

## Pickering particles as interfacial reservoirs of antioxidants

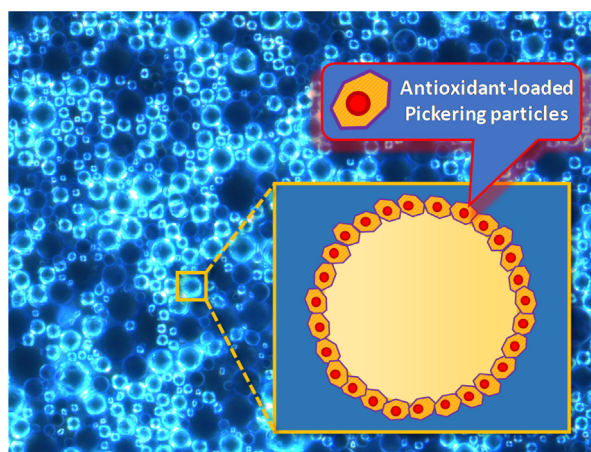
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### GRAPHICAL ABSTRACT



### ARTICLE INFO

#### Article history:

Received 24 March 2020

Revised 17 April 2020

Accepted 17 April 2020

Available online 21 April 2020

#### Keywords:

Pickering emulsion  
Lipid oxidation  
Lipophilic antioxidant  
Natural bioactive  
 $\alpha$ -tocopherol  
Biobased particle  
Encapsulation  
Interface  
Fat crystal

### ABSTRACT

**Hypothesis:** Emulsions are common structures encapsulating lipophilic bioactive molecules, both in biological systems and in manufactured products. Protecting these functional molecules from oxidation is essential; Nature excels at doing so by placing antioxidants at the oil-water interface, where oxidative reactions primarily occur. We imagined a novel approach to boost the activity of antioxidants in designer emulsions by employing Pickering particles that act both as physical emulsion stabilizers and as interfacial reservoirs of antioxidants.

**Experiments:**  $\alpha$ -Tocopherol or carnosic acid, two model lipophilic antioxidants, were entrapped in colloidal lipid particles (CLPs) that were next used to physically stabilize sunflower oil-in-water emulsions (“concept” Pickering emulsions). We first assessed the physical properties and stability of the CLPs and of the Pickering emulsions. We then monitored the oxidative stability of the concept emulsions upon incubation, and compared it to that of control emulsions of similar structure, yet with the antioxidant present in the oil droplet interior.

**Abbreviations:** CD, conjugated diene; CLP, colloidal lipid particle; CLSM, confocal laser scanning microscopy; DSC, differential scanning calorimetry; EDTA, ethylenediamine tetraacetic acid; HMP, high melting point; LOOH, lipid hydroperoxide; LDL, low density lipoprotein; MCT, medium chain triglycerides; p-AV, *para*-anisidine value; PUFA, polyunsaturated fatty acid; TEM, transmission electron microscopy.

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<https://doi.org/10.1016/j.jcis.2020.04.069>

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**Findings:** Both tested antioxidants are largely more effective when loaded within Pickering particles than when solubilized in the oil droplet interior, thus confirming the importance of the interfacial localization of antioxidants. This approach revisits the paradigm for lipid oxidation prevention in emulsions and offers potential for many applications.

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## 1. Introduction

Lipid droplets are omnipresent in living organisms [1], such as bacteria, yeasts, plants, animals, and in many man-made materials including a large range of food products (e.g., dairy products, sauces, dressings, beverages, infant formula, etc.). Depending on their origin, structure and function, they are referred to as oil bodies, lipid droplets, adiposomes, lipoproteins, or oil-in-water (O/W) emulsions [2,3]. All these colloidal systems share a common fate when they contain unsaturated lipids or other chemically labile molecules: they are susceptible to oxidation. The degradation of the substrate molecules and the formation of lipid oxidation products alter the quality and functionality of these systems (in particular, their sensory properties), and may even lead to the accumulation of potentially hazardous products [4,5].

Lipid oxidation in emulsions occurs primarily at the oil-water interface, where the oxidation-sensitive lipid molecules (e.g., glyceryl esters of polyunsaturated fatty acids (PUFAs), pigments, flavors) meet prooxidants, such as metal ions or reactive oxygen species [6–8]. In natural emulsions, this is usually mitigated by the presence of antioxidant molecules close to the oil droplet surface [9]. For instance, in oil bodies, i.e., storage containers for lipids in plants, antioxidants such as  $\alpha$ -tocopherol accumulate at the interface. This results in remarkable oxidative stability [10]. In the animal kingdom, similar strategies are also found; for instance, the protective effect of ascorbate in preventing the oxidation of low-density lipoproteins (LDLs) relies on its capability to regenerate  $\alpha$ -tocopherol radicals at the droplet surface, where the chromanol ring of  $\alpha$ -tocopherol faces the ascorbate-containing aqueous medium [11]. Accumulating antioxidants at the interface is thus presumably an excellent approach to inhibit lipid oxidation in emulsions [6,12–14]. At this locus, antioxidants can counteract the initiating radicals – those formed during the initiation step – before they begin propagating oxidation to other lipid molecules.

Surprisingly, this natural protective strategy has, to date, hardly been translated to the domain of man-made emulsions, despite their broad field of applications. Lipid oxidation is often prevented by using lipophilic antioxidants which locate inside the oil droplets, or hydrophilic antioxidants which are mainly distributed in the external aqueous phase. In both cases, this remote position to the interface results in a far-from-optimal activity. To overcome this, novel antioxidants have recently been synthesized through the covalent grafting of lipid chains on phenolic antioxidants. Using such homologous series, it was observed that the antioxidant activity in emulsions increases with the length of the alkyl chain grafted, until an optimum beyond which further lengthening leads to a collapse in antioxidant efficiency [15,16,17–20]. Antioxidants with the optimal activity were systematically those locating preferentially at the interface [13,15,16]. Even though these so-called phenolipids brought important mechanistic insights on the lipid oxidation phenomenon, their large-scale implementation is hampered by their cost, and by the fact that they are no longer natural molecules. Furthermore, the positioning of amphiphilic phenolipids at the interface is only transient, as they are moving from phase to phase, and are thus staying longer or more frequently at

the interface only from an average outlook [7]. Hence, the hypothesis underlying the present work is that imparting antioxidants with a long-term anchorage at the interface, while preserving their natural structure, can boost their efficiency in an unprecedented manner.

Recently, increasing attention has focused on developing food-compatible Pickering emulsions, i.e., systems where oil droplets are physically stabilized by biobased colloidal particles instead of conventional emulsifiers [21–23]. Among the range of suitable particles developed to date, colloidal lipid particles (CLPs) are promising stabilizers as they are simple to prepare from biobased building blocks, provide superior physical stability to the emulsions as compared to conventional emulsifiers, and are compatible with a range of emulsification methods including high pressure homogenization [24,25]. We herein describe a novel and bioinspired approach using CLPs as interfacial reservoirs of antioxidants. The extremely high adsorption energy of such particles [23,26] was exploited to anchor them at the interface for a much longer time than what normally applies for amphiphilic antioxidants. CLPs are prepared with high melting point (HMP) lipids, and thus possess a solid or semi-solid lipid interior which can be loaded with natural lipophilic antioxidants, such as  $\alpha$ -tocopherol or carnosic acid. In this way, antioxidants are forced to reside at the interface, where their efficiency to prevent lipid oxidation should be maximized. Hence, through the controlled hierarchical structuration of emulsions, we expect to achieve a dual functionality, where the Pickering particles act not only as physical stabilizers, but also as interface-anchored reservoirs of natural antioxidants.

## 2. Material and methods

### 2.1. Materials

Tripalmitin (purity > 99%), sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, iron(II) sulfate heptahydrate, ethylenediaminetetraacetic acid disodium salt dihydrate, *para*-anisidine, acetic acid, 25-NBD cholesterol and  $\alpha$ -tocopherol (purity 96%) were purchased from Sigma-Aldrich (Saint-Louis, USA). *N*-Hexane and methanol were obtained from Actu-All Chemicals (Oss, the Netherlands). 2-Propanol was purchased from Merck (Darmstadt, Germany). Sodium caseinate was supplied by DMV International (spray-dried; protein content 91.0%). Carnosic acid (purity 99%) was supplied by Chengdu Biopurify Phytochemicals Ltd (Chengdu, China). Medium chain triglyceride oil (MCT, Miglyol 812 N) was purchased from Cremer (Hamburg, Germany). Sunflower oil was purchased from a local supermarket, and was stripped from surface-active impurities and endogenous antioxidants prior to use, according to a previously described procedure [27].

### 2.2. Preparation of CLPs

CLPs were prepared according to Schröder et al. [24,25]. When CLPs were loaded with  $\alpha$ -tocopherol, the molten tripalmitin (80 °C) was added with a methanolic solution of  $\alpha$ -tocopherol, such that the final concentration in the CLP suspension was

446  $\mu\text{mol kg}^{-1}$ . In the case of carnosic acid, the molten tripalmitin (80 °C) was added with a methanolic solution of carnosic acid, such that the final concentration in the CLP suspension was 149  $\mu\text{mol kg}^{-1}$ . For control Pickering emulsions, CLPs were prepared without any antioxidant. The molten tripalmitin phase (5% w/w) was coarsely mixed with an aqueous phase (consisting of 1% w/w sodium caseinate in phosphate buffer 10 mM, pH 7.0) pre-heated at 80 °C using a rotor–stator homogenizer (Ultra-turrax IKA T18 basic, Germany) for 1 min at 11,000 rpm. The obtained coarse dispersion of molten tripalmitin was refined by passage through a high pressure homogenizer (Microfluidizer<sup>®</sup> Processor MF 110Y, Microfluidics, Newton, Massachusetts, USA) equipped with Y-shaped interaction chamber (F12Y; min. internal channel: 75  $\mu\text{m}$ ) at 400 bar, 5 cycles at 80 °C. The obtained fine dispersion was then cooled down to 4 °C to allow for tripalmitin crystallization, which formed a CLP suspension. We previously reported that this procedure leads to minimal residual amounts of non-adsorbed sodium caseinate [25,28].

### 2.3. Preparation of Pickering emulsions

Concept Pickering emulsions were prepared by mixing 10% w/w stripped sunflower oil with 45% w/w phosphate buffer (10 mM, pH 7.0) and 45% w/w CLP dispersion (containing 5% w/w tripalmitin, and  $\alpha$ -tocopherol or carnosic acid in the particles). The mixture was processed by high-speed stirring at 7000 rpm for one minute (Ultra-turrax IKA T18 basic, Germany), and the obtained coarse emulsion was then homogenized (Microfluidizer Processor MF 110Y with Y-shaped interaction chamber (F12Y; minimum internal dimension: 75  $\mu\text{m}$ ), Microfluidics, Newton, Massachusetts, USA) at 400 bar, 5 cycles at 0 °C. The resulting Pickering emulsions were stored at 4 °C. In control Pickering emulsions (i.e., with the antioxidant in the liquid oil droplet core), antioxidant-free CLPs were used; in that case, the antioxidant was incorporated in stripped sunflower oil prior to homogenization, via a methanolic solution as described previously, such that the final concentration in the Pickering emulsion was 200  $\mu\text{mol kg}^{-1}$  for  $\alpha$ -tocopherol and 67  $\mu\text{mol kg}^{-1}$  for carnosic acid, respectively, i.e., similar to that in the concept emulsion. One concept and one control Pickering emulsions were prepared with medium chain triglycerides (MCT) (i.e., containing only saturated fatty acids) instead of stripped sunflower oil. Some other Pickering emulsions were added with excess CLPs post-homogenization, which was achieved by mixing 66.7% w/w of a primary Pickering emulsion (prepared with 15% w/w sunflower oil, 45% w/w CLP dispersion, and 40% w/w buffer; similar homogenization conditions as previously described) with or without  $\alpha$ -tocopherol in the core of the oil droplets, with 33.3% w/w of CLP dispersion containing no or 300  $\mu\text{mol kg}^{-1}$   $\alpha$ -tocopherol, respectively. This led to emulsions with similar total  $\alpha$ -tocopherol concentration (200  $\mu\text{mol kg}^{-1}$  in the final emulsion), yet with a different initial localization of the antioxidant (either in both adsorbed and non-adsorbed CLPs, or exclusively in the excess, non-adsorbed CLPs).

### 2.4. Preparation of protein-stabilized emulsions containing non-adsorbed CLPs

Conventional protein-stabilized emulsions were prepared by homogenizing stripped sunflower oil (20% w/w) with a 2% w/w sodium caseinate solution, using the same homogenization procedure as in the previous section. After that, 50% w/w of the obtained emulsion were mixed with 45% w/w CLP dispersion, and 5% w/w buffer. This resulted in an emulsion containing liquid oil droplets covered by sodium caseinate, and exclusively non-adsorbed CLPs. As previously,  $\alpha$ -tocopherol was present either in the CLPs, or in

the core of the sunflower oil droplets. The concentration of  $\alpha$ -tocopherol in the final systems was 200  $\mu\text{mol kg}^{-1}$ .

### 2.5. Physical characterization of CLPs and emulsions

Characterization of particle dispersions and of emulsions (particle size distribution,  $\zeta$ -potential, transmission electron microscopy, polarized light microscopy and differential scanning calorimetry) was conducted as described previously [24,25]. To obtain larger oil droplets that allow for visualization by confocal laser scanning microscopy (CLSM), selected coarse emulsions were processed through a lab-scale colloid mill homogenizer with gap width of 0.32 mm (IKA Magic Lab, Staufen, Germany) operating for one minute at 15,000 rpm at 0 °C. CSLM images of O/W emulsions stabilized by CLPs were obtained with a Nikon C-2 confocal laser scanning microscope with a 60x oil immersion objective (Amsterdam, the Netherlands). In selected experiments, 25-NBD cholesterol was used instead of  $\alpha$ -tocopherol as a fluorescent analogue, as both molecules have close molecular structures and partition coefficients (Table S1). This fluorescent probe was incorporated in the CLPs or in stripped sunflower oil via a procedure similar to what was previously described for  $\alpha$ -tocopherol, and was used with a final concentration in the emulsions of 0.001% w/w. The probe was excited with the 488-nm line of an argon laser and the fluorescence light was collected with a bandpass filter between 490 and 505 nm.

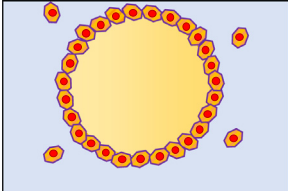
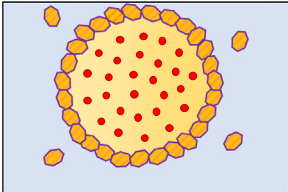
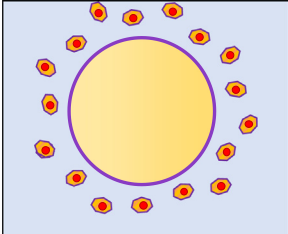
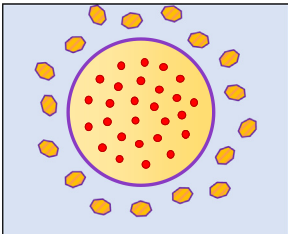
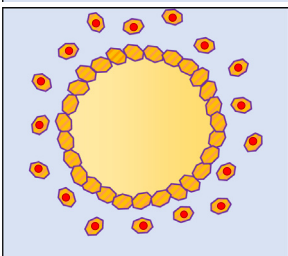
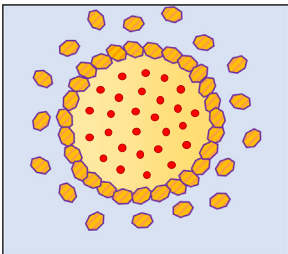
### 2.6. Assessment of oxidative phenomena

A metal-catalyzed oxidation system consisting of an equimolar mixture of  $\text{FeSO}_4$  and EDTA was prepared by separately dissolving both constituents (12 mM) in ultrapure water. Equivalent volumes of each solution were mixed, and the iron-EDTA complex was allowed to form under moderate stirring for 1 h in the dark [28]. Aliquots of emulsion (2 g) were distributed in 15-mL polypropylene centrifugation tubes. The oxidation initiator (100  $\mu\text{L}$ ) was added to the emulsions to obtain a final concentration of 200  $\mu\text{M}$  of both  $\text{FeSO}_4$  and EDTA. The tubes were rotated in the dark at 2 rpm at 25 °C for 0 to 336 h. Quantification of conjugated diene hydroperoxides and total aldehydes (*para*-anisidine value, pAV) was performed according to Schröder et al. (2019).  $\alpha$ -Tocopherol was also quantified after extraction from CLPs dispersions or emulsions. First, 4 mL chloroform, 3 mL methanol and 1 mL saturated sodium chloride solution were added to 2 mL of CLP dispersion or emulsion in a 15-mL polypropylene centrifuge tube, which were vortexed followed by centrifugation at 2000g for 8 min. The clear chloroform phase was then collected by cautiously boring a hole in the bottom of the centrifuge tube. Extracts were analyzed on a UltiMate 3000 liquid chromatography system (Thermo Scientific, Sunnyvale, CA, USA) using a C30 reversed phase column, 3  $\mu\text{m}$ , 150  $\times$  4.6 mm (YMC, Dinslaken, Germany). Extracts were eluted at 1 mL  $\text{min}^{-1}$  at 30 °C using a mobile phase with a linear gradient going from 81% methanol, 14% methyl *t*-butyl ether and 4% water to 74% methanol, 22% methyl *t*-butyl ether and 4% water within 8 min, and going back to its initial composition within 2 min.  $\alpha$ -Tocopherol was detected with a UV–VIS detector at 292 nm, and contents were calculated using a calibration curve that was linear in the range from 5 to 5000  $\mu\text{g mL}^{-1}$ .

### 2.7. Experimental design

All emulsions were prepared as at least independent duplicates, and multiple measurements ( $n \geq 3$ ) were performed upon incubation for each replicate. The following Table 1 summarizes the different emulsions that were investigated.

**Table 1**  
Different emulsion designs to assess the effect of antioxidant incorporation in CLPs on lipid oxidation. The red spots represent the lipophilic antioxidants ( $\alpha$ -tocopherol or carnosic acid). The antioxidant concentrations are given in the final systems ( $\mu\text{mol}$  per kg final emulsion).

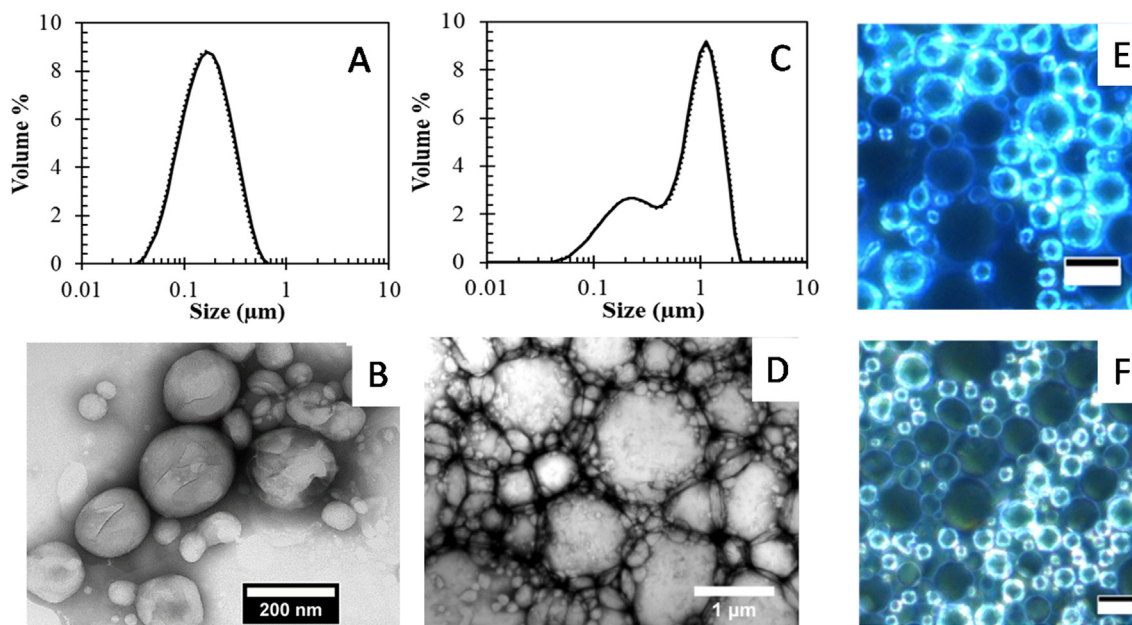
Denomination	Description	Antioxidant tested	Schematic representation
Concept Pickering emulsion (A)	Emulsion stabilized with antioxidant-loaded CLPs	$\alpha$ -Tocopherol ( $200 \mu\text{mol kg}^{-1}$ ) or carnosic acid ( $67 \mu\text{mol kg}^{-1}$ )	
Control Pickering emulsion (B)	Emulsion stabilized with empty CLPs, antioxidant in the liquid oil droplets	$\alpha$ -Tocopherol ( $200 \mu\text{mol kg}^{-1}$ ) or carnosic acid ( $67 \mu\text{mol kg}^{-1}$ )	
Conventional emulsion with antioxidant CLPs (C)	Sodium caseinate-stabilized emulsion with antioxidant-loaded CLPs in the continuous phase	$\alpha$ -Tocopherol ( $200 \mu\text{mol kg}^{-1}$ )	
Conventional emulsion with empty CLPs (D)	Sodium caseinate-stabilized emulsion with empty CLPs in the continuous phase, antioxidant in the liquid oil droplets	$\alpha$ -Tocopherol ( $200 \mu\text{mol kg}^{-1}$ )	
Pickering emulsion with excess antioxidant CLPs (E)	Emulsion stabilized with empty CLPs; excess antioxidant-loaded CLPs in the continuous phase	$\alpha$ -Tocopherol ( $200 \mu\text{mol kg}^{-1}$ )	
Pickering emulsion with excess empty CLPs (F)	Emulsion stabilized with empty CLPs; excess empty CLPs in the continuous phase, antioxidant in the liquid oil droplets	$\alpha$ -Tocopherol ( $200 \mu\text{mol kg}^{-1}$ )	

### 3. Results and discussion

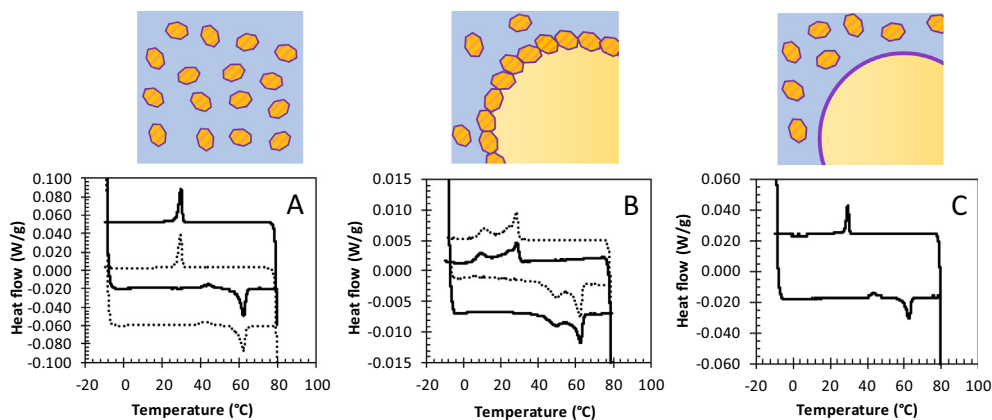
#### 3.1. Physical characteristics of CLPs and emulsions

CLPs (loaded with an antioxidant, or empty) were prepared by high-pressure homogenization of molten tripalmitin, a model biocompatible high melting point fat, in an aqueous sodium caseinate solution, and subsequent cooling to allow for tripalmitin to crystallize. The resulting caseinate-covered particles had a unimodal size

distribution with average diameter of 145 nm (Fig. 1A) and a negative surface charge, characterized by a  $\zeta$ -potential of  $-36$  mV (data not shown), as previously reported [28]. TEM images showed that CLPs exhibited an almost spherical morphology, with a complex internal structure of layered crystals (Fig. 1B), which was also well in line with previous findings [24,25]. In aqueous suspension, CLPs had crystallization and melting temperatures around 30 and 60 °C, respectively, as shown by DSC analysis (Fig. 2A). Interestingly, the aforementioned physical parameters were similar for



**Fig. 1.** (A) Particle size distribution of CLPs loaded with  $\alpha$ -tocopherol (dotted line) or empty (solid line); (B) TEM image of CLPs loaded with  $\alpha$ -tocopherol (scale bar is 200 nm); (C) Droplet size distribution of Pickering emulsions containing  $\alpha$ -tocopherol either in the CLPs (concept emulsion, dotted line) or in the core of the oil droplets (control emulsion, solid line); (D) TEM image of the concept emulsion (scale bar is 1  $\mu$ m); (E, F) Polarized light microscopy images of concept Pickering emulsions, freshly prepared (E) or after 72 h incubation at 25  $^{\circ}$ C (F) (scale bar on both images is 10  $\mu$ m).



**Fig. 2.** DSC melting and crystallization thermograms (bottom and top lines, respectively) of (A) CLPs loaded with  $\alpha$ -tocopherol (dotted line) or empty (solid line); (B) Pickering emulsions containing  $\alpha$ -tocopherol either in the CLPs (concept emulsion, dotted line) or in the core of the oil droplets (control emulsion, solid line); (C) a conventional emulsion stabilized with sodium caseinate, containing added CLPs in the aqueous phase.

empty or antioxidant-loaded CLPs (dotted or solid lines, respectively, in Figs. 1A and 2A), which indicates that the inclusion of a lipophilic antioxidant did not affect the physical properties of the particles. This can probably be explained by the antioxidant concentration used, which was too low to substantially affect the alignment and network of tripalmitin molecules upon crystallization.

We then produced CLP-stabilized Pickering emulsions by high pressure homogenization of an aqueous CLP suspension and sunflower oil. This resulted in emulsion droplets ranging from 0.3 to 3  $\mu$ m, with an average diameter around 1  $\mu$ m (Fig. 1C). The  $\zeta$ -potential of the emulsions was identical to that of the CLP suspension (i.e., around  $-36$  mV; data not shown). In these emulsions, a fraction of the particles adsorbs at the oil–water interface, as clearly visible by TEM (Fig. 1D), whereas the rest remains suspended in the continuous phase. This is in line with the particle size distribution determined for the Pickering emulsion (Fig. 1C),

which shows a minor peak around 0.2  $\mu$ m (presumably corresponding to the non-adsorbed CLPs) next to the main peak around 1  $\mu$ m corresponding the oil droplets. The formation of a crystalline shell around the liquid oil droplets is also clear on polarized light microscopy pictures, which show droplets surrounded by a bright ring, not only in freshly prepared emulsions (Fig. 1E), but also after several days of incubation (e.g., 72 h; Fig. 1F). In line with our previous work [25,28], we thus confirmed that sodium caseinate-covered CLPs are excellent physical stabilizers for Pickering O/W emulsions. Interestingly, the physical adsorption of CLPs at the surface of liquid oil droplets impacts their crystalline polymorphism: compared to the melting and crystallization thermograms of simple CLP suspensions (Fig. 2A), those of CLP-stabilized emulsions (Fig. 2B) showed a broadened main peak as well as an additional one at lower temperatures, revealing a less organized crystalline structure, and suggesting exchanges of triglyceride molecules between the crystallized CLPs and the liquid oil droplets.

Alternatively, we also prepared a simple sodium caseinate-stabilized emulsion, and added excess CLPs to this emulsion, post homogenization, to design a system in which the CLPs are exclusively located in the aqueous phase (Table 1C, D). In this system, no alteration of the DSC profile of the CLPs was detected compared to the starting simple CLP suspension (Fig. 2C). This seems to indicate that no exchange of lipid material takes place between the high melting fat of the CLPs and the liquid oil of the droplets, which opposes the situation previously described for CLPs adsorbed in Pickering emulsions.

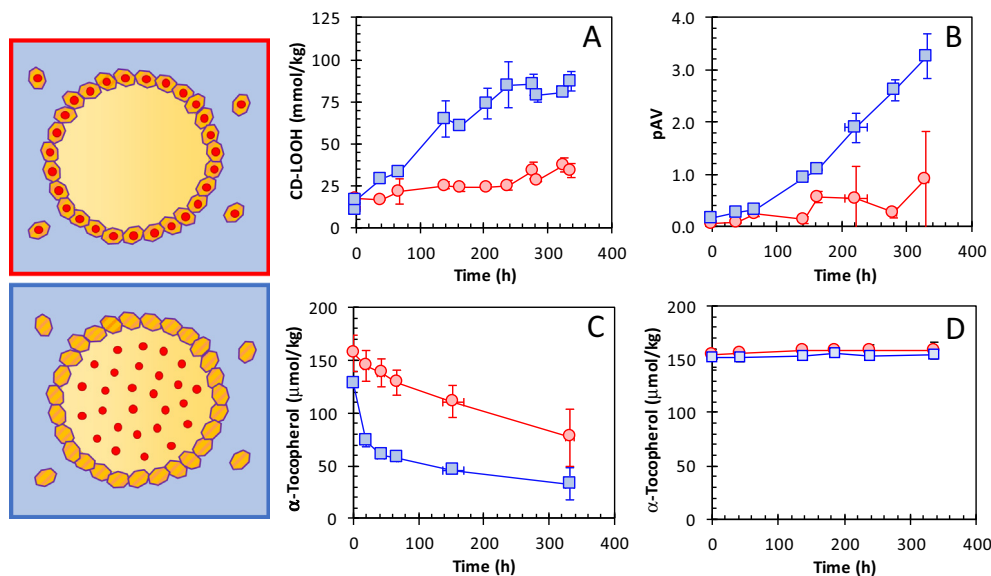
### 3.2. Oxidative stability of concept and control Pickering emulsions

CLP-stabilized emulsions (Table 1A, B) were incubated at 25 °C in the presence of a metal initiator, and we monitored the formation of primary (conjugated diene (CD) hydroperoxides (LOOH)) and secondary (aldehydes, measured by the *para*-anisidine value (pAV)) lipid oxidation products. Over 14 days of incubation, the accumulation of both CD LOOH and aldehydes was largely higher in the control Pickering emulsion containing  $\alpha$ -tocopherol in the oil droplets, than in the concept Pickering emulsion containing the antioxidant in the CLPs (Fig. 3A, B). It is thus clear that trapping antioxidants at the oil–water interface, i.e., the locus of initiation, largely boosts the oxidative stability of the emulsion. The fact that lipid oxidation products hardly form in the concept Pickering emulsion indicates that the autocatalytic pattern of the formation of lipid radicals (propagation) does not take place within the timescale of the experiment. By changing its location, the specific emulsion architecture that we propose may therefore confer  $\alpha$ -tocopherol – hitherto known as a chain-breaker [29] – with a new “initiation-breaking” role.

To dig deeper into the chemical role of  $\alpha$ -tocopherol in these emulsions, we monitored its loss during incubation (Fig. 3C). The concept Pickering emulsion containing  $\alpha$ -tocopherol in the CLPs showed a higher  $\alpha$ -tocopherol level at  $t_0$  compared to the control emulsion (157 vs. 127  $\mu\text{mol kg}^{-1}$ ). This implies that in addition to providing a boosted antioxidant activity in the emulsion, encapsulation in CLPs also protects the antioxidant itself against degradation during emulsion processing. Tocopherol can theoretically

be consumed in different ways: through exposure to high temperature [30], such as in our homogenization procedure; or in the presence of iron and/or radicals [31,32]. To distinguish between these potential causes, we prepared the exact same Pickering emulsions (concept and control) with medium chain triglycerides (MCT), a model saturated liquid oil that cannot oxidize, instead of stripped sunflower oil. When monitoring the  $\alpha$ -tocopherol level during incubation, we found that it was perfectly stable in both emulsions (Fig. 3D). Thus, the prooxidant ferrous iron by itself is not able to degrade  $\alpha$ -tocopherol in O/W emulsions that do not contain an oxidizable lipid phase. From this follows the hypothesis that in the control Pickering emulsions with PUFA-rich oil,  $\alpha$ -tocopherol is degraded during homogenization when present in the core of the droplets because it is in direct contact with lipid oxidation products. The CLPs protect the antioxidant from this contact, thereby offering stability upon processing.

Over incubation,  $\alpha$ -tocopherol was also degraded faster and to a larger extent in the control Pickering emulsion compared to the concept one (Fig. 3C), showing that its chemical stability aligns with that of the PUFAs. This is remarkable because chain-breaking antioxidants are traditionally considered sacrificial molecules that oxidize instead of unsaturated lipids; then, once most of the antioxidant has been consumed, the autocatalytic propagation of lipid oxidation may start. Here, we hypothesize that the use of Pickering particles as interfacial reservoirs of antioxidants confers  $\alpha$ -tocopherol with the ability to scavenge the first radicals generated during initiation, whereas when present in the core of the droplets,  $\alpha$ -tocopherol acts during the propagation phase by reducing peroxy radical chain-carriers coming from an exponentially growing stream of lipid oxidation products. This explanation supposes that lipid radicals react with  $\alpha$ -tocopherol at, or close to, their formation locus. There are actually experimental evidence and calculations that support the fact that alkoxy radicals are too reactive, and therefore have a too short half-life, to travel over a significant distance within oil droplets [7]. Peroxy radicals have longer half-lives, and therefore may diffuse within the oil droplets over time-scales relevant to oxidative reactions (as such, or after hydrogen abstraction from another lipid to form hydroperoxides, which are themselves surface-active). In all scenarios, the interfacial



**Fig. 3.** Formation of (A) conjugated diene hydroperoxides (CD LOOH) and (B) aldehydes (pAV), and (C) stability of  $\alpha$ -tocopherol during incubation of the concept and control Pickering emulsions (red circles and blue squares, respectively; see also schematic illustrations on the left) initially containing 200  $\mu\text{mol kg}^{-1}$   $\alpha$ -tocopherol and incubated with 200  $\mu\text{M}$   $\text{FeSO}_4/\text{EDTA}$ . (D) Stability of  $\alpha$ -tocopherol during incubation of Pickering emulsions containing non-oxidizable oil (medium chain triglycerides, MCT), with  $\alpha$ -tocopherol either in the CLPs (concept emulsion; red circles) or in the core of the oil droplets (control emulsion; blue squares).

anchoring of  $\alpha$ -tocopherol can be beneficial: alkoxy radicals are most likely formed at the interface, from the metal-catalyzed reduction of surface-active hydroperoxides, and can then immediately be inactivated; and if peroxy radicals tend to accumulate close to the interface, they can be rapidly taken care of too. Accordingly, a small amount of interface-anchored  $\alpha$ -tocopherol should be more efficient than many of the same molecule located within the oil droplets. This logically raises the question of how much the  $\alpha$ -tocopherol concentration may be reduced in the concept emulsion to obtain a protection equivalent to that attained in the control emulsion with  $200 \mu\text{mol kg}^{-1}$  of  $\alpha$ -tocopherol. Therefore, we reduced the initial  $\alpha$ -tocopherol concentration from  $200 \mu\text{mol kg}^{-1}$  to 100, then  $50 \mu\text{mol kg}^{-1}$  (2–4 times lower) in the concept emulsion. Around two to four times lower levels in antioxidants were sufficient to inhibit the production of CD-LOOHs and aldehydes (respectively) as effectively as  $200 \mu\text{mol kg}^{-1}$   $\alpha$ -tocopherol in the control emulsion (Fig. S1). This further substantiates the added value of the interfacial localization of antioxidants to boost their activity. While most known strategies of enhancing antioxidant activity are based on a synthetic approach wherein the antioxidant is chemically modified to improve their reactivity [33,34] or their location [20]; in our work, on the contrary, the natural structure of  $\alpha$ -tocopherol is preserved.

To validate if this approach could be extended to other lipophilic antioxidants, we performed the exact same experimental set, except that  $\alpha$ -tocopherol was replaced by carnosic acid, a moderately hydrophobic antioxidant widely used in food [35,36]. Again, the increase of both CD LOOHs and pAV was largely higher in the control Pickering emulsion containing the antioxidant in the oil droplets, than in the concept Pickering emulsion with carnosic acid in the CLPs (Fig. S2). The broad hydrophobicity range covered by  $\alpha$ -tocopherol ( $\log P = 10$ – $12$ ; Table S1) and carnosic acid ( $\log D$  at pH 7.0 = 2.4; Table S1) suggests that the hierarchical structure of emulsions we propose herein is applicable to a variety of antioxidants.

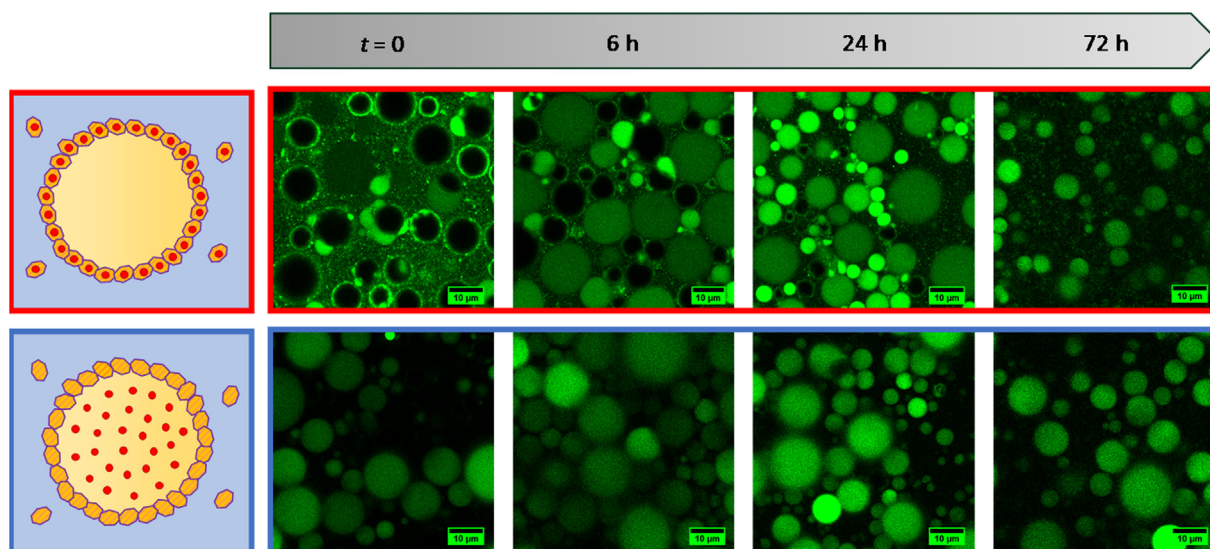
### 3.3. Mechanisms of action of antioxidant-loaded CLPs in emulsions

The aforementioned results raise two questions regarding the mechanisms by which the enhancement of the antioxidant efficiency works. First, as our emulsions are not at thermodynamic equilibrium, the localization of the lipophilic bioactive may change

within timescales relevant to the incubation period. Second, since only a fraction of CLPs is located at the surface of the oil droplets, whereas the rest remains in excess in the continuous phase, one may wonder what the role of the particles in the aqueous phase is, and if they contribute to the boosting effect. To investigate the dynamic aspects relative to the encapsulation of a lipophilic antioxidant in the CLPs, we selected 25-NBD cholesterol, a fluorescently labelled probe with a lipophilicity comparable to that of  $\alpha$ -tocopherol ( $\log P = 9$ – $10$ ; Table S1). This probe was used to prepare concept and control Pickering emulsions as described previously, except that we applied lower shear homogenization to obtain larger oil droplets that allow for visualization by light microscopy and confocal laser scanning microscopy (CLSM).

In the concept Pickering emulsions, immediately after homogenization, the fluorescent probe was present both in CLPs at the interface and the continuous phase, and not within the liquid oil droplets (Fig. 4, top). However, in time, we observed a progressive diffusion of the probe towards the core of the oil droplets. This reveals that the location of the fluorescent probe, and thus, by analogy, that of  $\alpha$ -tocopherol in the concept Pickering emulsions, evolves within timescales relevant to the incubation period. As could be expected, in the concept Pickering emulsions, the fluorescent probe was visible in the liquid oil droplets from  $t_0$ , without noticeable changes over time (Fig. 4, bottom). Interestingly, polarized light microscopy (Fig. 1E, F) and differential scanning calorimetry (Fig. S3) show that the CLPs, even when adsorbed at the oil–water interface, remained intact and crystalline over the entire experiment. This rules out the possibility that probe diffusion would be caused by solubilization of the CLPs themselves in the liquid oil droplets over time, which is in agreement with the long-term physical stability of such emulsions demonstrated in earlier work [28,37]. This dynamic transfer of lipophilic compounds implies that  $\alpha$ -tocopherol is not retained permanently at the interface and is slowly released to the oil droplet environment. The CLPs may therefore behave as transient antioxidant reservoirs, which boost the activity of antioxidants by imparting them with a certain residence time at the interface.

To evaluate the possible contribution of continuous phase CLPs to the high oxidative stability in the concept Pickering emulsions, we then prepared a simple sodium caseinate-stabilized emulsion, and added antioxidant-containing CLPs to this emulsion, post homogenization to design a system in which the CLPs are



**Fig. 4.** CLSM images of the Pickering emulsions with the fluorescent probe initially added in the CLPs (concept Pickering emulsion, top/red frame) or in the oil (control Pickering emulsion, bottom/blue frame), taken over incubation ( $t = 0$ – $72$  h). The scale bar on all images is  $10 \mu\text{m}$ .

exclusively located in the aqueous phase (Table 1C). When incubating this emulsion, and its counterpart emulsion with  $\alpha$ -tocopherol initially present in the oil droplet core (Table 1D), we found similar lipid oxidation and  $\alpha$ -tocopherol degradation rates (Fig. 5A–C). Thus, the encapsulation of  $\alpha$ -tocopherol in CLPs added to the continuous phase of a protein-stabilized emulsion does not improve the antioxidant's activity, which contrasts with the antioxidant boosting effect found in our concept Pickering emulsion. This is in line with the explanation that this latter effect is unequivocally due to the interfacial accumulation of antioxidants conveyed by the Pickering particles.

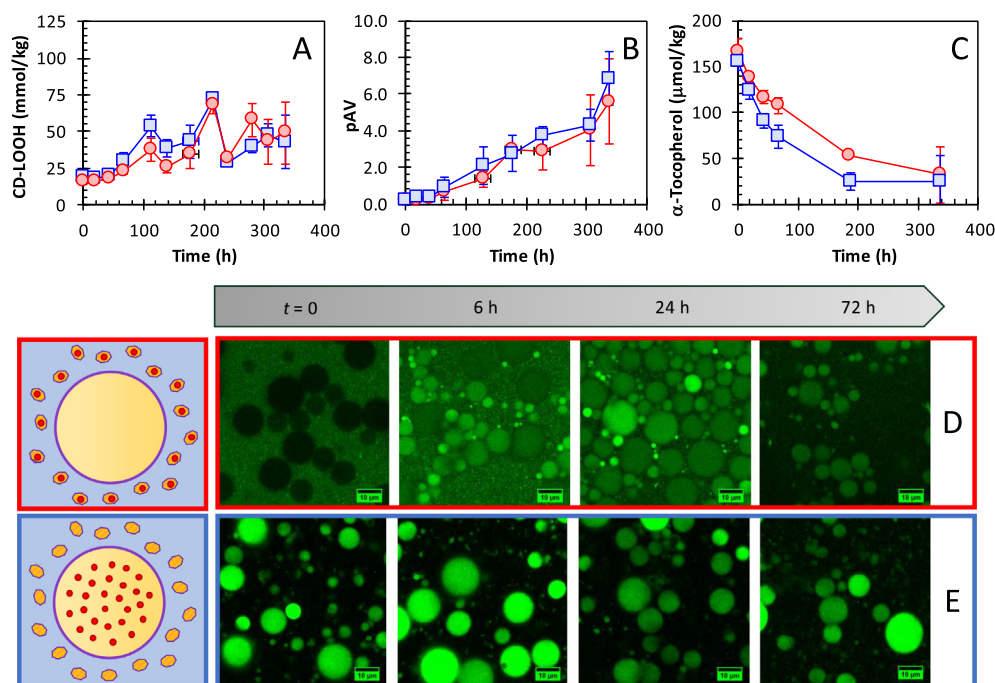
We then replaced  $\alpha$ -tocopherol by the fluorescent probe in similar emulsions. In the freshly prepared emulsions, a continuous green background surrounding black droplets shows that 25-NBD cholesterol was present only in the CLPs in the aqueous phase (Fig. 5E). Over incubation, the probe was rapidly transferred to the liquid oil droplets, which was not necessarily expected. In fact, both CLPs and oil droplets are negatively charged, which should thus prevent prolonged contacts between both structures. In addition, no alteration of the DSC profile of the CLPs was detected (Fig. 1), further indicating that no exchange of lipid material took place between the high melting point fat of the CLPs and the liquid oil of the droplets. It thus seems that in such a simple protein-stabilized emulsion, the diffusion of a lipophilic molecule initially loaded in the CLPs towards the liquid oil droplets does not require a prolonged contact and occurs very fast. Using the analogy of the probe's behavior, the antioxidant performance of  $\alpha$ -tocopherol regardless of its initial location in these emulsions (Fig. 5A, B) is probably explained by the fact that it rapidly diffuses in the oil droplets, thus erasing the initial architectural difference between the two emulsions. This finding is aligned with half time transfer values for  $\alpha$ -tocopherol between LDLs and other lipoproteins, which are in the range of 20–70 min [38].

Finally, to decipher the respective contributions of the adsorbed and non-adsorbed CLPs to the overall antioxidant activity while

sticking to the original interface structure, we designed a Pickering emulsion stabilized by  $\alpha$ -tocopherol-free CLPs, to which excess  $\alpha$ -tocopherol-loaded CLPs were added post homogenization, hence located in the continuous phase (Table 1E, F). Interestingly, lipid oxidation proceeded significantly faster in the Pickering emulsions containing  $\alpha$ -tocopherol exclusively in the continuous phase CLPs, compared to the corresponding control emulsion with the antioxidant in the oil droplets (Fig. 6A, B). This may be explained by the physical segregation of the PUFAs in the oil droplets from  $\alpha$ -tocopherol in the water phase, precluding the latter component to reduce PUFA-derived free radicals *in situ*. Importantly, this last data set confirms that the interfacial localization of our model antioxidant is essential to yield the boosting effect for antioxidant activity evidenced in our concept Pickering emulsions.

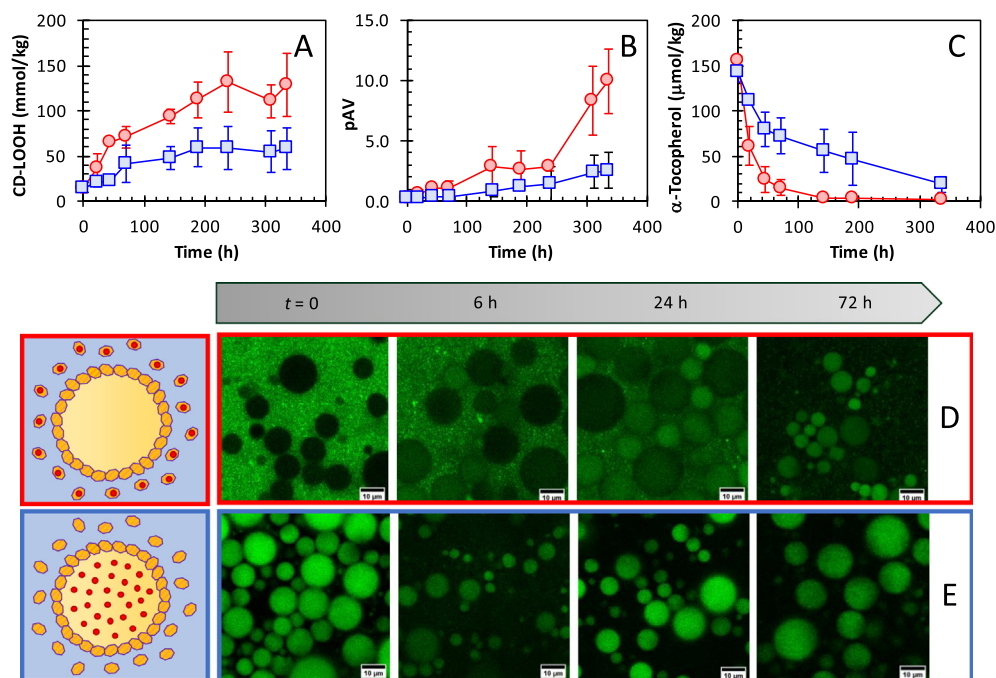
Along with the fast PUFA oxidation, the degradation of  $\alpha$ -tocopherol also proceeded very fast when located in the continuous phase CLPs (Fig. 6C). This was not expected because  $\alpha$ -tocopherol is only degraded in the presence of lipid oxidation products (Fig. 3C, D). Therefore, how to explain that an antioxidant localized in aqueous phase structures, and whose propensity to penetrate the oil droplets is largely blocked, may meet lipid oxidation products? Our hypothesis is that  $\alpha$ -tocopherol is reached by lipid oxidation products able to rapidly escape out of oxidized oil droplets. This would be in line with the relatively high concentration of hydroperoxides rapidly formed in this emulsion (Fig. 6A). Due to their relatively high surface activity, lipid hydroperoxides can, in theory, leak out from droplets, diffuse through the aqueous phase, and react with  $\alpha$ -tocopherol. Data presented here could thus constitute the first experimental proof of such a recently postulated scenario [7,8].

As previously, emulsions with a similar construction principle were also prepared with 25-NBD cholesterol. The diffusion of the fluorescent probe from the aqueous phase CLPs to the emulsion droplet core during incubation did occur (Fig. 6D), yet much slower compared to what we observed in the conventional



**Fig. 5.** Formation of (A) conjugated diene hydroperoxides (CD LOOH) and (B) aldehydes (pAV), and (C) stability of  $\alpha$ -tocopherol during incubation of conventional sodium caseinate-stabilized emulsions containing CLPs added post homogenization with  $\alpha$ -tocopherol incorporated either in the particles or in the liquid PUFA oil droplets (red circles and blue squares, respectively; see also schematic illustrations on the left), initially containing  $200 \mu\text{mol kg}^{-1}$   $\alpha$ -tocopherol. On the bottom, CLSM images of the corresponding emulsions, with the fluorescent analogue initially added in the CLPs (D) or in the oil (E), taken over incubation ( $t = 0$ –72 h). The scale bar on all images is 10  $\mu\text{m}$ .





**Fig. 6.** Formation of (A) conjugated diene hydroperoxides (CD LOOH) and (B) aldehydes (pAV), and (C) stability of  $\alpha$ -tocopherol during incubation of Pickering emulsions with extra CLPs added post homogenization, with  $\alpha$ -tocopherol incorporated either in the added particles or in the liquid PUFA oil droplets (red circles and blue squares, respectively; see also schematic illustrations on the left), initially containing  $200 \mu\text{mol kg}^{-1}$   $\alpha$ -tocopherol. On the bottom, CLSM images of the corresponding emulsions, with the fluorescent analogue initially added in the added CLPs (D) or in the oil (E), taken over incubation ( $t = 0$ –72 h). The scale bar on all images is  $10 \mu\text{m}$ .

protein-stabilized emulsion (Fig. 5D). The CLP-based interfacial layer can act as a physical barrier that slows down the diffusion of the aqueous CLPs' components to the oil droplets. This suggests that the interfacial CLP-based layer has a variable permeability to different colloidal structures depending on their size: CLPs and the components that they carry cannot rapidly be transferred due to their relatively large diameter (around  $150 \text{ nm}$ ), while lipid oxidation products, presumably self-assembled under the form of small colloidal objects ( $<150 \text{ nm}$ ) can. Other studies have reported that oxidation of dispersed lipids such as giant unilamellar vesicles or emulsified oil droplets can trigger the emission of small buds [39], debris [40], beads or lenticules [41]. If such small leaking objects were formed in our system, we can conclude that they are smaller than  $150 \text{ nm}$ , which constitutes the first approximation of their size, although it is a rough estimation. Thus, lipid oxidation products probably escape out of oxidized oil droplets as nanometric colloidal objects - possibly as microemulsion droplets or micelles - to oxidize  $\alpha$ -tocopherol incorporated in the continuous phase CLPs.

#### 4. Conclusions

On the basis of the previously established “cut-off effect”, postulating that phenolipids of intermediate lipophilicity position at the oil-water interface in emulsions where they can optimally prevent lipid oxidation [12,15,16], we have herein taken this principle one step further. In fact, we have proposed a hierarchical structure for designer emulsions in which the activity of natural antioxidants is substantially boosted, which is an advantage compared to chemically modified phenolipids. The use of antioxidant-loaded Pickering particles results in a dual functionality, providing both excellent physical and oxidative stability to biocompatible and food-grade emulsions. Besides, these interfacial reservoirs confer a substantial residence time to antioxidants at the interface during crucial stages in the chemical oxidation reaction cascade, scaveng-

ing the very first lipid radicals generated during the initiation step before the irreversible autooxidation chain reaction propagates. Accordingly, our proposed interfacial design can turn an archetypal chain-breaking antioxidant into a new type of initiation-breaking antioxidant. Such a shift in the antioxidant mode of action may augment the potential of oxidation inhibitors to an unprecedented level, and consequently lead to a large reduction of the antioxidant content needed to achieve protection of emulsions towards oxidative degradation. Beyond the potential of this concept for a broad range of applications, this work yields findings that could be pivotal in the field of lipid oxidation in discontinuous systems, such as the ability of lipid oxidation products to be transferred via the aqueous phase in the absence of any added surfactant. This approach can thus nicely complement other innovative current studies in the field [42,43], and without a doubt the combination of multiple approaches and visions will be an asset to solve this conundrum.

#### Author contribution

A.S., M.L and C.C.B.C conceived the experiments. A.S. performed the experiments. All authors discussed the data and their interpretation and co-wrote the manuscript.

#### 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.

#### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcis.2020.04.069>.

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