immersion in potassium  
501 chloride - hydrogen chloride (0 to 60 min) and PBS (60 to 240 min) solutions in a consecutive  
502 way, respectively. Different letters represent significantly different values ( $p < 0.05$ ).

503

504

505 Confocal microscopy was performed in order to evaluate the eventual loss of layers during  
506 the contact with the potassium chloride - hydrogen chloride and PBS media. As explained  
507 before, to test the presence of each coating only one coating was labeled in each experiment,  
508 in order to differentiate all the coatings that were labeled with FITC. Figure 8 shows that the  
509 three different coatings were still over each other and attached to the main structure. Those  
510 results are in accordance with other published works (Cui *et al.*, 2000; Kamalian *et al.*, 2014;  
511 Krasaekoopt *et al.*, 2006; Tam *et al.*, 2011).

512

513

514 Figure 8 – Microcapsules and coated microcapsules after 3 h into the PBS medium (with a  
515 previous contact of 1 h into potassium chloride - hydrogen chloride medium): a) alginate-  
516 based microcapsule (alginate labeled with FITC - scale 100  $\mu\text{m}$ ); b) APM ( $\epsilon$ -PLL labeled  
517 with FITC - scale 25  $\mu\text{m}$ ); c) APAM (alginate labeled with FITC - scale 100  $\mu\text{m}$ ); d) APACM  
518 (chitosan labeled with rhodamine - scale 100  $\mu\text{m}$ ). Independent experiments were performed  
519 where only the studied coating was labeled.

520

521

### 522 3.6. Release kinetics of folic acid into phosphate buffer

523 Mathematical modelling of transport phenomena is important for the design of carrier  
524 systems and of active compound carriers, since it may help predicting behavior *in vivo*. In  
525 this work, we studied the description of experimentally obtained data by the Linear  
526 Superimposition Model (LSM) (Eq. 5). The mechanisms of folic acid release from coated  
527 microcapsules were evaluated at 37 °C (temperature within the human body) and at pH 7 (pH  
528 of the small intestine).

529 In order to evaluate the physical mechanisms involved in folic acid release from coated  
530 microcapsules with different coatings it is important to use a model that successfully

531 describes the individual contributions of the diffusion and the relaxation processes. The LSM  
532 was fitted to the experimental data: concerning the Fickian part of the model ( $M_F$ , Eq. 2) and  
533 the relaxation part of the model ( $M_R$ , Eq. 4). As reported by other works, this model assumes  
534 that the transport mechanism from coated microcapsules: i) can be due only to the  
535 concentration gradient and polymer relaxation had no effect on the transport mechanism (i.e.  
536 Fick's behavior;  $i = 0$ ); or ii) transport can be due to the sum of concentration gradient and  
537 to the relaxation of the polymer matrix ( $i \neq 1$ ) (Pinheiro *et al.*, 2013).

538 Figure 9 clearly shows the effect of applying different coatings on coated microcapsules in  
539 the folic acid release profile. For each coating applied on the coated microcapsule, LSM  
540 fitting curves showed a good description of the experimental data. This indicates that this  
541 transport mechanism cannot be described by Fick's diffusion of folic acid in the coated  
542 microcapsules alone, but is governed by both Fickian and Case II transport. Also, it was  
543 observed that depending on the coating applied in the coated microcapsules, this system was  
544 governed by two or more relaxation processes.

545

546

547

548 Figure 9 – Profile of folic acid release from coated microcapsules at 37 °C in PBS;  
549 experimental data (×) and description of LSM (-) for coated microcapsules with A) first  
550 coating  $\epsilon$ -PLL, B) second coating alginate and C) third coating chitosan.

551

552 Table 1 presents the regression analysis results of the LSM fitting, showing that this model  
553 adequately describes the experimental data with relatively good regression quality ( $R^2 > 0.90$ )  
554 and that most parameters were estimated with good precision.

555

556 Table 1. Results of LSM fitting to experimental data of folic acid release from coated microcapsules.  
557 Quality of the regression based on RMSE and  $R^2$  evaluation. Estimates' precision is evaluated using  
558 the SHW% (in parenthesis).

559

560

561 The fitting of LSM model to experimental data shows that the mechanisms involved in folic  
562 acid release are composed by Fick diffusion contribution ( $M_F$ ) and two or more relaxation  
563 processes ( $M_R$ ). The application of the first coating ( $\epsilon$ -PLL) on the coated microcapsule  
564 suggests that Fick's diffusion is the main mechanism of folic acid release from the coated  
565 microcapsule. These results can be explained based on strong electrostatic interactions  
566 between alginate CR 8223 and  $\epsilon$ -PLL at pH 7 (pH of PBS). As mentioned above (section  
567 3.1), at this pH the interaction between these materials is mainly due to the high affinity of  
568 the charges between the molecules (alginate – negative charge;  $\epsilon$ -PLL – positive charge).  
569 Increasing the number of coatings on the coated microcapsule leads to a decrease of the Fick  
570 diffusion contribution and to the appearance of three relaxation steps. In fact, Figure 10  
571 shows that the anomalous transport considering two main relaxations ( $i=2$ ) was unable to  
572 predict the experimentally observed behavior and, hence, the physical mechanism of the  
573 transport phenomena involved for the coated microcapsule with the second and third coating  
574 was changed. This reflects that with these two last coatings the structure is more unstable,  
575 which means that the electrostatic interactions between alginate CR 8223| $\epsilon$ -PLL|alginate  
576 LFR5/60 and alginate CR 8223| $\epsilon$ -PLL|alginate LFR5/60|chitosan are weaker. This leads to  
577 the loosening of coated microcapsule' structure and promotes the release of folic acid due to  
578 polymer relaxation at different times. Also the relaxation rate constant ( $k_R$ ) decreased with  
579 the number of coatings, supporting this hypothesis. The rate at which folic acid molecules  
580 pass through the coated microcapsule layers decreases with the increase of the number of  
581 layers in the structure. Fickian rate constant ( $k_F$ ) also decreased with the number of coatings,  
582 reflecting the decrease of predominance of Fickian behavior.

583

584 Figure 10 – Fitting of Eq. 5 to the experimental data of controlled release of folic acid from  
585 coated microcapsules with: A) second coating and B) third coating (experimental results (x)  
586 and model-generated values for  $i=2$  (-) and  $i=3$  (-)). Inset shows the detail of the model fitting  
587 to the initial experimental data.

588

589 The diffusion coefficient ( $D$ ) was estimated based on Eq. 4 and it is possible to observe that  
590 this parameter was influenced by the composition of the coatings on the coated microcapsule.  
591 The application of a second coating decreases  $D$  from  $3.73 \times 10^{-13}$  to  $1.256 \times 10^{-13} \text{ m}^2 \cdot \text{min}^{-1}$  and

592 this last value remained constant for the third coating. These results suggest that the  
593 application of the second coating led to a slow, limiting step of the release of folic acid. This  
594 fact can also be explained by the utilization of two different alginates: the alginate used for  
595 the microcapsule production is more permeable, with a higher swelling capacity, while the  
596 one used as the second coating is more stable, with more G residues (more connection points  
597 leading to a stronger adhesion to the adjacent layers), being these characteristics the main  
598 differences of these two alginates (Draget & Taylor, 2011). Similar results in the release of  
599 folic acid from alginate-based capsules can be found in other works (Madziva et al., 2005;  
600 Pérez-Masiá et al., 2015)

601

602

#### 603 4. Conclusions

604 This work showed that it is possible to build coated microcapsules with three coatings ( $\epsilon$ -  
605 PLL, alginate and chitosan) with a good stability at different pH values. This allowed us to  
606 foresee that this coated microcapsule may have a good performance in probiotic protection  
607 under GIT conditions. The coated microcapsule has an average diameter smaller than  
608 100  $\mu\text{m}$ , thus being not detected in the mouth. Results also showed that its porosity will  
609 increase at neutral pH, allowing an increased exchange of nutrients and products e.g. between  
610 encapsulated cells and the intestinal medium, although further tests need to be done to show  
611 this. According to the folic acid release experiments it was proved that this coated  
612 microcapsule is permeable to folic acid, even when three coatings are applied on it, although  
613 it was also shown that the increase of the number of coatings decreases the folic acid  
614 diffusivity through capsules.

615

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