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# **Formation and stabilization of multiple w/o/w emulsions encapsulating catechin, by mechanical and microfluidic methods using a single pH-sensitive copolymer: effect of copolymer/drug interaction**

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## **Abstract**

Multiple w/o/w emulsions (MEs) are promising systems for protecting fragile hydrophilic drugs and controlling their release. We explore the capacity of a single pH-sensitive copolymer, PDMS<sub>60</sub>-*b*-PDMAEMA<sub>50</sub>, and salts, to form and stabilize MEs loaded with sucrose or catechin by a one-step mechanical process or a microfluidic method. ME cytotoxicity was evaluated in various conditions of pH. Using the mechanical process, the most stable emulsions were obtained with Miglyol<sup>®</sup>812N and isopropyl myristate in a final pH range of 8-12 and [0.3 M-1 M] NaCl concentrations. Conversely, with the microfluidic method, isopropyl myristate at pH

3 without salt was more efficient. Catechin strongly affected the formation of droplets by the mechanical process but did not modify the conditions of stability of MEs obtained by the microfluidic method. The antioxidant power of catechin was preserved in the inner droplets, [even in emulsions prepared by the mechanical method at pH 8](#). An incomplete release of sucrose and catechin from the emulsions was observed and attributed to the interaction of molecules with the copolymer through hydrogen bonding. This study highlights some of the barriers to break to formulate multiple emulsions stabilized by a PDMS-*b*-PDMAEMA copolymer or other polymers which can form hydrogen bonds interaction with encapsulated drugs.

**Keywords:**

block copolymer (PDMS-*b*-PDMAEMA), pH-sensitive polymer, microfluidics, catechin, antioxidant power

## 1. Introduction

Multiple water-in-oil-in-water (w/o/w) emulsions (MEs) are considered as promising systems for encapsulation and release of fragile hydrophilic active ingredients. The oil layer acts as a barrier against the external aqueous medium, and thus preserves drug properties during storage. It is also possible to co-encapsulate two molecules of opposite affinities, one in the inner aqueous phase and the other in the oil. Numerous examples of pharmaceutical compounds solubilized in multiple emulsions are found in the literature, to protect drugs from degradation, to avoid undesirable interactions with other compounds present in the formulation or the body, or to mask the unpleasant taste of drugs.<sup>1-17</sup> The release of the encapsulated compounds from these emulsions occurs by passive diffusion through the oil phase or by triggered destabilization of the multiple emulsion induced, for instance, by the coalescence of the internal droplets with the external aqueous phase. However, most multiple emulsions are unstable in the long term: They usually require the stabilization by two surfactants, and mutual diffusion occurs leading to spontaneous destabilization of the emulsion and release of the encapsulated drugs. Generally, multiple emulsions are formed by a two-step mechanical process, shearing the oil and aqueous phases together to form droplets.<sup>18,19</sup> It was shown recently that they can also be prepared mechanically by a one-step method using a single block copolymer.<sup>20</sup> This method avoids the destabilization which sometimes occurs during the second stage of emulsification.

The objective of the present study was to form and stabilize multiple w/o/w emulsions, encapsulating an active ingredient (catechin) thanks to a biocompatible copolymer. Copolymers may have different architectures (blocks, statistics, alternating, grafted, star, neutral, charged, etc.).<sup>21,22</sup> Depending on these architectures, they can adopt various conformations at the oil/water interface. The simplest case is that of diblock copolymers in which each block may adopt a brush conformation in the phase for which it has the greatest affinity, provided that the

surface per chain is small enough. Adsorption of polymers [at interfaces](#) is stronger than that of common surfactants, with a desorption energy per chain largely exceeding  $k_B T$ .<sup>23</sup> Furthermore, droplets coalescence is hindered by steric repulsion between the polymer chains covering the droplets.<sup>24</sup> Polymers also allow good control of the rheological properties of emulsions when chains are soluble in the continuous phase.<sup>25</sup> In 2008, Hanson et al.<sup>26</sup> proposed for the first time a system capable of forming multiple emulsions in a one-step process based on the use of a single copolymer of hydrophilic poly-lysine and hydrophobic poly-leucine blocks, without adding surfactants. Later, Zhang et al.<sup>27</sup> studied the effect of salts and observed that the number of water droplets per oil globule varied with the valence of the anion used: the higher the valence, the smaller the water droplets. The valence of the cation, on the other hand, had no influence, probably because the poly-lysine block was positively charged and interacted only with anions. Hong et al.<sup>28</sup> showed that poly (ethylene glycol)-polystyrene (PEG-PS) copolymers could stabilize water/toluene/water emulsions at toluene volume fractions ranging from 20 to 70%. Using a similar approach, w/o, o/w and w/o/w stable emulsions were formed in one step with copolymers of dimethylaminoethyl methacrylate (PDMAEMA) and styrene (PS-*b*-PDMAEMA)<sup>20,29</sup> or polydimethyl siloxane (PDMS-*b*-PDMAEMA).<sup>30</sup> PDMAEMA is a polymer sensitive to three physicochemical parameters: (i) pH, *via* its secondary amino groups positively charged at low pH and neutral at high pH, the pKa being around 7.5,<sup>31</sup>(ii) ionic strength at low pH when the polymer is protonated since salt induces the screening of the charges carried by the chain and the decrease in polymer hydrophilicity, and (iii) temperature, this polymer having a Lower Critical Solution Temperature (LCST) between 35 and 65°C, at pHs above its pKa, the exact temperature depending on the molar mass of the PDMAEMA segment.<sup>32,33</sup>

According to Besnard et al.,<sup>29</sup> at least three conditions must be met in their systems to form multiple emulsions stabilized by a single copolymer: (i) low interfacial tension ( $< 1.5$  mN/m),

(ii) limited transfer (< 25%) of the copolymer from the oil phase (where the polymer is soluble) to the aqueous phase, giving the system anti-Bancroft properties, and (iii) similar extension of the copolymer in each of the two phases, allowing stabilization of w/o and o/w curvatures.<sup>34</sup> PDMS-*b*-PDMAEMA with appropriate block lengths can meet these requirements. Emulsification by the mechanical method proved to be dependent on the pH and ionic strength. Dynamics of transitional phase inversion were studied by varying the pH or temperature.<sup>30</sup> The domain of stability of multiple emulsions when varying pH and ionic strength was identified. An optimal stability of 2 months was reached for an intermediate zone of pHs between pH 8 and pH 12.<sup>30</sup>

More recently, microfluidic methods have been developed to produce emulsions. Microfluidics has the advantage of forming droplets of perfectly controlled size and composition and, unlike the single-step mechanical process, it allows the composition of the internal and external aqueous phases to vary independently. Microfluidic methods make use of micrometric channels (about 100  $\mu\text{m}$  wide and 50  $\mu\text{m}$  high) to form droplets one by one. For multiple emulsions two successive cross junctions are required. First, droplets of the aqueous internal phase are formed in the intermediate oily phase, and then the globules encapsulating the aqueous droplets are formed in the external aqueous phase.<sup>35-37</sup> It is necessary to control the wettability of the channels during the two stages.<sup>38</sup>

In the present work, we chose PDMS<sub>60</sub>-*b*-PDMAEMA<sub>50</sub>, a copolymer with about the same length of hydrophobic and hydrophilic blocks (hydrophilic/hydrophobic ratio = 0.83) which is believed to be biocompatible. Biocompatible oils were also used such as Miglyol<sup>®</sup> 812N, and isopropyl myristate (IM). Salt concentration and pH were varied to obtain stable multiple emulsions at room temperature. One-step (mechanical) and two-step (through microfluidics) emulsification methods were tested, and the effectiveness of drug encapsulation was evaluated using model hydrophilic small compounds, like sucrose and catechin.

Whereas sucrose was merely used as a model, catechin has potential anticancer, anti-microbial, free-radicals scavenger and anti-inflammatory properties.<sup>39,40</sup> Most of these properties come from the presence of phenolic hydroxyl groups in the molecule. The main problem with this substance, however, is its low oral bioavailability, due to its poor permeability and stability in the gastrointestinal tract. Catechin is highly sensitive to oxidation. The high pH in the gut, in particular, is responsible for auto-oxidative reactions and degradation at  $\text{pH} \geq 6$ .<sup>2,40</sup> It is therefore important to prepare and stabilize catechin emulsions at a pH lower than 6 whenever it is possible unless a protective design is found. Encapsulation of catechin in w/o/w emulsions has been proposed for protection against the external aqueous medium. However, it has been reported that due to their amphiphilic properties, polyphenols including catechins can gradually diffuse through the oily phase. Therefore, the aim of the encapsulation of sucrose and catechin was to detect interferences with ME formation, compare their stability in the MEs, and study their interaction with the polymer adsorbed at the interface.

## **2. Materials and methods**

### *2.1. Materials*

Block copolymer, PDMS-*b*-PDMAEMA, was synthesized by Atom Transfer Radical Polymerization (ATRP) (see Ref. 30 for details) with a polydispersity of 1.08 and PDMS and PDMAEMA blocks were respectively composed of 60 and 50 monomer units. Miglyol<sup>®</sup>812N was generously provided by IMCD (France). Caco-2 cells were supplied by ATCC and fetal bovine serum by Gibco. Chloroform and methanol were obtained from VWR Prolabo (Leuven, Belgium). The K-SUFRG kit for sucrose assay was purchased from Megazyme. HT29-MTX cells, Dulbecco/Vogt Modified Eagle's minimal essential Medium (DMEM) and all other chemicals used were provided by Sigma Aldrich (St Louis, USA). Catechin was purchased as

(+)-catechin from Sigma Aldrich (St Louis, USA), a hydrated form of catechin whose mass proportion in water was estimated by  $^1\text{H}$  NMR to be about 3%.

## *2.2. Preparation of multiple emulsions*

The PDMS<sub>60</sub>-*b*-PDMAEMA<sub>50</sub> copolymer (5 mg/mL) was dissolved in 3 mL of oil (miglyol<sup>®</sup>812, or isopropyl myristate). Sucrose or catechin (1 mg/mL) were dissolved in water. The initial pH of the aqueous phase (3 mL) was adjusted between 3 and 6 by adding hydrochloric acid (HCl) or sodium hydroxide (NaOH) and the ionic strength was varied between 0 and 2 M by adding sodium chloride (NaCl). The two phases were brought into contact for 24 hours to allow the saturation of the aqueous phase by the oil and the copolymer. After 24h, the pH finally shifted to 8 regardless of the initial pH, except for Miglyol<sup>®</sup>812N at initial pH 3 with no added salt and for isopropyl myristate at initial pH 3 and a salt concentration of less than 0.5 M, where the final pH remained between 3 and 5. Nile red was added to tag the oil phase.

### *2.2.1. Mechanical one-step method*

The two phases were emulsified at 30,000 rpm for 40s with an Ultra-Turrax<sup>®</sup> T10 (Ika<sup>®</sup>) equipped with a S10N-8G dispersion tool. The emulsions were observed just after formation and at regular intervals by confocal microscopy (Olympus). Droplet diameters and polydispersity were estimated using an in-house MatLab software by J.B. Thomazo, which allows to distinguish between internal water droplets and oil drops in order to measure their size distribution: Only the smallest (below resolution) water droplets are not detected, which introduces a potential bias in the measurement.



### *2.2.2. Microfluidic method*

Multiple emulsions were formed using a single chip with two junctions as designed by Bodin-Thomazo et al.,<sup>38</sup> with modifications. The channel diameter between the two junctions was reduced to 50  $\mu\text{m}$  so that the water droplets formed at the first junction had a much smaller diameter than the oil droplets formed at the second junction. To prevent coalescence of the oil droplets when they arrive in the outlet chamber, a long serpentine outlet channel was created, allowing enough time to the polymer for adsorbing at the interface. Before injection, the aqueous phase (2 mL) at various salinities and pHs was left in contact for 24 h with 2 mL of oil containing 5 mg/mL of PDMS<sub>60</sub>-*b*-PDMAEMA<sub>50</sub>. The phases were then separated. The aqueous phase was divided in two, one part being used as the internal aqueous phase (supplemented with the targeted hydrophilic compound to encapsulate) and the other part as the external aqueous phase. The liquids were injected into the chip by syringe pumps whose flow rates were controlled by the neMESYS interface developed by CETONI.

### *2.3. Cell culture and assessment of emulsion cytotoxicity*

Caco-2 cells were grown at 37°C in DMEM with 10% of Fetal Bovine Serum, 1% amino acids and 1% antibiotics (penicillin and streptomycin) in a saturated water atmosphere with 5% CO<sub>2</sub>. After 3 days, the cell culture medium was renewed and 10,000 HT29-MTX cells were added to each well, dispersed in 100  $\mu\text{L}$  of cell culture medium. Both cell lines were left in the coculture incubator for 18 days, the culture medium being renewed three times a week. At the end of these 18 days, mucus developed uniformly onto the surface of the cells. The medium was then replaced by 100  $\mu\text{L}$  culture medium and 100  $\mu\text{L}$  emulsion. Copolymer cytotoxicity was determined by viability tests, according to the protocol described by Béduneau et al. and Antoine et al.<sup>41,42</sup> A sample of emulsion was left to cream. Then, its aqueous lower phase was removed and replaced by fresh aqueous phase at pH 1. After slight manual stirring, the multiple

emulsion turned to an o/w emulsion. Multiple and o/w emulsions diluted 2, 10 and 100 times were left in contact with the cells for 24 hours. The wells were then emptied by suction and filled with 200  $\mu$ L of fresh medium to which 20  $\mu$ L of (3-(4,5-dimethylthiazol-2-yl)-2,5-bromide) diphenyl tetrazolium) (MTT) solution (5 mg/mL) was added.<sup>43,44</sup> The absorbance of the wells was measured at 570 nm. Control experiments were performed on aqueous phases at pH 1 and pH 8, and 0.15 M NaCl, diluted 2 and 10 times. Triton X100 (0.1wt%) was used as positive cytotoxic control. Samples were also diluted 5 and 50 times in the cell culture medium to verify that dilution did not destabilize the cells.

#### *2.4. Interfacial tension measurements*

Interfacial tension measurements were carried out using a spinning drop tensiometer (SVT 20N, DataPhysics, Germany) fitted with a cylindrical quartz capillary thermostatically controlled at 20 °C, measuring 500  $\mu$ m in diameter and 11 cm long. The densities of oils and aqueous phases at different salt concentrations were determined using a DMATM 4500 Anton Paar densitometer, also thermostatically controlled at 20 °C.

#### *2.5. Analysis of catechin stability in aqueous solutions*

Catechins are very unstable compounds at pHs greater than 6,<sup>45-49</sup> Aqueous solutions of catechin (1 mg/mL) at pH 1; 4; 6 and 8 and 0.15 M NaCl were prepared and stored at room temperature in darkness for one month. Solutions at pH 1 and 4 were obtained by adding 1 M HCl to milli-Q water. Higher pHs (6 and 8) were stabilized by sodium phosphate buffer. At regular intervals, the UV/visible absorption of these solutions was measured at 230 and 280 nm using a UV2550 spectrophotometer (Shimadzu), after dilution to 5 mg/L in pure water. Catechin assay by HPLC was performed by injecting the samples (20  $\mu$ L) in a C18 Waters XBridge BEH300 column. A mixture of 0.09% trifluoroacetic acid (TFA) in acetonitrile (solvent A) and 0.1% TFA in water

(solvent B) was used as eluent in a linear gradient from 0 to 100% of A in 20 min. Detection was performed by mass spectrometry (LC-MS). Calibration was performed with 10 to 1000 pmol catechin in 0.1 M HCl solutions. <sup>1</sup>H-NMR measurements were carried out with 600 μL of a catechin (1 mg/mL) solution in deuterated phosphate buffer at pH 7.6 (without NaCl). To detect possible oligomers, steric exclusion chromatography was performed in aqueous phase with a mixed-bed poly (hydroxyethyl methacrylate) gel column (different pore sizes), using a Viscotek GPC max VE 2001 instrument equipped with a triple TDA 302 detector.

### *2.6. Evaluation of the antioxidant power of the catechin*

The antioxidant score of catechin was determined in pure aqueous phase, in aqueous phase surmounted by a polymer-free oil phase, and in aqueous phase surmounted by the oil phase containing 5 mg/mL PDMS<sub>60</sub>-b-PDMAEMA<sub>50</sub> with or without Butylated Hydroxy Toluene (BHT). It was also measured for internal water phases in multiple emulsions encapsulating catechin as follows: after emulsion creaming, the subphase was taken off by a syringe and replaced by an aqueous phase of same ionic strength at pH 1. This destabilized the multiple emulsion into a simple o/w emulsion. The continuous water phase of this destabilized emulsion was then measured at different times after multiple emulsion formation. The samples were stored at room temperature and protected from light for the duration of the study. Measurement of the antioxidant power of catechin was performed by the Oxygen Radical Absorbance Capacity (ORAC) method using fluorescein, 2,2'-Azobis(2-methylpropionamide) dihydrochloride (AAPH) (150 mM in phosphate buffer at pH 7.4) as radical initiator and 3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-carboxylic acid (Trolox) as reference.<sup>50,51</sup> Fluorescein intensity decay between 500 and 600 nm was measured every 1 min at 33°C on a Varian Cary Eclipse apparatus until fluorescence intensity was null. The

antioxidant power of catechin was expressed in Trolox Equivalent ( $\mu\text{mol TE}/\mu\text{mol}$ ), using a Trolox calibration plot.

### 3. Results and discussion

W/o/w multiple emulsions were prepared by the one-step or two-step emulsification process, using mechanical or microfluidic methods in various conditions of pH and salt concentrations.<sup>30,38</sup> The mechanical method allows obtaining large volumes of polydisperse emulsions in few minutes, whereas the microfluidic chip produces 1 mL of monodisperse multiple emulsion per hour. Two oils were tested. Miglyol<sup>®</sup> 812N mainly consists of a mixture of caprylic and capric triglycerides, whereas isopropyl myristate (IM) is an ester of myristic acid (Fig. 1). Both oils were used in the mechanical method, but IM, less viscous than Miglyol<sup>®</sup> 812N (5 mPa.s and 30 mPa.s, respectively), was preferred in the microfluidic method. In both processes, the oily and aqueous phases were emulsified after 24h contact. [This contact generally induces an increase in the aqueous pH to pH 8 \(as described in the Material and Methods section\), because the copolymer captures water protons at the interface.<sup>30</sup> The multiple emulsions were stable over longer periods of time when prepared at pH 8 rather than at pH 3.](#)

#### *3.1. Emulsions obtained by the mechanical method*

Fig. 2 presents images of stable multiple emulsions, 1 (resp. 2) year aged for Miglyol<sup>®</sup>812N (resp. isopropyl myristate), for an initial pH of 6 and a salt concentration increasing from 0 to 2 M. This figure is a summary of what was already reported in greater detail in Ref. 30. Using the mechanical process, and for both oils, the most stable MEs were obtained at initial pH between 6 and 14, and for NaCl concentrations in the [0.3 M-1.5 M] range. No stable multiple

emulsion could be obtained by this method at initial pH lower than 6, as already observed for PDMS<sub>38</sub>-b-PDMAEMA<sub>25</sub>.<sup>30</sup>

### 3.2. Assessment of the cytotoxicity of multiple emulsions stabilized by PDMS<sub>60</sub>-b-PDMAEMA<sub>50</sub>

The toxicity of multiple emulsions stabilized by PDMS<sub>60</sub>-b-PDMAEMA<sub>50</sub> was evaluated on co-cultures of Caco-2 and HT29-MTX cells. Methotrexate (MTX) induced the formation of a mucus layer at the surface of cells mimicking the digestive mucosa. Emulsions were prepared by the mechanical method at pH 8, then destabilized at pH 1. Some of the destabilized emulsions were diluted to study the effect on cells of emulsions at pH 2 and 3. The results in Table 1 show that cell viability was barely affected by the multiple emulsion at pH 8, or emulsions at pH 2 or 3. Conversely, emulsions at pH 1 led to a dramatic drop in cell viability (qualitatively comparable to detergent Triton X 100), due to the strong acidic pH rather than to a toxic effect of the copolymer.

**Table 1:** Cell viability measured for the aqueous phase (AP) at pH 8 and pH 1, for multiple emulsions at pH 8, multiple emulsions destabilized at pH 1 and for the destabilized multiple emulsions diluted to reach pH 2 and 3.

pH	AP pH 8	8	3	2	AP pH 1	1	Triton X100
Cell viability	100.6	94.1	92.3	90.9	9.5	9.7	4.9
(%)	+/- 7.8	+/- 8.4	+/-11.9	+/- 8.2	+/-0.5	+/- 0.4	+/- 0.4

### 3.3. Application to sucrose encapsulation

Sucrose was considered as a good model compound for testing drug encapsulation in the emulsions, because it is non-surface active and insensitive to pH changes.<sup>52,53</sup> It is also poorly oil soluble. Therefore, it should remain in the internal aqueous phase until destabilization of the multiple emulsions. Sucrose [1-100 mg/mL] was incorporated in emulsions prepared with

Miglyol<sup>®</sup>812N and 0.3 M NaCl. All mechanically prepared emulsions were multiple emulsions, with an average diameter of  $9.3 \pm 0.5 \mu\text{m}$  and  $2.4 \pm 0.4 \mu\text{m}$  for oil globules and internal water droplets, respectively. After 1-year aging, the size of emulsion droplets varied little (Fig. S1) except for emulsions containing 10 mg/mL sucrose, whose inner water droplets were slightly smaller. Sodium chloride concentration was also varied [0.15 - 1 M] with no significant consequence on the morphology, behavior, and aging of the emulsions (data not shown). Altogether, the multiple emulsions were barely changed by the encapsulation of sucrose compared to unloaded ones.

#### *3.4. Application to catechin encapsulation*

Following the successful encapsulation of sucrose, we tried to encapsulate catechin. However, no stable multiple emulsion could be obtained by Ultra Turrax mechanical mixing using isopropyl myristate (IM); direct o/w emulsions only were obtained. Conversely, it was possible to prepare multiple emulsions of catechin with Miglyol<sup>®</sup> 812N. This unexpected result could be explained by the destabilization of the interfacial film at IM-water interface by catechin. To check this assumption, the interfacial tensions at Miglyol<sup>®</sup> 812N-water and isopropyl myristate-water interface were measured in various conditions. From the results in Table S1, it is apparent that the interfacial tension was mostly controlled by the copolymer. Indeed, the interfacial tension for the pure oil-water interface was 24.3 and 27.1 mN/m for Miglyol<sup>®</sup> 812N and IM, respectively; These values were reduced to 1.0 and 0.2 mN/m in the presence of the copolymer at pH 8 and low salt concentration, and they did not vary when catechin was added. Accordingly, the surface tension is not the reason for the failure to form multiple emulsions with IM and catechin using the mechanical method. We suggest that a strong association between IM and catechin could modify the interfacial curvature (and not the tension), thus preventing the formation of MEs.

The stability of multiple emulsions prepared with Miglyol<sup>®</sup> 812N and catechin decreased as the salt concentration increased (Fig. 3 and Table 2). The emulsions formed with 1 M NaCl were destabilized after a few days. Those formed with a lower concentration of salt remained stable even after a year of storage, probably thanks to steric interactions between polymer chains.

Table 2: Catechin-loaded emulsions (1 mg/mL) prepared with Miglyol<sup>®</sup> 812N at pH 8 and with various NaCl concentrations and stored in darkness at room temperature.  $D_{oil}$  and  $D_{water}$  are mean diameters of oil and internal water droplets, respectively.

NaCl (M)	D0		1 year	
	D (oil) $\mu\text{m}$	D (water) $\mu\text{m}$	D (oil) $\mu\text{m}$	D (water) $\mu\text{m}$
0.15	21.8 $\pm$ 3.6	3.1 $\pm$ 1.2	12.7 $\pm$ 3.3	2.6 $\pm$ 1.3
0.3	34.4 $\pm$ 6.5	3.1 $\pm$ 1.5	44.8 $\pm$ 13.9	1.7 $\pm$ 0.8
0.6	43.3 $\pm$ 8.2	3.0 $\pm$ 1.5	65.3 $\pm$ 21.4	1.5 $\pm$ 0.6
1	70.9 $\pm$ 22.9	3.2 $\pm$ 1.4	emulsions destabilized after 3 days	

Our primary objective was to form the multiple emulsions by a one-step mechanical process. As mentioned before, the most stable multiple emulsions were obtained at pH 8. However, because catechin undergoes degradation at  $\text{pH} > 6$  (see section 3.5 for exact determination), alternative conditions for forming PDMS<sub>60</sub>-*b*-PDMAEMA<sub>50</sub>-stabilized w/o/w emulsions at intermediate pH levels were tried. The tests were carried out in the absence of catechin, after verifying that this compound did not influence the pH of the aqueous phase. Firstly, different salt concentrations and initial pHs of the aqueous phase were tried. However, none of them allowed obtaining multiple emulsions at final pHs below 8. Then, the concentration of the copolymer was decreased to reduce the pH shift, but the emulsions formed were either unstable (1 mg/mL) or of similar pH (3 mg/mL) than those formed with 5 mg/mL PDMS<sub>60</sub>-*b*-PDMAEMA<sub>50</sub>. An attempt was made to stabilize the pH at 4.7 or 6 by adding acetate or phosphate buffer, respectively, supplemented with 0.15 M NaCl. In these conditions, no pH

shift occurred, but emulsions were destabilized after few hours. Emulsification tests performed with catechin in citrate/phosphate buffers at pH 4.7 or 5.4 were also unsuccessful.

In conclusion, it was impossible to mechanically form and stabilize multiple emulsions at final pH lower than 8 and NaCl concentrations equal to - or higher than - 1 M. Catechin-loaded MEs were, however, prepared in these conditions to assess the actual effect of protection against pH 8, by solubilizing the polyphenol in the external and internal aqueous phases of MEs.

### *3.5. Assessment of catechin degradation in aqueous solutions*

In the multiple emulsions of catechin formed by the one-step mechanical method, catechin is encapsulated in the internal aqueous phase, but it is also present in the external phase where it could be degraded rapidly. To characterize the behavior of catechin in the external aqueous phase during emulsion aging, solutions of catechin were prepared at various pHs. As previously reported by Li et al. (2013)<sup>54</sup> and Sang et al.<sup>55</sup>, a change in the color of the solutions was observed, anticipating catechin degradation.<sup>56-58</sup> The mechanisms of degradation of catechin include epimerization leading to compounds with different conformations<sup>45,48</sup> and alkaline polymerization<sup>59</sup> resulting from its oxidation into quinone derivatives. This second mechanism is likely to alter the antioxidant properties of catechin.

Catechin degradation was assessed by UV/Visible spectroscopy change from the day of catechin solubilization till 7 and 30 days (Fig. S2). A broad band was observed around 400 nm, the intensity of which increased with time, while its maximum shifted towards shorter wavelengths. A decrease in catechin concentration was measured by LC-MS at all pHs, although the decrease was less pronounced at pHs 1 and 4 than at pHs 6 and 8. At pH 8, a dramatic drop in catechin concentration was observed, indicating complete degradation after 20 days (Fig. S3). These results are in good agreement with the literature.<sup>56-58</sup> However, no evidence of degradation products was found in LC chromatograms, even after changing elution



conditions. Our results suggest an oligomerization of catechin rather than a simple epimerization. NMR measurements confirmed the results obtained by LC-MS; The intensity of peaks decreased with time, without appearance, however, of new peaks corresponding to degradation products (Fig. S4). This, again, suggests the oligomerization of catechin. Indeed, the increase in mass of the analyzed compound would decrease relaxation times, which in turn would hamper molecule detection. A solution of catechin (4 mg/mL) prepared in a phosphate buffer at pH 8, stored for 50 days, lyophilized, and then hydrated by 0.2 M NaNO<sub>3</sub> solution was analyzed by steric exclusion chromatography (SEC). No oligomer could be detected. The limit of detection of the apparatus corresponded to about 6-7 units of catechin. It is possible that the oligomers adhered to the SEC column and were not eluted.

In conclusion, these stability experiments confirmed that in aqueous solution, catechin was degraded during storage in aqueous solutions at pH > 6. Degradation products were neither detectable by LC-MS nor by NMR or SEC. Our assumption is that catechin formed oligomers.<sup>60,61</sup> We then checked if catechin preserved its antioxidant power, when it was encapsulated in the internal water droplets of the multiple emulsions.

### *3.6. Evaluation of the antioxidant properties of catechin in solutions and in multiple emulsions*

The antioxidant properties of catechin solubilized at pH 8 in saline solution were verified by the ORAC method. Three series of samples were analyzed: (i) catechin aqueous solutions at various pHs, (ii) catechin solutions at pH 3 or 8 topped with oil containing the copolymer, and (iii) catechin solutions at pH 8, topped with oil containing the copolymer with or without an antioxidant (BHT). In the presence of the copolymer in the oily phase, the pH shifted from 3 to 8. In all studied solutions, the antioxidant power of catechin decreased (Fig. S5), even in the presence of BHT in the oily phase.<sup>9</sup> However, at low pHs (between 1 and 6) this decrease was weak and slow, confirming that catechin is indeed an antioxidant molecule. The decrease was

much faster at pH 8, the antioxidant power reaching a value close to zero within twenty days. This is in line with studies carried out with the LC-MS and demonstrates that the degradation of catechin was accompanied by a decrease in antioxidant power.

Antioxidant power measurements were also carried out on multiple emulsions encapsulating catechin prepared with Miglyol<sup>®</sup>812N (Fig. 4). The same trend as before was observed in the continuous phases of these emulsions at pH 8. However, the antioxidant power measured in the internal water phase after destabilization of the multiple emulsions was higher than in the external one, suggesting that catechin was protected in the aqueous droplets. Surprisingly, the antioxidant power was also found higher in the internal aqueous phase of freshly prepared emulsions ( $t = 0$ ). This could be explained by hydrogen bonding between catechin and the polymer at pH 8 and subsequent release of catechin at pH 1 when the polymer is charged: this would cause an error in the estimations of catechin concentration in both phases, overestimation for the external phase and underrating for the internal phase. However, the difference between the two values (12 and 4  $\mu\text{mol TE}/\mu\text{mol}$  in aqueous droplets and in the external phase, respectively) seems too high to be solely explained by the release of the catechin bound to the copolymer. [The properties of DMAEMA as internal reducing agent in ARGET ARTP have already been mentioned by Dong and Matyjaszewski and Flejszar et al.<sup>62,63</sup> The contribution of polymerized DMAEMA to the preservation of the antioxidant power of kaempferol was also suggested.<sup>64,65</sup> To verify whether under the conditions used PDMS<sub>60</sub>-\*b\*-PDMAEMA<sub>50</sub> had an antioxidant effect, we compared the fluorescence decay as a function of time of freshly prepared fluorescein solutions with and without copolymer, in the presence of AAPH. The results showed that indeed, in the presence of the copolymer, the fluorescence decrease was slowed down \(Figure S6a\). The same experiment was conducted on aqueous phases containing catechin and topped with oil. Again, the fluorescence decay of fluorescein induced by AAPH was slower when the solution contained the copolymer \(Figure S6b\). These results confirm the antioxidant](#)

property of the copolymer. Thus, the combined effect of the encapsulation, the interaction of the catechin with the copolymer and the antioxidant properties of the copolymer may explain the protection of the catechin in MEs.

Though the antioxidant power of catechin encapsulated in the internal water droplets was preserved for some time, high pH is unfavorable and another issue with the mechanical process is the degradation of the catechin solubilized in the external aqueous phase. These issues can be tackled by using the microfluidic method where all the catechin could be encapsulated in the inner aqueous phase.

### *3.7. ME formed by microfluidics and catechin encapsulation*

With the mechanical one-step process, pH and NaCl concentration were crucial parameters for the formation of stable multiple emulsions. They were obtained at pH 8 at a salt concentration below 1 M. However, with the two-step microfluidic method the conditions were found drastically different from those required in the mechanical method. With this method, multiple emulsions were formed with IM as oil and were found stable at initial pH 3 in the absence or at very low concentrations of salt (0-0.5 M). Those prepared at initial pH 6 or at higher NaCl concentrations could not be formed. In the first case where ME were formed, the pH after 24h contact of the aqueous and oil phase remained at 3, whereas in the second case where ME failed to form, the pH at 24h had shifted to 8. On the one hand, a pH of 3 means a priori moderately stable MEs as measured in Ref. 30. On the other hand, the obtention of MEs while maintaining a low pH is a great advantage of microfluidics over the mechanical method for catechin encapsulation. The most obvious parameters (i.e., oil/water interfacial tension, PDMS/oil or PDMS/water contact angles in the presence of the copolymer, or the viscosities of oil and water) that could explain the differences in the required conditions for forming the emulsions by the two processes were ruled out, because they were all found weakly affected by the changes in

pH or salt conditions (see Fig. S7 for interfacial tension measurements). Other parameters need to be considered like (i) the absence of stirring in the microfluidic chip, (ii) the time for the formation of droplets (about 1 ms) compared to the time needed for the copolymer to diffuse through the oil phase over a typical drop size (1000 s over 100  $\mu\text{m}$  as deduced from the value of the diffusion coefficient,  $2.7 \times 10^{-12} \text{ m}^2/\text{s}$ ). Two resulting effects can explain the discrepancy between the mechanical formation and the microfluidic one, besides the fact that emulsions are non-equilibrium objects whose final state may depend on the method of preparation. Firstly, in the mechanical process, many coalescence events are promoted by stirring. The results show that such events are unfavorable at pH 3 and lead to a single emulsion whereas, by microfluidics, no coalescence events are promoted except for the rare events where an internal water droplet meets the external o/w interface. Secondly, at pH 8 the polymer is neutral and could adsorb fast despite the long time given above for lowering the surface tension. The low surface tension could be responsible for the failure of ME formation in the microfluidic chip since the oil film does not break into droplets at the second microfluidic junction where the continuous oil phase is pushed through the external water phase (Fig. 5a). On the contrary, at pH 3, due to the high charge of the polymer, non-equilibrium effects could be stronger and lead to a favorable situation where the oil film readily breaks due to high surface tension (Fig. 5b). The most stable emulsions formed by the microfluidic method were obtained with IM, at pH 3 without salt. They remained stable for at least 2 years. Catechin-loaded multiple emulsions were prepared using the same conditions. On a month-scale, these emulsions appeared perfectly stable (Fig. 6).

### *3.8. Catechin and sucrose release*

To follow the release of hydrophilic compounds after destabilization of the multiple emulsions prepared by the one-step process, the external aqueous phase of the emulsions was washed, so

that it no longer contained the model drug. Successive dialysis at pH 8 were performed on freshly prepared emulsions, using membranes with a 6000-8000 cut-off. Three steps were sufficient to wash the emulsions. To maintain the multiple structure of the emulsions during washing, an osmotic imbalance favoring the swelling of the droplets was induced by dialyzing the emulsions containing 0.3 M in NaCl with aqueous phases containing 0.15 M NaCl only (Fig. S8). The concentration of catechin was determined by UV-visible spectroscopy at 230 nm after each dialysis step. After three washings, the total mass of the antioxidant recovered amounted to 80% of the total, meaning that 3 washing steps were enough (Table 3).

Table 3: Catechin concentration and mass determined after each dialysis step

Dialysis step	Catechin concentration ( $\mu\text{g/mL}$ )	Catechin mass (mg)
1	8.3	1.7
2	2.1	0.5
3	1.1	0.2

Following three washings of their external aqueous phase, emulsions were dialyzed at pH 1 with 0.15 M NaCl solution. This resulted in the destabilization of the emulsions as observed previously.<sup>66</sup> A noticeable difference was noticed between the catechin absorption spectra measured on the aqueous phases (Fig. 7). Whereas the spectrum for the catechin collected after emulsion washing (catechin in the external aqueous phase, dotted line) resembles the UV-vis spectra for catechin and degraded catechin as shown in Fig. S2, the spectrum of catechin after emulsion destabilization (plain line) is completely different. In addition to the change in concentration that can be roughly estimated by the ratio of curves at 230 nm (though this peak present in free catechin is hardly visible in the encapsulated catechin), the spectrum of the destabilized emulsion also lacks the characteristic peak at 280 nm. This modification of the

absorption spectrum again supports the hypothesis of an interaction between catechin and the copolymer, probably due to hydrogen bonding, as pictured in Fig. 8. This interaction is consistent with the synergistic effect of catechin and the polymer on the antioxidant power. The modification of the absorption spectrum could be explained by the formation and detection of copolymer/catechin complexes during salting out. A similar release process was observed for sucrose. Its release was probably hindered by hydrogen bonding with the hydrophilic block of the copolymer (Fig. 8).

When the microfluidic method was used, it was not necessary to dialyze the multiple emulsions prior to their destabilization at pH 1 because catechin was *a priori* only present in the internal droplets. However, about 55% of the catechin initially dissolved in the internal aqueous phase was detected in the external one. This indicates a rapid leakage of catechin during or after production of the emulsions, due to the difference in osmotic pressure between the two phases.<sup>5,9,67</sup> This leakage could be prevented by addition of salt (0.15 M) in the external aqueous phase. After emulsion destabilization at pH 1, 42% of the initial amount of catechin dissolved in the internal aqueous phase was released. In total, 96.7% of initial catechin amount was recovered, which was much higher than the 80% recovered in the emulsions prepared by the mechanical method. The difference in pH and salt concentrations between the two methods is significant: at pH 3 and minimum salt concentration used in microfluidics, hydrogen bonds can hardly be formed between catechin and PDMAMEA. It is then consistent to believe that the low recovery of catechin from mechanically prepared multiple emulsions was due to a strong interaction of catechin with the copolymer at pH 8.

#### **4. Conclusion**

It is possible to form multiple emulsions stabilized by the PDMS<sub>60</sub>-*b*-PDMAEMA<sub>50</sub> copolymer without addition of surfactants, using a one-step mechanical process or a microfluidic method.

The formation and stability of these emulsions are highly dependent on the pH and the salt concentration, due to the sensitivity of the hydrophilic block to pH. The formulation is also strongly impacted by the emulsification method used. Using the one-step method, the best results were obtained at pH 8, at various NaCl concentrations, depending on the chosen oil.

The encapsulation of water-soluble (sucrose, catechin) compounds brought new constraints. Sucrose and catechin developed hydrogen bonds with the copolymer, which partially hindered their release. Catechin underwent an oligomerization-like degradation in the aqueous phase when emulsions were prepared at pH 8, using the mechanical method. However, this degradation was less pronounced in the internal aqueous phase of the emulsions. Changes in the UV/visible spectra and the apparent low encapsulation rate suggest that interactions between catechin and the PDMAEMA moiety play a crucial role in the antioxidant power preservation. [The tests carried out with microfluidics required the use of a low viscosity oil and showed that it was possible to obtain stable multiple emulsions at pH 3, in the absence of salt. Microfluidics thus allowed to form multiple emulsions at a low pH that preserved the stability of catechin.](#)

The results of this study highlight the main barriers to break through in to encapsulate active ingredients in multiple emulsions stabilized by a PDMS-*b*-PDMAEMA copolymer, depending on the manufacturing method and the nature of the drugs to be solubilized in the aqueous and oily phases.

### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## **CRedit authorship contribution statement**

**Noémi Bodin-Thomazo:** Investigation, Formal analysis, Funding acquisition, Writing-original draft.

**Florent Malloggi:** Methodology, Supervision, Validation, Writing-review.

**Nadège Pantoustier:** Methodology, Supervision, Validation, Writing-review.

**Patrick Perrin:** Methodology, Supervision, Validation, Writing-review.

**Patrick Guenoun:** Conceptualization, Methodology, Validation, Formal analysis, Supervision, Resources, Writing-review & editing.

**Véronique Rosilio:** Conceptualization, Methodology, Validation, Formal analysis, Supervision, Resources, Writing-review & editing.

## **Appendix A: Supplemental material**

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## Legends to the figures

Figure 1: Chemical structure of the ingredients of multiple emulsions.

Figure 2: Aspect of multiple emulsions obtained at an initial pH of 6 using the one-step mechanical method, with Miglyol<sup>®</sup> 812N after 1-year aging for (a) [NaCl] = 0 M, ((b) [NaCl] = 1M, (c) [NaCl] = 2M, with isopropyl myristate after 1 year aging (except d) where it is 2 years) for (d) [NaCl] = 0.6 M, (e) [NaCl] = 1M, (f) [NaCl] = 2M. Scale bar = 30  $\mu\text{m}$ .

Figure 3: Aspect by confocal microscopy of catechin-loaded emulsions (1 mg/mL) with Miglyol<sup>®</sup> 812N at pH 8 obtained with various NaCl concentrations after 3-day or 1-year storage in darkness at room temperature. Scale bar = 30  $\mu\text{m}$ .

Figure 4: Evolution of the antioxidant power of catechin in ( $\blacktriangle$ ) the external aqueous phase prior to destabilization, and ( $\bullet$ ) the aqueous phase following destabilization.

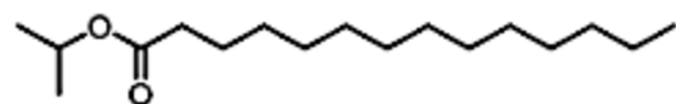
Figure 5: Images of the microfluidic circuit at the second junction where the oil phase including water droplets is pushed from right to left. (a) pH 8 case: the interface does not break, and oil drops do not form; (b) pH 3 case: the interface breaks and oil drops are formed. Scale bar = 100  $\mu\text{m}$ .

Figure 6: Catechin-loaded multiple emulsions formed by microfluidics at pH 3 in isopropyl myristate with no NaCl, after formation (left), aged 1 month (right). Scale bar = 100  $\mu\text{m}$ .

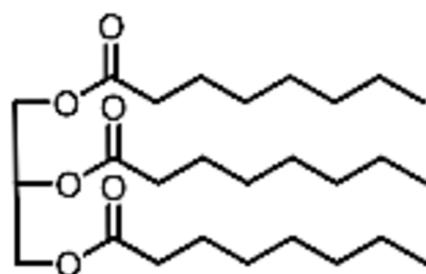
Figure 7: UV/vis. spectra for catechin after 2 washing steps (dashed blue) and after destabilization at pH 1 to a direct emulsion (red continuous).

Figure 8: Possible formation of hydrogen bonds between catechin (a) or sucrose (b) and the DMAEMA block of the copolymer.

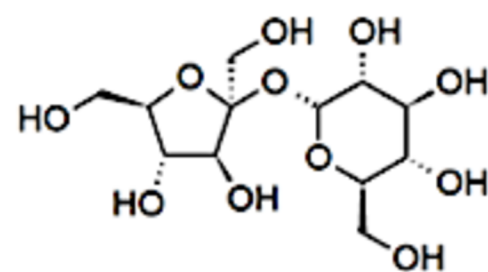
Fig 1



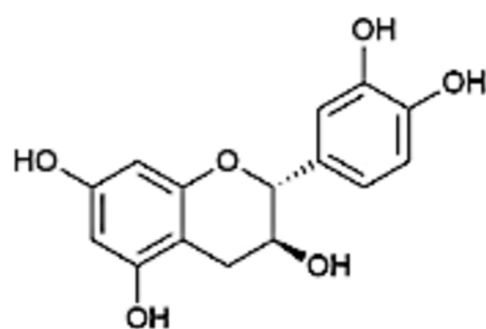
Isopropyl myristate



Medium chain triglyceride



Sucrose



Catechin

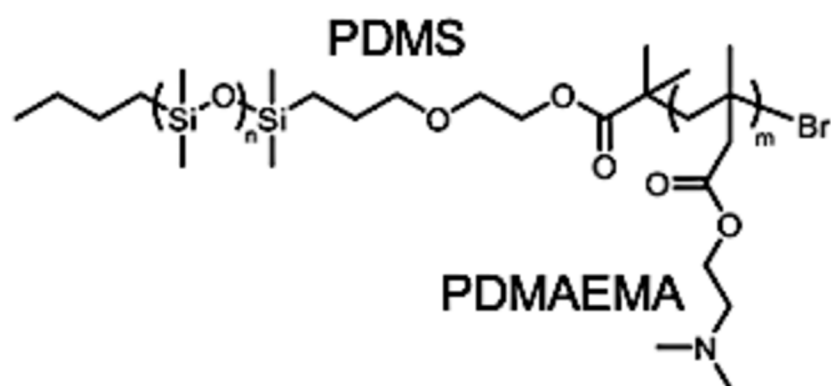
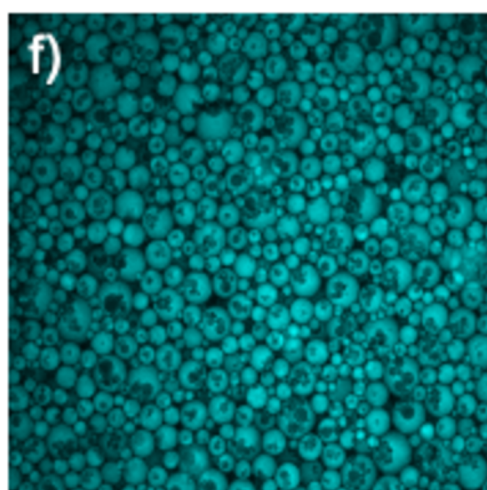
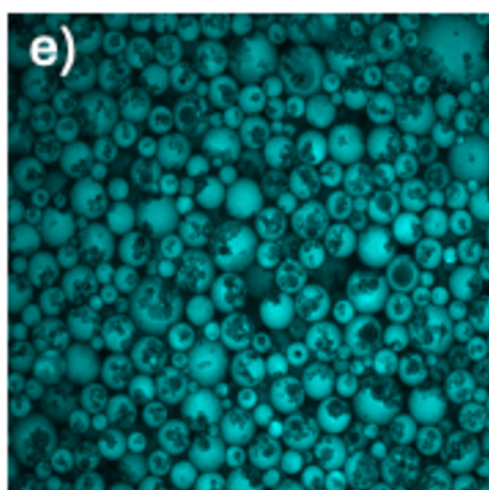
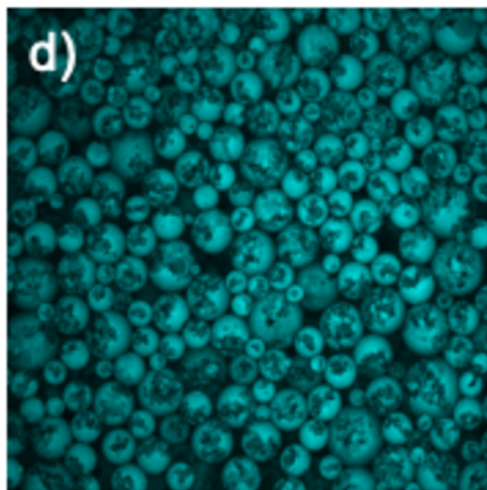
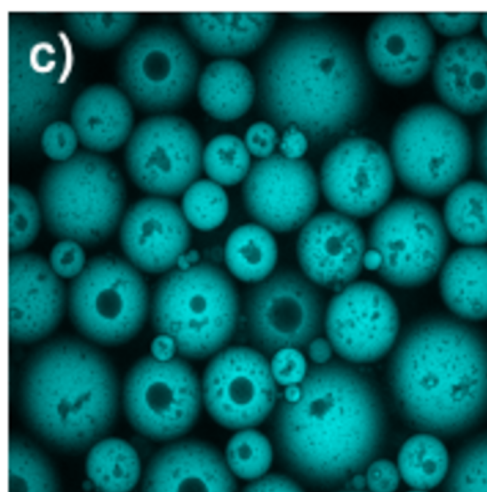
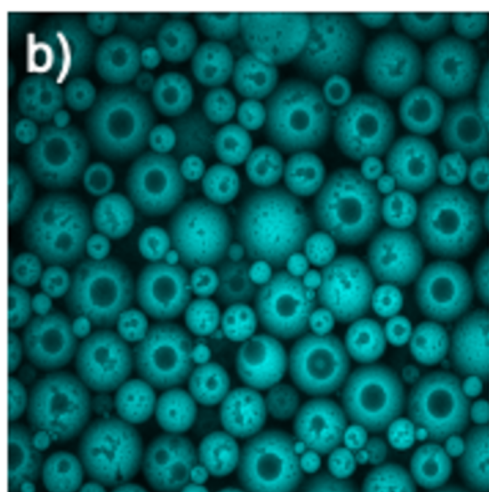
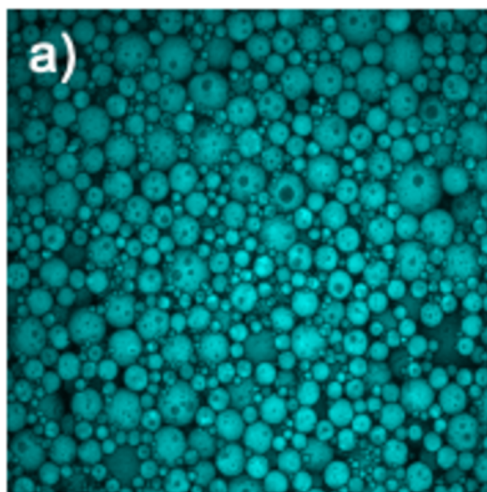


Fig. 2



340  $\mu\text{m}$

Fig. 3

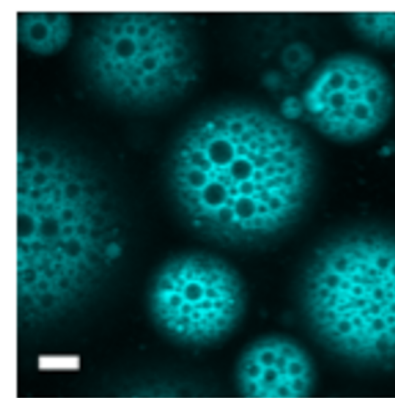
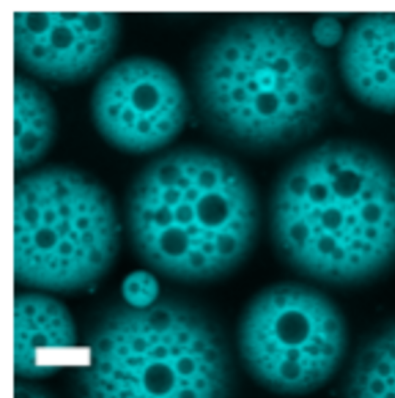
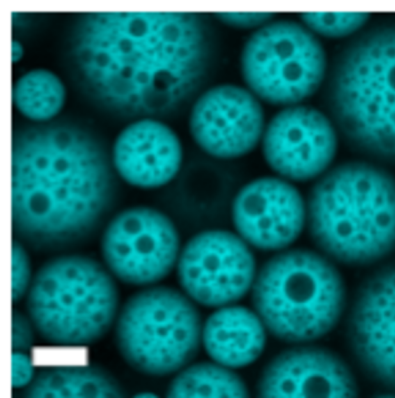
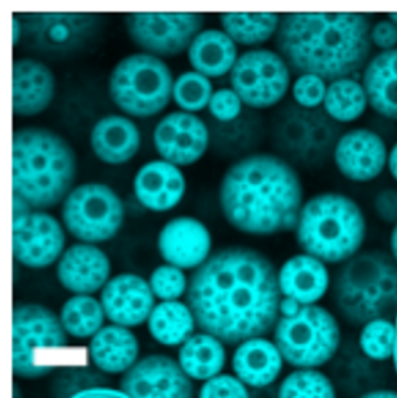
**[NaCl]  
0.15 M**

**0.30 M**

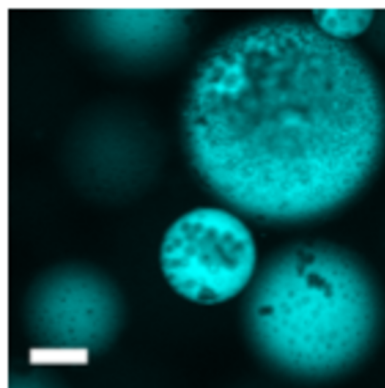
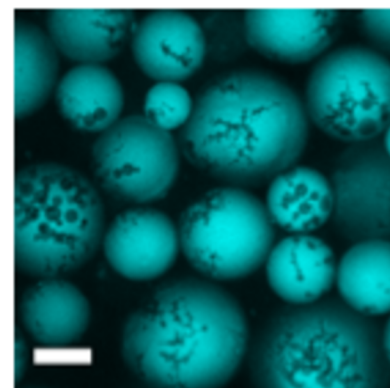
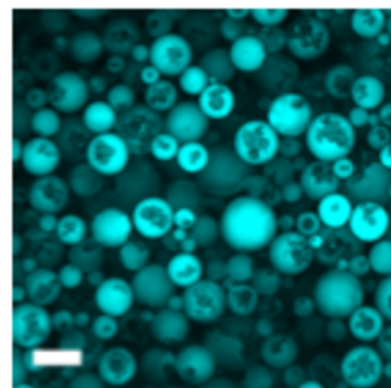
**0.60 M**

**1 M**

**Day 0**



**1 year aged emulsions**



**Day 3**

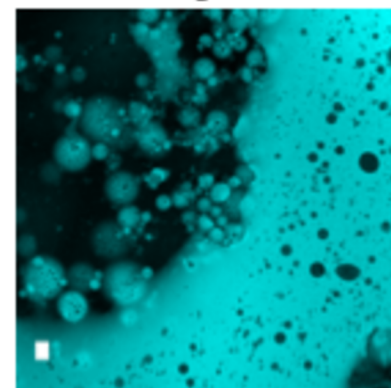


Fig. 4

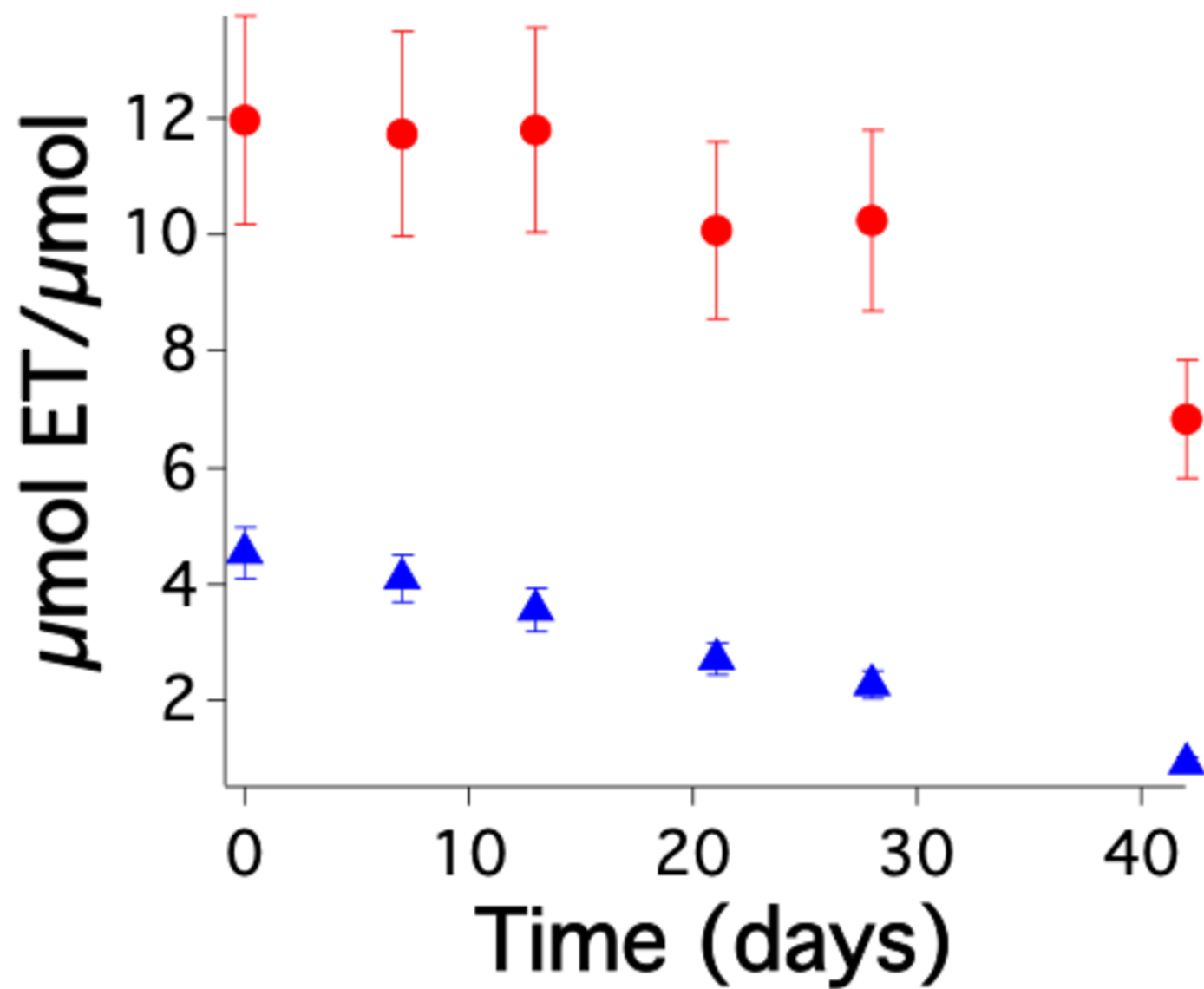


Fig. 5

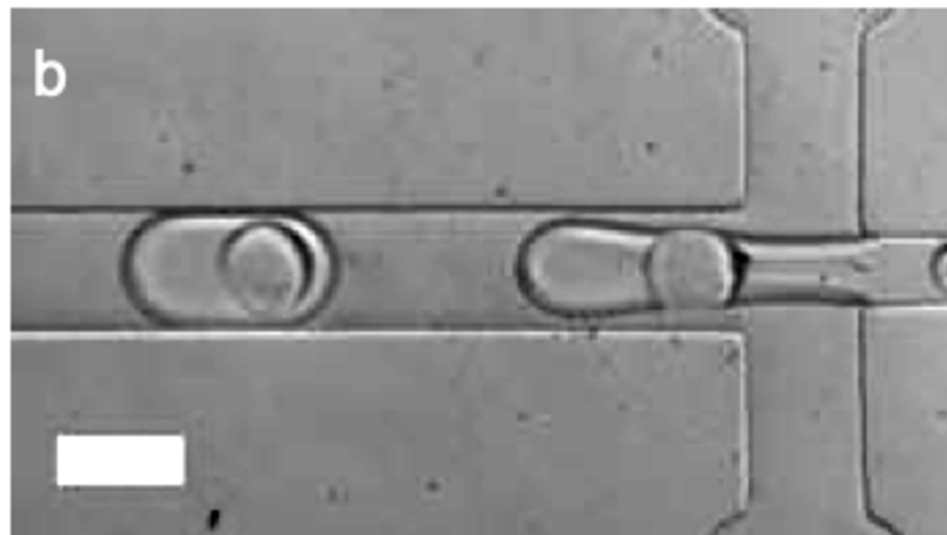
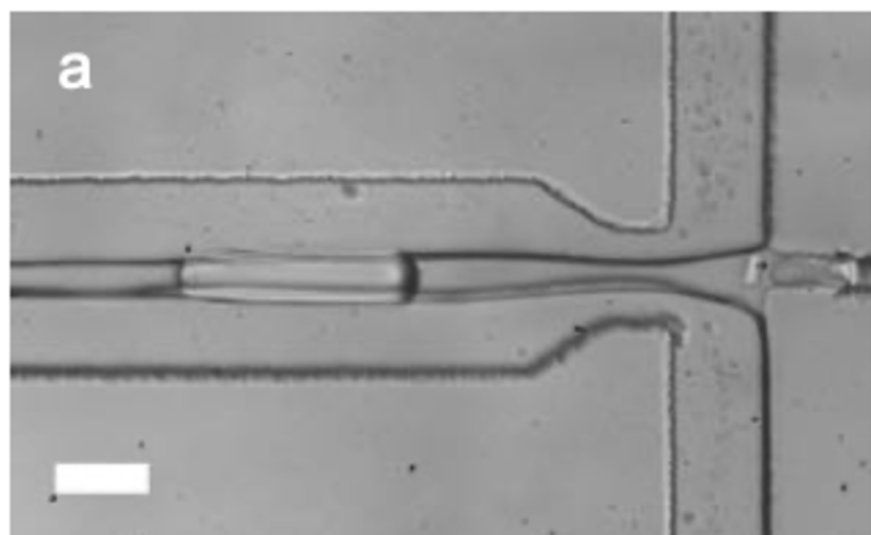
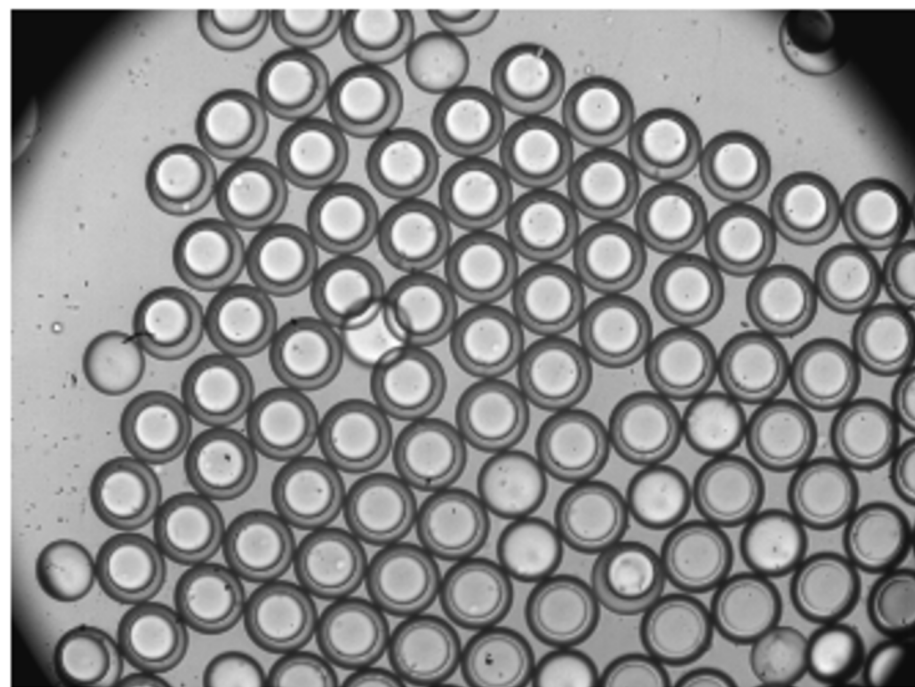


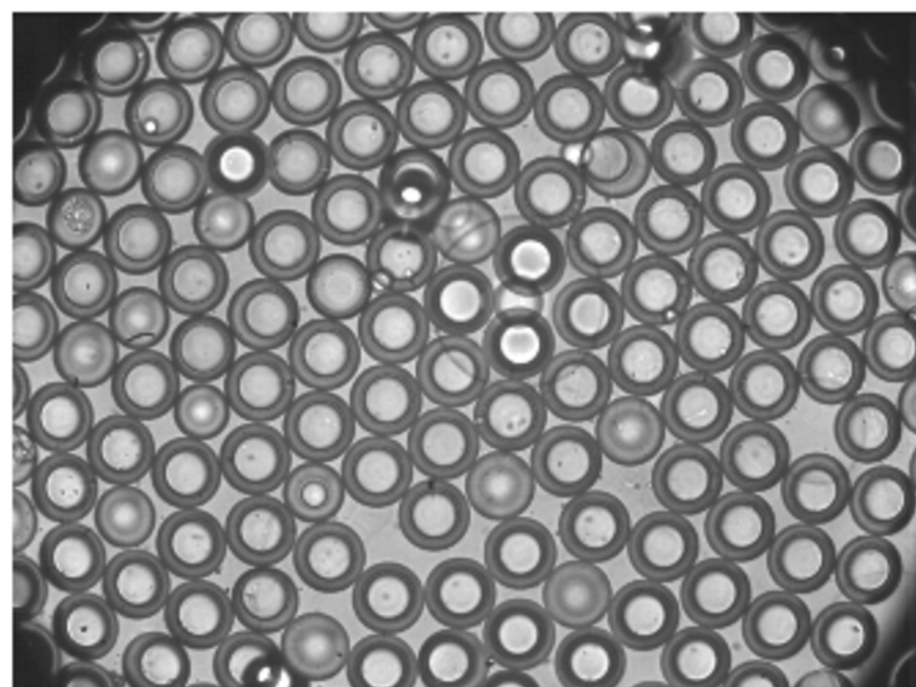


Fig. 6

$t = 0$



$t = 1 \text{ month}$



100  $\mu\text{m}$

Fig. 7

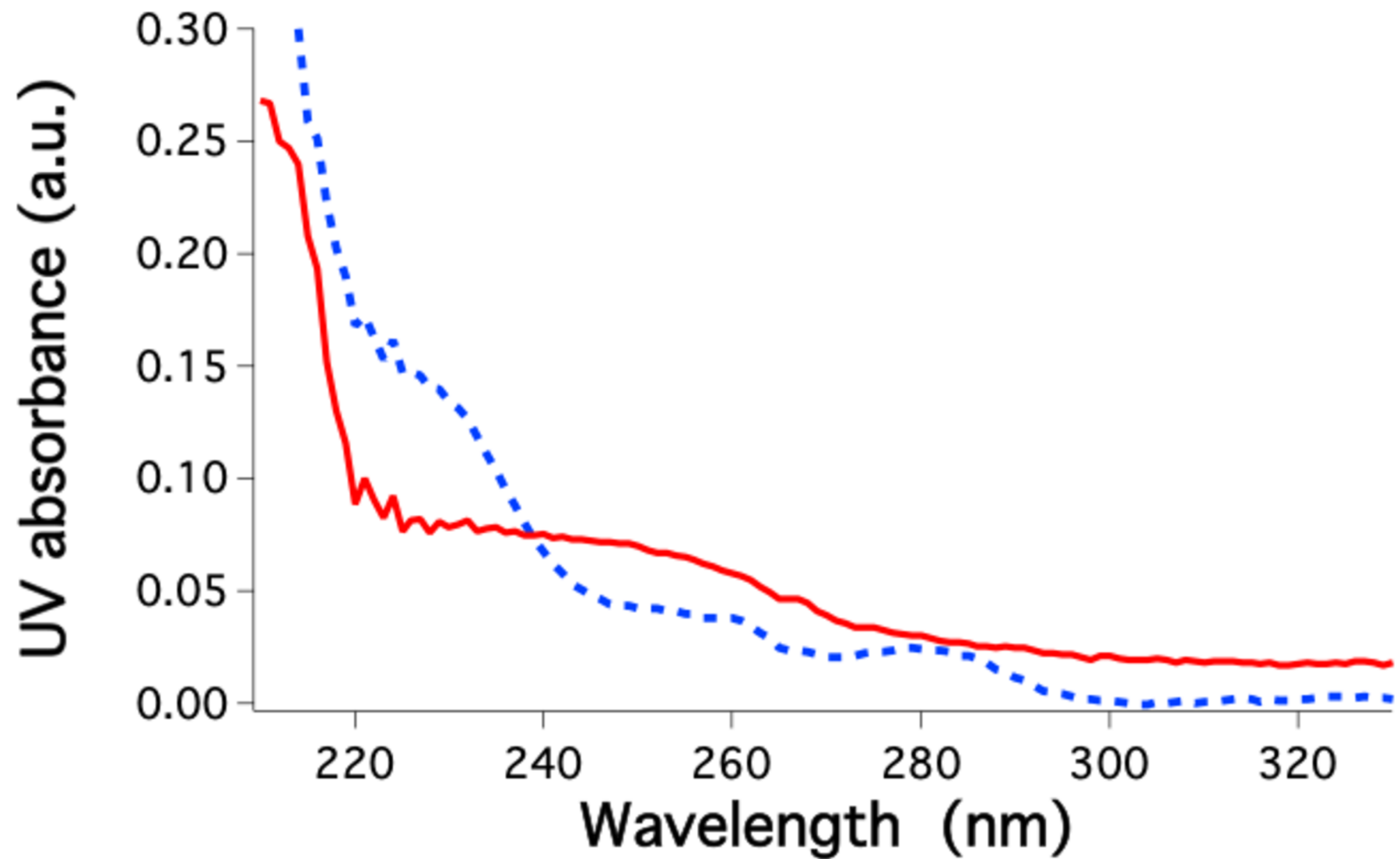


Fig. 8

