Characterization of Core-Shell Alginate Capsules

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CHARACTERIZATION OF CORE-SHELL ALGINATE CAPSULES GENERATED USING DROPLETS MILLIFLUIDIC

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Abstract:	A new droplets millifluidic/inverse gelation based process was used to produce core-shell alginate milli-capsules. Water-in-oil (W/O) emulsion dispersed phase containing Ca2+ ions was directly injected into a continuous alginate phase to generate a secondary W/O/W emulsion. Due to the cross-linking of alginate molecules by Ca2+ ions release, core-shell milli-capsules were formed with a very high oil loading. The influence of the curing time and of the storage conditions on capsules physico-chemical properties was investigated. It was first found as expected that alginate membrane thickness increased with curing time in the collecting bath. However, a plateau was reached for the higher curing times, in close relation with previous observations (Martins, Poncelet, Marquis, Davy, & Renard, 2017b) that an external oil layer surrounded the surface of W/O emulsion drops that acted as a barrier and hindered the release of aqueous CaCl2 droplets during curing time. Compression experiments on individual capsules revealed that alginate membrane thickness was inversely related to its mechanical properties, i.e. the thicker membrane, the lower surface Young modulus. Surface Young modulus ranged from 61 to 26 N/m at curing times of 3 and 45 min, respectively. This result was explained in terms of enhanced swelling properties of alginate membrane with curing time or storage conditions. Drying capsules led to much more resistant membranes due to the loss of water. Oil loading of 80 wt% was obtained for dry capsules whatever the conditions used.

Response to Reviewer 3 Comments

Point-by-point answers to reviewer 3 are given below and corrections appear in red in the revised manuscript.

Reviewer #3: I have reviewed this manuscript titled „CHARACTERIZATION OF CORE-SHELL ALGINATE CAPSULES GENERATED USING DROPLETS MILLIFLUIDIC" based on the innovativeness, quality, significance, and presentation of results. In general, the presentation of results is lacking. This paper could be published but only after the corrections and explanations.

Line 29. Change „was" to „were".

Changing was made in the revised manuscript.

Line 63. Change „consist" to consists".

« Consists » appears in the original manuscript.

Line 85. Change „the microcapsules production" to „the production of the microcapsules".

Changing was made in the revised manuscript.

Line 126. Change „were" to „was".

Changing was made in the revised manuscript.

Line 150. Were capsules washed before storage in Ca2+ or Na+ containing solutions?

According to reviewer's remark, the following sentence was changed in the revised manuscript "the capsules were sieved, rinsed with distilled water and suspended in a Ca^{2+} or Na^{+} solution".

Line 165. Change „microscopy" to „microscope".

Changing was made in the revised manuscript.

Line 224. Change „hydrophilic lipophilic" to „hydrophilic-lipophilic".

Changing was made in the revised manuscript.

Line 236. High-pressure homogenization? Where is this included in the paper? There is no mentioning of High-pressure homogenization throughout the paper.

We agree with reviewer 3 and the following changing was made in the revised manuscript « High shear mixing… »

Figure 5 and Figure 7. Are there statistically significant differences with regards to the curing time and membrane thickness/capsule diameter? Are there statistically significant differences with gelation time? These should be added to the figures and comment in the discussion.

Authors performed statistical analyses on data displayed in Figures 5 and 7. The following sentence was therefore added in the Figure captions of the revised manuscript : «At the 0.05 level, the populations means were significantly different ». In addition, authors added in the caption of Figure 5 « Solid line corresponded to the fitting of the data by a linear function; dotted line was just eye-guide. » Authors also replaced « Gelation time » by « Curing time » in Figure 7. Finally, additional comments regarding statistical analyses were added in the revised manuscript.

Table ~~1~~2 and Figure ~~8~~9 seem to have a repetition of the data for membrane thickness. Also are there significant differences found between 3h, 24h, and 48h in Figure ~~8~~9?

The repetition of the data between Table 2 and Figure 9 is only true for data obtained after 48h storage of capsules. Figure 9 therefore also displayed evolution of membrane thickness in time after 3, 24 and 48h storage of capsules.

Authors performed statistical analyses on data displayed in Figure 9. The following sentence was therefore added in the Figure caption of the revised manuscript : «At the 0.05 level, the populations means were significantly different ».

Regarding the writing, some corrections should be applied to add/remove articles the/a/an.

Changings were made according to reviewer 3 where articles « the/a/an » were in strikethrough text in the revised manuscript (or sometimes added in the revised manuscript).

Response to Reviewer 4 Comments

Point-by-point answers to reviewer 4 are given below and corrections appear in red in the revised manuscript.

General comments

The objects of the paper are interesting and it makes sense to investigate the droplet characteristics of capsules formed by inverse gelation in more detail. However as Ca-alginate gels are highly porous gels, I seriously doubt about the protective effect of a Ca-Alginate layer against oxygen and water. What is the mean pore size of this protective layer? How can the gel protect against the diffusion of water?

Authors have no doubt about the porosity of alginate beads for which values ranging from 5 to 200 nm were obtained using TEM (Andresen et al. ACS Symp. Ser. 1977; 48 361-381) while values ranging from 12 to 16 nm were obtained using size exclusion chromatography (Klein et al. Eur. J. Appl. Microbiol. Biotechnol. 1983 18 86-91). The differences in porosity were explained by a difference in porosity between outside (pores of small size) and inside (pores of large size) of alginate beads (Smidsrod, Carb. Res. 1973 27 107-118). Authors suppose that the differences in porosity identified by Smidsrod could also exist in alginate membrane of capsules. The wide distribution of pores size therefore gives alginate beads and capsules a wide range of applications and in particular in drug delivery applications.

Specific comments

p. 4 line 76: the solution could also be sprayed instead of dripped to get smaller capsules

Authors totally agree with reviewer 4. The following sentence was therefore added in the revised manuscript: “Alternative to reduce size is therefore to spray alginate solution rather than dripping to a bath of $CaCl_2$ solution [7].”

The objective of the work is not made clear.

According to reviewer 4, authors have modified lines 105 to 109 to make objective of the work clearer.

The objective of the present work is, therefore, to produce core-shell alginate milli-capsules using a new droplets millifluidic/inverse gelation based process. The contribution tends to analyze the effect of varying curing time and cation concentration of the storage solution on the final properties of the obtained capsules, including oil content and mechanical properties.

p. 5 line 107 To study the effect of cation concentration is not mentioned at all in the introduction part and it seems that this study was added to explain the change in young modulus with shell thickness

The effect of cation concentration is mentioned page 5 line 107 “…varying the curing time and the cation concentration of the storage solution…”.

2.1 line 117 ff: why is tween 20 not mentioned?

Tween 20 (Sigma Aldrich, France) was therefore added in the material section line 122 in the revised manuscript.

2.3.3 line 179: Is alginate solution Newtonian?

Authors have determined that low concentration solution of alginate at C = 1% and sunflower oil were both Newtonian while W/O emulsion displayed a shear-thinning behaviour (data not shown as authors thought that it did not bring useful information to understand the contribution).

Table 1: What is D

D corresponds to the capsule diameter. Table 1 was therefore corrected as follows in the revised manuscript:

	Diameter D (mm)	Membrane thickness m (μm)	D/m	Surface Young modulus (N/m)	Force at break (N)
Wet capsules	2.21± 0.07	297± 76	7.4± 0.3	26.1± 1.8	1.15± 0.27
Dry capsules	1.85± 0.14	27± 12*	60.1± 5.9	613± 163	1.45± 0.37

Fig. 3 curves are difficult to distinguish

Authors agree with reviewer 4 and Figure 3 was redrawn in the revised manuscript by plotting data with different colours.

p. 12 285: How was the oil content of the dried capsules quantified?

Oil content was determined by TGA for both wet and dried capsules. The sentence in the revised manuscript was rephrased as follows: "By this way, it was therefore possible to use this technique to determine the oil content of the wet and dried capsules between 300 and 510°C".

Fig.4: Why did you choose different regions of interest and different magnifications?

Micrographs were taken at different regions of interest in order to highlight membrane growth and heterogeneity as function of curing time. Scale bar was constant at 250 μm for all micrographs.

Fig. 5: How is polydispersity defined and where do I find this value in the plot? I don't see an exponential increase as indicated in the text but a linear increase and saturation above a certain curing time

Authors totally agree with remark of reviewer 4 as polydispersity was not properly defined in the manuscript. Size polydispersity resulted from size measurements of capsule diameters using microscopy and could be ascribed to the standard deviation divided by the average size. The following sentence was therefore added in the revised manuscript: "The average diameter of alginate capsules ranged from 1.9 to 2.6 mm depending on curing time (significant differences at $p < 0.05$), with a size polydispersity lower than 10% (i.e. standard deviation / average size)".

Regarding the evolution of membrane thickness versus curing time, authors totally agree with the remark of reviewer 4. Authors therefore rephrased the sentence s follows in the revised manuscript: "From Figures 4 and 5, it was concluded that capsule diameter and membrane thickness were linearly dependent on curing time; in addition, membrane thickness reached a plateau at high curing times due to restricted calcium diffusion."

Table 2: Units are missing

Authors corrected Table 2 in the revised manuscript as follows:

Storage solution (wt%)	Diameter (d, mm)	Membrane thickness (Mt, μm)	d/Mt	Surface Young modulus (Es, N/m)	Es/Mt (N/m/μm)
Distilled Water	$2.27 \pm 0.20^{a,c}$	479 ± 74^{a}	$4.7 \pm 0.3^{a,d}$	23.4 ± 2.64^{a}	0.049
$CaCl_2$ 0.1	$2.43 \pm 0.17^{a,b,c}$	$388 \pm 84^{a,b,c}$	$6.3 \pm 1.7^{a,b,c}$	28.7 ± 4.6^{a}	0.073
$CaCl_2$ 0.3	$2.33 \pm 0.18^{a,b,c}$	$316 \pm 44^{b,c}$	$7.4 \pm 1.4^{b,c}$	$39.5 \pm 3.3^{b,c}$	0.125
$CaCl_2$ 1	$2.40 \pm 0.19^{a,b,c}$	$392 \pm 61^{a,b,c}$	$6.1 \pm 1.1^{a,b,c}$	$29.1 \pm 6.6^{a,b}$	0.074
$CaCl_2$ 1.5	2.60 ± 0.14^{b}	345 ± 48^{c}	7.5 ± 1.1^{c}	42.6 ± 6.9^{c}	0.123
NaCl 1.5	2.11 ± 0.14^{c}	748 ± 96^{d}	2.8 ± 0.5^{d}	2.3 ± 0.1^{d}	0.003

Fig. 8a: I can't see a linear decrease and the linear regression line is not justified at all!

Authors agree with remark of reviewer 4 where linear fitting of data in Figure 8a was highly speculative. The following figure was included in the revised manuscript where a discontinuous line was added as an eye-guide:

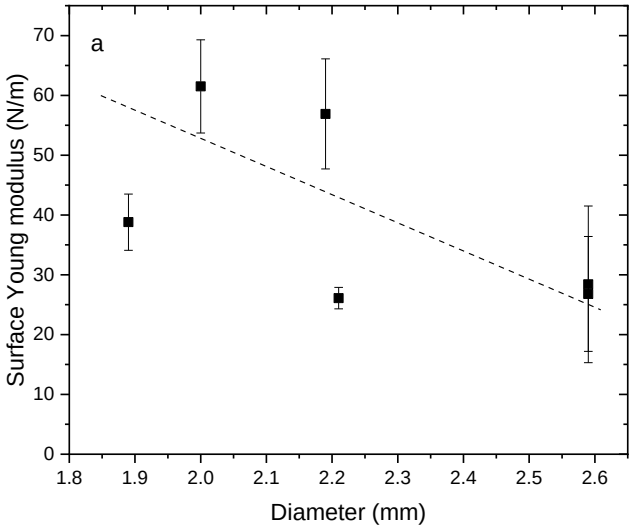

Fig. 8b and 8c: No plateau can be found as indicated in the text

Authors agree with remark of reviewer 4. This mistake was therefore corrected in the revised manuscript as follows: “This phenomenon was clearly seen on Figures 8b and 8c where a linear decrease was noticed for the surface Young modulus with the increase of membrane thickness and diameter / thickness ratio.”

p. 18 line 419: How does the young modulus change with curing time for dried microcapsules?

Measurements of the Young modulus were performed on dried capsule only at constant curing time (30 min).

CHARACTERIZATION OF CORE-SHELL ALGINATE CAPSULES GENERATED USING DROPLETS MILLIFLUIDIC

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ABSTRACT

A new droplets millifluidic/inverse gelation based process was used to produce core-shell alginate milli-capsules. Water-in-oil (W/O) emulsion dispersed phase containing Ca^{2+} ions was directly injected into a continuous alginate phase to generate a secondary W/O/W emulsion. Due to the cross-linking of alginate molecules by Ca^{2+} ions release, core-shell milli-capsules were formed with a very high oil loading. The influence of the curing time and of the storage conditions on capsules physico-chemical properties were investigated.

It was first found as expected that alginate membrane thickness increased with curing time in the collecting bath. However, a plateau was reached for the higher curing times, in close relation with previous observations (Martins, Poncelet, Marquis, Davy, & Renard, 2017b) that an external oil layer surrounded the surface of W/O emulsion drops that acted as a barrier and hindered the release of aqueous $CaCl_2$ droplets during curing time. Compression experiments on individual capsules revealed that alginate membrane thickness was inversely related to its mechanical properties, i.e. the thicker membrane, the lower surface Young modulus. Surface Young modulus ranged from 61 to 26 N/m at curing times of 3 and 45 min, respectively. This result was explained in terms of enhanced swelling properties of alginate membrane with curing time or storage conditions. Drying capsules led to much more resistant membranes due to the loss of water. Oil loading of 80 wt% was obtained for dry capsules whatever the conditions used.

KEYWORDS**:** Millifluidic; encapsulation; inverse gelation; alginate; sunflower oil.

1. INTRODUCTION

The study of microcapsules based on biodegradable polymers is of great importance in current scientific research worldwide due to the unusual combination of properties and versatility of these materials.

Microencapsulation avoids the degradation and volatilization of bioactives, protecting and preserving their biological activity and active functional ingredients. The microencapsulation technique has been mainly described as a process in which small particles or droplets are surrounded by a homogeneous or heterogeneous coating, forming beads or capsules with various applications [1]. There are three major fields of application: food production, e.g. ~~the~~ enhancement of nutrition by encapsulated vitamins, minerals or even pro-biotic bacteria; ~~the~~ cosmetic industry, where encapsulation of colors and flavors became more and more popular within the last years; and ~~the~~ drug delivery field.

Oils are widely applied in the formulation of foods, pharmaceutical and cosmetic products; however, they are often volatile, labile and sensitive to environmental factors such as heat, light, water and oxygen [2]. An efficient strategy to decrease their sensitivity towards environmental conditions consists of its encapsulation in inert polymer matrix using gelation/emulsification technique [3-5].

Alginate is one of the most widely used biocompatible polymers in microencapsulation, as it forms a highly versatile, biocompatible and nontoxic matrix of gel that protects the active components of factors such as heat and moisture thereby enhancing stability and bioavailability. Alginates are algal polysaccharides consisting of a linear chain of (1-4) linked residues of β-D-mannuronic acid (M) and α-L-guluronic acid (G) in different proportions and sequential arrangements [6]. This polymer has a remarkable property of quick gelation in presence of divalent ions such as calcium ions (Ca^{2+}).

Among the different methods used to prepare beads of gelled alginate, the most widely used encapsulation method is the simple extrusion/external gelation [7-8], wherein the formation of beads containing the solution of alginate and the active to be encapsulated is accomplished by using an extruder device dripping to a bath of $CaCl_2$ solution which induces alginate gelation [1]. The main limitations of this technique are the large size of beads formed, which depends on the diameter of the nozzle extruder, the large quantity of oil used to generate emulsion drops which is generally wasted, and the difficulty of large scale production because beads are formed one by one. One alternative to reduce size is therefore to spray alginate solution rather than dripping to a bath of $CaCl_2$ solution [7].

On the other hand, in the inverse gelation mechanism, oil and $CaCl_2$ solution are emulsified and added dropwise into an alginate solution bath [9], where Ca^{2+} ions diffuse from the emulsion drop to the alginate bath cross-linking the surrounding alginate molecules [9]. This technique allows producing core-shell capsules with an oil loading of nearly 100% (v/v) for dry capsules by using the dispersion-inversion gelation technique [10]. In addition, the production of microcapsules using droplets millifluidic is a promising strategy for oil encapsulation and allows the easy production of highly controlled and uniform microcapsules from W/O emulsions [11]. A similar droplets millifluidic set-up was used to produce core-shell alginate capsules with an aqueous core instead of an oil core [12]. The millifluidic device used in [11] consisted in a co-axial flow focusing geometry; water-in-oil (W/O) emulsion drops containing Ca^{2+} are directly injected through a capillary or needle into the co-flowing continuous phase (alginate solution) to form ~~the~~ micro-capsules downstream of the circuit. The oil encapsulation using this gelation mechanism is a very recent approach and is still scarcely studied [9, 2, 11]. For example, Martins et al. (2017b) studied the different factors affecting ~~the~~ emulsion stability, ~~the~~ capsule size and ~~the~~ membrane thickness. This study demonstrated the success of the production of (micro)-capsules based on the inverse

gelation mechanism using droplets millifluidic. Monodisperse (micro)-capsules with sizes ranging from 1.4 mm down to 140 μm were produced. The present work can be considered as a continuation of the work of Martins et al (2017b), where the focus was on the final properties of capsules and more specifically on the mechanical properties and oil content of the capsules. In many cases, the capsules are subjected to mechanical stresses exerted by their environment that induce deformation and potential breakup. It is important to control the mechanical properties of capsules to ensure protection and/or release of the encapsulated substances under proper conditions and also to avoid breakup during processing. The present study focused on capsules made of a deformable membrane with an internal liquid core.

The objective of the present work is, therefore, to take advantages of the new droplets millifluidic/inverse gelation based process to produce core-shell alginate milli-capsules and to analyze the effect of varying ~~the~~ curing time and ~~the~~ cation concentration of the storage solution on the final properties of the obtained capsules, including oil content and mechanical properties.

The use of millifluidic devices paves the way to an integrative formulation of core-shell materials with a broad size range of monodisperse capsules (from μm to mm) and new complex architectures. This should lead to the rapid emergence of new products in cosmetics or food, where the texture and visual aspect play a key role for sale.

2. MATERIALS AND METHODS

2.1. Materials:

Sodium alginate powder (Saltialgine S 60 NS) with a mannuronic (M) to guluronic (G) acid unit ratio (M/G) and a molar mass equal to 1.37 and 1.57×10^5 g/mol, respectively, was kindly donated by Cargill (France). Calcium chloride powder ($CaCl_2 \cdot 2H_2O$) (Panreac Quimica Sau, Spain), sunflower cooking oil (Associated Oil Packers, France), PGPR 90

(Danisco, France), Tween 20 (Sigma Aldrich, France) were used to prepare the W/O emulsions. Other chemicals reagents were obtained from Sigma Aldrich (France).

2.2. Methods:

2.2.1. Preparation of alginate and calcium chloride solutions

Ten grams of alginate powder was dissolved in 1 L of demineralized water using a magnetic stirrer. Tween 20 (0.5%, v/v) was added to the alginate solution. The calcium chloride solution was prepared by dissolving 240 g of $CaCl_2 \cdot 2H_2O$ in 1 L of demineralized water.

2.2.2. Preparation of the W/O emulsions

Emulsions were prepared according to Martins et al. (2017b). One hundred millilitres of sunflower oil containing 0.96 g of surfactant (PGPR 90) was mixed using a high shear mixer (Ultra-Turrax T25, IKA, Germany) at 18 000 rpm for 1 min. Sixty millilitres of calcium chloride solution (240 g/L) was then added slowly and a new shear mixing at 18 000 rpm for 3 min was performed.

2.2.3. Capsules production by droplets millifluidic

A millifluidic device with a co-axial flow focusing geometry was used as described in [11] (Figure 1, Supplementary data). The dispersed phase, a W/O emulsion freshly prepared (see section 2.2.2.) was pumped (Harvard Apparatus PHD 2000, France) through a fused silica capillary tube (interior diameter (ID) 530 µm and outside diameter (OD) 660 µm) at a rate (Q_{emul}) of 4 mL/h. The continuous phase, an alginate solution added with Tween 20, was pumped through a Teflon tube (ID=1.57 mm and OD= 0.5 mm) at a rate (Q_{alg}) of 10 mL/h. The W/O emulsion and the alginate solutions co-flowed in the glass tube (ID= 2 mm, OD= 4 mm and length of 10 cm). As the generation of capsules using W/O emulsion requires

hydrophilic surfaces, the glass tube was previously immersed in a saturated-NaOH solution for 5 min at room temperature and rinsed using tap water [13].

W/O emulsion drops were formed and dispersed in the continuous alginate phase. Calcium ions (Ca^{2+}) diffused from the W/O emulsion drop cross-linking the alginate molecules. The capsules were collected in an alginate bath stirred at 50 rpm. After a curing time (time of contact between the capsules and the alginate solution) varying between 3 and 50 min, the capsules were sieved, rinsed with distilled water, suspended in a Ca^{2+} or Na^{+} solution or in distilled water and stored at 4°C until use.

2.3. Characterization of the W/O emulsions

2.3.1. Stability

~~the~~ W/O emulsion stabilities were performed as described by Martins et al. (2015). Hundred millilitres of W/O emulsions were placed in tubes of 100mL with graduation of 1mL. The emulsions were kept at ambient temperature (20± 2 °C) and visually inspected as a function of time in order to assess the critical time for which 1% of phase separation occurred (i.e. corresponding to 1 mL of phase separated liquid).

2.3.2. Microscopic observations

W/O emulsions were diluted ten times in sunflower oil and observed under the microscope. The diluted emulsions were brought on a microscope slide using a capillary after which a cover slip was gently applied. Image acquisition was conducted using an optical microscope (Leica Microsystems, France). The size of $CaCl_2$ solution droplets (dispersed phase) was determined by image analysis using the ImageJ 1.47v freeware (National Institutes of Health, Bethesda, Maryland, USA). ~~the~~ Measurements were carried out in triplicate with three repetitions. In other words, three W/O emulsions of each formulation were analyzed by

optical microscopy. Three different zones (1.1 × 1.5 mm) for each sample were imaged and approximately one hundred droplets per zone were measured.

2.3.3. Rheological measurements

The viscosity of sunflower oil, alginate solution and water in-oil emulsions were investigated with a stress-controlled rheometer (AR-2000, TA Instruments, New Castle, DE) equipped with a Couette geometry (DIN 412). The temperature was controlled with a Peltier system. ~~the~~ Dynamic viscosity and shear stress as a function of shear rate between 0.01 and 1000 s^{-1} were measured. Viscosity of alginate solution at C = 1% (w/v) gave η = 35.9 ± 3.9 mPa.s while that of sunflower oil was of 70.2 ± 1.7 mPa.s. W/O emulsion had a viscosity η = 212.5 ± 7.4 mPa.s.

2.4. Characterization of capsules

2.4.1. Microscopic observation

~~The~~ External diameter (D), and membrane thickness (m) of capsules were performed under an optical microscope (Olympus IX51, France) equipped with a camera (Olympus DP70). A 40X objective was used to image microcapsules.

Dp controller software (version 2.1.1) was used for images acquisition. Shell thickness (M_t) and capsules diameter (d) were therefore calculated from image analyses on 10 capsules per experimental condition using the imageJ 1.47v freeware (National Institutes of Health, Bethesda, Mayland, USA).

2.4.2. Total oil content of the capsules

A TGA Q5000 instrument was used for the thermo-gravimetric analysis (TGA). An isothermal step at 60° C during 30 minutes was applied before the ramp up to 800° C at

10 °C/min under a nitrogen atmosphere (25 mL/min). The capsules were dried 48 h at room temperature and a_w=0.59 (NaBr) before TGA analyses. Oil content was determined by analyzing the thermograms of pure components and capsules.

2.4.3. Mechanical study

A Dynamic Mechanical Analyzer (Rheometric Scientific, EEUU) equipped with a plate-plate geometry (diameter of 20 mm) was used. The upper plate had a diameter of 20 mm and the normal force was measured as a function of the compression in a range between 0.001 and 20 N. A force gap test was used to compress the capsules with a linear compression speed of 0.6 mm / min. The gap and the normal force being imposed were measured simultaneously at the upper plate. The surface Young modulus was calculated by a quantitative analysis of force–displacement curves at small deformations [14-15]. Five replicates were considered for each capsule.

2.4.4 Statistical analysis

Data values obtained in the experiments were statistically analyzed by one-way analysis of variance (ANOVA) employing OriginLab 8.5 software. Differences between pairs of means were assessed on the basis of confidence intervals using the Tukey test. The least significance difference was $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Characterization of ~~the~~ W/O emulsions

Emulsions containing sunflower oil, $CaCl_2$ solution (240 g/L) and PGPR 90 were prepared using a high shear mixer. The dispersed W/O phase contained the calcium content that allows

to crosslink the alginate and form the membrane of the capsules. ~~the~~ Knowledge of the stability is very important as it is directly related with the Ca^{2+} release, and therefore the membrane formation.

PGPR 90 was chosen as emulsifier due to its low hydrophilic-lipophilic balance value (HLB=1.5), which made it prone to stabilize W/O emulsions [16].

Several images of the emulsion in function of storage time, formed by dispersing calcium aqueous phase into the sunflower oil containing the surfactant PGPR 90, were shown in Fig. 1. Minimal phase separation and droplet coalescence occurred during storage over 24 h. Martins et al (2017b) studied the effect of surfactant concentration on the stability of the emulsions. By increasing emulsifier concentration, W/O emulsions with stabilities increasing from 0.9 to 132 h were found. Marquez et al. [17] and Su et al. [18] found that higher the emulsifier concentration was, lower was the size of water droplets in ~~the~~ W/O emulsion, increasing both its viscosity and stability.

In addition to visual inspections of the emulsion stability, the drops size of the dispersed aqueous phase was measured after 10 times dilution with oil by optical microscopy. Figure 2a showed an image of a W/O emulsion stabilized by PGPR 90 at 6 g/L. High shear mixing led to emulsions with a narrow size distribution and a mean diameter of the dispersed aqueous phase drops of about 1.4 μm.

In order to confirm that it is a W/O emulsion, a drop of the emulsion was dispersed in water (Figure 2b) and analyzed under optical microscopy. A double W/O/W emulsion was formed by an oil continuous phase and a first dispersed $CaCl_2$ solution droplets internal phase surrounded by a second dispersed $CaCl_2$ external phase.

3.2. Characterization of components by TGA

~~the~~ Thermal degradation behavior of pure components that form the capsules (alginate, calcium and sunflower oil) was performed and analyzed by TGA up to 800°C, with the idea of identifying the degradation temperature of each component. Figure 3 showed the TGA thermograms and derivative thermograms dTGA of sunflower oil, $CaCl_2$ and alginate powders.

By analyzing the TGA of $CaCl_2 \cdot 2H_2O$, a considerable weight loss was clearly observed between 25°C and 130°C that was ascribed to the endothermic removal of crystalline and loosely bound surface water, as it was also observed by Karunadasa et al. [19]. There was a first endothermic dehydration at around 130 °C accounted for the emergence of monohydrate calcium chloride ($CaCl_2.H_2O$) (Figure 3). A significant reduction in weight of calcium chloride was observed during the second endothermic dehydration (between 150 and 220 °C, dTGA curve, Fig. 3) that further ensured the complete removal of crystalline water from the material. Although the magnitude of second endothermic dehydration was significantly larger than the first one, it was concluded that the removal of crystalline water from the monohydrate was rather difficult compare to the dihydrate calcium chloride (Fig. 3, dTGA curve). This was a good indication of the strong association of crystalline water in monohydrate that requires a considerable amount of thermal energy to eliminate it from the monohydrate [19].

~~The~~ TGA/dTGA curves for sunflower oil displayed two thermal decomposition steps in the range of 300 to 510°C, with no residue remaining at 800°C (see Figure 3). It was observed that the first step (300 to 420°C) corresponded to the decomposition of the polyunsaturated fatty acids. During heating, the triglycerides, which form 96 to 98% of the edible oils, produce volatile compounds, which are constantly removed by vapor generated during heating. These products (dimers, trimers, polymers) are formed principally by thermal reactions of unsaturated fatty acids, such as linoleic acid. The first step is the most important for the

thermal stability of edible oils, because this is the step where decomposition of the unsaturated fatty acids begins. The thermal stability of sunflower oils is dependent on the composition of the fatty acids, as it is influenced by artificial antioxidants [20]. The beginning of oxidation in edible vegetable oils is characterized by absorption of oxygen through the fatty acid chain, subsequently forming the oxidation product as peroxides. This behavior is generally identified by an increase in the initial mass of the sample. The second step in the thermal decomposition of sunflower oils (420 to 520°C) corresponds to the decomposition of monounsaturated fatty acids such as oleic acid and saturated fatty acids, such as palmitic acid. During this reaction, the double bonds are broken, causing the triglyceride molecules in the edible vegetable oils to become saturated [20].

Figure 3 also showed ~~the~~ TGA and dTGA curves for ~~the~~ sodium alginate. It is shown that the salt decomposed by dehydration followed by degradation to Na_2CO_3 and ~~a~~ carbonized material that decomposed slowly from 550-750 °C in N_2 (Figure 3), as also noticed by other authors [21-22].

Taking into account that ~~the~~ alginate content in the dried capsules as well as the degradation peaks observed by dTGA curves are negligible, compared to the sunflower oil alone, it was considered that temperature range of the oil degradation did not overlap with those of alginate and $CaCl_2$ pure components. By this way, it was therefore possible to use this technique to determine the oil content of the wet and dried capsules between 300 and 510°C.

3.3. Microcapsules formation

Based on Martins et al (2017b) previous results, the mechanism of capsule formation would occur in three steps:

I- ~~the~~ Emulsion drops characterized by a continuous oil phase containing $CaCl_2$ solution droplets contacted the alginate solution

II- ~~the~~ $CaCl_2$ solution droplets near the oil-alginate solution interface were able to migrate from the emulsion to the alginate phase by a mechanism of diffusion/permeation through the oil layer [23]

III- ~~the~~ Ca^{2+} ions cross-linked the alginate chains resulting in the formation of a membrane.

3.3.1. Effect of curing time

W/O emulsions containing 90 g/L of $CaCl_2$ and 6g/L of PGPR 90 were co-flowed with the alginate-Tween 20 continuous phase in order to generate monodisperse W/O emulsion drops that finally reached the alginate bath. These capsules were kept in contact with the alginate bath by increasing curing time from 3 to 50 minutes. After these curing times, capsules were filtered, rinsed with distilled water and stored in a $CaCl_2$ solution (15 g/L) at 4°C before ~~the~~ microscopy observations.

3.3.1.1. Size of capsules and membrane thickness

Microscopic observations revealed that spherical capsules with a well-defined alginate membrane were collected in the alginate bath whatever the curing time (Figure 4).

Figure 5 showed the dependence of ~~the~~ capsule diameter and ~~the~~ membrane thickness with ~~the~~ curing time. The average diameter of alginate capsules ranged from 1.9 to 2.6 mm depending on curing time (significant differences at $p < 0.05$), with a size polydispersity lower than 10% (data not shown). Other authors also found that microcapsules produced using microfluidics had extremely narrow size distributions [24]. Using droplets millifluidic process, capsule size depends on droplet dimension before getting into contact with the alginate phase, ~~the~~ droplet dimension depending on both the internal diameter of the Teflon tube (which was constant and equal to 1.57 mm throughout the experimental assays) and the dispersed over continuous flow rates ratio (also constant and equal to 0.4).

Membrane thickness measurements (made from pictures obtained by optical microscopy) showed a curing time-dependent thickness of alginate capsules which increased with curing time (from 98 ± 28 to 306 ± 21 μm at 3 and 45 minutes, respectively) (significant differences at $p < 0.05$) (see Figure 5). The increase of ~~the~~ membrane thickness was significant at low curing times and reached a pseudo-plateau at high curing times. The capsules reached therefore the maximum of membrane thickness after 30 min of curing time. This result was consistent with the increase of diffusion resistance and the decrease of calcium chloride concentration in time [25]. Blandino *et al*. [26] obtained nearly the same evolution for the thickness of gel layer capsules as a function of time for an alginate-CMC capsule system where thickness was measured by cutting the capsules into halves and examining the membranes with a microscope.

From Figures 4 and 5, it was concluded that capsule diameter and membrane thickness were linearly dependent on curing time; in addition, membrane thickness reached a plateau at high curing times due to restricted calcium diffusion. Martins et al. [11] checked that the emulsion drop surface was surrounded by an oily layer that limited the contact of the $CaCl_2$ solution droplets with the external media. This external oil layer could act as a barrier hindering the release of aqueous $CaCl_2$ droplets during the capsule production.

3.3.1.2. Capsules oil content

In this study, droplets millifluidic was used as a rapidly expanding technology with a unique ability to "package" materials in the form of millicapsules, and a natural polysaccharide from brown seaweed as a stable container of sunflower oil. Therefore, it was very important to ascertain the capacity of the polymeric matrix to hold the bioactive compounds.

Oil content was around 80% for all the analyzed dried millicapsules, a content quite high compare to other encapsulation processes based on the external and on the internal gelation

mechanisms [27]. Once the emulsion was in contact with alginate, the process of membrane formation occurred but the oil was already encapsulated. As the contribution of the membrane weight was negligible with respect to the oil content, the increase in the curing time did not represent a substantial change of the final core content (Figure 2, supplementary data). This was the main reason why, despite the high reproducibility of the method to produce monodisperse core-shell capsules, the oil content was always constant.

3.3.1.3. Mechanical properties of capsules

Mechanical behavior is a very important characteristic, as capsules or beads have to withstand mechanical stress caused by shear forces, compression or osmotic pressure, during the manufacture of the food product, packaging, transport, storage, handling and consumption. In particular, mechanical stiffness of alginate gel millicapsules, controlled by the strength and number of cross-links between the polymer chains, influences the permeability of the capsules, property highly important for solutes diffusion in cell encapsulation [28].

Numerous methods exist to determine the mechanical properties of capsules [29]. A well-established method consists in uniaxial compression of a single capsule between two plates and recording the force-displacement curve. Compression experiments are generally sufficiently sensitive to characterize the mechanical behavior of liquid-filled alginate capsules.

Elastic characteristics such as Young modulus are calculated from the initial slope of the force-displacement curve by means of different approaches (e.g. Hertz model) [15, 24, 29, 30]. The advantage of single-bead measurement consists in detailed information of a single object and variations between beads of one production batch can be thoroughly analyzed. Otherwise, it is time consuming to collect substantial, statistical significant data [24, 31].

Alginate capsules are sensitive to deformations that may lead to their rupture or to undesirable early release of their content. It was demonstrated from the analyses of force-indentation curves obtained by AFM that alginate microspheres, beads and capsules had almost a pure elastic behavior [32]. Surface Young modulus, or two-dimensional Young modulus, is therefore an interesting parameter to quantify mechanical stability as it describes the elastic properties of the capsules. From Equation (1), compression curves at small deformations were linearly fitted in order to extract the two-dimensional (or surface) Young modulus *Es* under point loading close to the pole [15]:

$$F = \frac{4Es\,m}{a\sqrt{3(1-vs^2)}}dD \qquad \text{Eq. 1}$$

In this equation, *dD* is the capsule displacement, *F* the measured force, *Es* the surface Young modulus, *m* the membrane thickness, *a* the radius of the capsule and *vs* the surface Poisson ratio for which a value of 1/3 was assumed according to [25]. Other more complicated methods of resolution by solving numeric calculations were presented in further citations [33, 34]. It was therefore important to recall that Eq. 1 only holds for spherical capsules in the very small deformations regime.

A typical force (F) versus displacement (D) curve from compression of single dry and wet alginate capsules were shown in Figure 6. As expected, the drying step induced an approximately ten-fold reduction of the membrane thickness due to water loss and, consequently, a huge increase of the surface Young modulus was noticed (see Table 1 and Figure 6).

Figure 7 summarized surface Young modulus values of wet alginate capsules in function of curing time (calculated by linear regression from compression curves at small displacements). Surface Young moduli (significant differences at $p < 0.05$) approximately two-fold decreased

with increasing curing time, result in apparent contradiction with the increase of membrane thickness with curing time. ~~The~~ Surface Young modulus values were in the same order of magnitude that those obtained by Leick et al. [25] for alginate capsules obtained from two types of alginate with different guluronic acid content: 6.5 N/m and 32.9 N/m, for the higher and lower content of guluronic acid, respectively. From another study, Leick et al. [35] found surface Young moduli of 10 and 5 N/m for pure alginate and alginate/PLL capsules taking a Poisson ratio of zero, values consistent with the assumption of a lower cross-linking degree of the alginate/PLL composite capsules. More interestingly, each additional electrolyte adsorption layer (PLL or alginate) gave roughly an increase of the surface Young modulus of about 5 N/m for the investigated alginate/PLL/alginate capsule system under the used preparation conditions. Messaoud et al. [36] investigated the influence of pH for alginate capsules, and they obtained surface Young moduli ranging from 14.9 to 22 N/m at pH 7 and pH 2, respectively, indicating that a decrease in pH resulted in a stiffer alginate membrane. The same pH-dependence thickness of composite pectinate/shellac capsules was found by Leick et al. [37]. The dependence of the pH value for composite capsules on the deformation was also consistent with variations of the membrane thickness. Membranes prepared with additional shellac seemed to be thinner but stiffer. Furthermore, at the lowest pH value, the highest amount of precipitated shellac led to the strongest inhibition of calcium ion diffusion and therefore to the thinnest membranes.

Figure 8 displayed the evolution of the surface Young modulus with the capsule dimensions. Apart from the slight decrease of surface Young modulus with the increase of wet capsule diameter (Figure 8a) and the linear decay with membrane thickness (Figure 8b), it was noticed that the surface Young modulus linearly increased with the diameter / thickness ratio of capsules (Figure 8c). Rachik et al. [14] reported that the increase in membrane thickness was linked to an increase in intrinsic membrane rigidity as it was expected that the density of

crosslinking was larger in thicker membranes. Consequently, the surface Young modulus *Es* that measures the whole ability of the membrane to deform upon deformation increased. Contrary, in our study, surface Young modulus ranged from 68 to 21 N/m at 3 and 50 min, respectively, (see Table 1), indicating that an increase of curing time led to an increase of membrane thickness (see Figure 8b) and to a decrease of the mechanical properties (lower surface Young modulus E_s values) resulting in softer alginate membranes. This decrease of the two-dimensional modulus could be explained by a higher hydration level of the membrane leading therefore to a decrease of the crosslinking degree, i.e. the density of ~~covalent~~ ionic bonds between Ca^{2+} and alginate would be smaller in the thicker membrane due to water uptake. Following this hypothesis, ~~the~~ calcium content from the W/O droplets quickly diffused from the emulsion-core to the alginate solution and remained constant with time. This phenomenon was clearly seen on Figures 8b and 8c where a linear decrease was noticed for the surface Young modulus with the increase of membrane thickness and diameter / thickness ratio. This result therefore gave more confidence to the hypothesis of Martins et al. [11] who clearly identified by optical microscopy that the emulsion drop surface was surrounded by an oily layer that limited the contact of ~~the~~ $CaCl_2$ solution droplets with ~~the~~ alginate phase. This external oil layer therefore acted as a barrier hindering the release of aqueous $CaCl_2$ droplets during curing time.

3.3.2. Effect of cation concentration

In order to understand the effect of the storage solution on both membrane thickness and surface Young modulus, capsules with a curing time of 30 min in an alginate bath were stored in NaCl solution (1.5%wt) and several $CaCl_2$ solutions at concentrations ranging from 0 to 1.5%wt.

Microscopic observations were performed during a 48 h period to see the evolution of membrane thickness (see Figure 9) (significant differences at $p < 0.05$). The first important observation was that membrane thickness was higher when capsules were stored in distilled water contrary to $CaCl_2$ solutions, result in agreement with the swelling of alginate membrane in time. After 24 h of storage in $CaCl_2$ solution, ~~the~~ membrane thickness reached a plateau whatever ~~the~~ $CaCl_2$ concentration (Figure 9). This result was in accordance with the absence of diffusion of calcium ions from inside to the outside of the capsules and conversely hindering the growth of alginate membrane in time. Finally, the storage of capsules in NaCl solution led to a huge increase of membrane thickness in time, result in accordance with the competition of calcium and sodium ions for alginate cross-linking and the resulting swelling of alginate membrane due to the loss of calcium ions in the cross-links. The membrane thickness measured at 48 h was used to evaluate the mechanical properties immediately after the microscopic observations.

Table 2 showed the surface Young modulus values obtained from the analyses of the force displacement curves. Surface Young modulus values were constant with an average value of 35 N/m whatever the calcium concentration used for the storage of alginate capsules, result in agreement with the constant value of membrane thickness (Mt ~ 360.3 µm).

In order to eliminate the influence of membrane thickness on the surface Young modulus and to facilitate the comparison between the different storage conditions, Table 2 also showed a normalized Surface Young modulus defined as *Es*/Mt. Even if it was confirmed that Es/Mt was constant whatever the calcium concentration used for capsules storage with Es/Mt ~ 0.099, it was noticed that Es/Mt two-fold decreased when capsules were stored in distilled water and more important thirty three-fold decreased when capsules were stored in NaCl. The increase of membrane thickness according to the decrease of Es/Mt was of 33% in water and of 107% in NaCl. Swelling process of alginate membrane could be evoked to explain the

increase of membrane thickness and therefore the decrease of surface Young modulus depending on ~~the~~ storage conditions. This swelling process would be considerably favored in presence of sodium salt compare to distilled water in particular due to the competition of sodium and calcium ions for the guluronic acid residues on alginate chains favoring the large decrease of cross-linking density in the alginate network and the huge increase of water uptake within the membrane. Authors recently clearly demonstrated, using spinning drop techniques, capsule squeezing experiments and interfacial shear rheology, changes in the mechanical properties of alginate membrane after storage of capsules in calcium or sodium salt solutions [38]. For instance, surface Young modulus determined by compression experiments decreased from 41 to 6.0 N/m when capsules were stored in calcium and sodium salt solutions, respectively. This effect was ascribed to changes of the cross-linking density and therefore a reduction of cross-linking density due to ion exchange in the case of sodium salt. It was also found recently that sugar concentrations above 15% (wt) reduced extensibility of alginate molecules and lead to a more open or less connected gel network with aggregated alginate strands [39]. In addition, authors also demonstrated the high swelling rate of alginate capsules without sugar in presence of simulated intestinal fluid, fluid containing sodium hydroxide and potassium phosphate. The effect of sodium salt could be similar to sugar or intestinal fluid by changing the alginate network and in particular pore diameter allowing a higher uptake of water in the membrane. Pore diameters ranging from 2.52 to 3.94 nm were recently reported, using low temperature N2 adsorption-desorption analysis, depending on alginate sample and sodium content [40].

CONCLUSIONS

Stable W/O emulsions with a narrow size distribution were obtained by homogenization using PGPR 90 as surfactant. Spherical millimetric capsules were successfully obtained by inverse

gelation using droplets-based millifluidic. The effect of curing time and cation concentration on the oil content, structure and mechanical properties of capsules was analyzed. Alginate membrane thickness increased with curing time in the collecting alginate bath until a plateau was reached, in close relation with the presence of an oily layer surrounded the surface of W/O emulsion drops therefore limiting the contact of ~~the~~ $CaCl_2$ solution droplets with ~~the~~ alginate phase. This external oil layer therefore acted as a barrier hindering the release of aqueous $CaCl_2$ droplets during curing time. Compression experiments on individual capsules revealed that alginate membrane thickness was inversely related to its mechanical properties, i.e. the thicker membrane, the lower surface Young modulus. This result was explained in terms of enhanced swelling properties of alginate membrane with curing time or storage conditions. Drying capsules led to much more resistant membranes due to the loss of water. Oil loading of 80 wt% was obtained for dry capsules whatever the conditions used.

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Table 1. Mechanical properties of wet and dry capsules. Curing time = 30 min. conditions.

	Diameter D (mm)	Membrane thickness Mt (μm)	D/Mt	Surface Young modulus (N/m)	Force at break (N)
Wet capsules	2.21± 0.07	297± 76	7.4± 0.3	26.1± 1.8	1.15± 0.27
Dry capsules	1.85± 0.14	27± 12*	60.1± 5.9	613± 163	1.45± 0.37

*Membrane thickness was estimated from other capsules that were obtained and dried in the same

Table 2. Surface Young modulus values of capsules after 48h storage in distilled water, $CaCl_2$ or NaCl solutions. Curing time in alginate solution = 30 min.

Storage solution (wt%)	**Diameter (d, mm)**	**Membrane thickness (Mt, μm)**	**d/Mt**	**Surface Young modulus (Es, N/m)**	**Es/Mt (N/m/μm)**
Distilled Water	$2.27 \pm 0.20^{a,c}$	479 ± 74^{a}	$4.7 \pm 0.3^{a,d}$	23.4 ± 2.64^{a}	0.049
$CaCl_2$ 0.1	$2.43 \pm 0.17^{a,b,c}$	$388 \pm 84^{a,b,c}$	$6.3 \pm 1.7^{a,b,c}$	28.7 ± 4.6^{a}	0.073
$CaCl_2$ 0.3	$2.33 \pm 0.18^{a,b,c}$	$316 \pm 44^{b,c}$	$7.4 \pm 1.4^{b,c}$	$39.5 \pm 3.3^{b,c}$	0.125
$CaCl_2$ 1	$2.40 \pm 0.19^{a,b,c}$	$392 \pm 61^{a,b,c}$	$6.1 \pm 1.1^{a,b,c}$	$29.1 \pm 6.6^{a,b}$	0.074
$CaCl_2$ 1.5	2.60 ± 0.14^{b}	345 ± 48^{c}	7.5 ± 1.1^{c}	42.6 ± 6.9^{c}	0.123
NaCl 1.5	2.11 ± 0.14^{c}	748 ± 96^{d}	2.8 ± 0.5^{d}	2.3 ± 0.1^{d}	0.003

^{a,b,c,d} Different letters in the same column indicate significant differences among the different storage solutions ($p < 0.05$).

Figure captions

Figure 1. Images of W/O emulsions as function of storage time.

Figure 2. Micrographs of a) oil diluted W/O emulsion (scale bar 25 µm) and b) water diluted W/O emulsion (scale bar 250 µm) resulting in the formation of a double W/O/W emulsion.

Figure 3. Thermogravimetric analyses of sunflower oil, alginate and calcium chloride powders used to prepare capsules.

Figure 4. Micrographs of wet capsules as function of curing time. Scale bar 250 µm.

Figure 5. Diameter d (mm) and membrane thickness Mt (µm) of wet capsules as function of curing time. Solid line corresponded to the fitting of the data by a linear function; dotted line was just eye-guide. At the 0.05 level, the populations means were significantly different.

Figure 6. Compression behavior of wet and dry capsules. Curing time = 30 min.

Figure 7. Surface Young modulus (N/m) of wet capsules as function of curing time. At the 0.05 level, the populations means were significantly different.

Figure 8. Evolution of the surface Young modulus with wet capsule dimensions: a) diameter; b) membrane thickness and c) diameter/thickness ratio.

Figure 9. Evolution of the membrane thickness after different storage times in distilled water, $CaCl_2$ or NaCl solutions. Curing time in alginate solution = 30 min. At the 0.05 level, the populations means were significantly different.

Figure 1

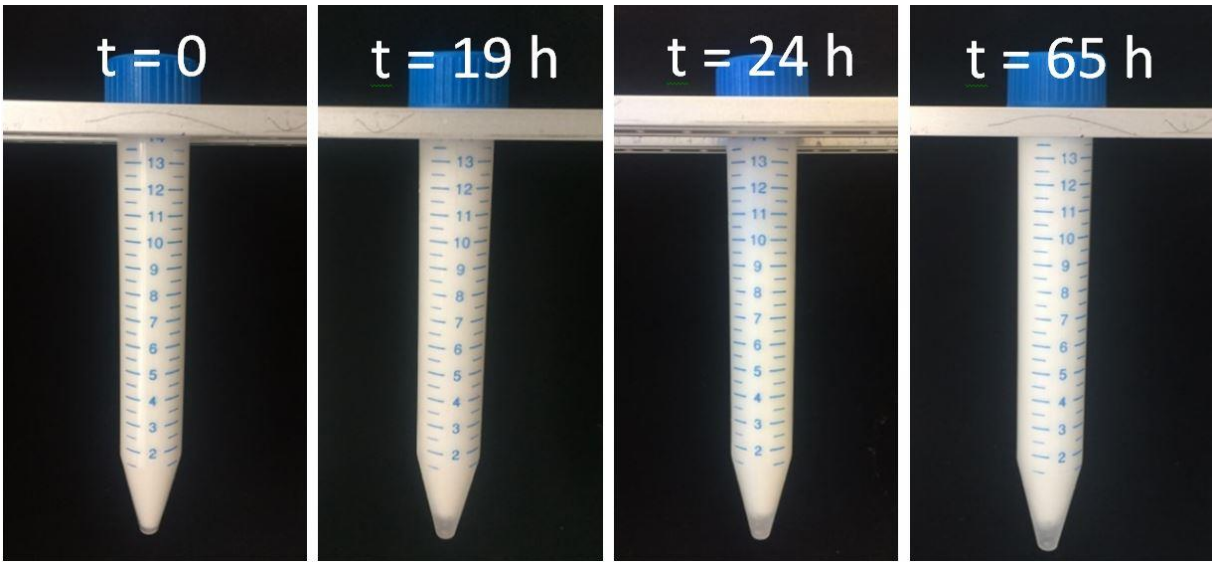


Figure 2

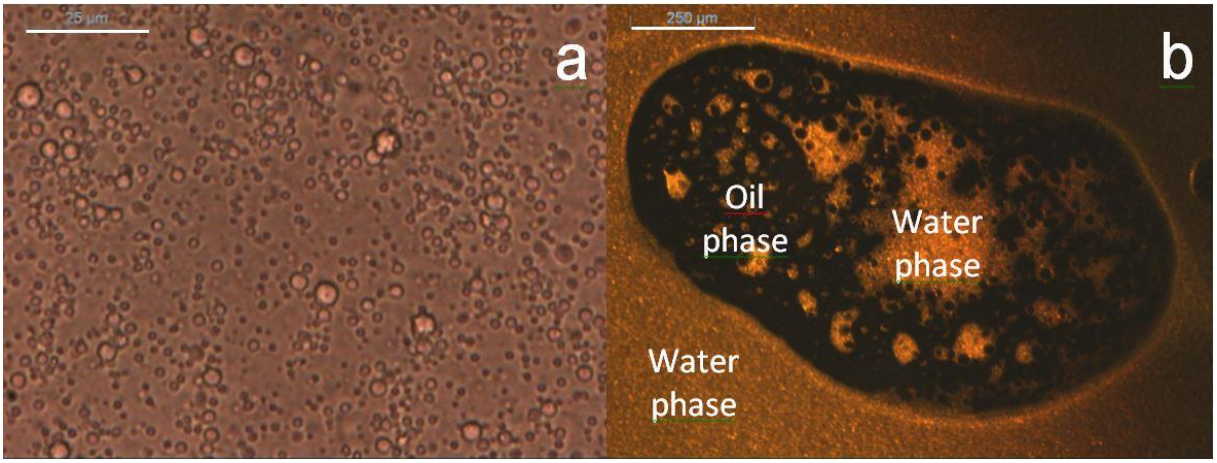


Figure 3 (revised)

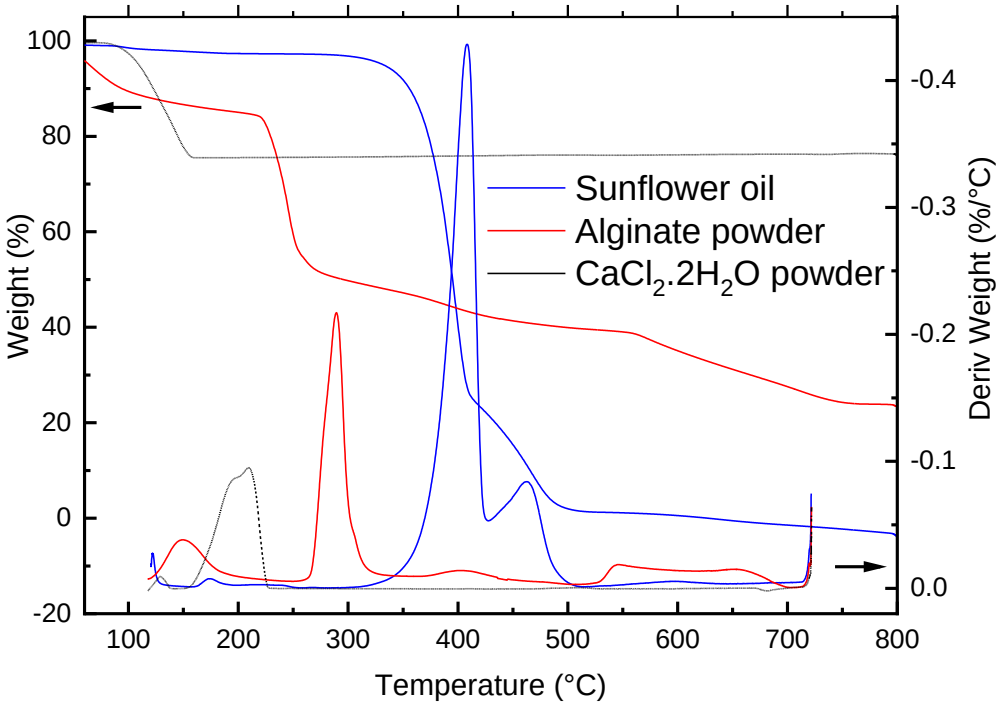


Figure 4 (revised)

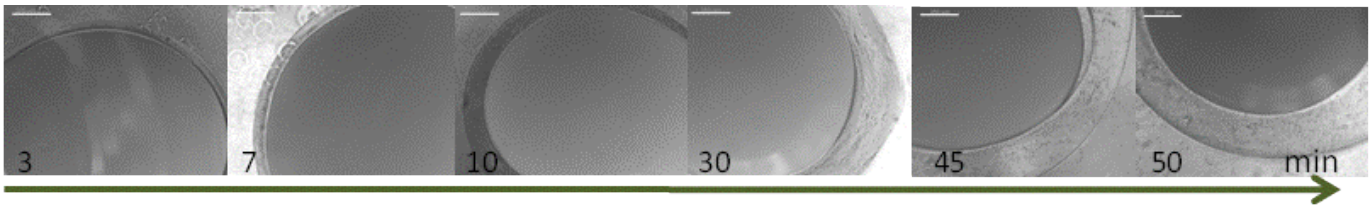


Figure 5 (revised)

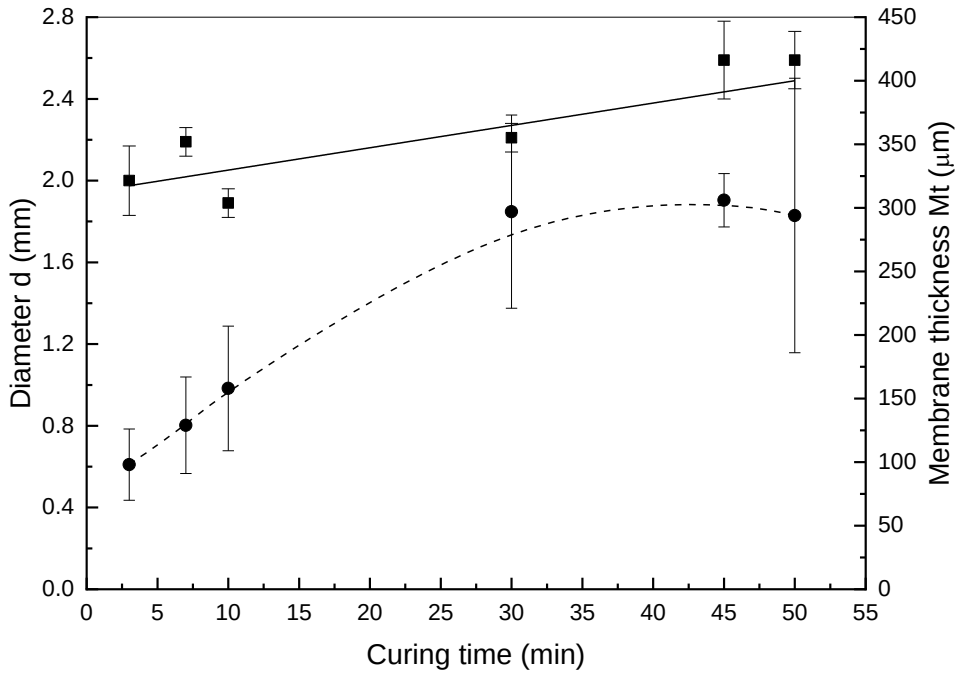


Figure 6

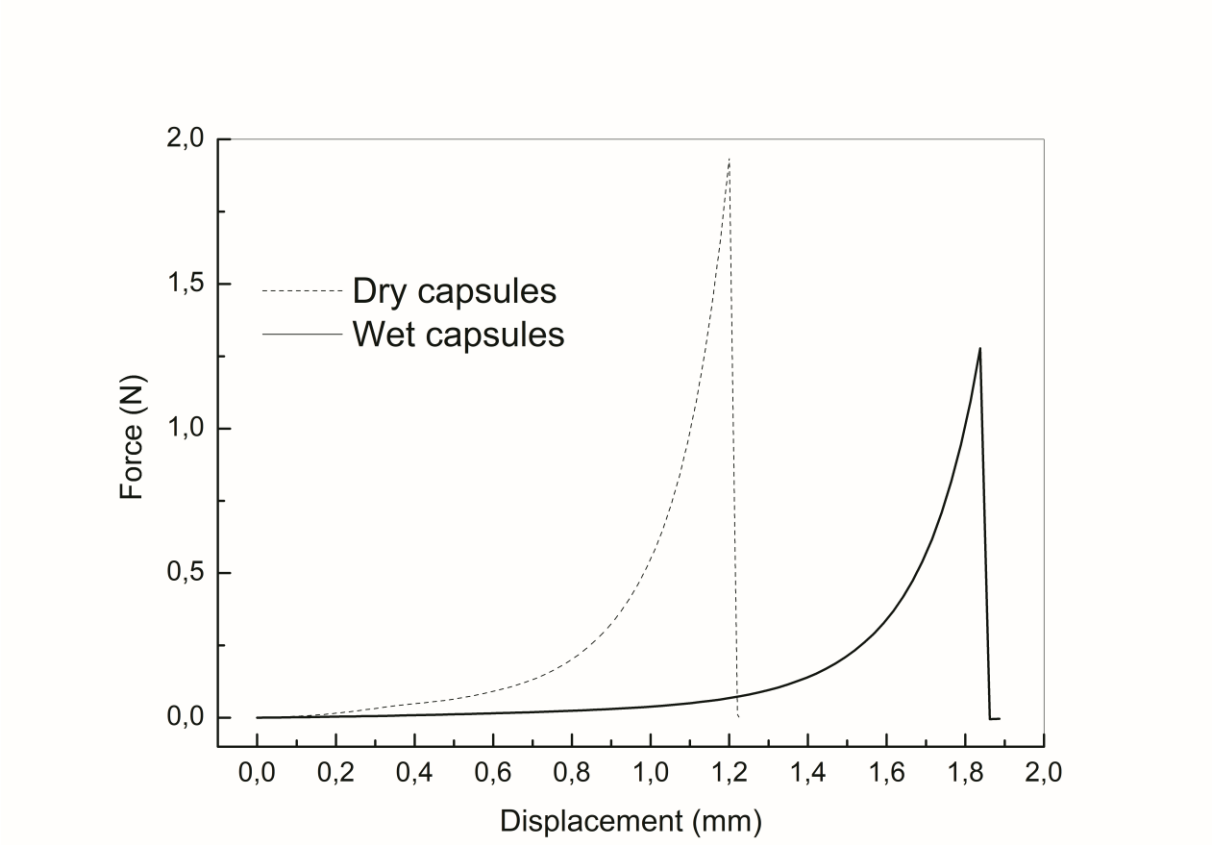

Dry capsules
Wet capsules
Force (N)
Displacement (mm)
2,0
1,5
1,0
0,5
0,0
0,0
0,2
0,4
0,6
0,8
1,0
1,2
1,4
1,6
1,8
2,0

Figure 7 (revised)

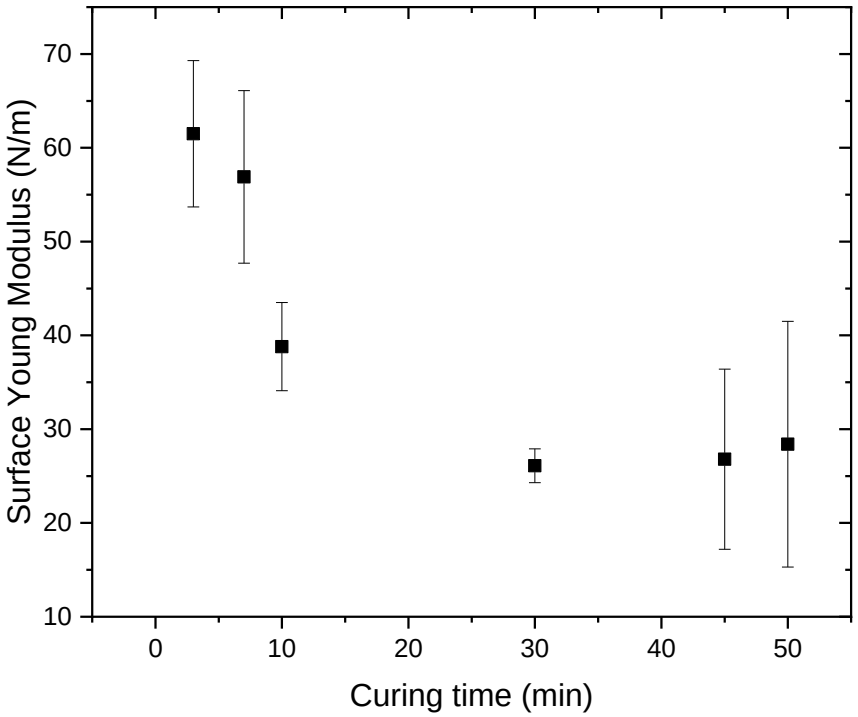


Figure 8 (revised)

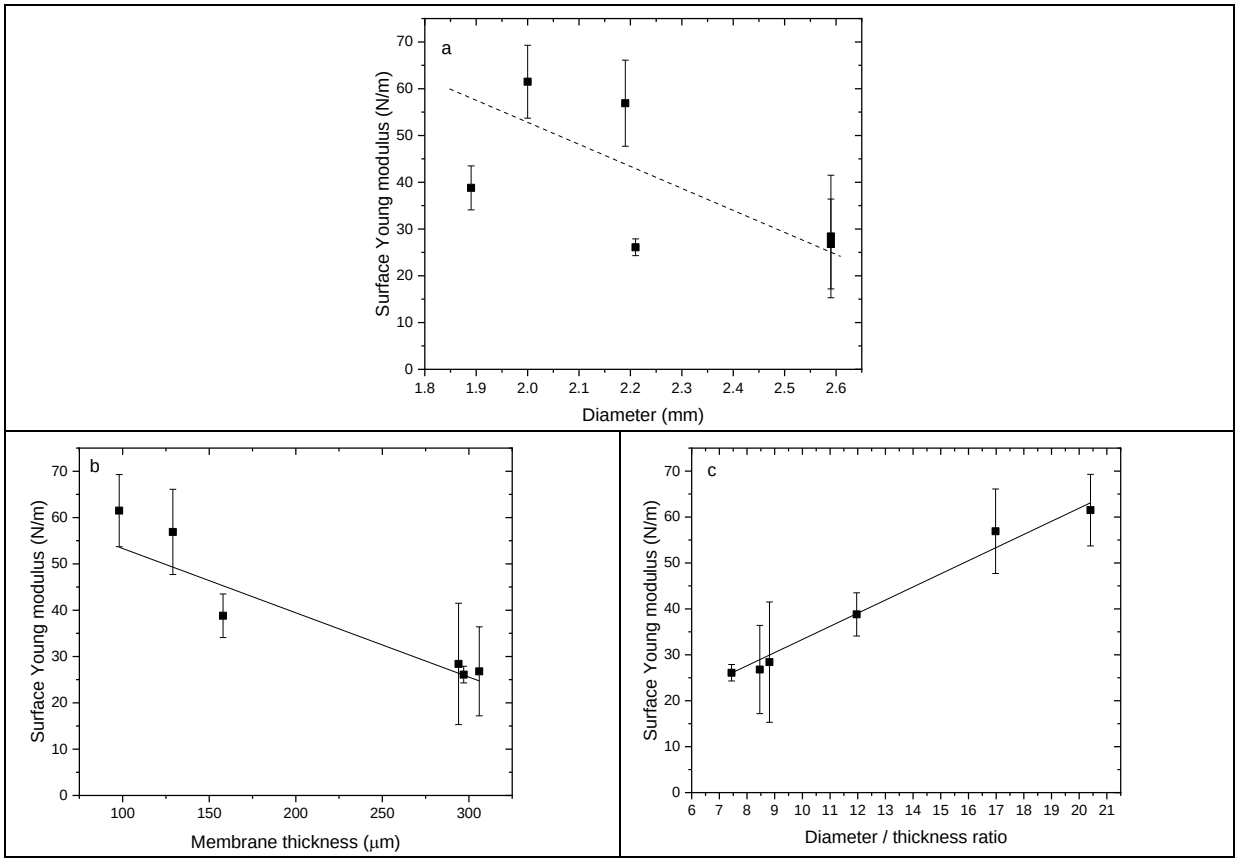
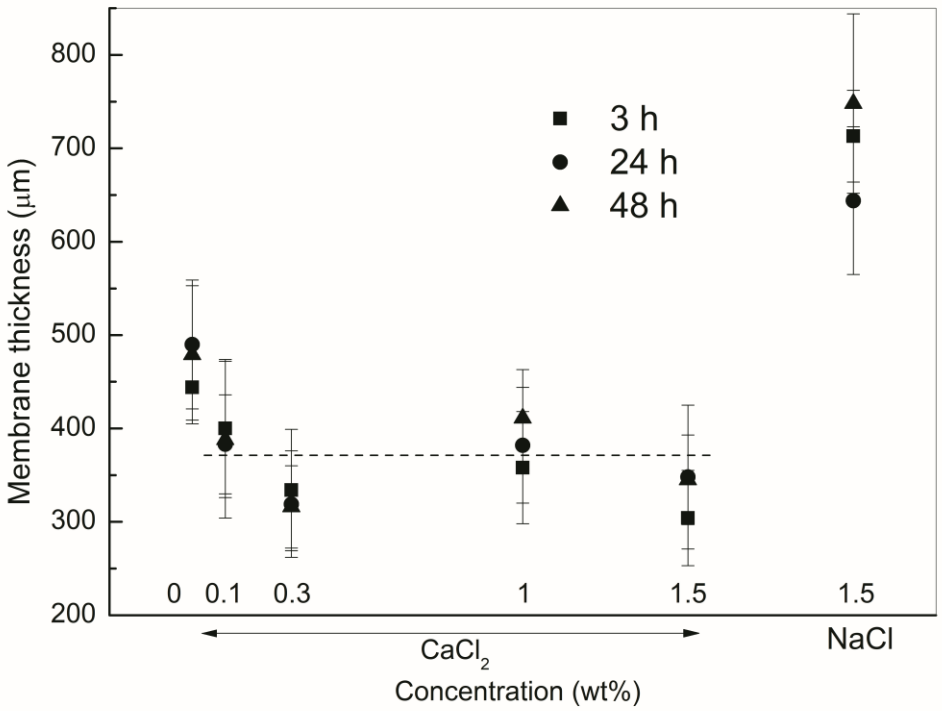


Figure 9


Membrane thickness (μm)
800
700
600
500
400
300
200
3 h
24 h
48 h
0
0.1
0.3
1
1.5
1.5
CaCl2
NaCl
Concentration (wt%)