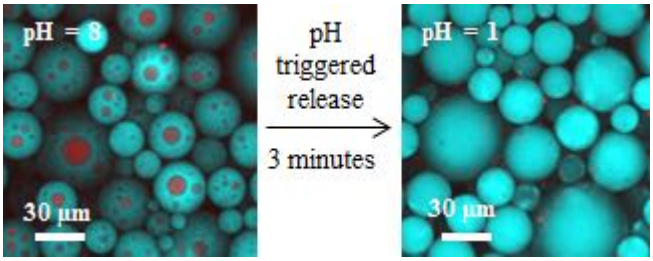
pH and ionic strength triggered destabilization of biocompatible stable water-in-oil-in-water (W/O/W) emulsions

Marine Protat, Noémie Bodin-Thomazo, Frédéric Gobeaux, Florent Malloggi, Jean Daillant, Nadège Pantoustier, Patrick Perrin, Patrick Guenoun

pH and ionic strength triggered destabilization of biocompatible stable water-in-oil-in-water (W/O/W) emulsions

GRAPHICAL ABSTRACT



Graphical abstract: Triggered release of a hydrophilic dye encapsulated in a biocompatible W/O/W emulsion, formed by one-step mixing and stabilized by a single block copolymer using pH and ionic strength (*I*) stimuli. Oil phases appear in blue, dye-loaded aqueous phases appear in red and dye-free aqueous phases appear in black.

AUTHOR NAMES

Marine Protat [1,3], Noémie Bodin-Thomazo[1,3], Frédéric Gobeaux[3], Florent Malloggi[3], Jean Daillant[4], Nadège Pantoustier[1,2], Patrick Perrin[1,2], Patrick Guenoun[3]*,*

AUTHOR INFORMATION

* **Corresponding Authors**

E-mail addresses: marine.protat@espci.org (M. Protat), noemi.bodin@gmail.com (N. Bodin-Thomazo), frederic.gobeaux@cea.fr (F. Gobeaux), florent.malloggi@cea.fr (F. Malloggi), jean.daillant@synchrotron-soleil.fr (J. Daillant), nadege.pantoustier@espci.fr (N. Pantoustier), *patrick.perrin@espci.fr (P. Perrin), *patrick.guenoun@cea.fr (P. Guenoun)

AUTHOR ADDRESSES

1. Sciences et Ingénierie de la Matière Molle, PSL Research University, ESPCI Paris, CNRS, 10 rue Vauquelin, 75231 Paris cedex 05, France

2. Sciences et Ingénierie de la Matière Molle, Sorbonne Universités, Université Pierre et Marie Curie, Paris 06, 10 rue Vauquelin, France.

3. Université Paris-Saclay, CEA, CNRS, NIMBE, LIONS 91191, Gif-sur-Yvette, France.

4. Synchrotron Soleil, L'Orme des Merisiers, Saint-Aubin – BP 48, 91192 Gif-sur-Yvette Cedex, France.

ABSTRACT

The design of biocompatible multiple emulsions is an important challenge in the field of controlled delivery systems for protecting and delivering compounds encapsulated and protected in the innermost phase. In this paper, we use biocompatible water – Miglyol®812 water-in-oil-in-water (W/O/W) emulsions stabilized by a stimuli-responsive diblock copolymer consisting of poly(dimethylsiloxane) (PDMS) and poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) to design an easy-to-process new

delivery W/O/W system. Such emulsions are formed in a single emulsification step. They present a high encapsulation yield and are shown to be stable over months. As such, the encapsulation of a hydrophilic dye (Alexa fluor) in the innermost water phase is successfully demonstrated over months. These emulsions are stimulable either by a shift in pH level or in ionic strength. The former destabilizes the multiple emulsion and leads to a simple one while the latter partly maintains the multiple character. Eventually both stimulations are effective in the dye release and molecular mechanisms are proposed for explaining the observed two-**stage** kinetics of release.

KEYWORDS

Multiple emulsion, W/O/W, stimuli-responsive interface, block copolymer, encapsulation, triggered release

INTRODUCTION

The potential of application of multiple emulsions, especially under the W/O/W form, is promising in different domains like in food, cosmetics or biomedical industries to name a few.[1,2,3] Until recently, pharmaceuticals have primarily been formulated as fast-acting chemical compounds dispensed orally or by injection. Since 1970, drug delivery systems that release the encapsulated drug at a targeted place with controlled delivery kinetics have become increasingly elaborate and complex.[4,5] Multiple W/O/W emulsions are one of the various ways that have been investigated to protect the compounds from oxidation or other external stresses during storage thanks to the oil phase and to make long-term release drug delivery systems. Food industry, which also highlights the importance of multiple emulsions, targets with success either the encapsulation of various aroma, sensitive food or bioactive compounds[6,7], or the production of low-fat products.[8,9] On a fundamental basis, the formation of stable multiple emulsions arises from an appropriate

combination of suitable process and emulsifying agents meaning that such an achievement strongly depends on the emulsification method and emulsion composition.[10] In most studies, W/O/W emulsions are made by a two-step emulsification method and stabilized by several emulsifiers.[11,12,13,14,15] Even if this method enables good encapsulation efficiency and control of the encapsulation yield, the preparation of these emulsions is often long and complicated. Furthermore, the stability offered by those methods is often too limited in time to lead to a precise understanding of the release mechanisms and concrete industrial applications. Exceptions do exist like in several studies of Grossiord's group which was able to evaluate the release of encapsulated compounds by shearing a stable W/O/W emulsion.[16,17,18,19] Passive release of electrolytes was also studied for multiple emulsions stabilized by **silicone**-based surfactants, suggesting that reverse micelles transport and diffusion were two mechanisms operating for the release [20]. Strategies for slowing down multiple emulsions destabilization were discussed in that context.[21] Except for shear-induced release[22], most release mechanisms were passive ones and occur along with the progressive destabilization of the emulsion[23] like by progressive evaporation of one compound.[24] This is clearly a major issue for applications as products often need to be stored before being used.

In the recent years, new one-step methods of preparation of W/O/W emulsions were developed using polymers or particles as emulsifiers.[25,26,27,28] These studies focused on the control of the physico-chemical properties of the system to obtain W/O/W emulsions with long-term stability. However, even if these emulsions have potential to make delivery systems, most of them are still not biocompatible[29,30] or do not provide an easy way to control and trigger the release of entrapped compounds.[25]

Consequently, very few studies were carried out so far about the encapsulation potential and the triggered release of encapsulated species in one-step formed multiple emulsions. Recently, Tu et al. obtained W/O/W emulsions that are pH-responsive.[31] These emulsions were made in one emulsification step and stabilized by Janus particles made of styrene and acrylic acid. Interestingly, the water encapsulation rate of W/O/W emulsions was calculated using a method based on macroscopic measurements on creamed emulsions. Even if no compound was actually encapsulated, the possibility to turn the W/O/W emulsions into O/W ones to release the inner water droplets using pH was established. In a previous work[32], we described the physico-chemical conditions under which biocompatible water – Miglyol® 812 and water – isopropyl myristate emulsions form with respect to their stability and type i.e., direct (O/W), inverse (W/O) or multiple (W/O/W, O/W/O). Emulsions are formed in a one-step emulsification process and stabilized by a single biocompatible emulsifier, a $PDMS_{38}$-*b*-$PDMAEMA_{25}$ block copolymer. The biocompatibility of the complete emulsion has been recently demonstrated **using** 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, making this test a real test of biocompatibility of emulsions in digestive conditions (submitted). In this paper, we describe how to use these emulsions to entrap and release a hydrophilic molecule, the Alexa Fluor fluorescent dye, in the internal water droplets using various stimuli. We report on the stability of the dye-loaded W/O/W emulsions during storage and on the capability of the system to encapsulate the dye in a one-step method of preparation. The controlled triggered destabilization of the multiple emulsion is demonstrated by using pH or ionic strength stimuli. The mechanisms for destabilization are discussed to explain observations.

MATERIALS AND METHODS

Materials

We synthesized the $PDMS_{38}$-*b*-$PDMAEMA_{25}$ and $PDMS_{60}$-*b*-$PDMAEMA_{50}$ polymers as described in ref. 32. PSMAEMA is a ionizable polymer at low pH with a pKa around 7.[33] Sodium chloride (NaCl) and Nile Red were purchased from Sigma-Aldrich. Miglyol®812 was generously provided by IMCD. Alexa Fluor 647 was purchased from Life Technologies. Dialysis membranes (MWCO 6000 to 8000) were purchased from Spectrum® Laboratories. HCl and NaOH solutions were prepared in the lab from NaOH crystals (Sigma Aldrich) and concentrated HCl supplied by Merck. Milli Q water with a resistivity of 18.2 MΩ cm was used to prepare aqueous solutions for emulsions.

Emulsions Preparation

For both polymers, the polymer was dissolved in Miglyol®812 at a concentration of 5 g L^{-1}. Miglyol®812 is a biocompatible oil already used in formulations in both cosmetic and pharmaceutical industries. NaCl, HCl and NaOH solutions were used to prepare aqueous solutions at different pH and ionic strengths (*I*) using Milli Q water. A fixed amount of 3 mL of polymer-containing oil and 3 mL of aqueous solution were placed into contact without mechanical mixing for 24 hours at room temperature. All the pH values reported below were measured after a contact of 24 hours between aqueous and oil phases for all samples. Nile red dissolved in toluene was added to the oil phase, and a small amount of water-soluble Alexa Fluor 647 was added to the aqueous phase of W/O/W emulsion samples prior to emulsification to obtain dye-loaded emulsions.

Emulsions were obtained by homogenizing the two phases left in contact for 24 hours using an Ultra Turrax T10 homogenizer (8 mm head) operating at 24000 rpm for 40 seconds.

Emulsion Washing

For washing the dye out from the external phase after the emulsions are made, the aqueous phase under the creamed emulsion was taken out and gently replaced by the same volume of an aqueous solution at the same pH and ionic strength as the removed one. Four washing steps were necessary in order to completely eliminate any sign of the Alexa dye from the external water phase, i.e. until it was no longer detectable in confocal images. Between each washing step, the sample was carefully agitated manually to homogenize the emulsion without modifying its morphology as checked by confocal imaging.

Emulsion destabilization and Dye Release

The emulsion destabilization and release of a dye molecule when it is encapsulated in the internal aqueous phase of W/O/W emulsions was triggered by a pH or ionic strength shift. For pH-triggered destabilization and release, multiple emulsions were dialyzed against an aqueous solution at the same ionic strength and temperature but with a much more acidic pH (pH = 1, 2, 3 or 5 instead of pH = 8.2 initially). A volume of 8 mL of emulsion was dialyzed versus 250 mL of aqueous phase at the given pH. We proceeded the same way for ionic strength-triggered destabilization for which the emulsion was dialyzed against an aqueous solution at the same pH and temperature but containing no salt.

Observations of emulsions during release were made by confocal microscopy. Small samples of 150 μL were taken out from the vial containing the emulsion and observed at room temperature, for the two types of release. Samples observed before destabilization are put arbitrarily on the logarithmic timescale of destabilization (Fig. 8) at t=1s for delineating the initial plateau of internal water content.

Determination of the encapsulation yield

The encapsulation yield is defined as the volume ratio of the internal water droplets to that of the initial total volume of the aqueous phase (i. e. 3mL). It was estimated using two different methods.

As a first method, a macroscopic evaluation of the encapsulation yield was made using the method established by Tu et al.[31] for which two emulsion samples, an O/W and a W/O/W one, were compared. The packing volume fraction P, determined from the simple O/W emulsion, is defined as follow:

$$P = \frac{V_{oil\ phase}}{V_{simple\ emulsion}} \qquad (1)$$

where $V_{oil\ phase}$ is the identical volume of the oil phase used to make both emulsions and $V_{simple\ emulsion}$ is the volume occupied by the simple O/W emulsion after creaming. Then, the encapsulation yield, τ, can be calculated as follows:

$$\tau = \frac{V_{internal\ water}}{V_{water\ phase}} = \frac{V_{creamed\ multiple\ emulsion} \times P - V_{oil\ phase}}{V_{water\ phase}} \qquad (2)$$

where $V_{internal\ water}$ is the volume of the internal water droplets of the W/O/W emulsion, $V_{water\ phase}$ is the volume of the total water phase of the emulsions and $V_{creamed\ multiple\ emulsion}$ is the volume occupied by the multiple W/O/W emulsion after creaming. These volumes were calculated from the measurements of the height of the different phases for emulsions made in identical cylindrical vials. This formula assumes that both types of emulsions have the same drop size distribution and same packing. The O/W emulsion that was chosen as a reference for the determination of τ was consequently checked to exhibit a drop size distribution quite similar to that of the external W/O/W drops.

The encapsulation yield was also determined by a second method using a chloride ion-selective electrode. First, a W/O/W emulsion was made using NaCl to obtain the desired

ionic strength (C_w). Directly after emulsification, 5 mL of this emulsion were transferred into a dialysis bag (total water volume V_w) and were dialyzed under magnetic stirring against 50 mL of $NaNO_3$ aqueous solution (V_d) at the same pH and ionic strength as the emulsion. The water solution was changed four times a day keeping the total volume of water constant. The decrease of the NaCl concentration was followed using the chloride ion-selective electrode. When the remaining NaCl concentration reached a background level (C_b), the emulsion was considered to be washed, i.e. the external phase of the W/O/W emulsion is ion chloride free. The emulsion was then broken using a pH change as stimulus and the released NaCl concentration (C_r) was measured (assuming a total release) using the electrode one week after the beginning of the destabilization to be sure that the equilibrium is completely reached. Assuming that no leakage of chloride ions has occurred outside of the internal water droplets during the dialysis process, the encapsulation yield can then be calculated as:

$$\tau = \frac{V_{internal\,water}}{V_{total\,water}} = \frac{V_w + V_d}{V_w} \times \frac{C_r - C_b}{C_w - C_b} \qquad (3)$$

Stability Measurements

Dye-loaded emulsion stability was assessed one day, two months and four months after emulsification. Emulsions were stored in the dark at room temperature.

Characterization

Emulsion type, dye encapsulation, dye release and emulsion stability were assessed by Confocal Laser Scanning Microscopy (CLSM) using Nile Red as a hydrophobic fluorophore. Confocal imaging was done in a quartz cell (0.5 mm light path) through an Olympus Fluoview FV1000 inverted confocal microscope. 150 μL emulsion samples were placed in the cell and studied at room temperature.

The following criterion was used to discriminate between O/W and W/O/W emulsions. W/O/W emulsions are defined as emulsions for which the surface (of the 2D confocal image) of the oil drops containing water droplets covers more than 50% of the surface of the total emulsion image (which contains pure oil drops and oil drops containing water). The surfaces were all being measured from a series of four confocal microscopy pictures of a given sample. If the criterion is not fulfilled, then the emulsions are called O/W.

Diameters of emulsions oil drops were calculated from the images obtained by confocal microscopy (depth of field is much smaller than drop diameters). For each W/O/W emulsion, we measured the mean oil drop size and standard deviation (SD) of a panel of 500 oil drops using Image J (NIH software) with a systematic error of 10 %, which occurs mostly because some drops are visible albeit their largest diameter is not perfectly in the z plane of focus. For dye-loaded W/O/W emulsions, the mean number of internal water droplets per oil globule (N_{ID}) was determined by counting the number of internal water droplets for a panel of 100 oil globules. The uncertainty measurement, which is about 20 %, mainly arises from the small size internal water droplets which are not resolved: the water droplet distribution is then truncated.

NaCl concentrations were measured using a chloride ion-selective electrode (combined electrode, Vernier). For each measurement, the electrode was previously soaked into a standard solution at 1000 mg mL^{-1} of NaCl for at least one hour and then the electrode was calibrated using three different standard solutions of NaCl: 1 mg mL^{-1}, 10 mg mL^{-1} and 1000 mg mL^{-1}. Finally, the potential of the sample solution was measured. The concentration of chloride ions [Cl^-] in the sample solution was calculated as follows:

$$[Cl^-] = 10^{[E(sample)-E(1)]/S_m} \; and \; S_m = [E(1000) - E(10)]/2$$

where S_m is the electrode slope, E(1000), E(10) and E(1) are the potentials measured in the 1000, 10 and 1 mg mL^{-1} standard solutions respectively and E(sample) the potential measured in the sample solution. The concentration of chloride ions in the sample solution was then converted into a NaCl concentration by assuming a total salt release.

Image analysis:

The analysis relies on the Auto Local Procedure provided by ImageJ, NIH (https://imagej.net/plugins/auto-local-threshold.) It enables thresholding a picture on a local basis by adjusting a radius r within which the threshold is computed; different algorithms are provided within ImageJ and for each picture it was tested which algorithm reveals at best (by comparison with the original image) either the oil+internal water domains or the internal water domains. In most cases, the Bernsen, Otsu or Phansalkar algorithms were used. In a second step, the particles analysis of ImageJ was used to delineate domains and measure their total surface. The surface area was then computed in both cases for providing estimation of the oil+internal water or internal water enclosed surface ratio. As a final check, for each image, a composite image made of the thresholded oil domains (see Fig. 7 c) as an example) multiplied to the thresholded image of internal water domains (see Fig. 7 d) as an example) was created and compared to the original picture (see results in Fig. 7 e))

RESULTS AND DISCUSSION

A) EMULSION FORMULATION AND LOADING

A-1) Emulsion formulation in presence of Alexa Fluor

Emulsions were made in a one-step emulsification process for various pH and ionic strength conditions and stabilized by a unique block copolymer of $PDMS_{38}$-*b*-$PDMAEMA_{25}$ or $PDMS_{60}$-*b*-$PDMAEMA_{50}$. For several experiments, Alexa Fluor was incorporated in the emulsion, serving as a probe for encapsulation/release studies. Alexa Fluor is a hydrophilic dye frequently used as a cell and tissue label. It is also a fluorescent dye, detectable in confocal microscopy and, for these reasons, it has been chosen to study loading and delivery capabilities of stable water - Miglyol® 812 W/O/W emulsions. Alexa Fluor was encapsulated into an emulsion at pH = 8.2 and $I = 2$ mol L^{-1} by dissolving aliquots of the fluorescent dye into the water phase prior to emulsification. The confocal microscopy images of emulsions made in the presence of Alexa Fluor are presented in Figure 1.

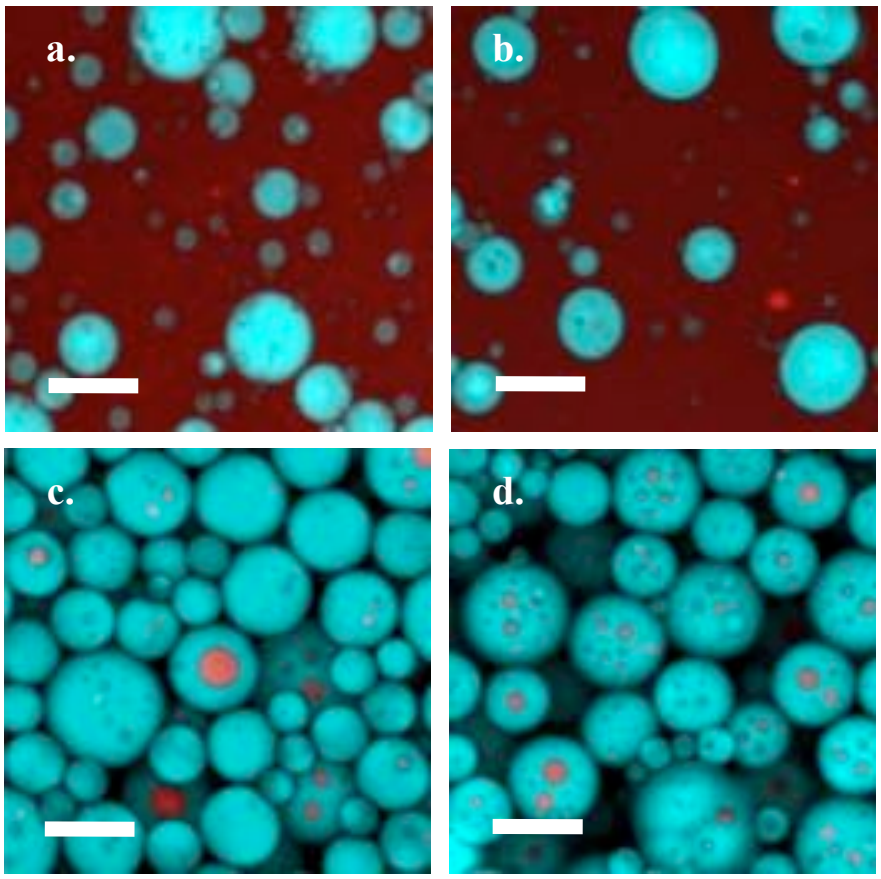


Figure 1: Morphology of water - Miglyol® 812 emulsions stabilized by $PDMS_{38}$-*b*-$PDMAEMA_{25}$ and formulated in presence of Alexa Fluor. Fluorescence confocal microscopy images of emulsions prepared **a.** at pH = 1 and I = 0.1 mol L^{-1}, **b.** at pH = 7.4 and I = 0.1 mol L^{-1}, **c.** at pH = 8 and I = 1 mol L^{-1} and **d.** at pH = 8.2 and I = 2 mol L^{-1}. For pictures **c** and **d**, the external phase was washed and appears black. The Nile Red containing oil phase appears in blue, the Alexa Fluor containing water phase in red and the blank water phase in black. All scale bars are 30 μm.

At I = 0.1 mol L^{-1}, direct O/W emulsions are formed with our system (pictures **a** and **b**, Figure 1). W/O/W emulsions are formed when the pH is close to 8 and the ionic strength as low as 0.1 mol L^{-1} (Figure 1 **c**-**d** for I= 1 or 2 mol. L^{-1}). The emulsion formulated at I = 2 mol L^{-1} (Figure 1 **d**) has a significantly higher encapsulation yield than the one formulated at I = 1 mol L^{-1} (Figure 1 **c**).

From the previous results, pH of 8.2 and an ionic strength of 2 mol L^{-1} (Figure 1 **d**) were chosen as optimal conditions to formulate a W/O/W emulsion stabilized by $PDMS_{38}$-*b*-$PDMAEMA_{25}$ copolymer in presence of Alexa Fluor in the water phase. For the copolymer $PDMS_{60}$-*b*-$PDMAEMA_{50}$, multiple emulsions without encapsulated dyes were formulated at a smaller ionic strength of 0.3 M.

In Fig. 1, it must be noticed that quite different volume fractions of oil drops are observed: this is due to the altitude of observation and creaming effect. As in Fig. 1 c), quite a large volume fraction of the oil dispersed phase can be reached because of polydispersity. This large volume fraction is consistent with the possible 30% water content which is encapsulated (see A-4) implying a 80% volume fraction of the dispersed phase.

A-2) Emulsions washing

As emulsification proceeds in one step, for dye-containing emulsions, the concentration of Alexa Fluor is the same in both internal and external water phases. In order to perform release studies, Alexa Fluor needs to be encapsulated only within the inner water droplets, thus requiring the removal of the dye from the aqueous outer phase. This was achieved as described in Materials and Methods section where the dye-containing external water phase was progressively replaced by a water phase with similar pH and ionic strength.

The emulsion morphology was then controlled throughout the washing steps using confocal microscopy in order to follow the decrease of dye intensity in the external water phase and the possible evolution in the mean size of oil drops (Figure 1 **c**-**d** and Table 1).

Emulsion	Before washing	After washing	2 months after washing	4 months After washing
∅ ± SD (μm)	22 ± 9	21 ± 9	22 ± 7	21 ± 7
N_{ID}	6 ± 1	6 ± 1	5 ± 1	6 ± 1

Table 1: Washing and aging of a water – Miglyol® 812 emulsion (pH = 8.2 and *I* = 2 mol L^{-1}) formulated in presence of Alexa Fluor and stabilized by $PDMS_{38}$-*b*-$PDMAEMA_{25}$. Mean diameter (ø), standard deviation (SD) of oil drops and average number of water droplets per oil globule (N_{ID}) for a freshly prepared emulsion (before washing), for an emulsion that was washed four times and for emulsions two months and four months after the end of the washing procedure. For each sample, the error in the determination of mean oil drop diameters can be estimated to be around 10 %.

As shown in Figures 1**c**-**d**, after four washing steps, the external water phase observed by confocal microscopy appears entirely empty of its Alexa Fluor content. The mean size of oil drops and the average number of water droplets per oil globule are identical within error bars before and after washing (Table 1), which shows that the morphology of the dye-loaded W/O/W emulsion is not affected by the washing process. In the following, the washed emulsion encapsulating Alexa Fluor in the internal water droplets will be called "Alexa Fluor loaded emulsion".

A-3) Stability of Alexa Fluor loaded emulsions

Stability of Alexa Fluor loaded emulsions was assessed one day, two months and four months after the end of the washing steps by confocal microscopy (Figure 2) as well as by drop size measurements (Table 1). Clearly, no significant variation in the oil drop size

or in the number of water droplets per oil drop could be observed up to four months, therefore indicating the remarkable stability of the loaded emulsion during storage.

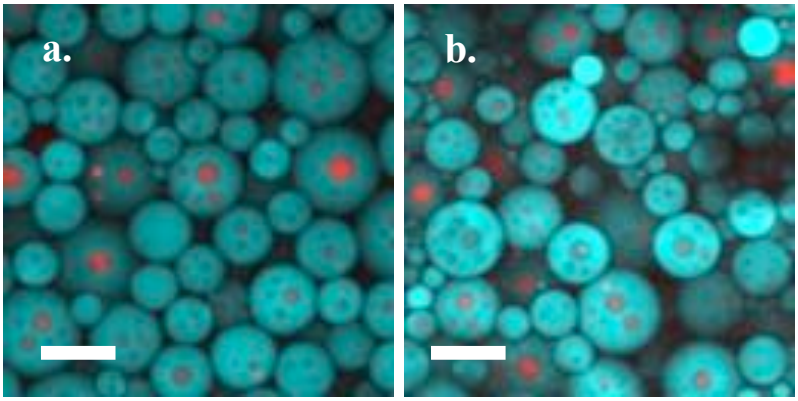


Figure 2: Stability of an Alexa Fluor loaded emulsion stabilized by $PDMS_{38}$-*b*-$PDMAEMA_{25}$ (pH = 8.2 and I = 2 mol L^{-1}). Fluorescent confocal microscopy images of the emulsion **a.** immediately after emulsification and **b.** after four months of storage in the dark at room temperature. Oil phases appear in blue and water phases containing Alexa Fluor appear in red. All scale bars, 30 μm.

Moreover, the inner water droplets and the external water phase stay red and black, respectively, four months after the external phase was washed. This means that the dye does not diffuse appreciably through the oil globule such as any change can be detected by confocal microscopy. Overall, these results show the high stability of the Alexa Fluor loaded emulsion with respect to the dye encapsulation.

A-4) Determination of the encapsulation yield of multiple emulsions

As described in the experimental section, the encapsulation yield, τ, defined as the ratio between the volume of the internal aqueous phase and the total volume of water in the emulsion was here determined using two methods, referred hereafter as the Tu's method and the chloride-ion selective electrode method.

Measures of the encapsulation yield using the two different methods for an Alexa Fluor loaded emulsion stabilized by $PDMS_{38}$-*b*-$PDMAEMA_{25}$ (pH = 8.2 and I = 2 mol L^{-1}) were obtained. Errors in the determination of the encapsulation yield were estimated from experimental protocols. The Tu's method leads to an encapsulation yield of about 20 ± 5 %. A value of 30 ± 10 % for the encapsulation yield was determined using the chloride-ion sensitive electrode method. The two methods are in agreement given the experimental uncertainties but the macroscopic Tu's method returns a lower value than the electrode-based one. This cannot be due to leakage in the electrode method which would give the opposite result. Tu's method assumes identical packing which could be wrong because of differences in polydispersity between simple and multiple emulsions: actually in Ref. 32, Figure 5 **a**, it was observed that polydispersity of multiple emulsions is somewhat larger than for simple emulsions. This leads to underestimate the packing parameter, leading to underestimate the encapsulation yield as measured. Overall, the value of the encapsulation yield for this W/O/W emulsion, τ, is thus at least 15 % (20-5 %), a value that is certainly high enough to target interesting applications in drug delivery. For instance, in some cases, the external phase can be either washed and the compound of interest recycled by concentrating or the system can be used as a two-stage delivery system for non-fragile compounds: a first dose is immediately delivered where the system is injected and the second one will be specifically delivered to the low pH region.

B) pH STIMULUS

B-1) pH triggered release of multiple emulsions without dye

pH was used as a stimulus to destabilize multiple emulsions stabilized by copolymer $PDMS_{38}$-*b*-$PDMAEMA_{25}$ (pH = 8.2 and I = 1 mol L^{-1}) or copolymer $PDMS_{60}$-*b*-$PDMAEMA_{50}$ (pH = 8.2 and I = 0.3 mol L^{-1}). The pH of the external aqueous phase was decreased to different values using dialysis while both the temperature and ionic strength were kept constant at 25°C and 1 or 0.3 mol L^{-1} respectively during the destabilization. The process was followed by confocal microscopy. Figure 3 presents comparative pictures of the emulsions stabilized by $PDMS_{38}$-*b*-$PDMAEMA_{25}$ before and after pH changes for pH 1 and 5

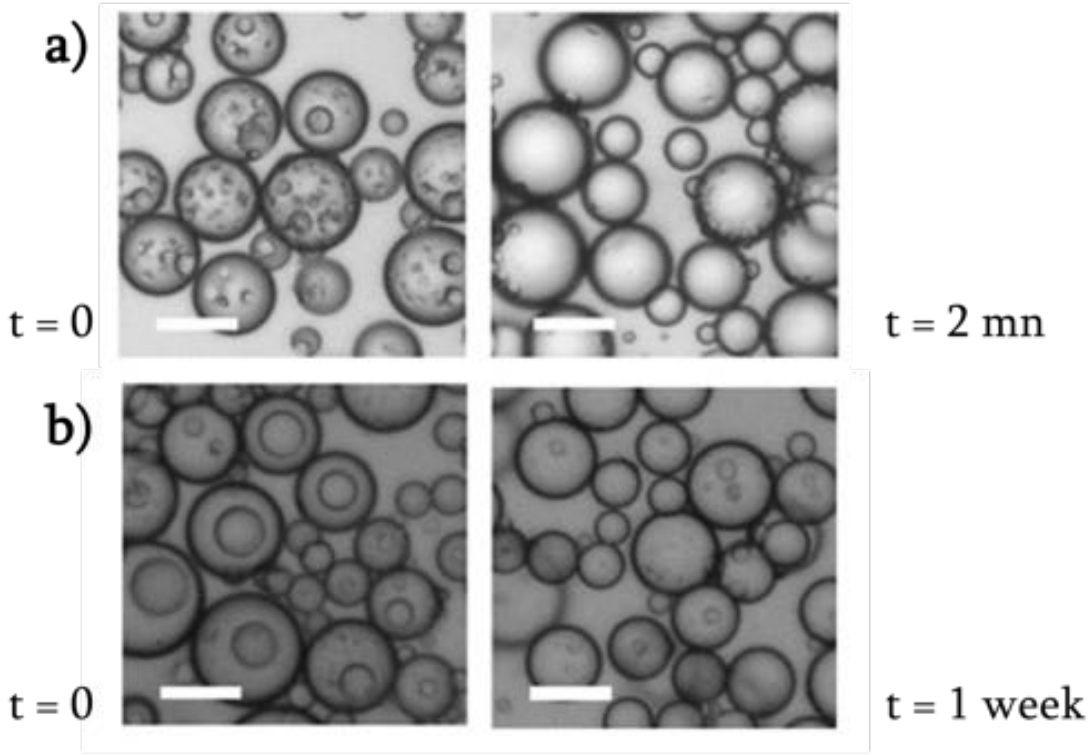


Figure 3: Simple optical microscopy images of pH destabilization of multiple emulsion without dye stabilized by $PDMS_{38}$-*b*-$PDMAEMA_{25}$ for a final pH of 1 (**a)**) and for a final pH of 5 (**b)**). Scale bar is 20 μm.

Similar comparative images at destabilization pHs of 1, 2 & 3 are shown in Figure 4 at a different spatial scale for emulsions stabilized by copolymer $PDMS_{60}$-*b*-$PDMAEMA_{50}$. Some scatter in size distribution is observed from one pH value to another: this is due to sampling of the emulsion where at each pH and each time, a particular zone is imaged.

In all cases, lowering the pH drives the multiple emulsion to a direct one, except for pH 5 where the emulsion stays partly multiple. Kinetics of destabilization is also markedly different according to targeted pH and slows down as pH is increased. The kinetics was not observed to be dependent on copolymer used.

It must be also noted, that the dialysis procedure, is not limiting the observation in time, since, at pH 1 for instance, changes in two minutes of time can be observed. However, at these short times, it is possible that the pH in the emulsion is not fully equilibrated.

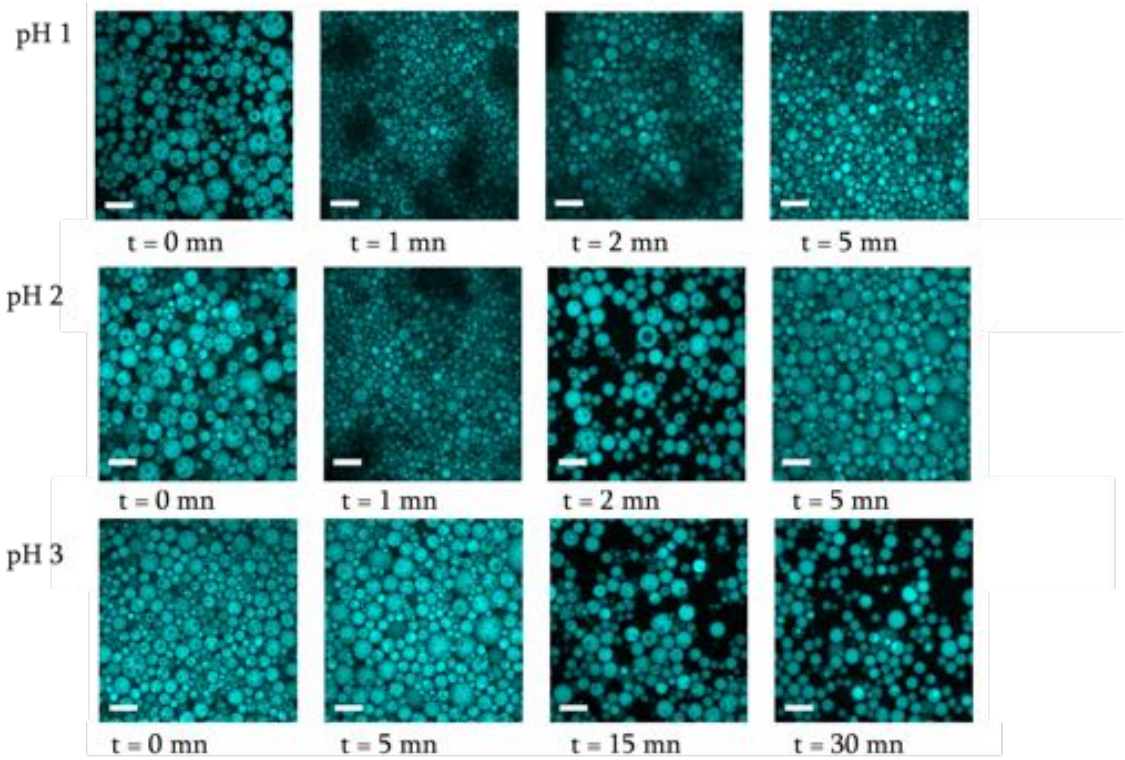


Figure 4: Destabilization of bare multiple emulsion stabilized by $PDMS_{60}$-*b*-$PDMAEMA_{50}$. Note the slowing down in kinetics as pH increases. Scale bar is 30 μm.

B-2) pH triggered release of dye loaded multiple emulsions

pH was also used as a stimulus to destabilize multiple emulsions containing the Alexa dye and trigger its release from a loaded emulsion stabilized by copolymer $PDMS_{38}$-*b*-$PDMAEMA_{25}$ (pH = 8.2 and I = 2 mol L^{-1}). The pH of the external aqueous phase was decreased to pH = 1 or pH = 2 (Figure 5) using dialysis. Both the temperature and ionic strength were kept constant at 25°C and 2 mol L^{-1} respectively during the release experiment of the dye. The release process was followed by confocal microscopy.

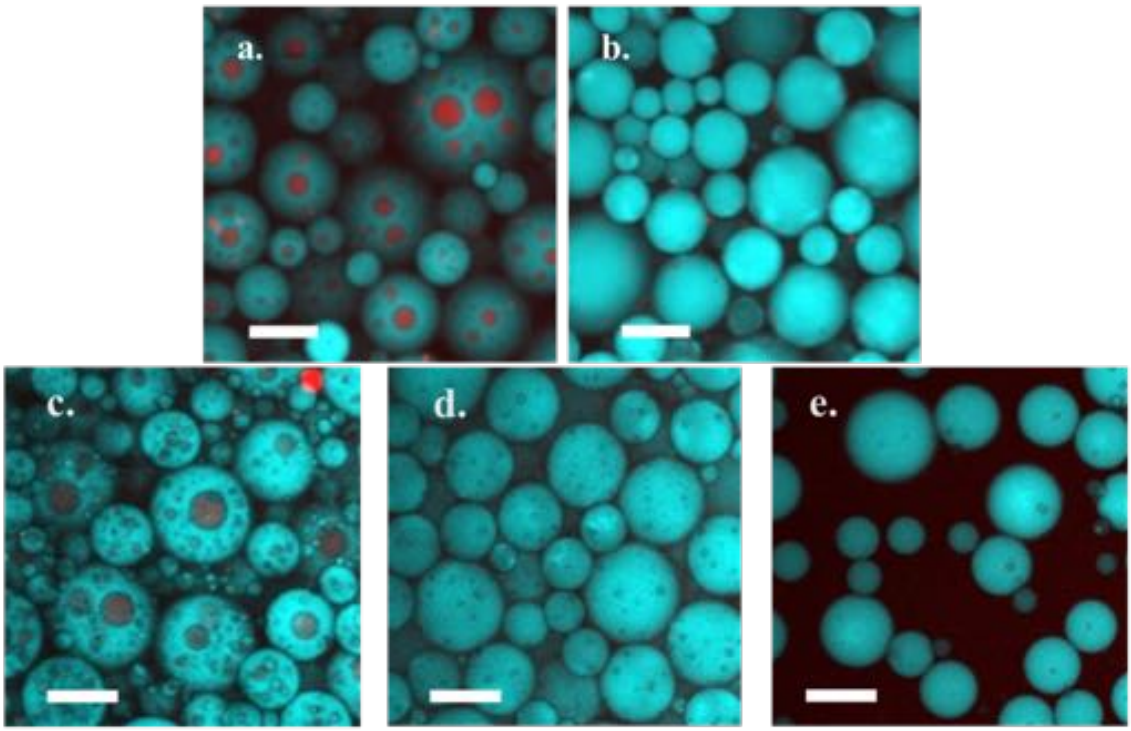


Figure 5: pH triggered release of the encapsulated Alexa Fluor (at pH = 1 and 2). Fluorescent confocal microscopy images of an Alexa Fluor loaded emulsion (pH = 8.2 and I = 2 mol L^{-1}). **a.** Initial emulsion at pH = 8.2; **b.** Emulsion observed after 3 minutes of release at pH = 1; **c.** Emulsion observed after 5 minutes at pH 2; **d.** Emulsion observed after 15 minutes at pH 2; **e.** Emulsion observed after 2 hours at pH 2. Oil phases appear in blue and water phases containing Alexa Fluor appear in red. All scale bars are 30 μm.

As shown in Figures 5, at the end of the destabilization processes, the W/O/W emulsion has turned into a single O/W leading to the release of the dye from oil globules (Figure 5 **b** and **e**).

As observed with bare emulsions, the kinetics of Alexa Fluor release can be tuned by changing the pH release value. Indeed, when the release is processed at pH = 1 the

W/O/W emulsion appears fully destabilized into an O/W emulsion about three minutes after the application of the pH stimulus (Figure 5 **b**). At pH 2, slight changes of the emulsion morphology are only visible five minutes after the beginning of the pH change. After 15 minutes, the emulsion morphology has turned to a direct emulsion one (Figure 5 **d**) and stops evolving about two hours later (Figure 5 **e**). It is worth noticing that at that time, the release is not fully achieved as some small internal water droplets are still encapsulated inside oil globules in contrast to the release processed at pH = 1 (Figure 5 **b** and **e**). In the course of destabilization, the Alexa fluor coloration fades away and the dye is no more detectable.

B-3) Discussion about pH induced release:

The destabilization of the multiple emulsion by lowering the pH can be explained by different molecular mechanisms. The first one is based on molecular diffusion of water through the oil where it is partly soluble at an amount of about 2360 μg/g.[34] Lowering pH should cause an imbalance of water molecular flux through the oil, emptying droplets by an osmotic flux of water. The osmotic pressure induced by lowering the pH to 1 is of order 5 atm.. as estimated by a simple perfect gas formula, counting the ionic concentrations. However, this mechanism does not explain the dye disappearance from inner droplets.

It is then worth considering a second possible mechanism which is the exchange of water through the oil by water-swollen reverse micelles as already discussed in literature for two-steps formed W/O/W emulsions.[20,35,36] This mechanism would be inoperant at pH 8 because of an equal inward and outward flux of water (the Laplace pressure is too small to induce a disequilibrium because of the large size of droplets and weak surface tension). When pH is lowered on the outer side, a neat flux of micelles would lower the inner pH

by bringing protons to the inner droplets (Figure 6). This mechanism could alternatively empty the inner water if the inbound flux of micelles is less that the outbound flux: this is very likely since the formation of reverse micelles curved towards water for low pH values where the PDMAEMA chains are fully charged and rather extended, is *a priori* less favorable than for more flexible conformations. This extension of chain conformation at low pH was recently demonstrated for similar copolymers by neutron reflectivity at oil-water interface[37] where it was also shown that conformation is essential for dictating the nature of emulsions. In particular a balanced conformation between the hydrophilic and hydrophobic moieties is requested for reaching a multiple emulsion. Moreover, the decrease in pH for internal water will destabilize interfaces curved towards water and promote coalescences of inner droplets with the water-continuous phase: this is a more direct mechanism for transferring water to the continuous phase.

The direct water transfer through the oil by whichever mechanism is in fair agreement with images shown in Figure 3 where the largest water inner droplets are decreasing in size between t= 0 and t = 1 week for the pH 5 case. At pH 3, Figure 4, small size inner droplets seem to disappear with time, a phenomenon which can be equally attributed to direct water transfer or to coalescences.

Examining now dye-loaded emulsions, it must be noted that at pH 8 where stable emulsions are observed for months, the first mechanism (water molecular diffusion) preserves dye-loaded emulsions whereas the second mechanism could empty the inner dye unless no dye is transported in the micellar core at pH 8. At lower pHs where destabilization occurs, the first mechanism alone would empty inner droplets but should preserve or even increase (by concentrating the dye) fluorescence which on the contrary is not observed anymore (Figure 5). As explained before, the second mechanism cannot

explain the transfer of the dye out of the water droplets by incorporating the dye into the micelles (except maybe when pH is low enough for incorporating dye into micelles). We then suggest that inverse micelles are necessary for lowering the pH of inner droplets, then destabilizing the interfaces to induce coalescences, diluting the dye out. Note that the dye is no more visible when out because of dilution effect though it was independently checked that it still can emit at pH 1 (Figure 1 **a**). In Figure 3, due to the smaller difference in pH, it may happen that water transfer, either direct or by inequal flux of micelles is more effective than coalescences, leading to incomplete destabilization.

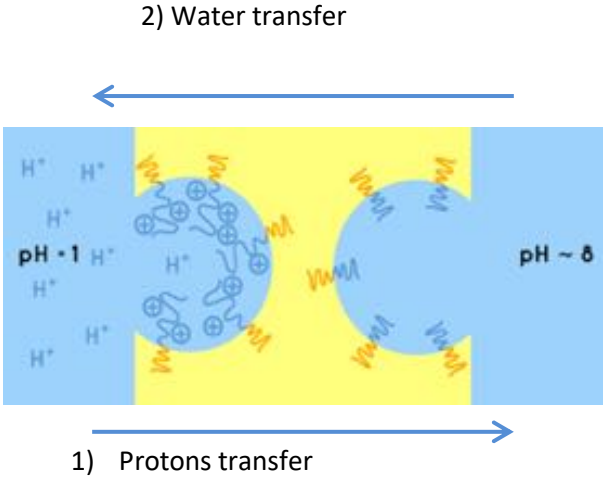


Figure 6: Mechanisms of destabilization by unbalanced reverse micelles transfer either 1) carrying protons or 2) carrying water for turning a multiple emulsion into a direct (O/W) one. At equal pHs both fluxes would be balanced while at low external pH (case of the Figure 5), flux 1) would be much higher than 2).

To be more quantitative, the series of pictures taken over a large enough range of time, when pH was lowered at 3 (Figure 4), were analyzed. At each time, the multiple emulsion picture was locally thresholded (ImageJ, NIH) for letting appear only white oil drops including black

inner water droplets as shown in Figure 7 **a**) followed by a particle analysis providing the

total surface of oil (Figure 7 **b**) including inner water) and the total surface of internal water (Figure 7 **c**):

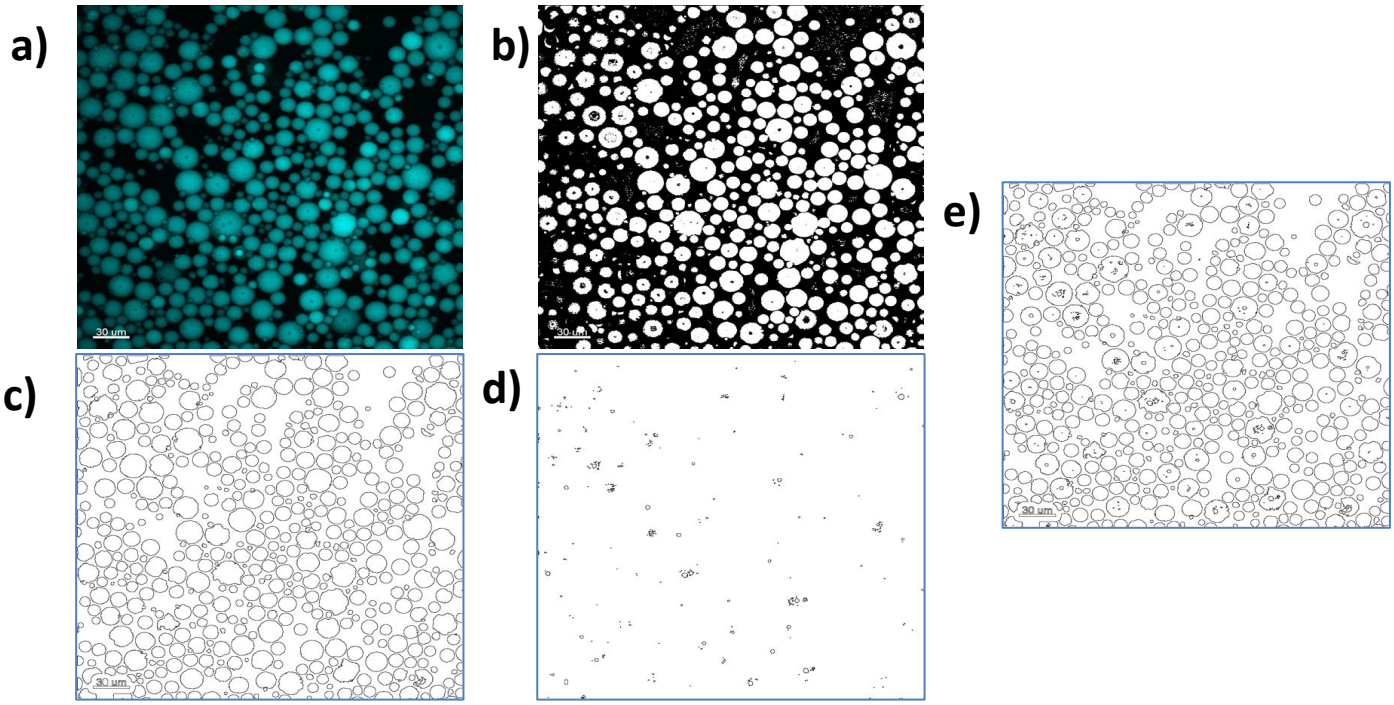


Figure 7: After 45' from left to right: a) original image, b) locally thresholded image by algorithm Bernsen, c) oil domains as analyzed from image b), d) internal water domains as analyzed from image b), e) composite image from images c) & d) for comparison with the images a) & b). This latter comparison helps judging whether the analysis procedure is correct.

The ratio of internal water (estimated as a surface in Figure 7 **c**) to the oil+internal water quantity (surface of drops in Figure 7 **b**) was followed by this method as a function of time (Figure 8).

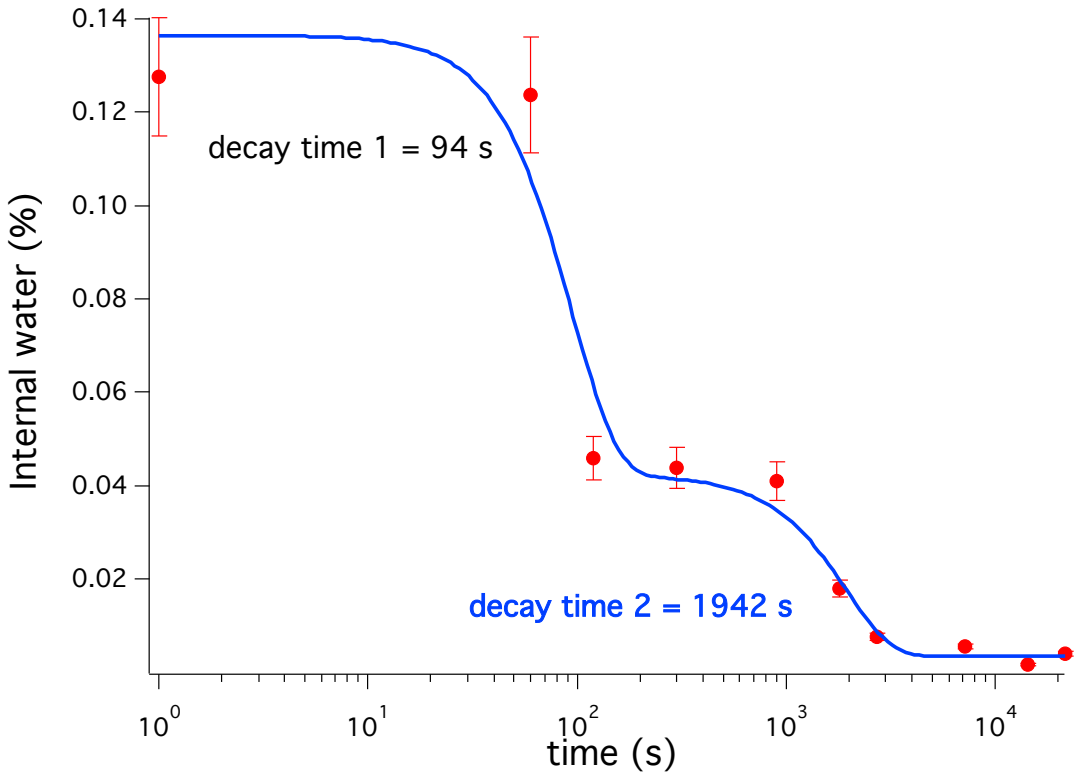


Figure 8: Kinetics of internal water disappearance with time (in seconds). Data were fitted **to the sum of two compressed exponential** providing two well separated kinetics times **of decay**, t_1=94 s and t_2= 1942 s. Conditions are a jump at pH 3 at initial time

The first data point at t = 1s is arbitrary in time and actually corresponds to the initial emulsion at t = 0 and helps defining the initial plateau of encapsulation. On this plateau the encapsulation yield of water is about the ratio Internal Water/(Internal Water+Oil)= 12.5% where this 2D determination compares fortuitously well with the encapsulation measurements at 3D given above. Indeed the 2D determination excludes many small off-

resolution water droplets and cannot be converted to a 3D yield. A striking point in Figure 8 is the two-**stage** kinetics which is apparent from the data and has been quantified by fitting the whole kinetics by the sum of two compressed exponential with a compressed exponent of 2. Such a compressed exponential is generically defined as $f(t)= a*\exp(-(t/t_0)^{\beta})$ where β is an exponent which is equal to 2 in our case. The fit nicely reveals the two times which are around 100 and 2000 s (the two decay time values were checked to be insensitive to the exact choice of arbitrary time chosen for the first data point). This analysis points to two different mechanisms successively at play. The first one can be attributed to the fast diffusion of water molecules through the oil as previously explained. Typically, 100 s corresponds to the diffusive time of a water molecule over a distance of 23 μm through Miglyol, a size which compares favorably with typical oil drops sizes. This diffusive time was calculated by a Stokes-Einstein formula of the water diffusion coefficient $kT/6\pi\eta R$ where η is the Miglyol viscosity and R the radius of a water molecule. The second slower mechanism could be attributed to the micelle-induced coalescence process which was also described previously.

C) IONIC STRENGTH STIMULUS

C-1) Ionic strength triggered destabilization and release

According to their phase diagram, emulsions can be also *a priori* turned from multiple ones to direct ones by decreasing the ionic strength. This was first carried out for bare emulsions stabilized by $PDMS_{38}$-*b*-$PDMAEMA_{25}$ starting from a pH = 8.2 and I = 1 mol L^{-1} situation. A dialysis is carried out versus a solution at pH = 8.2 and no added salt.

Images of the destabilization are shown in Figure 9 and reveal a spectacular osmotic swelling of the internal water droplets.

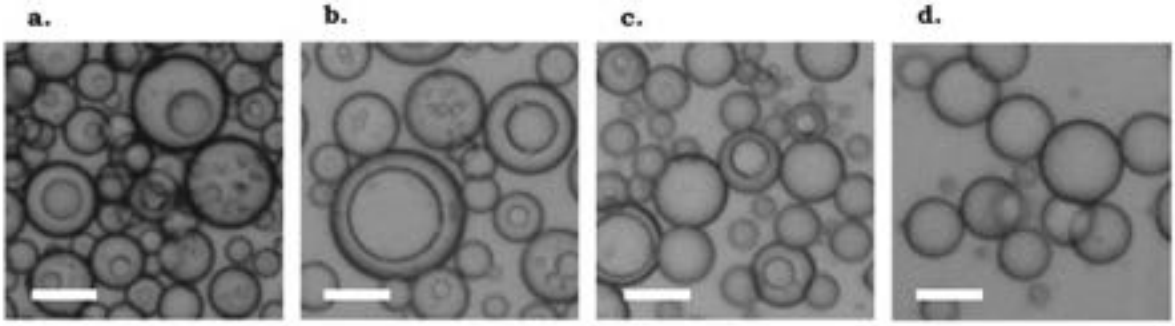


Figure 9: Simple optical microscopy of a temporal sequence of bare multiple emulsion destabilization following a lowering of ionic strength from an initial I=1 mol. L^{-1} down to zero.

A water flux from the external water phase towards the internal one swells the internal droplets and ultimately turns the emulsion to a direct one after coalescence of internal and external water phase.

C-2) Ionic strength triggered release of Alexa Fluor

Alexa Fluor release was attempted from the loaded emulsion (pH = 8.2 and I = 2 mol L^{-1}) using ionic strength as a stimulus. The ionic strength of the external aqueous phase was decreased to a value close to zero (Figure 10) using dialysis against a salt-free aqueous solution. Both the temperature and pH were kept constant at 25°C and 8.2 respectively during the release experiment of the dye. Figure 10 presents confocal microscopy images of the emulsion before and during the ionic strength change.

Five minutes after the beginning of the destabilization attempt, oil globules start to swell due to the osmotic pressure gradient between the external and the internal water phase (Figure 10). Internal water droplets swell progressively until 2 hours after the ionic strength was changed, which generates coalescences between internal droplets (Figure 10 **a**-**e**). Indeed, Figure 10 **e** shows that the majority of oil globules now contains a single water droplet in contrast to picture **a** where globules contain many water droplets. The decrease of ionic strength in the internal water droplets during swelling also induces droplet coalescence with the external phase: some oil droplets without water inside are observed (Figure 10 **f**). Indeed, the polymer is less able to stabilize W/O interfaces when the ionic strength is low (Figure 1).[32] However, contrary to what occurred for the bare emulsion at comparable times, less coalescence between internal water droplets and external aqueous phase are observed, meaning that the W/O/W morphology of the emulsion is mostly preserved during the ionic strength change

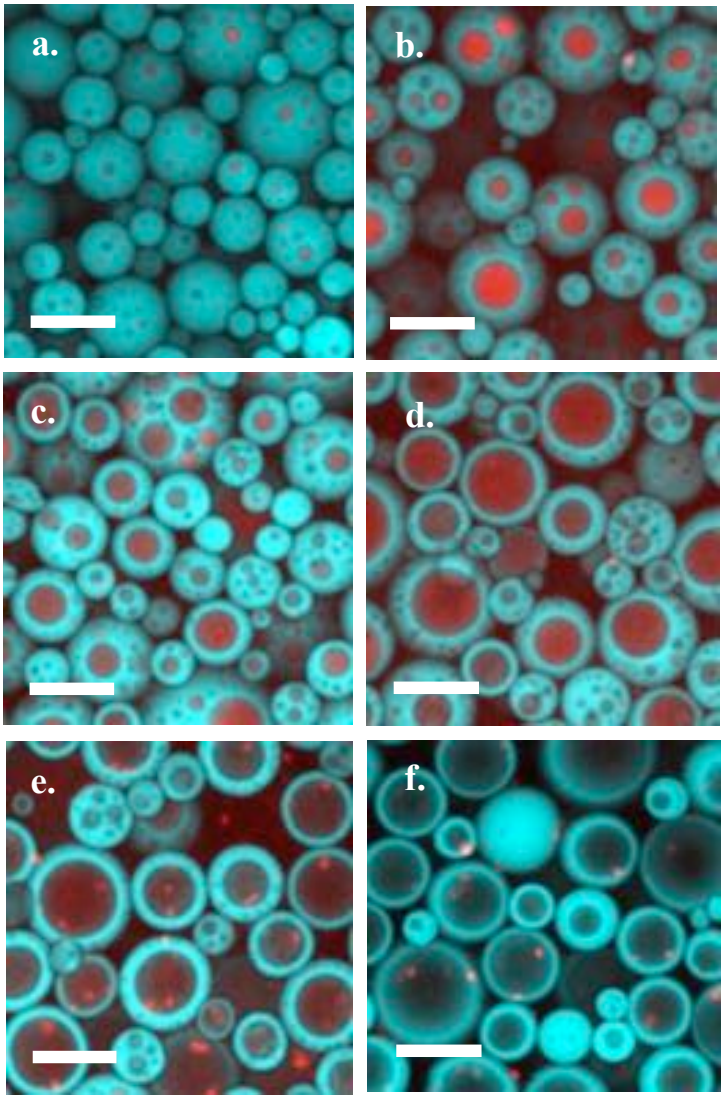

a.
b.
c.
d.
e.
f.

Figure 10: Ionic strength triggered release of an Alexa Fluor loaded emulsion (pH = 8.2 and I = 2 mol L^{-1}). Fluorescence confocal microscopy images **a.** for the initial emulsion (I = 2 mol L^{-1}) and **b.** after 5 minutes, **c.** after 15 minutes, **d.** after 1 hour, **e.** after 2 hours and **f.** after 5 days in contact with a water phase for which I = 0 mol L^{-1}. Oil phases appear in blue and water phases containing Alexa Fluor appear in red. In **d** an arrow is pointing to possible phase separation of dye (see text). Scale bar is 30 µm.

About the dye release, two different features are noted. Fırst, the general dye emission fades away like by dilution in the external phase. Second, some bright red isolated spots are visible, some of them being clearly localized in the external water phase. The occurrence of these spots seems to be preceded in some cases by the appearance of heterogeneities which look like phase separation within droplets (see arrow in Fig. 10 d).

Discussion about ionic strength triggered release:

Molecular diffusion of water or reverse micelles transport through oil is of course here also a candidate mechanism for transporting water from the outer phase making inner droplets swell. However, the dye modification and release cannot be explained by this feature alone as in the pH trigger case. Coalescences of inner water droplets with external

phase are observed, though rarer than in the bare case. They are favored by the decrease in ionic strength mediated by inverse micelles, which destabilizes interfaces curved towards water and would explain the dilution of the dye in the continuous phase. Localized red spots appear as a result of phase separation and as fluorescence disappear. This means that red spots are due to precipitation of the dye in interaction with polymer. From our previous work it is known that the polymer is barely soluble in water but micelles do exist in water as evidenced by cryoTEM experiments. Red spots would be the result of precipitation of a dye-polymer complex triggered by the lowering of the ionic strength. A characterization of the first part of the process (t<25h)) can be obtained by computing the internal water surface and the oil surface in the same manner as previously done for pH-induced destabilization. Results are presented in SM section and confirm an osmotic swelling mechanism (Fig. SM1).

CONCLUSIONS

In this paper, we have demonstrated that biocompatible water – Miglyol® 812 W/O/W emulsions stabilized by PDMS-*b*-PDMAEMA are able to encapsulate a test molecule for times comparable to typical storage times. Although this is not necessary for many practical uses, we also demonstrated that washed multiple emulsions (where the continuous phase is devoid of the encapsulated molecule) can be of the same temporal stability. However, by studying the destabilization mechanisms, it must be noted that this stability aspect of washed multiple emulsions is probably very dependent on the nature of the encapsulated molecule since reverse micelles are suspected to be an important vector of transfer through the oil. Using a dye molecule such as Alexa Fluor 647 allowed

us to reveal long-time encapsulation and triggered release mechanisms. These latter mechanisms were particularly important to evidence and characterize since many triggers can *a priori* destabilize W/O/W emulsions and induce the dye release. This is in particular true for pH change which is an important stimulus for biocompatible emulsions. Indeed, destabilizing multiple emulsions upon acidification is a major feature for designing an oral drug that would be successfully and selectively released in the acidic stomach.

pH lowering was precisely shown to turn our multiple emulsions to direct ones (O/W emulsions), enabling the release of the encapsulated molecule in the water continuous phase. The study of the mechanisms led us to propose a two-**stage** mechanism where molecular diffusion and transfer through the oil by reverse micelles plays a key role in the release. The kinetics was shown to be highly dependent on the final targeted pH, i.e. slowing down as the final pH is increased.

Lowering of the ionic strength is another way of perturbing our multiple emulsions, in this case by osmotic swelling of the internal water droplets. Bare multiple emulsions are destabilized by turning them into direct ones but it was observed that the inclusion of a dye molecule can prevent this destabilization pathway. In our case, the dye, although diluted away from the internal droplets, was shown to precipitate probably because of interaction with polymer micelles.

Overall, the system we examined here is of great interest for the development of oral active vectors in pharmaceutics of functional food. Biocompatible and easy to form, our W/O/W emulsions could encapsulate and protect a hydrophilic drug, and be destabilized in the stomach at low pH into O/W emulsions, which could allow a controlled release of the encapsulated drug. It is worth noticing that for a real application, the washing of the external water phase is not always a requirement so that our system thus presents a really

simple way to formulate stable and usable W/O/W emulsions. An important aspect that is put forward by our study is however the interaction of the encapsulated species with the copolymer stabilizing the emulsion: release mechanisms reveal that this interaction is central to control the mode of release and must consequently more deeply evaluated. This is the goal of a next study devoted to the encapsulation and release of a molecule of therapeutic interest.

ACKOWLEDGEMENTS

We thank the RTRA Triangle de la Physique for its funding through the Imagemul project and CEA for funding both M. Protat and N. Bodin-Thomazo PhD projects.

REFERENCES

[1] C. Charcosset, Preparation of emulsions and particles by membrane emulsification for the food processing industry, J. Food Eng. 92 (2009) 241-249. https://doi.org/10.1016/j.jfoodeng.2008.11.017

[2] E. Dickinson, Double Emulsions Stabilized by Food Biopolymers, Food Biophys. 6 (2011) 1-11. https://doi.org/10.1007/s11483-010-9188-6.

[3] J. O'Regan, D.M. Mulvihill, Sodium caseinate-maltodextrin conjugate hydrolysates: Preparation, characterisation and some functional properties, Food Chem. 123 (2010) 21-31. https://doi.org/10.1016/j.foodchem.2010.03.115.

[4] Ranade, V. V.; Cannon, J. B. *Drug Delivery Systems, Third Edition*; CRC Press, 2011.

[5] Y. Zhang, H.F. Chan, K.W. Leong, Advanced materials and processing for drug delivery: The past and the future, Adv. Drug Deliv. Rev. 65 (2013) 104-120. https://doi.org/10.1016/j.addr.2012.10.003.

[6] N. Garti, Double emulsions - Scope, limitations and new achievements, Colloids Surfaces A Physicochem. Eng. Asp. 123-124 (1997) 233-246. https://doi.org/10.1016/S0927-7757(96)03809-5.

[7] D.J. McClements, E.A. Decker, Y. Park, J. Weiss, Structural Design Principles for Delivery of Bioactive Components in Nutraceuticals and Functional Foods, Crit. Rev. Food Sci. Nutr. 49 (2009) 577-606. https://doi.org/10.1080/10408390902841529.

[8] C.H. Choi, D.A. Weitz, C.S. Lee, One step formation of controllable complex emulsions: From functional particles to simultaneous encapsulation of hydrophilic and hydrophobic agents into desired position, Adv. Mater. 25 (2013) 2536-2541. https://doi.org/10.1002/adma.201204657.

[9] D.J. McClements, E.A. Decker, J. Weiss, Emulsion-based delivery systems for lipophilic bioactive components, J. Food Sci. 72 (2007) 109-124. https://doi.org/10.1111/j.1750-3841.2007.00507.x.

[10] M. Serdaroglu, B. Öztürk, A. Kara, Turkish J. of Agriculture-Food Sci. and Technol., 3, (2015) 430-438 https://doi.org/10.24925/turjaf.v3i6.430-438.336

[11] M. Gallarate, M.E. Carlotti, M. Trotta, S. Bovo, On the stability of ascorbic acid in emulsified systems for topical and cosmetic use, Int. J. Pharm. 188 (1999) 233-241. https://doi.org/10.1016/S0378-5173(99)00228-8.

[12] R.K. Owusu, Q. Zhu, E. Dickinson, Controlled release of L-tryptophan and Vitamin B2 from model water/oil/water multiple emulsions, Top. Catal. 6 (1992) 443-453. https://doi.org/10.1016/S0268-005X(09)80030-1.

[13] R. Bou, S. Cofrades, F. Jiménez-Colmenero, Physicochemical properties and riboflavin encapsulation in double emulsions with different lipid sources, LWT - Food Sci. Technol. 59 (2014) 621-628. https://doi.org/10.1016/j.lwt.2014.06.044.

[14] N.P. Aditya, S. Aditya, H. Yang, H.W. Kim, S.O. Park, S. Ko, Co-delivery of hydrophobic curcumin and hydrophilic catechin by a water-in-oil-in-water double emulsion, Food Chem. 173 (2015) 7-13. https://doi.org/10.1016/j.foodchem.2014.09.131.

[15] M. Kaimainen, S. Marze, E. Järvenpää, M. Anton, R. Huopalahti, Encapsulation of betalain into w/o/w double emulsion and release during invitro intestinal lipid digestion, Lwt. 60 (2015) 899-904. https://doi.org/10.1016/j.lwt.2014.10.016.

[16] S. Raynal, J.L. Grossiord, M. Seiller, D. Clausse, A topical W/O/W multiple emulsion containing several active substances: formulation, characterization and study of release, J. Control. Release. 26 (1993) 129-140. https://doi.org/10.1016/0168-3659(93)90112-I.

[17] L. Olivieri, M. Seiller, L. Bromberg, M. Besnard, T.N.L. Duong, J.L. Grossiord, Optimization of a thermally reversible W/O/W multiple emulsion for shear-induced drug release, J. Control. Release. 88 (2003) 401-412. https://doi.org/10.1016/S0168-3659(03)00013-0.

[18] G.M. Tedajo, S. Bouttier, J. Fourniat, J.L. Grossiord, J.P. Marty, M. Seiller, Release of antiseptics from the aqueous compartments of a w/o/w multiple emulsion, Int. J. Pharm. 288 (2005) 63-72. https://doi.org/10.1016/j.ijpharm.2004.09.012.

[19] A. Djedour, F. Boury, J.L. Grossiord, Modulation of the release from a w/o/w multiple emulsion by controlling the viscoelastic properties of the two interfaces, J. Drug Deliv. Sci. Technol. 19 (2009) 197-203. https://doi.org/10.1016/S1773-2247(09)50036-6.

[20] Y. Sela, S. Magdassi, N. Garti, Release of markers from the inner water phase of W / O / W emulsions stabilized by silicone based polymeric surfactants, J. Control. Release. 33 (1995) 1-12. https://doi.org/10.1016/0168-3659(94)00029-T.

[21] A. Benichou, A. Aserin, N. Garti, Double emulsions stabilized with hybrids of natural polymers for entrapment and slow release of active matters, Adv. Colloid Interface Sci. 108-109 (2004) 29-41. https://doi.org/10.1016/j.cis.2003.10.013.

[22] C. Goubault, K. Pays, D. Olea, P. Gorria, J. Bibette, V. Schmitt, F. Leal-Calderon, Shear rupturing of complex fluids: Application to the preparation of quasi-monodisperse water-in-oil-in-water double emulsions, Langmuir. 17 (2001) 5184-5188. https://doi.org/10.1021/la010407x.

[23] L. Sapei, M.A. Naqvi, D. Rousseau, Stability and release properties of double emulsions for food applications, Food Hydrocoll. 27 (2012) 316-323. https://doi.org/10.1016/j.foodhyd.2011.10.008.

[24] Y.A. Jaimes-Lizcano, Q. Wang, E.C. Rojas, K.D. Papadopoulos, Evaporative destabilization of double emulsions for effective triggering of release, Colloids Surfaces A Physicochem. Eng. Asp. 423 (2013) 81-88. https://doi.org/10.1016/j.colsurfa.2013.01.054.

[25] J.A. Hanson, C.B. Chang, S.M. Graves, Z. Li, T.G. Mason, T.J. Deming, Nanoscale double emulsions stabilized by single-component block copolypeptides, Nature. 455 (2008) 85-88. https://doi.org/10.1038/nature07197.

[26] L. Hong, G. Sun, J. Cai, T. Ngai, One-step formation of W/O/W multiple emulsions stabilized by single amphiphilic block copolymers, Langmuir. 28 (2012) 2332-2336. https://doi.org/10.1021/la205108w.

[27] L. Besnard, F. Marchal, J.F. Paredes, J. Daillant, N. Pantoustier, P. Perrin, P. Guenoun, Multiple emulsions controlled by stimuli-responsive polymers, Adv. Mater. 25 (2013) 2844-2848. https://doi.org/10.1002/adma.201204496.

[28] L. Besnard, M. Protat, F. Malloggi, J. Daillant, F. Cousin, N. Pantoustier, P. Guenoun, P. Perrin, Soft Matter Breaking of the Bancroft rule for multiple emulsions stabilized by a single stimulable, (2014). https://doi.org/10.1039/c4sm00596a.

[29] L. Hong, G. Sun, J. Cai, T. Ngai, One-step formation of W/O/W multiple emulsions stabilized by single amphiphilic block copolymers, Langmuir. 28 (2012) 2332-2336. https://doi.org/10.1021/la205108w.

[30] Y. Zhang, J. Gou, F. Sun, S.C. Geng, X. Hu, K. Zhang, X. Lin, W. Xiao, X. Tang, Impact of electrolytes on double emulsion systems (W/O/W) stabilized by an amphiphilic block copolymer, Colloids Surfaces B Biointerfaces. 122 (2014) 368-374. https://doi.org/10.1016/j.colsurfb.2014.07.008.

[31] F. Tu, D. Lee, One-step encapsulation and triggered release based on Janus particle-stabilized multiple emulsions, Chem. Commun. 50 (2014) 15549-15552. https://doi.org/10.1039/c4cc07854c.

[32] M. Protat, N. Bodin, F. Gobeaux, F. Malloggi, J. Daillant, N. Pantoustier, P. Guenoun, P. Perrin, Biocompatible Stimuli-Responsive W/O/W Multiple Emulsions Prepared by One-Step Mixing with a Single Diblock Copolymer Emulsifier, Langmuir. 32 (2016) 10912-10919. https://doi.org/10.1021/acs.langmuir.6b02590.

[33] Bütün, V.; Armes, S. P.; Billingham, N. C. Synthesis and aqueous solution properties of near-monodisperse tertiary amine methacrylate homopolymers and diblock copolymers, Polymer. 42 (2001) 5993-6008. https://doi.org/10.1016/S0032-3861(01)00066-0

[34] L.M. Land, P. Li, P.M. Bummer, The influence of water content of triglyceride oils on the solubility of steroids, Pharm. Res. 22 (2005) 784-788. https://doi.org/10.1007/s11095-005-2595-6.

[35] N. Gaiti, A. Aserin, Y. Cohen, Mechanistic considerations on the release of electrolytes from multiple emulsions stabilized by BSA and nonionic surfactants, J. Control. Release. 29 (1994) 41-51. https://doi.org/10.1016/0168-3659(94)90120-1

[36] A.T. Florence, D. Whitehill, Some features of breakdown in water-in-oil-in-water multiple emulsions, J. Colloid Interface Sci. 79 (1981) 243-256. https://doi.org/10.1016/0021-9797(81)90066-7.

[37] M. Protat, N. Bodin-Thomazo, F. Malloggi, J. Daillant, R.A. Campbell, G. Fragneto, E.B. Watkins, P. Perrin, N. Pantoustier, P. Guenoun, Neutron reflectivity measurements at the oil/water interface for the study of stimuli-responsive emulsions, Eur. Phys. J. E. 41 (2018). https://doi.org/10.1140/epje/i2018-11693-8.