



# Mixed interfaces comprising pea proteins and phosphatidylcholine: A route to modulate lipid oxidation in emulsions?

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## ABSTRACT

Many food emulsions, such as mayonnaise or infant formula, are stabilized by combinations of proteins and phospholipids that are concomitantly present at the oil-water interface. It is expected that the physical, as well as the oxidative stability of emulsions are affected by the interfacial composition. Controlling the interfacial composition can therefore be a means to improve emulsion stability, and in particular lipid oxidation.

In this work, we varied the ratio of pea protein and sunflower phosphatidylcholine and investigated the resulting interfacial composition and oxidative stability of oil-in-water (O/W) emulsions prepared at fixed pea protein concentration. Increasing the phospholipid concentration led to a monotonic decrease in the adsorbed proteins and to an increase in the adsorbed phospholipids. Cryo-transmission electron microscopy revealed a slight decrease in interfacial thickness, measuring 2.8 nm for pea protein alone, compared to 2.3 nm at the highest phosphatidylcholine-to-pea protein ratio (PC/sPPI) of 0.8 (w/w), and a slight decrease in interfacial roughness, albeit that the differences are small.

The oxidative stability of the emulsions globally increased with increasing the PC/sPPI ratio, with the exception of the system with a PC/sPPI ratio of 0.6, which was more sensitive to oxidation than all the other PC-containing emulsions. This peculiar behavior is discussed in the context of model interfacial films of comparable mixed compositions, which exhibit discrete spherical structures and strands. Our results suggest that the PC/sPPI ratio determines the specific interfacial structure that is formed (i.e., packing and patchiness), and it can be expected that thereby the oxidative stability of O/W emulsions stabilized by proteins and phospholipids is modulated.

## 1. Introduction

Lipid oxidation deteriorates food quality and thus needs to be mitigated, not only efficiently but also in ways that comply with consumer wishes for sustainable and natural food products (Aimutis, 2022; Aschemann-Witzel & Peschel, 2019). To this end, natural antioxidants have been considered, although they are less efficient than synthetic antioxidants (e.g., EDTA) (Ghorbani Gorji, Smyth, Sharma, & Fitzgerald,

2016; Laguerre, Tenon, Bily, & Birtić, 2020). It has been stressed that the localization of the antioxidants greatly determines their effectivity (Laguerre et al., 2015; Phonsatta et al., 2017), yet this point is often overlooked. Furthermore, in nature, effective strategies to mitigate lipid oxidation can be found and these go beyond the classic use of a single antioxidant component. For instance, in plants, lipids are stored as natural oil-in-water (O/W) emulsion droplets, called oleosomes. These droplets are stabilized by a monolayer of phospholipids and membrane proteins, mostly oleosins (Nikiforidis, 2019; Wijesundera et al., 2013).

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## Abbreviations

sPPI	'soluble' fraction of pea protein isolate
PC	phosphatidylcholine

The observed high oxidative stability has been attributed to the interfacial architecture, more specifically, it has been suggested that oleosins form a barrier against reactive oxygen species (ROS) (Ratnayake & Huang, 2015; Rayner, 2015; Wijesundera et al., 2013). This barrier effect may be reinforced by radical scavenging and metal chelation by proteins (Elias, Kellerby, & Decker, 2008), as well as interfacial binding of lipophilic antioxidants, such as tocopherols. In addition, phospholipids have been reported to act as antioxidants through regeneration of oxidized tocopherols, and chelation of metal ions (Cui & Decker, 2016; Hennebelle et al., 2024; Lu, Nielsen, Baron, & Jacobsen, 2017; Samdani, McClements, & Decker, 2018; Zamora & Hidalgo, 2005).

A relevant starting point for the design of oxidation-resistant emulsions could thus be the combination of proteins and phospholipids, that actually can be found in many food emulsions, such as mayonnaise and infant formula. Previous research conducted on model O/W emulsions has demonstrated that when proteins were replaced or supplemented with phospholipids, the resulting emulsions exhibited comparable or reduced physical stability and generally lower oxidative stability (Berton-Carabin, Genot, Gaillard, Guibert, & Ropers, 2013; García-Moreno, Horn, & Jacobsen, 2014; Yesiltas, Sørensen, et al., 2019) whereas in other studies, partial substitution led to emulsions with higher oxidative stability (Yesiltas, García-Moreno, Sørensen, Akoh, & Jacobsen, 2019). Nevertheless, a clear connection between the interfacial structure and oxidative stability has not been established yet. Most research on such systems has been done on dairy protein/phospholipid mixtures (Berton-Carabin et al., 2013; García-Moreno et al., 2014; Yesiltas, García-Moreno, et al., 2019; Yesiltas, Torkkeli, et al., 2019), whereas studies involving plant protein emulsifiers are only rarely done in combination with phospholipids; the few available examples (Bourgeois, Couëdelo, Subirade, & Cansell, 2020; Li et al., 2018, 2020) mostly focused on the physical stability of the systems, with oxidative stability seldomly investigated (Chen et al., 2020; Li et al., 2020).

To address this knowledge gap, the present work used mixtures of pea protein and sunflower phosphatidylcholine (PC) to assess whether they could be rationally tailored to form interfacial structures which control the oxidative stability of O/W emulsions. Pea proteins were selected as an industrially relevant representative of pulse proteins given their functional (emulsifying) properties (Grossmann, 2023; Shanthakumar et al., 2022). Besides, they were previously shown to form emulsions with a higher oxidative stability compared to that of emulsions formulated with other plant proteins (Gumus, Decker, & McClements, 2017). The soluble fraction of pea protein isolate has been shown to have a higher binding capacity of iron and, at high protein concentrations, a similar free radical scavenging capacity compared to whey protein isolate (Hinderink, Schröder, Sagis, Schroën, & Berton-Carabin, 2021). PC is the most abundant phospholipid type in oil bodies (Tzen, Cao, Laurent, Ratnayake, & Huang, 1993) and in lecithin, and was therefore chosen in the present work.

The interfacial composition was modulated by keeping the total concentration of pea protein constant and varying the mass ratio with PC. It was expected that at higher phospholipid-to-protein ratio (PL-P), the concentration of phospholipids at the interface would increase at the expense of the proteins. Two microscopy techniques were used to study the formed interfacial structures: atomic force microscopy (AFM) and cryogenic transmission electron microscopy (cryo-TEM). AFM provides insights into flat/2D organization (Morris & Gunning, 2008), whereas cryo-TEM gives an impression of 3D emulsion systems with curved interfaces (Friedrich, Frederik, de With, & Sommerdijk, 2010; Waninge,

Kalda, Paulsson, Nylander, & Bergenstähl, 2004). The interfacial composition and structures are linked to lipid oxidation in emulsions and the results are discussed in terms of sustainable food design that fits clean label strategies.

## 2. Materials and methods

### 2.1. Materials

Rapeseed oil (kindly supplied by Unilever, Wageningen, the Netherlands) was stripped from impurities and tocopherols by using alumina powder (MP EcoChrome™ ALUMINA N, Activity: Super I, Biomedicals) (Berton, Genot, & Ropers, 2011). Pea protein isolate (PPI) was obtained from Roquette (NUTRALYS® S85F, purity 78 %; N x 5.6) and phosphatidylcholine (PC) was donated by LIPOID (LIPOID H 100, purity 90%). For sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Mini-PROTEAN gels (12% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well comb, 30 µl/well, Bio-Rad), Bio-safe Coomassie G-250 stain, Laemmli sample buffer, Tris/Glycine/SDS-buffer (running buffer) and precision plus protein standard (Bio-Rad, Richmond, CA, USA) were used. Sodium phosphate dibasic, sodium phosphate monobasic, sodium dodecyl sulfate (SDS), bromophenol blue R-250 (BPB), and 2-mercaptoethanol were purchased from Sigma Aldrich (Saint Louis, MO, USA) and were of at least analytical grade. 2,2,4-Trimethylpentane (isooctane) and 2-propanol were obtained from Alfa Aesar (Kandel, Germany) and Actu-All Chemicals (Oss, the Netherlands), respectively. Deuterated chloroform (CDCl<sub>3</sub>) with 0.03% tetramethylsilane (TMS), deuterated dimethylsulfoxide (DMSO-*d*<sub>6</sub>), and deuterated 4 Å molsieves were purchased from Euriso-top (Saint-Aubin, France). Ultrapure water was obtained from a Milli-Q system (Millipore Corporation, Billerica, Massachusetts, USA) and used throughout all experiments.

### 2.2. Methods

#### 2.2.1. Preparation of the pea protein solution

An aqueous solution of pea proteins was prepared as previously described by Hinderink, Münch, Sagis, Schroën, and Berton-Carabin (2019). Briefly, a 10 wt% PPI suspension was prepared in a 10 mM phosphate buffer (pH = 7.0) and hydrated for at least 48 h at 4 °C. The soluble protein fraction was obtained by centrifuging the suspension (16,000×g, 30 min), and collecting the supernatant, which was in turn centrifuged again in the same conditions. The protein concentration in the supernatant was determined with the Dumas method (Shea & Watts, 1939), applying a nitrogen-to-protein conversion factor of 5.6. The supernatant, further referred to as 'soluble pea protein' (sPPI) was then diluted to a concentration of 1.11–1.12 wt%, giving a concentration of 1 wt% in the final emulsion.

#### 2.2.2. Dissolving phosphatidylcholine in the oil

A stock solution of PC in methanol was prepared (0.2 w/v.%) and added at increasing concentration to the stripped rapeseed oil to obtain 0.2–0.8 wt% in the final emulsion, while keeping the oil volume fraction constant. After incorporation of the methanolic solution into the oil (and prior to emulsion preparation), the methanol was evaporated under nitrogen flow at 25 °C (Reacti-Therm III, Thermo Fisher Scientific, US) and stirred for 1.5 h. It was reported in literature that this procedure leads to emulsions that are physically more stable compared to dispersing the phospholipids in the aqueous phase (Magnusson, Nilsson, & Bergenstähl, 2016; Yamamoto & Araki, 1997).

#### 2.2.3. Preparation of the emulsion

A coarse emulsion was prepared by mixing 10 wt% stripped rapeseed oil (+0–0.8 wt% PC) with the aqueous phase (1 wt% sPPI) using a high-speed blender (S18N-19G, Ultraturrax R, IKA-Werke GmbH & Co., Staufen, Germany) at 11,000 rpm for 1 min. The coarse emulsion was

then further passed five times through a high-pressure homogenizer (M-110Y Microfluidizer, Microfluidics, Massachusetts, USA) equipped with a F-12Y interaction chamber at 400 bars to obtain the final emulsion. The coil of the system was cooled throughout homogenization by ice water to prevent heating up of the emulsion during preparation. Sodium azide (0.02 wt%) was added and the emulsions were stored in 20-mL vials (6 mL per vial), and horizontally rotated (3 rpm) in an oven at 40 °C, in the dark. Samples were taken at day 0, 1, 2, 7 and 14 for physical and oxidative stability analysis; at least three emulsions were prepared independently, for each formulation.

## 2.2.4. Physical stability

**2.2.4.1. Droplet size distribution.** The emulsion droplet size distribution was measured by static light scattering (Mastersizer 3000, Malvern Instruments Ltd.; Worcestershire, UK) using the refractive indexes for water (1.330) and rapeseed oil (1.473), and an absorption index of 0.01. All emulsions were measured as such, and after dilution in a 1 wt% SDS solution (1:1 v/v) to distinguish between the apparent droplet size distribution of possible aggregated droplets, and the actual droplet size distribution. The average droplet size is reported as the Sauter mean diameter ( $d_{3,2}$ ). Each result is the mean of at least two independent emulsion samples, of which each is the average of five measurements.

**2.2.4.2. Emulsion morphology.** The emulsion morphology was studied using light microscopy (Axioscope, Zeiss, Germany) at 40 × magnification, without dilution.

**2.2.4.3. Zeta-potential.** The zeta potential was determined by measuring the electrophoretic mobility of droplets via laser Doppler electrophoresis and Phase Analysis Light Scattering (PALS) using a Zetasizer Nano ZS (Malvern Instruments Ltd.; Worcestershire, UK). The zeta potential was calculated using the Smoluchowski model with refractive indices of 1.330 and 1.473 for water and rapeseed oil, respectively. Samples were 101 times diluted with MilliQ water and measured after 3 min of equilibration at room temperature with three measurements per sample. The reported zeta potentials are the average values for two independent emulsions.

## 2.2.5. Protein surface load

The surface load was determined as described by [Hinderink, Sagis, Schroën, and Berton-Carabin \(2021\)](#). The serum phase of the emulsions was separated from the creamed phase by centrifugation at 15,000×g for 1.5 h, and collected by cautiously making a hole at the bottom of the tube. The creamed phase was re-dispersed in buffer (under gentle agitation) to wash out unadsorbed proteins entrapped in the creamed phase, rotated for 1 h and again centrifuged at 15,000×g for 1.5 h. The creamed phase obtained after centrifugation was re-dispersed into 1 wt % SDS solution (under gentle agitation), and then re-centrifuged. The continuous phase, containing the proteins that were initially adsorbed, was collected in the same way as previously explained for the serum phase, and analyzed together with sPPI protein standards (protein solutions diluted to 1–4 g/L) using SDS-PAGE under reducing conditions, as described by [Hinderink et al. \(2019\)](#). The lipid content in the cream was determined by mixing an aliquot of cream in isooctane:isopropanol (3:2) and extracting the oil as described in section 2.2.6. The obtained lipid mass ( $m_{oil}$ ) was determined by subtracting the weight of the empty tube from the weight of the tube containing the extracted lipids. The surface load  $\Gamma$  was calculated using (equation 5.1):

$$\Gamma = \frac{C_{prot} \cdot V_{SDS} \cdot d_{3,2}}{6 \cdot m_{cream} \cdot V_{cream}} \quad (1)$$

where  $C_{prot}$  is the protein concentration measured via SDS-PAGE,  $V_{SDS}$  and  $m_{cream}$  are the volume of the added SDS solution, and the mass of cream, respectively,  $d_{3,2}$  the Sauter mean diameter. The lipid volume in

the cream,  $V_{cream}$  is defined by (equation 5.2):

$$V_{cream} = \frac{m_{oil,cream}}{\rho_{oil}} \quad (2)$$

where  $m_{oil,cream}$  is mass of oil ( $m_{oil}$ ) per gram of cream and  $\rho_{oil}$  is the density of the oil (0.915 g/cm<sup>3</sup>).

To calculate the concentration of proteins in the serum phase, the amount of adsorbed proteins was subtracted from the total amount of proteins in the emulsion. This concentration was also determined experimentally via SDS-PAGE as described for the concentration in the cream ( $C_{prot}$ ). An example for SDS-PAGE-based determination of the protein concentration in the solutions can be found in [Fig. S1](#).

## 2.2.6. Sample preparation for cryo-TEM and cryo-TEM imaging

Prior to cryo-TEM sample preparation, the TEM grids were glow-discharged to make the surface of the carbon TEM support film hydrophilic. Cryo-TEM samples were prepared by applying either 3 µL of sPPI dispersion, or of emulsion (50 times diluted using 10 mM phosphate buffer, pH = 7.0), on a 200 mesh Cu grid with a R2/2 Quantifoil® carbon support film (Quantifoil MicroTools GmbH). The emulsion free of sPPI was prepared the same way as the other emulsions containing only buffer and stripped rapeseed oil with 1 wt% PC (section 2.2.3). The sample vitrification was performed using an automated vitrification robot (Vitrobot™ Mark IV, Thermo Fisher Scientific) to blot and plunge the samples into liquid ethane. The emulsions were stored for 3 weeks at 4 °C before TEM sample preparation. Cryo-TEM imaging was conducted using the TU/e CryoTitan (Thermo Fisher Scientific) operated at 300 kV and equipped with a Field-Emission Gun, a post column Gatan Energy Filter (GIF, model 2002), and a post-GIF 2k × 2k Gatan CCD camera (model 794). Images were acquired at an electron dose rate of 10 e<sup>-</sup>/Å<sup>2</sup> • s<sup>-1</sup> using an exposure time of 2 s at a nominal magnification of 24000 × and a nominal defocus of −1.5 µm. At these defocus conditions the first cross-over point in the contrast transfer function of the microscope is at ~0.58 nm<sup>-1</sup>, which allows direct interpretation of objects of 1.7 nm; or larger which is adequate for resolving the interfacial layers of the emulsion droplets. The interfacial thickness was deduced from a line intensity profile (20 pixel in width) from the cryo-TEM images using DigitalMicrograph®. Line scans were taken at random locations with a distinguishable interface.

## 2.2.7. Langmuir-Blodgett films

Langmuir-Blodgett (LB) films were prepared using a Langmuir trough (KS NIMA/Biolin Scientific Oy, Finland) filled with buffer. The sPPI films were formed by spreading either 80 or 115 µL sPPI solution (1 g/L) onto the air-water interface. In the case of the PPI/PC mixtures, the PC solution (0.5 g/L in chloroform) was first spread onto the interface and, after an equilibration time of 30 min, the sPPI solution was introduced. The volume of PC solution varied from 12.0 to 207.5 µL corresponding to 0.2–0.8 wt% PC in emulsion (section 3.2), depending on the required PC- sPPI ratio ([Table 1](#)), while keeping the protein concentration constant.

The formed layers were left to equilibrate for 30 min, after which they were compressed by moving the barriers (5 mm/min) to the desired

**Table 1**

Overview of the ratios between pea protein and PC used to prepare Langmuir-Blodgett films corresponding to various surface loads (mg/m<sup>2</sup>) of both constituents.

sPPI (mg/m <sup>2</sup> )	PC (mg/m <sup>2</sup> )	Mass ratio (PC/sPPI)
1.47	–	0
	0.076	0.052
	0.38	0.26
0.76	–	0
	0.198	0.26
	0.7885	1.04
	0.988	1.3

surface load. The surface pressure was measured using a platinum Wilhelmy plate (perimeter 20 mm, height 10 mm); the surface pressure at the desired surface load was kept constant while directly loading the interfacial layers on freshly cleaved mica sheets (Highest Grade V1 Mica, Ted Pella, USA) at a withdrawal speed of 1 mm/min. The LB films were dried in a desiccator at room temperature. All films were produced in duplicate and analyzed in at least two locations with an atomic force microscope (AFM; MultiMode 8-HRTM, Bruker, USA). AFM images were acquired in tapping mode using a Scanasyt-air model non-conductive pyramidal silicon nitride probe (Bruker, USA) with a nominal spring constant of 0.40 N/m (Bruker, Billerica, US). A lateral scan frequency of 0.977 Hz was used, and the resolution was set at  $512 \times 512$  pixels in a scan area of  $2 \times 2 \mu\text{m}$  or  $5 \times 5 \mu\text{m}$ . AFM images were analyzed with the NanoScope Analysis 1.5 software by also determining the roughness of the films.

### 2.2.8. Lipid oxidation

Lipid oxidation in the emulsions was measured with  $^1\text{H}$  NMR according to the method of Merckx, Hong, Ermacora, and van Duynhoven (2018). Prior to the measurement, the oil was extracted by adding isooctane:isopropanol (3:2) to the emulsion (4:1 v/v), vortexing 3 times for 20 s each, and centrifuging for 8 min at 4700 rpm. The isooctane layer was collected, and the solvent was evaporated under nitrogen flow (Reacti-Therm III, Thermo Fisher Scientific, US) at  $25^\circ\text{C}$ . Each data point is the average of at least three individual emulsion samples (0–2 days) or of at least two individual emulsions (7/14 days), of which each was measured 3 times. Standard deviations are calculated based on all measurements ( $n = 6$  or  $9$ ) of the different replicates combined.

### 2.2.9. Statistics

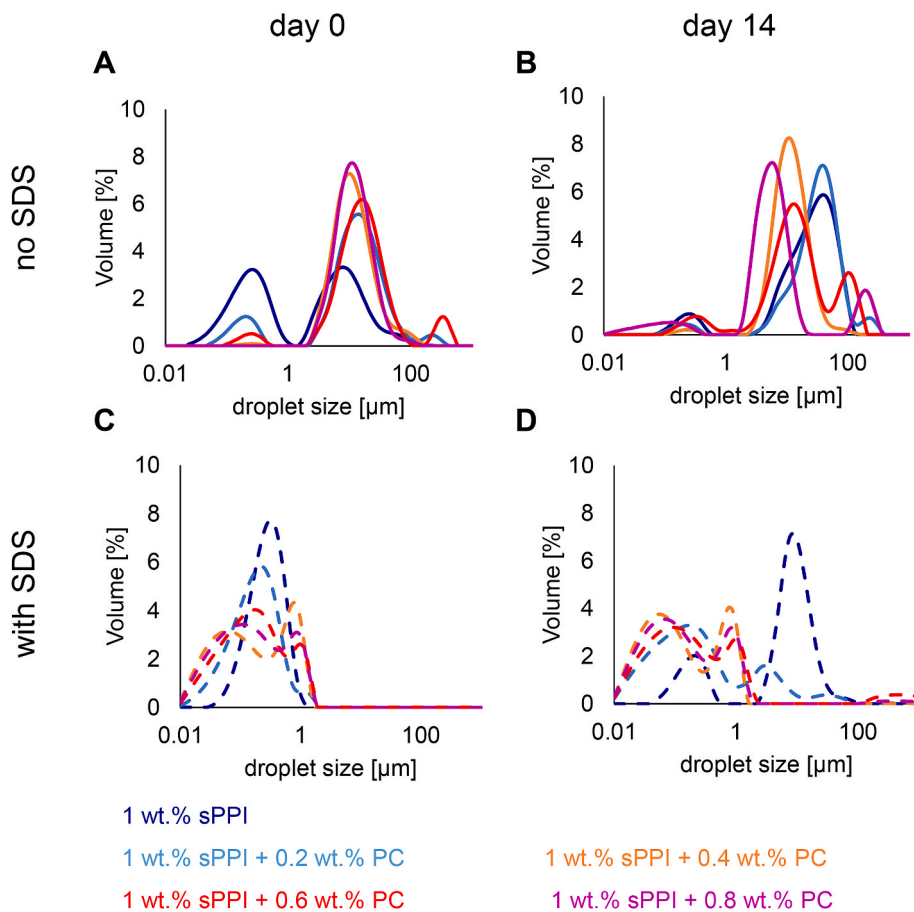
The significance of the differences in the serum protein concentrations was determined using IBM SPSS statistics software with one-way ANOVA and post-hoc with the Tukey HSD method to compare means. Significance was established with  $p < 0.05$ .

## 3. Results and discussion

### 3.1. Droplet size distribution in emulsions stabilized with sPPI or sPPI/PC mixtures

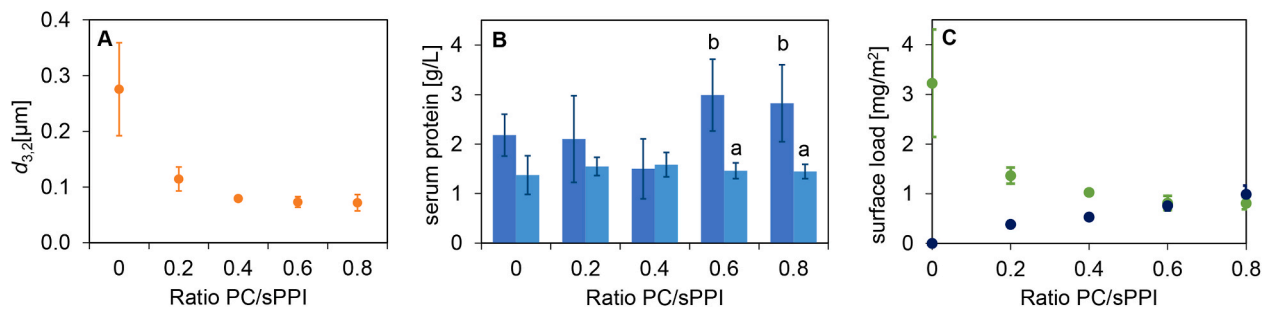
The droplet size distributions (Fig. 1) of all fresh emulsions showed a multimodal distribution that shifted to smaller droplet sizes when the emulsions were diluted with 1 wt% SDS, which implies that the emulsions were flocculated (Schroën & Berton-Carabin, 2016). For emulsions with 0 and 0.2 wt% PC, the droplet size distributions were monomodal after addition of SDS but remained bimodal for emulsions with higher PC concentrations (0.4–0.8 wt%).

The average droplet sizes ( $d_{3,2}$ ) measured after SDS dilution ranged from 0.07 to  $0.17 \mu\text{m}$ , and from 0.22 to  $1.4 \mu\text{m}$  for emulsions stabilized by PPI/PC mixtures, or PPI only (Fig. 1C & D; Fig. 2A). This implies that droplet breakup during homogenization is facilitated by PC, forming droplets with enhanced stability against flocculation and coalescence (see also Fig. S2 A–C). The results are in line with the findings of Mantovani, Cavallieri, Netto, and Cunha (2013), Garcia-Moreno et al. (2014), McSweeney (2008), and Fang and Dalgleish (1996), and can be explained by the higher total emulsifier concentration (1.2–1.8 vs. 1 wt %) during emulsion preparation, most probably in conjunction with a higher surface activity of PC. The combination with PC hardly affected



**Fig. 1.** Droplet size distribution of the emulsions at day 0 (A, C) and day 14 (B, D). The distribution was measured as such (solid line) or after two-fold dilution of the emulsion in 1 wt% SDS (dashed) to assess possible flocculation ( $n = 5$ ). For clarity, the displayed data correspond to a representative result for each sample; similar trends were obtained for three independent replicates.





**Fig. 2.** (A) Average  $d_{3,2}$  for the creamed phases re-dispersed in SDS at day 0, (B) serum protein concentrations (calculated: dark blue or determined by SDS-PAGE: light blue) and (C) the measured pea protein surface load ( $\text{mg}/\text{m}^2$ ) (green) and calculated PC surface load (assumption: 100% adsorption of PC; blue) of emulsions stabilized by sPPI and PC at different ratios. The bars show the standard deviation of independent replicates (mostly within marker size for surface load, PC concentration, and  $d_{3,2}$ ). Small letters indicate significant differences between the two methods to determine the serum protein concentration (between one method concentrations were not significantly different).

the zeta potential (Fig. S3), which is aligned with the findings of McSweeney, Healy, and Mulvihill (2008).

Emulsions containing 0.4–0.8 wt% PC maintained a constant droplet size distribution during 14 days of storage. In contrast, emulsions stabilized with 0 or 0.2 wt% PC developed a bi- or multimodal distribution containing larger droplets over time (as shown in Fig. 1C and D; dark and light blue lines) suggesting that SDS is no longer able to disrupt the large structures formed, which can be due to droplet coalescence, or to strongly bound flocs. When checking the morphological appearance of the SDS-diluted emulsions by optical microscopy (Fig. S2 D-F), irregularly shaped structures consisting of small sub-structures can be seen for the emulsions with 0 and 0.2 wt% PC, pointing to the fact that strongly bound flocs were probably formed.

### 3.2. Protein surface load in emulsions stabilized with sPPI or mixtures of sPPI and PC

Please note that the mean droplet diameter ( $d_{3,2}$ ) decreased with increasing the PC/sPPI ratio (Fig. 2A), resulting in similar protein concentrations in the serum (Fig. 2B) when compared within one method ( $\sim 1.5$ – $3$  or  $1.4$ – $1.6$  g/L). Since the total protein concentration in the emulsions is 10 g/L, this implies that substantial amounts of protein are adsorbed. The emulsion made with sPPI had a surface load of  $3.2 \text{ mg}/\text{m}^2$ , which is close to values reported for comparable emulsions (e.g.,  $3.5 \text{ mg}/\text{m}^2$ ; Hinderink et al., 2019). The protein surface load decreased with increasing PC concentration (Fig. 2C), and might level off at a PC/sPPI ratio of 0.6–0.8, reaching its lowest value at  $0.8 \text{ mg}/\text{m}^2$ . This indicates that in the concentration range studied, PC is able to only partially prevent pea protein adsorption (Courthaudon, Dickinson, & Christie, 1991). At all ratios, mixed pea protein-PC layers were formed, which have been reported to possibly adopt various structural organizations: homogeneously mixed or phase separated monolayers, and/or mixed multilayers with primary layer(s) composed predominantly of PC and secondary layer(s) composed predominantly of sPPI (Waninge et al., 2005).

### 3.3. Interfacial structure of sPPI and sPPI-PC at oil-water interfaces

Cryo-TEM was used to study the nanoscale structure of the sPPI dispersion and adsorbed layers in emulsions (Fig. 3).

The 3–5 nm dark granular structures visible in Fig. 3B (white arrows) are expected to be pea proteins (legumin and/or vicilin) (Burger & Zhang, 2019; Gueguen, Chevalier, Barbot, & Schaeffer, 1988). Emulsion droplets made with 1 wt% sPPI only, or with the same PPI concentration with 0.8 wt% PC are shown in Fig. 3C–F. The emulsion droplets in the sPPI-based emulsion show a heterogeneous interface with dark particles at the droplet surface (Fig. 3D) that are similar to those observed in the sPPI dispersion, which are thus probably adsorbed proteins. The

interfacial film of the emulsion droplets made with sPPI and 0.8 wt% PC showed some heterogeneity but had a relatively smooth surface (Fig. 3F). The large spherical objects in Fig. 3C and E are probably oil droplets trapped and flattened in the vitrified ice layer.

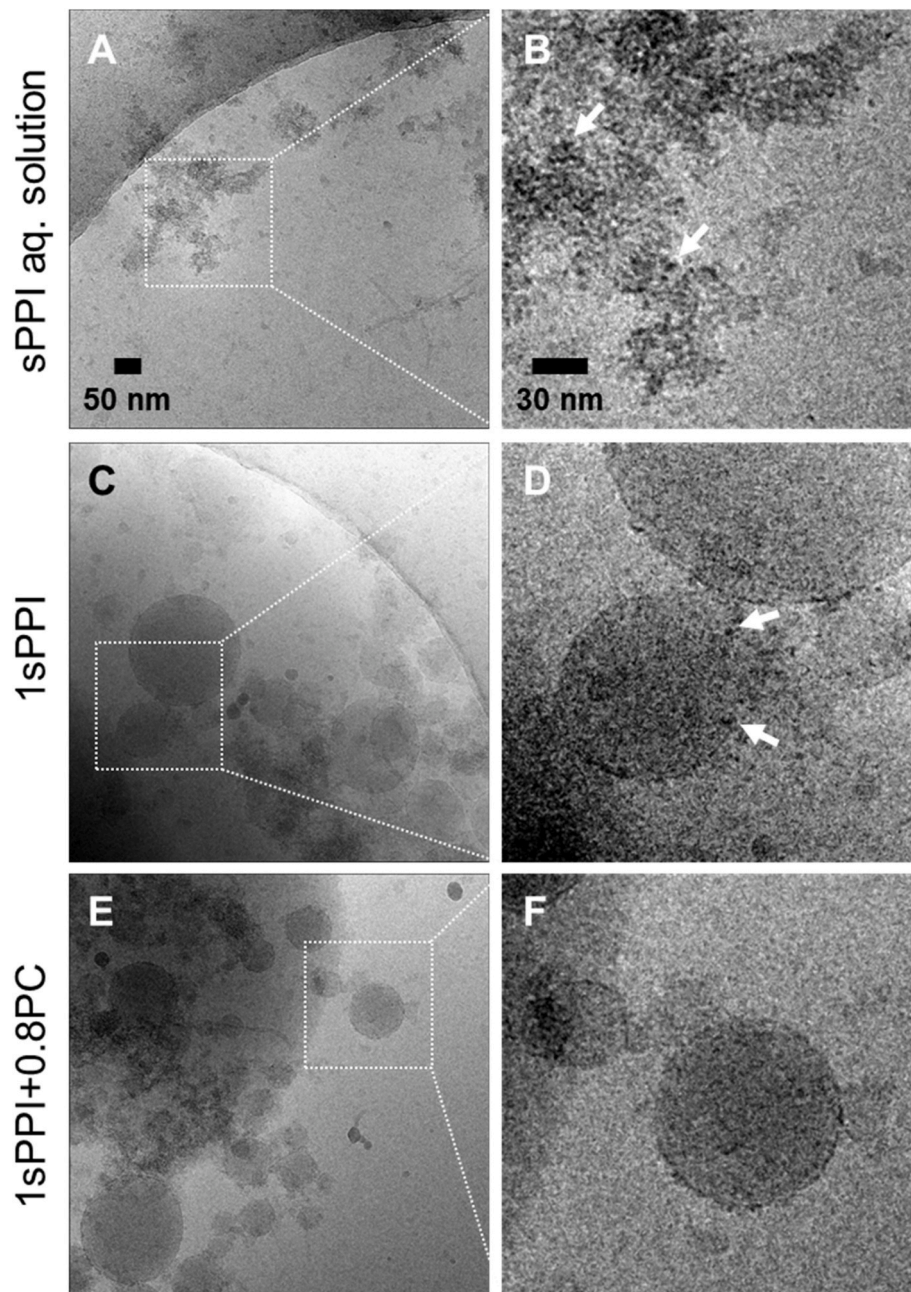
To quantify the interfacial structures in terms of thickness and heterogeneity, line intensity profiles across the droplet interface were analyzed at various locations (Fig. 4). In each region, the line intensity profile averaged over 7.6 nm (20 pixel) in width was measured, and at half height of the intensity valley (droplet interfaces are dark) the interfacial thickness of the selected region was determined (see Table 2 for the values found).

The mean interfacial thickness of 1 wt% sPPI emulsions was  $2.8 \pm 0.8 \text{ nm}$ , which is similar to 1 wt% sPPI emulsion with 0.4 wt% PC ( $2.6 \pm 0.7 \text{ nm}$ ) or 0.6 wt% PC ( $2.7 \pm 0.7 \text{ nm}$ ). The emulsion with 0.8 wt% PC had a slightly lower mean thickness ( $2.3 \pm 0.6 \text{ nm}$ ; standard errors of means 0.08 vs. 0.11/0.10). In the absence of sPPI, the interfacial layer in the emulsion was difficult to distinguish and therefore the bilayer thickness of a PC liposome was measured ( $4.9 \pm 0.5 \text{ nm}$ , see Table 2), and it is assumed that half this value is similar to the thickness of an interfacial PC layer without sPPI. The distribution of the interfacial thicknesses (Fig. 5) may seem wider for emulsions that contain sPPI, but we think that the differences are very small, and too close to call. The PC double layer thickness is less likely to substantially vary.

Which component is present at the interface depends on their adsorption kinetics (both diffusion and binding). In general, PC does not require conformational changes as would be typical for proteins. It is also expected that the average diffusion distance will be lower for PC given their localization in the oil (droplets), which may have contributed to their prominence in the interface of our emulsions. Furthermore, interfacial displacement may take place; for example, low molecular weight emulsifiers have been reported to (partly) displace adsorbed proteins post-emulsification. Such protein displacement was assessed by Waninge et al. (2005) who studied the surface load of milk proteins ( $\beta$ -casein or  $\beta$ -lactoglobulin) and membrane phospholipids in emulsions. The authors found that the emulsion preparation method greatly influenced the interfacial composition, which can be very complex. Protein-based emulsions are often non-equilibrated systems and it can be expected that the aqueous phase and surface composition (especially in the presence of proteins and phospholipids) would also be non-equilibrated and driven by (slow) kinetic processes.

### 3.4. Interfacial structure of sPPI or sPPI-PC mixtures at air-water interfaces

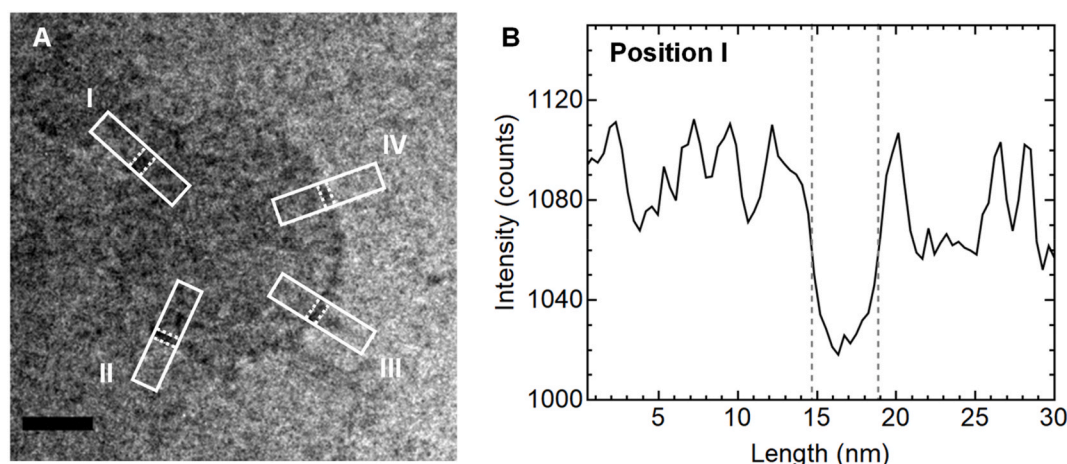
Langmuir films prepared at the air-water interface (Mackie, Gunning, Wilde, & Morris, 2000; Morris & Gunning, 2008) were used here to reach deeper insights into the structure of mixed protein-phospholipid interfacial films. Fig. 6 shows AFM images of such dried pea



**Fig. 3.** Representative cryo-TEM images of (A) soluble pea protein isolate (sPPI), and emulsions prepared with (C) 1 wt% sPPI, and (E) 1 wt% sPPI with 0.8 wt% PC, in 10 mM phosphate buffer (pH = 7.0). The white arrows point to granular aggregates possibly originating from proteins. The magnification is  $24000\times$  with a nominal defocus of  $-1.5\text{ }\mu\text{m}$  (B, D, F) are zoomed-in (white dashed box) regions of (A), (C), and (E), respectively. A 9 pixels ( $3\times 3$ ) median filter was applied for (B–F) to reduce noise. Contrast and brightness were adjusted for better visibility. The large spherical features seen in images (A) and (C) correspond to the edge of the hole in the carbon film of the TEM grid.

protein-PC interfaces (i.e., Langmuir-Blodgett – LB – films) for fixed protein surface load of either  $1.4\text{ mg/m}^2$  (0.2 wt% PC) or  $0.8\text{ mg/m}^2$  (0.6 or 0.8 wt% PC). The surface pressure was higher at higher PC concentration for both protein surface loads. At low protein surface load ( $0.8\text{ mg/m}^2$ -films; panels (i)–(iv) in Fig. 6), two regions could be distinguished. Firstly, PC is filling in the gaps within the protein network which leads to a limited increase in surface pressure and later to a steep increase when the protein network is compressed by the higher interfacial concentration of PC, as described by Wilde, Mackie, Husband, Gunning, and Morris (2004). Upon reaching the saturation surface pressure, the proteins are displaced by PC, possibly leading to structures where proteins are adsorbed onto the PC layer or bilayer formation of PC (Waninge et al., 2005; Yesiltas, Torkkeli, et al., 2019). As picture (iv)

shows higher interfacial structures (please see color code that is different for the panels) compared to picture (iii), it might be possible that we are looking at the transition from squeezing proteins out in favor of multi-layer formation at a saturation surface pressure of  $41\text{ mN/m}$ . For the films with a higher protein surface load ( $1.4\text{ mg/m}^2$ ; panels (I)–(III) on Fig. 6), as expected, the starting surface pressure was higher than for the films with a low surface load. The protein network was already compressed by PC at 20% of the total concentration, which was further enhanced by the higher PC concentration (panel III). In both  $0.8$  and  $1.4\text{ mg protein/m}^2$ -films, displacement of proteins led to more interfacial structures with strands and spherical shapes, and less protein aggregates. Similar types of structures have previously been observed for rapeseed protein concentrate-based LB films (J. Yang et al., 2021).

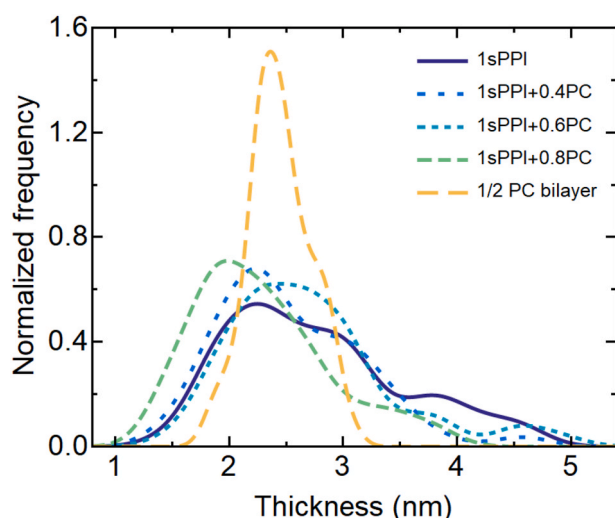


**Fig. 4.** (A) Cryo-TEM image (zoomed-in) of an oil droplet from an emulsion containing 1 wt% sPPI. The TEM image was taken at a magnification of  $24000\times$  with a nominal defocus of  $-1.5\ \mu\text{m}$ . Position I–IV are the regions where line intensity profiles across the interface were taken. Contrast and brightness were adjusted to optimize visibility; the scale bar is 20 nm. (B) Example of a line profile at position I. Integration width was set to 20 pixels (0.38 nm/pixel). Gray dashed lines show the approximate half width of the valley.

**Table 2**  
Interfacial thickness analyzed by intensity profiles in cryo-TEM images.

Emulsion	Number of measured line profiles	Interfacial thickness (mean $\pm$ standard deviation; nm)
1% sPPI	45	$2.8 \pm 0.8$ (SEM: 0.11)
1% sPPI + 0.4% PC	47	$2.6 \pm 0.7$ (SEM: 0.10)
1% sPPI + 0.6% PC	52	$2.7 \pm 0.7$ (SEM: 0.10)
1% sPPI + 0.8% PC	53	$2.3 \pm 0.6$ (SEM: 0.08)
1% PC	44	$4.9 \pm 0.5^*$ (SEM: 0.08)

SEM: standard error of the mean. \*The reported value is the measured thickness of the bilayer of a PC liposome.



**Fig. 5.** Smoothed histogram of the interfacial thickness for 1 wt% soluble fraction of pea protein isolate (1sPPI) emulsions mixed with different concentrations (0–0.8 wt%) of phosphatidylcholine (PC).

Protein displacement was previously reported for interfacial films with  $\beta$ -lactoglobulin and  $\beta$ -casein in combination with Tween 20 and rapeseed protein concentrate with oleosomes (Mackie et al., 2000; Wilde et al., 2004; J. Yang, Berton-Carabin, Nikiforidis, van der Linden, &

Sagis, 2022).

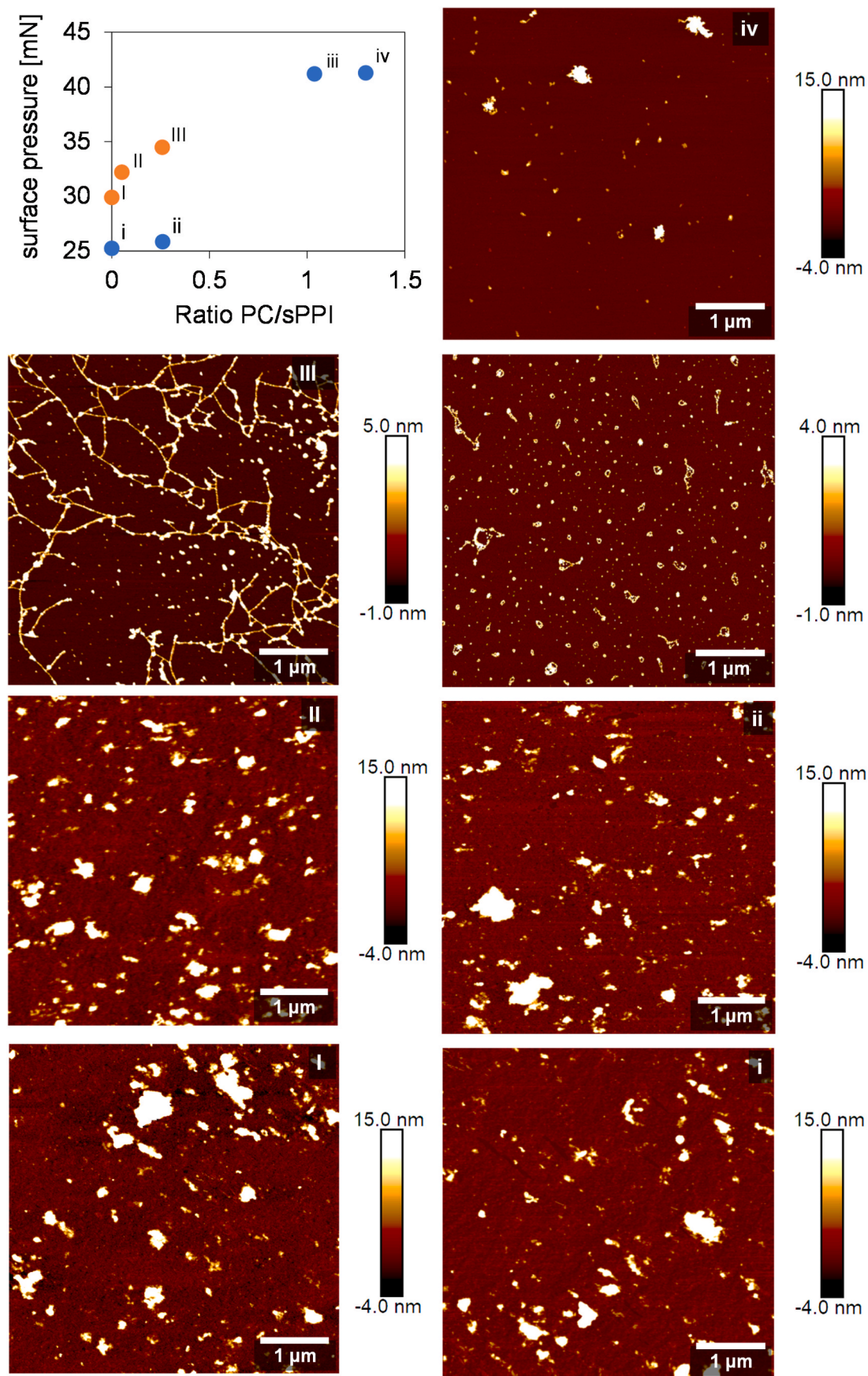
When looking in greater detail at the LB films (Fig. 7), it is clear that with increasing the PC/sPPI ratio, the interfaces became smoother. This is consistent with the previously described process of PC permeating the protein network and filling in the gaps which results in the formation of domains that are rich in both protein and PC. As the PC concentration increases these PC domains become more and more dominant, and multilayers may form (Wilde et al., 2004). The onset of protein displacement is expected to be represented by the reduced roughness at PC/PPI ratios  $>0.3$ . From the roughness measurements, no clear distinction could be made between films with PC/sPPI ratios of 1.03 or 1.3 (points (iii) and (iv), respectively), whereas this is possible for panel iv that shows higher interfacial structures, most probably protein aggregates, which are even more abundant in panels i–ii and I–II.

### 3.5. Oxidative stability of emulsions stabilized with sPPI or mixtures of sPPI and PC

The oxidative stability was assessed by monitoring the concentration of hydroperoxides (primary oxidation products) and aldehydes (secondary oxidation products) formed in the emulsions over a period of 14 days under accelerated storage conditions ( $40^\circ\text{C}$ ; horizontally rotating). In general, the emulsions exhibited a higher oxidative stability with increasing the PC concentration (Fig. 8), for both oxidation markers. Yet, in contrast to this general trend, the emulsion with 0.6 wt% PC showed rather high levels of oxidation products compared to the other emulsions with PC. It is important to emphasize that this result was obtained on multiple independent replicates, suggesting that samples having this specific PC to protein ratio have some particular characteristics that make them sensitive to oxidation – hence a composition that is not preferred.

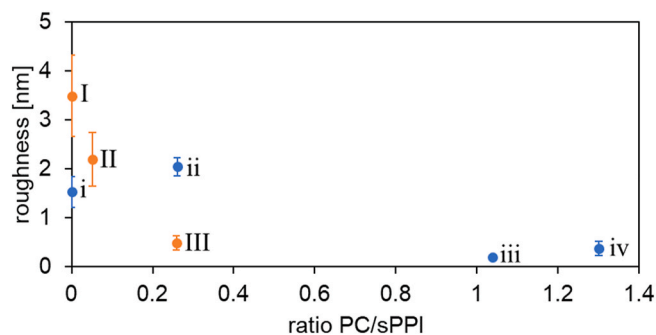
Thus, while the physical stability of the emulsions was consistently improving with increasing PC concentration, the oxidative stability followed a non-monotonic pattern, generally improving with higher PC concentration except for the system with 0.6 wt% PC that led to emulsions that oxidized similarly as the 1 wt% PPI and 1 wt% PPI – 0.2 wt% PC emulsions. Protein in the bulk water phase can chelate metal ions and scavenge radicals (Berton, Ropers, Viau, & Genot, 2011; Faraji, McClements, & Decker, 2004; Gumus et al., 2017; Münch, Schroën, & Berton-Carabin, 2024) and thus affect oxidative emulsion stability. In our work, the protein concentrations in the aqueous phase were low (1.4–3 g/L) compared to the total protein concentration in the emulsion (10 g/L) (section 3.2), and not that different between emulsions through



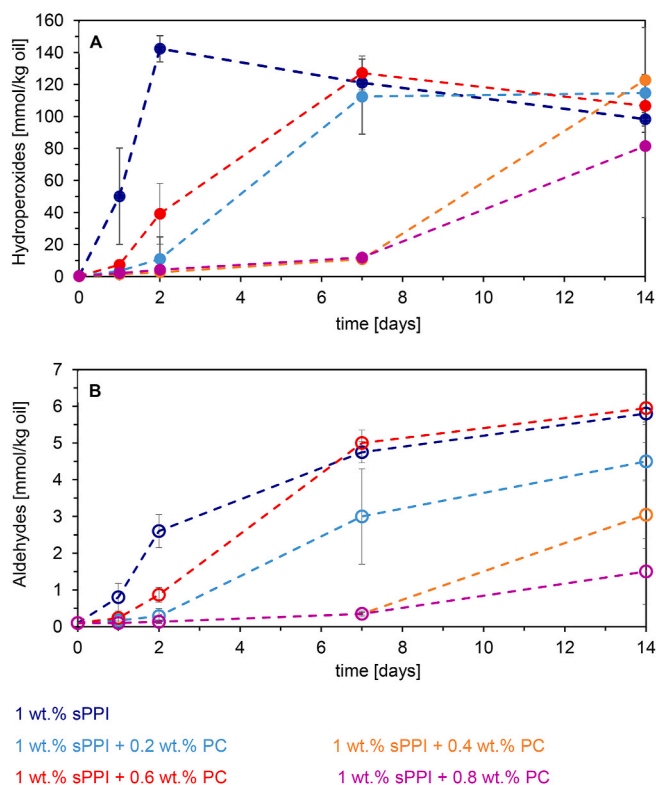


**Fig. 6.** AFM analysis of Langmuir-Blodgett (LB) pea protein-PC films with a high (1.4 mg/m²; orange; I-III) or low protein load (0.8 mg/m²; blue; i-iv). The surface pressures at which the LB films were sampled are given in the graph.





**Fig. 7.** Roughness of Langmuir-Blodgett (LB) pea protein-PC films analyzed with AFM for films with a high ( $1.4 \text{ mg/m}^2$ ; orange; I-III) or low protein surface load ( $0.8 \text{ mg/m}^2$ ; blue; i-iv). The error bars represent the standard deviation of at least three independent pictures.



**Fig. 8.** Oxidative stability of emulsions A) hydroperoxide (closed symbols) and B) aldehyde concentration (open symbols) in mmol per kg of oil over time for emulsions stabilized by 1 wt% sPPI and emulsions stabilized with 1 wt% sPPI and 0.2, 0.4, 0.6 or 0.8 wt% PC. The error bars show the standard deviation of at least two independent duplicates and measurements ( $n \leq 2$ ;  $i \leq 2$ ). The lines are drawn to guide the eye.

a concomitant change in droplet size. The differences in oxidative stability are expected to be independent of the protein concentration in the serum, and it stands to reason that the non-monotonic change in oxidative stability is rather due to a change in the interfacial composition and structure (cryo-TEM and AFM results).

Increasing the PC concentration at the interface may result in an alteration of the interfacial organization of the proteins (Ohtsuru, Yamashita, Kanamoto, & Kno, 1979), in a change in the overall interfacial structure above the saturation surface pressure (section 3.3 & 3.4) (Waninge et al., 2005), and the formation of multilayers with proteins attached onto the PC layer (section 3.2 & 3.4) (Fig. 9). In Fig. 6, pea protein aggregates disappear and appear again with increasing PC/sPPI

ratio (section 3.4). If the aggregates relate to oxidative reaction sites, this may explain changes in oxidative stability given differences in proximity to the interface. However, considerable further research effort would be needed to substantiate this interpretation. PC is always in relative proximity to lipids and the chelation of metal ions by PC might promote lipid oxidation as discussed in our recent review (Münch et al., 2024). The formation of multilayers is not likely given the TEM results that showed hardly any effect on interfacial thickness, and variation therein.

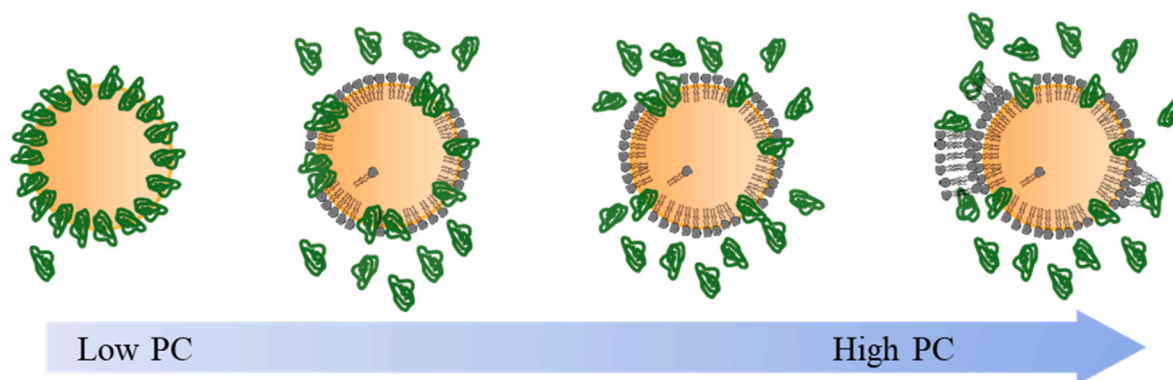
In literature, higher (García-Moreno et al., 2014) or lower oxidative stability (Berton-Carabin et al., 2013; García-Moreno et al., 2014) were reported for emulsions stabilized by protein-phosphatidylcholine mixtures compared to emulsions stabilized by proteins only. These divergent outcomes may be explained by the fact that these examples vary widely in their total emulsifier concentration (0.32–2.8), type and purity of phospholipids, ratio of phospholipids to protein that were considered (0.06–2.5), and the oil contents (10–70 wt%) also making a comparison to our study difficult if not impossible. The present results advance the understanding of interfacial structure by establishing a connection with oxidative stability. Yesiltas et al. (2020; 2019) reported on different interfacial structures in a 70 wt% emulsion stabilized by sodium caseinate and PC. At low PC, a monolayer PC with sodium caseinate loosely bound, and for high concentration PC bilayers were reported. The structure difference was here likewise proposed to have an influence on the oxidative stability, although not experimentally examined as in the present study.

It is good to point out that depending on the phase in which PC is initially dissolved or dispersed prior to emulsion preparation, different structures may be formed. In the work of Yesiltas, García-Moreno, Sørensen, Akoh, and Jacobsen (2019) (PL-P phospholipid-protein-ratios: (PL-P): 0.5/0.83/2.5), PC was added to the aqueous phase, which has implications for adsorption during emulsion formation (Benjamins, Thuresson, & Nylander, 2005; Waninge et al., 2005; Yamamoto & Araki, 1997). Introducing the PC through the oil phase, as we did, is expected to lead to higher interfacial PC concentration due to a better solubility in the oil compared to the aqueous phase, and to a higher bulk concentration of PC in the oil as compared to when the PC is dispersed in the aqueous phase (given a fixed PC concentration in the emulsion). This was also the case in the study of Berton-Carabin et al. (2013), who found a lower oxidative stability for emulsions with a mixed interface compared to single protein interfaces, even though in that case, only one very low PC-to-protein ratio (PL-P: 0.06) was considered which was much lower than in this study (PL-P: 0.2/0.4/0.6/0.8).

To conclude, from the results presented here and the information available in literature, it can be expected that the oxidative stability of emulsions is related to specific interfacial structures, in combination with the aqueous phase protein concentration (Gumus et al., 2017). The underlying mechanisms, however, still remain elusive. In spite of the detailed investigations carried out here, more research will be needed. A good starting point could be to investigate the amount of reaction sites of those interfacial structures and localization of radicals via spatiotemporal mapping (S. Yang, Verhoeff, Merckx, van Duynhoven, & Hohlbein, 2020).

#### 4. Conclusions

We investigated the influence of the pea protein-phospholipid ratio on emulsion stability and were particularly interested in their effect on lipid oxidation. These emulsions were resistant to coalescence, and had somewhat smaller droplet sizes compared to the reference sPPI-emulsion where no PC was added. The inclusion of PC in the emulsion formulation further enhanced its stability by preventing flocculation. The emulsions exhibited a non-monotonic trend in terms of oxidative stability. In certain cases, this resulted in both elevated physical and oxidative stability, whereas specific ratios of phospholipids and proteins decreased the oxidative stability. This could potentially be attributed to



**Fig. 9.** Schematic drawing of the interfacial structure with different phospholipid-to-protein ratios. The green structures represent pea proteins (e.g., globulins) whereas the small gray structures represent PC molecules.

the development of particular interfacial structures near the saturation surface pressure, suggesting phase separation and domain formation. Our findings can have important implications for food emulsions containing mixed proteins and phospholipids, as modifications of the formulations can lead to unexpected alterations in the oxidative stability, that may be extremely difficult to predict due to its non-monotonic nature. The applications that would be impacted by our findings are those in which the initial materials are not well characterized and contain naturally occurring phospholipids. This will become increasingly relevant as the shift towards plant-based and other biobased sources for sustainable circular food production takes place. In order to provide a comprehensive understanding, more research is required to unveil the intricate interfacial structure and its impact on the oxidative stability of emulsions.

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### CRediT authorship contribution statement

**Katharina Münch:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Machi Takeuchi:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Remco Tuinier:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Simeon Stoyanov:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Karin Schroën:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Heiner Friedrich:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Claire Berton-Carabin:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, 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.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2024.109962>.

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