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Jenny Marie Andersson, Olivier C Masbernat, Kevin Roger. Emulsions stabilized by phospholipids. Journal of Colloid and Interface Science, 2025, 678 (Part B), pp.410-418. 10.1016/j.jcis.2024.08.249 . hal-04755502

**HAL Id: hal-04755502**

**<https://hal.science/hal-04755502v1>**

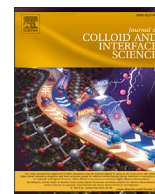
Submitted on 27 Oct 2024

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## Regular Article

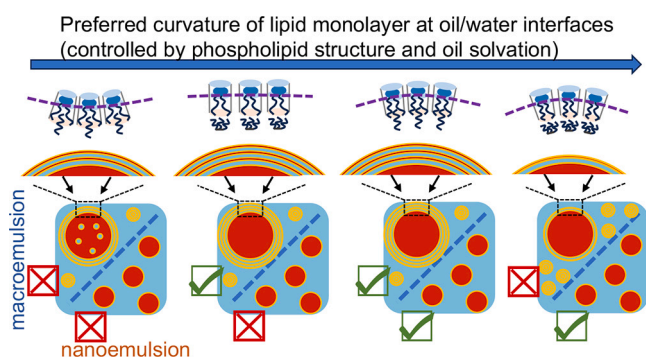
## Emulsions stabilized by phospholipids

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## GRAPHICAL ABSTRACT



## ARTICLE INFO

## Keywords:

Parenteral  
Emulsion  
Phospholipid  
Phase diagrams  
Self-assembly  
Curvature  
Macroemulsion  
Nanoemulsion  
Metastability

## ABSTRACT

**Hypothesis:** Phospholipids are amphiphilic molecules able to adsorb at oil/water interfaces and thus used to stabilize parenteral emulsions. Yet, their low preferred curvature, which sensitively depends on molecular structures and interactions, favors the formation of lamellar phases and sets constraints on the system formulation. Combining phase studies, structural interfacial characterizations, and stability monitoring for different water/phospholipid/oil systems should shine a light on the mechanisms at play and thus tools to optimize formulations.

**Experiments:** Four phase diagrams were established for ternary aqueous systems containing either DOPC or POPC as the phospholipid and hexadecane or miglyol 812 as the oil. Droplet interfaces were probed using small-angle neutron scattering and the amount of adsorbed lipid was determined using separation and Raman spectroscopy. The metastability of both nano and macro emulsions was systematically assessed over weeks using light scattering.

**Findings:** We show that nanoemulsion droplets are stabilized by a lipid monolayer and display excellent metastability if the preferred curvature is positive and large enough, even without any added charges or at high ionic strengths. In contrast, macroemulsion droplets are stabilized with a lipid multilayer, which should possess a positive preferred curvature but also a good enough interfacial anchorage, which is lost upon increasing the preferred curvature. Overall, we provide a rationale for understanding the impact of molecular changes in the formulation on emulsion metastability, through the analysis of the lipid film preferred curvature, layering, and interfacial anchorage.

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Received 17 April 2024; Received in revised form 29 August 2024; Accepted 29 August 2024

Available online 3 September 2024

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## 1. Introduction

Phospholipids are key amphiphilic molecules in biological membranes. Indeed, their hydrophilic and hydrophobic parts are rather balanced, which promotes low-curvature supramolecular assemblies such as bilayers and thus lamellar phases [1]. Abundant, biosourced, and biocompatible, phospholipids would be desirable emulsion stabilizers, notably in applications requiring large amounts of stabilizers with a high biocompatibility. For instance, intravenous administration requires small enough droplets to enter the bloodstream and a steric-type stabilization, as an ionic stabilization would be inefficient at the corresponding high ionic strengths. Phospholipids are thus the natural choice to stabilize these parenteral nanoemulsions [2,3]. However, formulating a metastable emulsion is in practice rather challenging when using phospholipids, as any small composition change, notably in phospholipid or oil, can lead to destabilization. Empirical strategies have typically used additives such as oleic acid, and a control of processing steps which notably involve two sequential emulsification steps performed to produce first a macroemulsion and then a nanoemulsion [4], followed by a heat treatment. Overall, a rational understanding of the relationship between global composition, processing, and emulsion metastability remains lacking.

This problem has actually been under investigation for half a century with scientists trying to unravel the relationship between emulsion metastability and interfacial properties, as a progressive understanding of amphiphilic self-assembly emerged. In the 1970's, Friberg et al. observed that the formation of liquid crystalline phases at oil/water interfaces could significantly hinder droplet coalescence in macroemulsions [5–7]. In the 1980's Rydhaag et al. pursued this approach when phospholipids were used as stabilizers and argued that only phospholipids that were able to form multilayers could stabilize macroemulsions [8]. In the 1990's, Shchipunov et al. further investigated the formation of multilayers at oil/water interfaces in the presence of phospholipids [9]. This was later pursued by Pautot et al. [10]. Meanwhile, Sjölund et al. [11] and later Angelico et al. [12] investigated the phase behavior of ternary oil/water/phospholipid systems, evidencing that equilibrium mesostructures are determined by the balance of all the intermolecular interactions taking place at the amphiphilic-covered oil/water interfaces. This interplay has been thoroughly described in the self-assembly literature using either the mesoscopic concept of spontaneous/preferred curvature(s) [1], which corresponds to the local curvature(s) of the film that optimizes all the interactions, or alternatively the molecular concept of spontaneous packing parameter [13], which is the ratio between a 'sterical' area linked to the volume over the length of the amphiphile and a 'spontaneous' area corresponding again to optimizing the interactions. A direct correspondence exists between these concepts, as shown quantitatively for non-ionic surfactants by Kunz et al. [14] and qualitatively for phospholipids by Chen and Rand [15]. Typically, a preferred curvature turned towards the oil ('positive' by convention) corresponds to a preferred packing parameter smaller than 1.

The relationship between emulsion metastability against coalescence and the surfactant layer preferred curvature was proposed by Kabalnov and Wennerström [16] with the argument that if the preferred curvature was opposite to that of the forming pore between two droplets, coalescence would be hindered. Furthermore, this theory was shown to quantitatively agree with experimental metastability data for surfactant-stabilized emulsions [17]. At the same time, Kabalnov investigated the case of phospholipid-stabilized emulsions and argued that similarly to any surfactant-stabilized emulsion, the monolayer preferred curvature dictated metastability [18,19]. Furthermore, he also claimed that the formation of multilayers could also significantly enhance metastability. These findings were confirmed by a following study by Saito et al. [20]. Norden et al. consistently observed that the addition of drugs could significantly impact the metastability of the carrier emulsion if they were able to modify the preferred curvature of the amphiphilic layer [21]. Overall, the composition of the global formulation (oil, phospholipid...)

is known to impact drastically the formulation [22,23], which represents a challenge for industrial applications and calls for a rational understanding.

In this work, we investigated the relationship between formulation, self-assembly, interfacial structure, and droplet metastability for model emulsions formulated with four different oil/phospholipid/water model systems. We first present equilibrium phase diagrams of the four systems and show how they shine a light on the value of the phospholipid preferred packing/curvature, which notably controls self-assembly. We then first examine the metastability and interfacial structure of nanoemulsions produced in surfactant-poor conditions [24] by the high-pressure homogenization of macroemulsions, using respectively light scattering and small-angle neutron scattering (SANS). These conditions correspond to creating a larger amount of oil/water interfaces than what can be covered by the phospholipid available. We then turned to the case of macroemulsions produced in contrast in surfactant-rich conditions [24] by rotor-stator processing of oil and water. In these conditions, the amount of phospholipids exceeds what is needed to cover the oil/water interfaces produced through fragmentation. We finally investigated the location of this phospholipid excess, using separation and Raman spectroscopy for analysis, before performing similar light scattering and SANS experiments to assess their metastability and interfacial structure.

## 2. Materials and methods

### 2.1. Chemicals

POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) and DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) (99% pure) were purchased from NOF Europe GmbH, Germany. Oleic acid (99% pure), Hexadecane (99% pure), Chloroform (HPLC standard, 99.8% purity) and NaCl (99% pure) were purchased from Sigma-Aldrich. Miglyol ©812N (C8-C10 triglycerides) was a kind gift from Ioi Oleo GMBH. We used MilliQ grade water from a MilliPore purifier, Merck, Germany.

### 2.2. Emulsion preparation

To make the macro-emulsions, stock solutions of 24.2wt% lipids in oil (either Miglyol or hexadecane) were prepared by adding dry lipids (either DOPC or POPC) to the oil fraction of the emulsion after which the surfactant (oleic acid) if used, was added at 1.4 surfactant to lipid wt%. The lipid-oil mixture was then mixed by a vortex and heated to 60 °C for 5 minutes to solvate the lipids. The lipid-oil stock solution was diluted to 12.1, 6.1, 3.0, 1.5, and 0.75 wt% lipid to oil. To make the emulsions 1.4 mL of lipid-oil solution was carefully added on top of 5.6 mL of the NaCl solution (1 mM or 500 mM) and processed with an ultra-turrax at a speed of 25000 rpm for 3 min to create a 20:80 oil in water emulsion. In the case of the POPC-Miglyol macro-emulsions the samples were processed with an ultra-turrax at temperatures up to 80 °C using a water bath to investigate if the temperature would affect the emulsion droplet outcome. Macro-emulsions of each of the DOPC/POPC-Miglyol/Hexadecane combinations were also prepared by mixing the lipids in the water first and then adding the oil on top before processing with the ultra-turrax to investigate if the lipid starting location had any influence on the emulsion outcome. Nano-emulsions were prepared from the macro-emulsions by passing them through a high-pressure homogenizer at 207 bars four times.

### 2.3. Amount of non-adsorbed lipids

We assessed the amount of lipids wasted as vesicles in the aqueous phase and not adsorbed at oil/water interfaces by Raman spectroscopy. Emulsion droplets were separated from the supernatant by gentle centrifugation (1000 rpm for 5 minutes) after which the supernatant was collected with a micropipette. This concentrated emulsion was washed with MilliQ water and centrifuged again twice to ensure that vesicles

were removed. We collected and pooled together the different aqueous rinsing fractions. We freeze-dried both the washed emulsion and the rinsing fraction to remove water and then dissolved the dry extract in chloroform. As POPC and DOPC are unsaturated lipids, they display a specific peak at  $1661\text{ cm}^{-1}$  corresponding to the stretching of the double bonds of the acyl chains in the lipids. A calibration curve was thus established for lipid/oil mixtures, using for both oils the broad peak between  $2800$  and  $3100\text{ cm}^{-1}$  corresponding to  $\text{CH}_2$  and  $\text{CH}_3$  stretches, after normalization by the peak area of chloroform at  $671\text{ cm}^{-1}$ . The amount of all species in both the emulsion and the rinsing fraction was then quantified.

## 2.4. SANS

Small-angle neutron experiments were performed on the PAXY small-angle diffractometer located at the Orphee reactor in Saclay, France. Samples were dissolved in  $20\text{ mM NaCl}$  solution in  $\text{D}_2\text{O}$ . Spectra were recorded using four different spectrometer configurations:  $\lambda = 6\text{ \AA}$  (incident wavelength),  $d = 1\text{ m}$  (distance of the sample to the detector),  $\lambda = 6\text{ \AA}$  and  $d = 3\text{ m}$ ,  $\lambda = 8.5\text{ \AA}$  and  $d = 5\text{ m}$ , and  $\lambda = 15\text{ \AA}$  and  $d = 6.7\text{ m}$ . The wave vector  $q$  range covered was from  $6.5 \times 10^{-3}$  to  $4.5 \times 10^{-2}\text{ \AA}^{-1}$ . Contributions from the Quartz Helma cells and ambient noise were subtracted. Data was normalized using the  $\text{H}_2\text{O}$  spectra and set to absolute scale thanks to normalization factor measurements [25]. The incoherent background subtraction was performed using the sample transmission at high  $q$  values and a calibration of  $\text{D}_2\text{O}/\text{H}_2\text{O}$  solutions at different ratios (transmission as a function of scattered intensity). Emulsion droplets were washed before the measurements, by the same protocol as for the lipid loss determination, to get rid of any vesicles in the emulsions. To prevent creaming during measurements, we used tumbling cells designed by Rennie and coworkers [26], who kindly provided them at our disposal for the beamtime.

## 2.5. Phase diagram and SAXS

Samples were prepared by mixing the phospholipid, the oil, and water with a vortex and left to equilibrate for at least 7 weeks. Then samples were analyzed using small-angle X-ray scattering to characterize mesostructures through analysis of structure factors. SAXS measurements were performed on a Xeuss 2.0 laboratory instrument from XENOCs. Samples were either injected in disposable quartz capillaries ( $1.5\text{ mm}$  inner diameter) or in between kapton windows if too viscous.

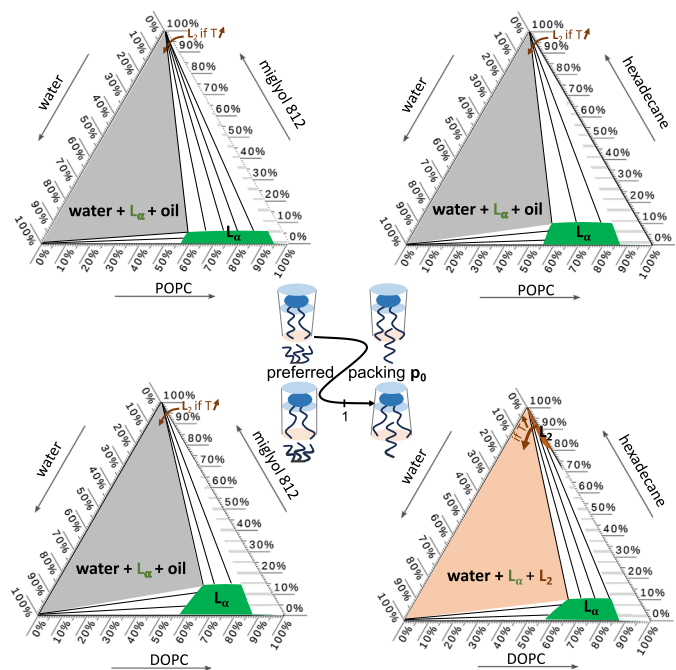
## 2.6. Light scattering and emulsion metastability

Droplet size distributions of emulsions were measured using a static light scattering instrument (Mastersizer 3000, Malvern) with a  $633\text{ nm}$  laser wavelength. An emulsion sample was diluted in circulating water until an obscuration value between 1 and 10% was obtained, to avoid multiple scattering. The average droplet diameter was determined as the volume-surface mean diameter  $d[3,2]$  or Sauter diameter. Each measurement was repeated five times at room temperature. Emulsion metastability was assessed by monitoring the increase in droplet size over time. Emulsions were hand-shaked before the measurements.

## 3. Results

### 3.1. Phase diagrams: the interplay between lipid structure and oil solvation

Isothermal equilibrium phase diagrams of oil/amphiphile/water ternary systems tell us how species self-assemble in a given mesostructure when tuning composition. Phase diagrams thus contain precious thermodynamic information about the intermolecular interactions at play, and can notably directly yield information about preferred curvature/packing at interfaces. Fig. 1 displays the ternary phase diagrams of the four water/phospholipid/oil systems used in this study,



**Fig. 1. Lipid/Oil/Water ternary phase diagrams.** We established four different phase diagrams corresponding to two different phospholipids, POPC and DOPC, and two different oils, hexadecane and Miglyol 812. All four diagrams display a lamellar phase region originating from the lipid/water binary diagram (green area). However, its extent and thus lamellae' swelling depends on the lipid/oil couple. Overall, the diagram is dominated by a three-phase coexistence of water, lamellar phase, and oil except (gray area) for the DOPC/Hexadecane/water system in which the oil phase is replaced by a reverse micellar phase (dark orange). Comparing these four phase diagrams thus testifies to the change in preferred packing/curvature when switching oils and lipids, as indicated by the central curved arrow. Note that increasing temperature favors the formation of the reverse micellar phase in the oil-rich corner, evidencing that the preferred curvature turns towards the oil ( $p_0 > 1$ ). (For interpretation of the colors in the figure(s), the reader is referred to the web version of this article.)

which correspond to two different phospholipids (DOPC and POPC) and two different oils (Miglyol 812N and hexadecane). These diagrams were obtained by combining SAXS measurements of the obtained mesostructures, Raman microscopy measurements of the number of phases and location of the three-phase corner, and visual inspection. At first glance, all diagrams may seem rather similar. A pure lamellar phase is obtained at high lipid content (green area), which highlights that phospholipids display a close-to-zero preferred curvature (preferred packing close to 1). Over most of the diagram, the three-phase separation of pure water, oil, and the lamellar phase is observed (gray area). Yet, taking a closer look yields several important observations. For instance, the location of the three-phase corner touching the lamellar phase quantifies the swelling extent of the lamellar phase as oil molecules penetrate phospholipids tails. Increasing the swelling thus corresponds to increasing the preferred packing, eventually leading to a topological change as the preferred curvature, initially oriented towards oil, turns towards water. We see a sharp increase of the lamellar phase swelling following the sequence POPC/Miglyol ( $\approx 6\%$  oil) < POPC/Hexadecane ( $\approx 10\%$  oil) < DOPC/Miglyol ( $\approx 14\%$  oil), which corresponds to what is expected from both lipid and oil structures. Indeed, as DOPC is more unsaturated than POPC, its preferred packing parameter,  $p_0$ , is higher than the one of POPC ( $p_0[\text{DOPC}] > p_0[\text{POPC}]$ ), which favors a curvature turning towards water [27]. Hexadecane solvates better lipid tails than Miglyol, which increases oil penetration and thus also increases the preferred packing parameter of a given lipid ( $p_0^{\text{hexadecane}}[\text{DOPC}/\text{POPC}] > p_0^{\text{Miglyol}}[\text{DOPC}/\text{POPC}]$ ), which also favors a curvature turning towards water. Strikingly, the swelling

stops increasing when further increasing the packing parameter in the DOPC/hexadecane system ( $\approx 9\%$  oil). This can be understood from the appearance of a reverse micellar phase in the oil-rich corner, evidencing that  $p_0^{\text{Hexadecane}}[\text{DOPC}] > 1$ , which corresponds to a preferred curvature that is then turned towards water. Overall, phase diagrams thus allow the direct comparison of phospholipid packing parameters at oil/water interfaces:

$$p_0^{\text{Miglyol}}[\text{POPC}] < p_0^{\text{Hexadecane}}[\text{POPC}] < p_0^{\text{Miglyol}}[\text{DOPC}] < 1 < p_0^{\text{Hexadecane}}[\text{DOPC}].$$

It is worth stressing that all these systems exhibit the formation of the reverse micellar phase if heated up sufficiently, which means that increasing temperature also increases the packing parameter and promotes topological changes. Interestingly, this can lead to a sharp viscosity decrease of the oil/phospholipid solution in temperature conditions typical of the conditions achieved industrially in the rotor-stator to obtain a macroemulsion.

Overall, phase diagrams tell us about key parameters such as the variation of the preferred packing/curvature with composition and temperature and the extent of the lamellar phase and thus lipid solubility in the different phases.

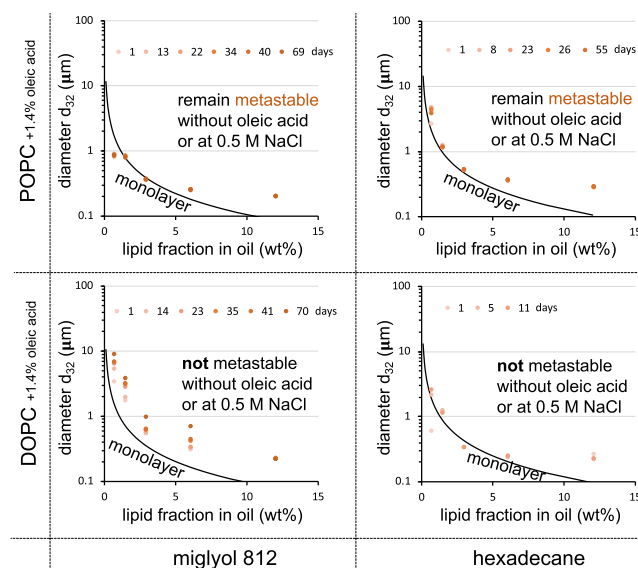
### 3.2. Nano-emulsions

#### 3.2.1. Size and metastability

Nanoemulsions were obtained by high-pressure homogenization of macroemulsions at increasing phospholipid concentrations in the oil and their droplet size, the inverse of effective curvature, was monitored over time by light scattering (Fig. 2). We systematically used the Sauter diameter,  $d[3,2]$ , which corresponds to the equivalent diameter of a collection of equisized droplets of the same total area and volume as the real polydispersed droplet collection [28]. In the presence of oleic acid, a commonly added anionically charged surfactant, all formulations yielded metastable emulsions. This is expected for any nanoemulsion stabilized by ionic surfactants at low ionic strength, as long-range ionic repulsions prevent the Brownian droplets from colliding. Still, one might note that metastability is lower for systems associated with a higher packing parameter such as POPC/Miglyol and POPC/Hexadecane (corresponding to preferred curvatures turning less towards oil). Furthermore, long-range repulsions can be suppressed by removing oleic acid or increasing ionic strength through salt addition. Emulsion metastability is then determined by the success rate of opening and growing a hole between two colliding droplets. This interface-related metastability is in our experiments directly related to the preferred packing/curvature: the lower the packing, and thus the more turned towards the dispersed phase the preferred curvature, the higher the metastability.

Moreover, we observed a decrease in droplet size (Sauter diameter) with increasing concentration that can be split qualitatively into two regimes. At low lipid concentrations, the droplet size matches the theoretical prediction that assumes that all the lipid is adsorbed at oil/water interfaces. This is the same law observed when preparing some particle-stabilized emulsions [29,30], through limited coalescence, or nanoemulsions through microemulsion agitation, superswelling, and quenching, as in the sub-PIT (Phase Inversion Temperature) method [31–33]. In this surfactant-poor regime [24], the initial amount of interfaces created by mechanical fragmentation is higher than what can be covered by the available amphiphiles. In contrast, at larger lipid concentrations, emulsification proceeds in the surfactant-rich regime in which the size is determined by the mechanical input, such as the operating pressure here) and some phospholipid is not adsorbed at oil/water interfaces but rather wasted in water.

Overall, in oil/phospholipid/water systems corresponding to sufficiently low packing parameters, phospholipids are remarkable stabilizers yielding highly metastable nanoemulsions, efficiently used at interfaces paving the way to a straightforward droplet size control.



**Fig. 2. Nanoemulsions metastability.** Sauter mean diameter obtained by laser granulometry for nanoemulsions prepared via high-pressure homogenization at different lipid concentrations. The measured droplet size is well-described by a simple model (black line) assuming that all phospholipids are adsorbed as a monolayer on oil/water interfaces. Only two systems yield metastable emulsions, in the absence of ionic repulsions, POPC/miglyol and POPC/hexadecane. These systems correspond to the lower preferred packing parameter and a preferred curvature that is thus sufficiently turned toward the oil.

#### 3.2.2. Interfacial structure

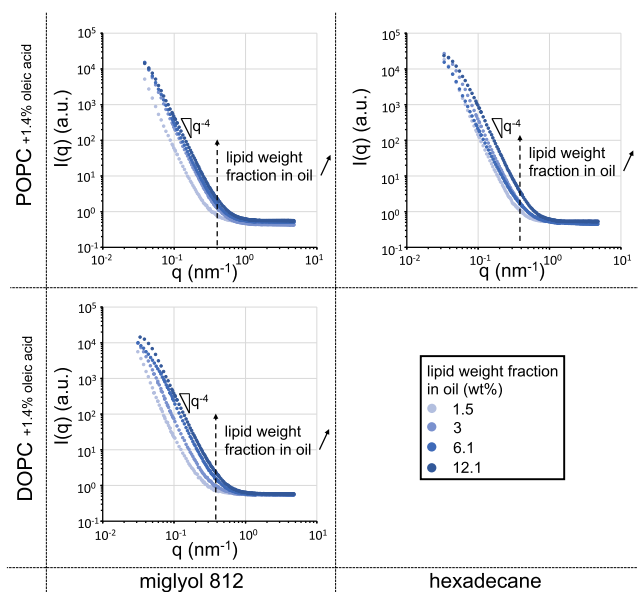
The above data suggests that nanoemulsion droplets are covered by a phospholipid monolayer, which calls for direct confirmation as literature proposes the existence of interfacial multilayers. We thus characterized interfaces by performing small-angle neutron scattering (SANS) experiments, an in situ and ensemble method. By using deuterated water, the contrast of the lamellar phase (multilayer) is also tremendously enhanced compared to the set electronic contrast of small-angle X-ray scattering (SAXS). SANS spectra of the different nanoemulsions are displayed in Fig. 3 and all display a similar shape that consists of two parts: a decay in  $q^{-4}$  (Porod law), and a constant plateau related to incoherent scattering determined by the number of hydrogens in the system (oil and phospholipids). As emulsions were prepared at the same oil volume fraction, this plateau is approximately the same for all samples, with a small difference stemming from the varying amounts of phospholipids. The specific area determined by the Porod law increases with increasing lipid content, consistently with a decrease in the droplet size (see Fig. 2 and Supporting Information).

Furthermore, we do not observe any signature of phospholipids mesostructures such as multilayers in a lamellar arrangement. Interfaces are thus covered with a phospholipid monolayer, consistent with the simple model described in the previous section.

### 3.3. Macroemulsions

#### 3.3.1. Size and metastability

Macroemulsions were produced using a rotor/stator setup (Ultra-Turrax), at constant power and duration. Samples were then immediately measured through light scattering, which yields an estimate of the droplet size distribution from which we extracted the Sauter diameter  $d_{32}$ , which corresponds to the inverse of the effective droplet curvature. Fig. 4 displays measurements that were repeated over time after gentle homogenization of the creaming emulsions. Here, it is worth stressing that emulsion metastability is in our experiments unrelated to the rheology of the continuous phase, which has been observed with similar systems in other conditions [34].



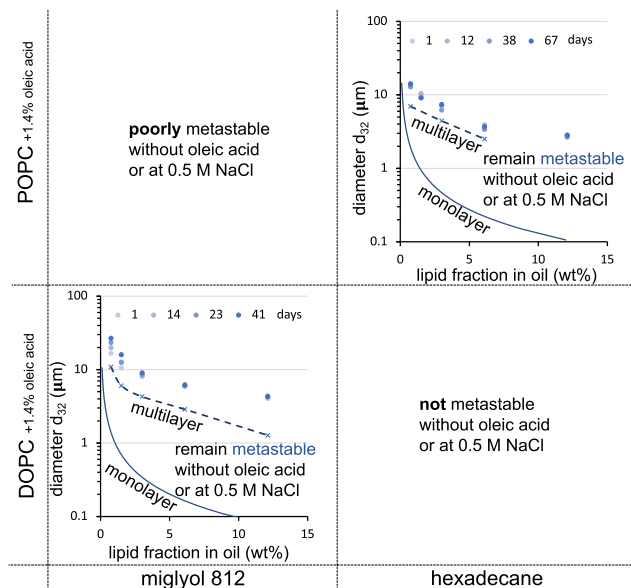
**Fig. 3. Small Angle Neutron Scattering of nanoemulsions.** The Porod regime,  $I(q) \sim S \cdot q^{-4}$  with  $S$  the total interfacial area, is observed for all emulsions and we observe no structure peak in all prepared nanoemulsions, consistently with Fig. 2 that suggests that lipids only form a monolayer at the oil/water interface.

Emulsifying the DOPC/hexadecane/water system led to immediate macrophase separation, which prevented any measurements. The POPC/Miglyol/water system did yield some droplets with limited metastability and broad size distribution, with an oil layer quickly forming on top. They were thus not systematically monitored. For the two other systems, we observed that the droplet size decreased with increasing phospholipid concentration, which is consistent with a surfactant-poor regime. We also investigated the impact of having oleic acid in the formulation, a standardly added ionic surfactant in commercial emulsions (See Supporting Information). However, we did not observe significant differences between emulsions prepared with or without oleic acid (even at 10 wt% in oleic acid), which suggests that ionic repulsions do not provide metastability in macroemulsions, in contrast to nanoemulsions. Indeed, macroemulsion droplets are pushed into contact by gravity, in contrast to nanoemulsion droplets that undergo Brownian collisions.

Similarly to the nanoemulsion case, a simple model in this regime is thus to assume that all phospholipids adsorb as a monolayer at oil/water interfaces, which determines the total interfacial area and thus droplet size. In contrast to the nanoemulsion case, the corresponding blue curve in Fig. 4 fails to describe the experimental data by an order of magnitude. This discrepancy can indicate that phospholipids assemble as multilayers at oil/water interfaces and/or are partly ‘lost’ as vesicles in water. We will now show that these two effects are both at play and can be deconvoluted and quantified through separately characterizing interfaces and assessing the amount of lipid dispersed in water.

### 3.3.2. Interfacial structure

Interfacial structuration, such as multilayering, can be assessed using small-angle neutron scattering (SANS). In addition to providing a high contrast of the lamellar phase when using deuterated water, SANS is well-suited for the characterization of macroemulsions as it samples the emulsion over a much larger volume than its x-ray counterpart (SAXS), which is crucial to avoid droplets confinement and destabilization. Yet, gravity leads to droplet creaming, which we prevented gravity-induced droplet creaming by using rotating quartz cells designed and kindly provided by Rennie and coworkers [26]. To avoid the possible scattering contribution of phospholipid self-assembly structures in bulk, such as vesicles in water, we washed emulsions before measuring them with



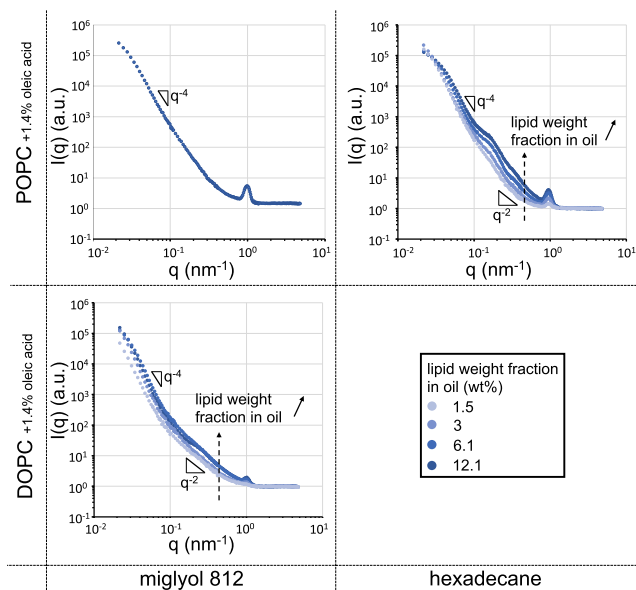
**Fig. 4. Macroemulsions metastability.** Sauter mean diameter obtained by laser granulometry for macroemulsions prepared via fragmentation in a rotor-stator device (ultra-turrax) at different lipid concentrations. Only two systems yield metastable emulsions with neutral or screened interfaces. In all cases, the droplet size is much larger than expected if lipids are all adsorbed as a monolayer (blue curve). The dotted line shows the calculation for a multilayer arrangement of lipids calculated from Fig. 6 data (crosses), and the difference with the measured size corresponds to the amount of non-adsorbed lipids that are lost as vesicles in water. Overall, smaller droplets produced at higher lipid concentrations and corresponding to a thicker interfacial multilayer display a higher metastability.

SANS using cycles of gentle centrifugation (1000 rpm for 5 minutes) followed by the dilution of the concentrated emulsion. This washing procedure ensures that the measured scattering only originates from the oil/water interfaces. Typical SANS spectra are displayed in Fig. 5. Spectra were deliberately not subtracted but rather rescaled by the droplet volume fraction, which usually slightly varies after rinsing cycles.

The most important feature is the presence of a correlation peak at reciprocal distances of around  $1 \text{ nm}^{-1}$ , for all samples. We verified by measuring separately pure lamellar phases (by SANS and SAXS) in these systems that this correlation peak matched perfectly the first-order peak of the lamellar phase. Furthermore, we verified by subsequent rinsing that this peak could not originate from the remaining lipid excess in water. Note that non-rinsed emulsions yield similar spectra but display a much larger correlation peak. We can thus conclude that this correlation peak corresponds to a lipid multilayer adsorbed at oil/water interfaces, with a typical repeat distance or bilayer thickness of around 6 nm. The lamellar peak intensity increases with increasing lipid fraction in the oil, as the total amount of adsorbed lipids also increases. Interestingly, a shoulder is visible for the POPC/hexadecane around  $0.16 \text{ nm}^{-1}$ , which gives a correlation distance of around 42 nm that amounts to 7 bilayers (14 monolayers). Overall, these direct structural measurements demonstrate that macroemulsions are stabilized by an interfacially-adsorbed lamellar phase. This interfacial structure notably explains the four-fold birefringence pattern that has been observed for these emulsions [35], which we also observe for our systems.

### 3.3.3. Quantifying how much lipid adsorbs at oil/water interfaces

While the previous SANS measurements evidence the formation of phospholipid multilayers at oil/water interfaces, they also show that non-rinsed emulsions display a stronger structure peak related to this lamellar organization. This means that some phospholipids are rather dispersed in water as multilamellar vesicles. We thus systematically quantified the amount of lipid in both dispersed and continuous phases following emulsification. To this end, the two phases were first sepa-

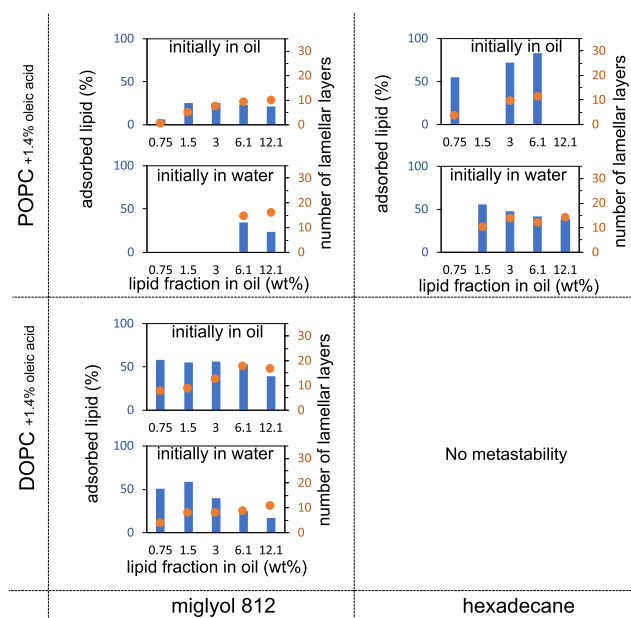


**Fig. 5. Small Angle Neutron Scattering of macroemulsions.** Replacing water by  $D_2O$  allows probing concentrated emulsions with a high neutronic contrast. For all emulsions, we observe the Porod regime with the intensity decaying as  $q^{-4}$ , but we also observe a structure peak at the expected location for the first order of the lamellar phase, corresponding to the repeat distance between two layers. We also observe that its magnitude increases with increasing lipid concentration. Since all emulsions were washed to remove lipids lost in water, the observed lamellar structure can thus only be an interfacial one. We can thus conclude that multilayers form at the oil/water interface for the prepared macroemulsions.

rated through gentle centrifugation (1000 rpm for 5 minutes) and then freeze-dried. They were subsequently dissolved in chloroform, a solvent for phospholipids, and we measured their Raman spectra. Through an experimentally determined calibration curve, we obtained the amount of phospholipid in each chloroform solution and thus ultimately in both dispersed and continuous phases of the emulsion, the sum of which was verified to match the expected total amount in the formulation. The phospholipid amount in the dispersed phase is the interfacial amount since phospholipid solubility in the oil in the presence of water is negligible according to the phase diagrams shown in Fig. 1. The total interfacial area can be separately obtained through light scattering measurements, directly from the simultaneous knowledge of the Sauter diameter and the oil volume fraction. We can then obtain the number of lipid layers in the multilayered interfacial structure (See Supporting Information). The corresponding data is displayed in Fig. 6 for two different emulsification protocols, which differed only in the initial lipid location (oil or water).

We observe that the percentage of adsorbed lipids strongly varies with the oil/phospholipid couple, and weakly with the lipid amount and initial location. For example, the percentage of adsorbed lipids for POPC/hexadecane/water and DOPC/miglyol/water systems is above 50% and thus much larger than for the POPC/miglyol/water system. This result is consistent with the different phase diagrams displayed in Fig. 1, which show a lower oil swelling of the lamellar phase for the POPC/miglyol/water system. Therefore, degrading the lipid tail solvation by the oil is a double-edged change. Indeed, it simultaneously decreases the preferred packing parameter, shifting the preferred curvature further towards the oil, but also enhances lipid waste in water through the loss of anchorage of the lamellar phase at oil/water interfaces.

Another observation from this data set is that the initial lipid location does impact lipid adsorption. Overall, lipid adsorption is higher when lipids are initially located in the oil than in the water phase, which is in line with an ellipsometric study of miglyol/water interfaces in the



**Fig. 6. Quantifying the adsorbed lipids amount (%).** By separating droplets from the continuous phase and using Raman spectroscopy, we can obtain the amount of lipids adsorbed on oil droplets. As we also measured droplet size, we can also compute the number of interfacially adsorbed lipid layers. If the preferred curvature is too strongly turned towards the oil (POPC/miglyol), most lipids are wasted in water as vesicles. Decreasing preferred curvature enhances lipid adsorption as interfacial multilayers. We also observe that overall lipid location matters and that more lipid is wasted when initially placed in water. Overall, the number of adsorbed layers is higher at larger lipid concentrations and smaller droplet sizes.

presence of DOPC by Benjamins et al. [36]. Whilst heavily influenced by thermodynamics, multilayering is thus partially influenced by kinetics through the emulsification pathway.

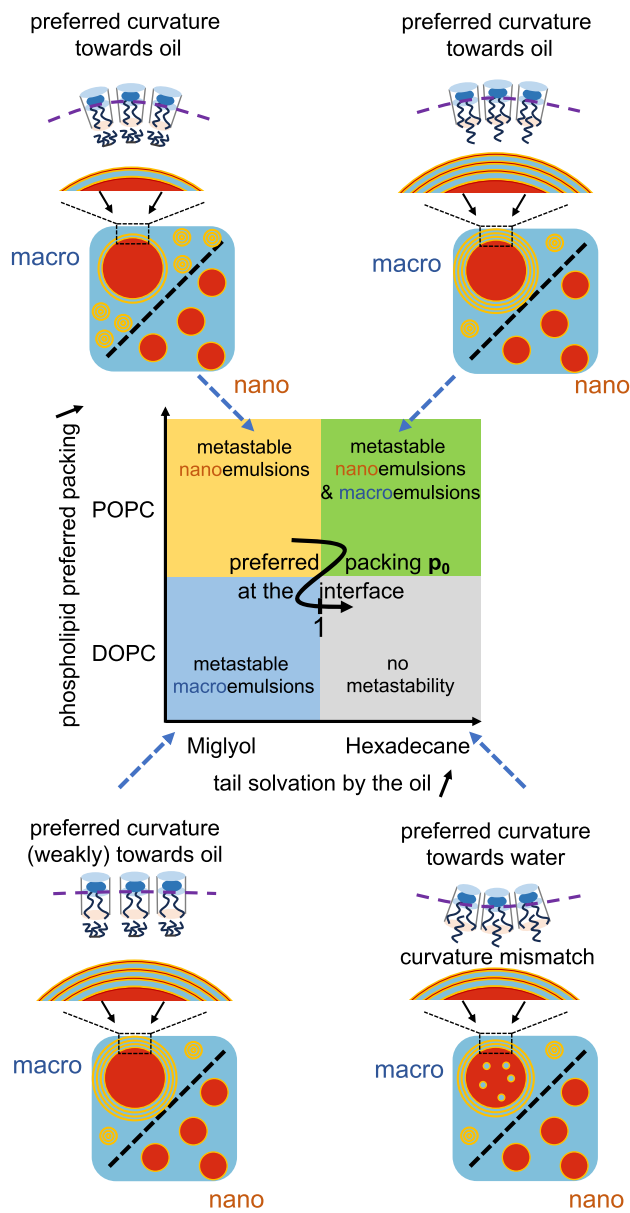
We can also directly compute the number of lipid layers adsorbed at the oil/water interface and observe that it reaches a maximum value of around 16, which matches the correlation distance observed in SANS. We also observed that the number of layers decreases with decreasing the amount of phospholipid in the formulation. Interestingly, this decrease obtained via direct titration was highly consistent with the estimate obtained from the lamellar peak amplitude in SANS measurements (see Supporting Information). This means that the extent of the multilayered structure obtained spontaneously at the interfaces of oil/water macroemulsions depends on both formulation and process conditions.

#### 4. Discussion

We now turn towards a discussion of the important parameters controlling the metastability of emulsions stabilized by phospholipids. For this purpose, we summarized in Fig. 7 the main observations regarding emulsion metastability, preferred packing/curvature, and molecular characteristics for the four systems investigated.

##### 4.1. Preferred packing/curvature and metastability of emulsions stabilized by phospholipid monolayers

The amphiphilic layer's preferred packing/curvature is an important thermodynamic parameter that notably controls self-assembly. Phase diagrams displayed in Fig. 1 evidence that lamellar phases are the main self-assembly structure in all investigated systems. This shows that the preferred packing of phospholipids at oil/water interfaces is close to unity, and that the preferred curvature of the monolayers is close to zero. Yet, phase diagrams also show that slight differences in the phospholipid molecular structure and solvation by the oil lead to changes of



**Fig. 7.** Summary of the molecular parameters at play, the resulting preferred packing/curvature variation, and consequences on both macroemulsion and nanoemulsion metastability. Since nanoemulsions are processed from macroemulsions, a formulation optimum is observed for systems with a preferred curvature of the same sign that the effective droplet curvature but not too large. This delicate balance explains the sensitivity to the formulation of phospholipid-stabilized emulsions, which can be controlled systematically via the approach described in this work.

the preferred packing in the following sequence:

$p_0^{\text{Miglyol}}[\text{POPC}] < p_0^{\text{Hexadecane}}[\text{POPC}] < p_0^{\text{Miglyol}}[\text{DOPC}] < 1 < p_0^{\text{Hexadecane}}[\text{DOPC}]$ . This sequence corresponds to increasing lipid tail solvation by the oil or increasing the lipid hydrophobicity. From a preferred curvature standpoint, our four systems lie on each side of phase inversion conditions, where the preferred curvature is zero.

Kabalnov and Wennerström proposed a hole activation mechanism in which the preferred curvature plays a crucial role in the coalescence of emulsion droplets [18]. Indeed, they argued that the preferred curvature controls the growth of the hole connecting two contacting droplets. For example, a hole connecting two oil droplets displays a curvature turned towards water, the continuous phase. Holes would thus be stabilized by an amphiphilic monolayer displaying a preferred curvature that

is also turned towards water ( $p_0 > 1$ ). In contrast, if the preferred curvature is turned toward oil ( $p_0 < 1$ ), then a curvature free energy barrier hinders the hole growth and thus coalescence.

Our results for the DOPC/hexadecane system show indeed that negative preferred curvatures, evidenced by the emergence of inverted structures, compromise the metastability of both nano and macroemulsions. Avoiding negative preferred curvatures is thus the most important condition that formulations should meet. Yet, this condition is not a sufficient one, since as pointed by Kabalnov and Wennerström [18,19], the preferred packing should sufficiently deviate from unity to hinder hole growth in between two droplets. Indeed, nanoemulsions prepared with the DOPC/miglyol system are unstable, showing the preferred packing is too close to unity and the preferred curvature for this system is too close to zero. In contrast, POPC/miglyol and POPC/hexadecane systems yield metastable nanoemulsions with an extremely long shelf-life. Interestingly, macroemulsions prepared with the DOPC/miglyol system are in contrast metastable. There it is worth recalling that macrodroplets are surrounded by a lipid multilayer, in contrast with nanodroplets, which are only surrounded by a lipid monolayer.

#### 4.2. Multilayer size and anchorage: interplay between composition and process in macroemulsions

This distinction was already discussed by Kabalnov and Wennerström who showed that an adsorbed multilayer is a powerful means to amplify the value of the preferred curvature, since each layer needs to be opened [16]. If the preferred curvature is of the same sign to the hole curvature, there is no free energy barrier, independently of the number of layers. In contrast, if the preferred curvature is of the opposite sign to the hole curvature, the free energy barrier quickly increases with the number of layers in the interfacial lamellar structure [18]. In the case of the DOPC/Miglyol system with a preferred packing parameter smaller but close to unity, droplets stabilized by a phospholipid monolayer undergo coalescence, while droplets stabilized by a phospholipid multilayer are metastable. Our experiments are thus consistent with this amplification of the curvature free energy barrier by interfacial multilayers, leading to metastability. The interfacial multilayered structure, which originates from a preferred packing parameter close to unity, thus compensates for the associated weakness of the curvature free energy barrier.

Yet, the poor metastability of macroemulsions prepared with the POPC/miglyol system, which displays the highest preferred curvature, highlights an additional phenomenon at play. Indeed, in this system, most phospholipids form vesicles in water rather than interfacial multilayers, as shown in Fig. 6. Interestingly, this system also displays the lamellar phase with the lowest oil swelling (Fig. 1), stemming from a reduced oil penetration in the phospholipid tails. This could lead to a weaker anchorage of the phospholipid multilayer during emulsification and a decrease in the interfacial lamellar phase cohesiveness. Still, it is worth noting that it was possible to obtain macroemulsions displaying some metastability, albeit much lower than for the POPC/hexadecane or DOPC/miglyol systems, contrasting with the DOPC/hexadecane system that leads to immediate macroscopic phase separation. This increase in the amount of lipids wasted upon decreasing lipid solvation by the oil limits the simple strategy that would consist of increasing as much as possible the preferred curvature while keeping the lamellar phase as the dominant self-assembly structure. Indeed, oil/lipid interactions also underlie the value of the preferred curvature. Therefore, there exists only a narrow range within which direct macroemulsions stabilized with phospholipids are metastable. This range corresponds to a preferred curvature turned towards the dispersed phase (oil), but not too large as this would also correlate with a weaker interfacial anchorage of the lamellar phase.

It is worth stressing again that we did not observe this optimum with nanoemulsion metastability. Indeed, we showed that nanoemulsions were stabilized by a phospholipid monolayer. In this case, all the



lipid is adsorbed at oil/water interfaces, and only the sign and absolute value of the preferred curvature matter.

Overall, nanoemulsions can display outstanding metastability, even though the corresponding macroemulsions may still undergo slow coalescence despite interfacial multilayers when the droplet size increases above dozens of microns. This difference likely relates to creaming when droplets become large enough, which promotes inter-droplet contacts, thus requiring further lowering of the coalescence probability per unit area.

#### 4.3. Rational formulation strategies

At this stage, we have provided the mechanisms through which both nano and macro oil-in-water emulsions can be stabilized by phospholipids. Of primary importance is the sign of the preferred curvature, which must be positively turned toward the oil. As this value depends on both phospholipid and oil molecular structures, the formulation should be first checked to meet this requirement. For nanoemulsions, the idea would be to have this curvature as large as possible. Yet, this can compromise the metastability of the corresponding macroemulsion, which is first formed as an intermediate system before processing in the high-pressure homogenizer. Some processing issues can also arise due to self-assembly, such as a large viscosity increase of the oil/lipid solution, which potentially requires heating the system. Yet, the key question is then a processing one. If the delay between macroemulsion formation and its processing into the high-pressure homogenizer is small enough, then the poor macroemulsion metastability observed when the preferred curvature is too large is still sufficient to avoid macroscopic phase separation during the transfer from the first emulsification step to the second one. This requires designing a continuous flow process in which storage times are very small. Of course, if the final aim is rather to produce macroemulsions, then one should rather consider the optimum curvature described in this work as a guiding framework for formulation.

## 5. Conclusion

Throughout this work, we have investigated the stabilization of oil-in-water macro and nanoemulsions by phospholipids. Our approach was to investigate systematically the interplay between formulation, self-assembly, interfacial structure, and droplet metastability. We have shown that the formation of interfacial multilayers, an emblematic characteristic of emulsions stabilized by phospholipids [5–10], is not systematic and mostly depends on the emulsification method and thus droplet size. Indeed, nanoemulsion droplets prepared by high-pressure homogenization are rather covered by a phospholipid monolayer, paving the way to efficient use of the amphiphile, a feature that has been reported for particle-stabilized emulsions in the limited-coalescence regime [29,30] and surfactant-stabilized emulsions obtained by non-equilibrium self-assembly [31–33]. Furthermore, the stabilization of these nanoemulsions does not require long-range ionic repulsions, which questions the ubiquitous use of fatty acids in formulations to supposedly enhance metastability [2]. Rather, the metastability rests on a value of the amphiphilic layer preferred or spontaneous curvature that is sufficiently turned towards the oil, which is in line with conclusions by Kabalnov [18,19]. When increasing the droplet size above the micron and moving to the realm of macroemulsions, the requirement changes, and droplets are only metastable if a multilayer is adsorbed, which can be rationalized from the Kabalnov-Wennerström coalescence model [16]. We have shown that the thickness of this multilayer results from a complex interplay of emulsification history, including the initial lipid location as was previously reported for model interfaces [36], and lipid concentration. The preferred curvature should also be turned towards the oil, but strikingly not too much to avoid the detachment of the interfacial layer as vesicles in water. The formulation of macroemulsions is thus much more drastic than the formulation of nanoemulsions and should be adapted to the desired target. If macroemulsions are the

final target, then the metastability should be optimized within the optimum conditions stated above. If nanoemulsions are the final target and macroemulsions intermediates, as in parenteral applications, then time scales are crucial. Macroemulsions should be processed into nanoemulsions as quickly as possible. In practice, this requires continuous flow processing at high flow rates. Overall, our results provide a rational strategy to design metastable emulsions stabilized with phospholipids and thus adapt to any formulation change, such as oil and phospholipids sources, a major issue when designing parenteral emulsions [2,3,22,23].

#### CRediT authorship contribution statement

**Jenny Marie Andersson:** Methodology, Investigation, Data curation. **Olivier Masbernat:** Investigation, Conceptualization. **Kevin Roger:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

#### 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.

#### Data availability

Data will be made available on request.

#### Acknowledgements

Gabriel Brossard and Léo Garcia are acknowledged for discussions and first experiments on the topic. The LLB is acknowledged for providing us with SANS beamtime, with the precious support of Alexis Chennevière. We are also grateful to Adrian Rennie for kindly lending us copies of the rotating SANS cells that he designed and manufactured. The research federation FERMAT (Université de Toulouse, France) is acknowledged for providing access to the Xeuss 2.0 SAXS instrument (Xenocs) through the CPER IMATECBIO grant, with support from Pierre Roblin. Agence Nationale de la Recherche (ANR) is acknowledged for support through grants COATING (ANR-17-CE-09-0004-01) and PROSALIDE (ANR-23-CE06-0017-01).

#### Appendix A. Supplementary material

Supplementary material related to this article can be found online at <https://doi.org/10.1016/j.jcis.2024.08.249>.

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