Research Article

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Oxidative stability of emulsions fortified with iron: the role of liposomal phospholipids

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Abstract

BACKGROUND: Interest in supplementing food with iron to counteract dietary deficiencies has been on the rise in recent years. A major challenge is the pro-oxidant activity of soluble iron, which compromises the chemical stability of the enriched food products. This problem could be mitigated by encapsulating iron, to physically keep it separated from oxidizable substrates, such as unsaturated fatty acids. In the present work, the physical and chemical stability of surfactant- or protein-stabilized oil-in-water emulsions fortified with iron was investigated.

RESULTS: Iron (ferrous sulfate) was successfully incorporated in liposomes at high encapsulation efficiency (89%). The liposomes obtained were added to emulsions stabilized with either Tween 20 or whey protein isolate (WPI), and its oxidative stability was monitored and compared with emulsions with free iron. Tween 20-stabilized emulsions were more stable against oxidation than WPI-stabilized emulsions, and furthermore lipid oxidation was substantially higher in emulsions containing iron (either free, or encapsulated in liposomes) than in blank emulsions. This shows that liposomal encapsulation did not inhibit the pro-oxidant activity of iron.

CONCLUSION: Despite the high encapsulation efficiency of iron in our liposomes, these systems are not suitable to supplement model foods with iron because of the associated deleterious chemical reactivity. This is most probably due to the phospholipids used as encapsulation material being prone to oxidation, which may actively contribute to the oxidative process. These aspects are normally not taken into account but we showed that they are of utmost importance, and should be taken as a starting point in the design of delivery systems.

Keywords: iron; liposomes; encapsulation; oil-in-water emulsions; lipid oxidation; whey proteins; Tween 20

INTRODUCTION

Iron is an essential trace element, which is of major importance for biological systems. Iron deficiency causes anemia, which is the most common nutritional deficiency worldwide, affecting about 30% of the world population.[1] Food fortification with iron salts is an effective and cost-efficient long-term strategy that can be applied for the prevention of anemia.[2,3]

However, fortification of foods with iron can, in turn, affect the chemical stability of the food products. For instance, lipid oxidation has become a major concern in emulsion-based food products, such as milk, beverages, and infant formula. Iron catalyzes oxidative processes by forming and decomposing lipid hydroperoxides (ROOH) into highly reactive peroxyl (ROO•) and alkoxyl (RO•) radicals. These radicals further react with unsaturated lipids, leading to the formation of more lipid radicals, thus propagating the radical chain reaction.[4] Decomposition of intermediate products such as alkoxyl radicals results in the generation of a broad range of secondary products, such as aldehydes, ketones, or alcohols, some of which are volatile and hence responsible for the formation of 'off' flavors.[5] This causes the quality of food to deteriorate and makes it unsuitable for consumption.

Iron-catalyzed lipid oxidation can be retarded by preventing hydroperoxides from coming into close proximity of pro-oxidants, or by adding metal chelators such as ethylenediamine tetraacetate (EDTA).[4,6] Ethylenediamine tetraacetate is a synthetic chelating agent, which can bind transition metals (e.g., Fe^{+2}, Cu^{+2}) on its negatively charged sites,[4] and has been shown to retard lipid oxidation dramatically in oil-in-water (O/W) emulsions.[7,8] Alternatively, one could argue that iron ions should be prevented from coming into close contact with lipid substrates, which are susceptible to oxidation, using an appropriate encapsulation

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system. Beside the prevention of iron-food interaction, encapsulation may also enhance the absorption of dietary iron.[9,10] It is therefore relevant to investigate whether iron encapsulation can prevent its pro-oxidant activity in model food systems, such as emulsions.

Liposomes are submicron-scale spherical vesicles composed of phospholipid bilayers, which surround an aqueous compartment. This makes them suitable for the encapsulation and dispersion of both hydrophilic (compartment) and lipophilic (in bilayer) components in aqueous media.[11] They can also be prepared with food-grade ingredients and are non-toxic.[11] Some studies have already successfully attempted encapsulating soluble iron in liposomes,[12–14] and there is even one available example of incorporation of such iron-loaded liposomes in milk.[12] In the latter work, no sensory defect could be detected in milk enriched with iron-loaded liposomes, compared to non-enriched milk, over one week of refrigerated storage. However, evidence for the potential of iron-loaded liposomes to be used for iron supplementation in polyunsaturated fatty acid (PUFA)-rich matrices is still scarce.

The objective of this study was therefore to evaluate the ability of liposomes to encapsulate iron and thereby limit its pro-oxidant activity when incorporated into protein- or surfactant-stabilized emulsions rich in PUFAs. The oxidative stability of O/W emulsions fortified with liposomal ferrous sulfate was compared with that of emulsions containing either empty liposomes or non-encapsulated ferrous sulfate. The physical properties (droplet size, ζ-potential, and emulsion stability) and chemical stability of all emulsions were determined as functions of time.

MATERIALS AND METHODS

Materials

Sunflower oil from a local supermarket was stripped by means of alumina to eliminate impurities and tocopherols.[15] Whey protein isolate (WPI; 97.5 wt% protein) was obtained from Davisco (Minneapolis, MN, USA). L-α phosphatidylcholine (PC) from egg yolk (purity ~60%), cholesterol (purity ≥ 99%), Tween 20, and iron (II) sulfate heptahydrate and chemicals mentioned later were obtained from Sigma-Aldrich (St Louis, USA). Phosphate–citrate buffer (0.1 mol L^{-1}, adjusted to pH 6.8) was used for both emulsion and liposome preparation. All chemicals were used as purchased unless otherwise stated. The fatty acid composition of stripped sunflower oil and lecithin and their content in tocopherols were determined as described elsewhere.[16,17] Their initial chemical status was assessed by measuring their content in conjugated diene hydroperoxides and their *p*-anisidine value, as described in the following sections. The corresponding values are reported in Tables 1 and 2.

Table 1. Initial contents in endogenous lipid oxidation products and antioxidants in stripped sunflower oil and phosphatidylcholine

	Conjugated diene content (mmol eq HP kg oil^{-1})	*p*-Anisidine value (AU)	Tocopherol content (μg g oil^{-1})
Stripped sunflower oil	5.43 ± 0.16	0.03 ± 0.01	<7
Phosphatidylcholine	9.41 ± 0.52	0.34 ± 0.01	<7

Table 2. Fatty acid composition (ppm) of stripped sunflower oil and phosphatidylcholine

Fatty acids	Stripped sunflower oil (ppm)	Phosphatidylcholine (ppm)
Myristic acid (C14:0)	3.91 ± 0.28	4.01 ± 1.15
Palmitic acid (C16:0)	366.21 ± 20.20	1060.52 ± 0.24
Stearic acid (C18:0)	202.96 ± 10.55	573.59 ± 1.41
Arachidic acid (C20:0)	83.11 ± 28.61	21.91 ± 3.12
Behenic acid (C22:0)	4.24 ± 4.24	ND
Lignoceric acid (C24:0)	0.26 ± 0.14	ND
Total SFA	**660.68**	**1660.01**
Palmitoleic acid (C16:1*n-7*)	2.71 ± 0.19	11.41 ± 2.71
Oleic acid (C18:1*n-9*)	1247.75 ± 36.27	636.1 ± 4.48
Erucic acid (C22:1*n-9*)	ND	1.09 ± 0.01
Total MUFA	**1250.46**	**648.58**
Linoleic acid (C18:2*n-6*)	1746.24 ± 0.51	142.49 ± 3.93
α-Linolenic acid (C18:3*n-3*)	1.41 ± 1.04	0.67 ± 0.11
Total PUFA	**1747.65**	**143.15**
Total FA	**3658.79**	**2451.74**

ND, not detected; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.
Results are expressed as mean ± SD of duplicate determination.

Liposome preparation and physical characterization

Liposome dispersions were prepared fresh before each set of experiments by a reverse-phase evaporation technique as previously described,[18] with some modifications. The liposome composition included cholesterol and Tween 80; cholesterol was shown to reduce leakage of encapsulated core material and enhance the rigidity of the bi-layer,[9,19] whereas the polyoxyethylene chains of Tween 80 cover the liposome vesicles leading to a hydrophilic surface layer. L-α Phosphatidylcholine (0.45 g) and cholesterol (0.018 g) were initially dissolved in diethyl ether (30 mL). Then the organic phase that was obtained was mixed with 10 mL buffer solution (pH 6.8) containing 61 mmol L^{-1} $FeSO_4$, $7H_2O$ (except for the empty liposomes). The mixture was sonicated with a probe sonicator (Microtip 3 mm, Branson Sonifier 250, the Netherlands) for 10 min at a frequency of 20 kHz, power of 150 W and amplitude of 40%. The mixture was kept on ice during sonication to minimize temperature increase and any associated chemical degradation. Then, the organic solvent was removed using a rotary evaporator (at controlled reduced pressure and 45 °C), after which 20 mL aqueous phase containing Tween 80 (2.5%, pre-heated at 50 °C) was added under gentle vortexing. The suspension thus obtained was passed through filter paper (0.45 μm, Whatman, Sigma-Aldrich, St Louis, USA) to obtain liposomes that were stored at 4 °C in a refrigerator.

The size distribution, polydispersity index (PDI) and surface charge (zeta potential) of the liposomes were measured with a dynamic light-scattering instrument (Zetasizer Nano ZS, Malvern Instruments Ltd, Cambridge, UK). The refractive index of the dispersant was set to 1.33 for water and that of the dispersed phase of liposome to 1.47. Samples were diluted 1000-fold with ultrapure water prior to measurement. Samples were prepared three times independently, then each replicate was measured three times. For the zeta potential analysis, the results are expressed as a mean value in mV ± standard deviation.

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Determination of encapsulation efficiency of liposomes

The so-called ferene method was used and adapted to determine the content of iron entrapped in the liposomes. Ferrous iron reacts with the chromogen ferene to form a blue chromophore.[20] The intensity of the color is proportional to the amount of iron present in solution and can therefore be determined spectrophotometrically. The wavelength of maximum absorbance of the chromophore (λ_{max}) was determined to be 593 nm. Calibration curves were prepared by appropriate dilution of stock iron solution in ultrapure water. The ferene triazine solution (6 mmol L^{-1}) was prepared in ultrapure water, and a dissociating agent solution was prepared mixing the reducing agent, which was L-ascorbic acid solution (0.25 mol L^{-1}) and acetate buffer (1.4 mol L^{-1}) adjusted at pH 4.5, in a 1:1 ratio (v/v).

To determine the concentration of free (non-encapsulated) iron (Fe_f, ppm), a 2 mL liposomal ferrous sulfate suspension was placed in a centrifugal ultrafiltration device (Amicon® Ultra-4, 3000 MWCO, Merck Millipore, Darmstadt, Germany) and centrifuged at 4200×*g* for 1 h at 20 °C. The filtrate was collected and diluted 100 times with ultrapure water. To determine the total iron concentration in the liposomal suspension (Fe_T, ppm), 1 mL liposomal ferrous sulfate suspension was added to 5 mL hydrochloric acid (37%), and boiled for 20 min. The samples obtained were mixed with 1 mL HCl solution (10%). Before analysis by the ferene method, samples were diluted 100 times with ultrapure water.

Half a milliliter of sample (ultrapure water for the blank, ferrous sulfate calibration solutions or samples) was mixed with 1 mL dissociating agent solution, and then 0.1 mL ferene stock solution was added and vortexed. The mixture was kept in the dark for 5 min, after which the absorbance was measured at 593 nm. The encapsulation efficiency (EE) of liposomes was calculated as shown in Eqn (1):

$$EE\ (\%) = \left[\left(Fe_T - Fe_f\right)/Fe_T\right] * 100 \quad (1)$$

Emulsion preparation and physical characterization

The aqueous phase consisted of phosphate–citrate buffer (0.18 mol L^{-1}, pH 6.8) and either Tween 20 (1.5 wt%) or WPI (2 wt%). Tween 20 and WPI were dissolved in the aqueous phase the day before emulsion preparation and gently stirred overnight at room temperature. A coarse emulsion containing 30 wt% stripped sunflower oil was prepared using a rotor stator homogenizer (UltraTurrax T25 Basic Disperser with 25 mm (diameter) blade, (Janke & Kunkel, IKA, Staufen, Germany) at 7000 rpm for 2 min, followed by three passes at 800 bar through a high-pressure homogenizer (Microfluidizer M-110Y High Pressure Pneumatic, Microfluidics, Massachusetts, USA). The cooling jacket of the homogenizer was filled with iced water to limit the temperature rise during the emulsification process. The experiments were performed twice as independent duplicates.

After preparation, emulsions were distributed in 50 mL tubes (40 g per tube). Three different systems were studied: emulsions fortified with liposomal ferrous sulfate suspension ($FeSO_4$ loaded liposomes), emulsions with empty liposome suspension (empty liposomes), and emulsions fortified with non-encapsulated ferrous sulfate solution (free $FeSO_4$). For both iron-containing systems, the final iron concentration in the emulsions was 200 µmol L^{-1}. Samples were incubated at 40 °C, on a rotating agitation device (5 rpm) for up to 13 days to prevent creaming.

The droplet size distribution of emulsions was determined using static light scattering (Mastersizer 2000, Malvern Instruments Ltd). The refractive indices of sunflower oil (1.47) and water (1.33) were used as particle and dispersant index, respectively. Five measurements were conducted per sample. Results are reported as DeBrouckere mean diameters ($d_{4,3}$). On the last day of incubation, WPI-stabilized emulsions were also diluted in concentrated SDS solution (10%, fourfold dilution) prior to the measurement to allow possible droplet flocs to disintegrate. They would otherwise be measured as single large droplets.

For the measurement of the zeta potential of emulsions, we used the same instrument and procedure as described above. Measurements were taken immediately after emulsification, and at the end of the incubation period.

Oxidation experiments

Primary oxidation products

The formation of conjugated diene hydroperoxides was assessed using the method described previously.[21] Briefly, emulsion aliquots were diluted in isopropanol, and the mixture was centrifuged at 1200×*g* for 4 min. The absorbance of the supernatant was recorded between 200 and 310 nm using a UV-visible spectrophotometer (DU 720 Beckman Coulter, Brea, CA, USA). Results are expressed in mmol of equivalent hydroperoxides per kg of oil (mmol eq HP kg^{-1} oil) with 27 000 M^{-1} cm^{-1} as the molar extinction coefficient of conjugated dienes at 233 nm.

Secondary oxidation products

The formation of secondary oxidation products was monitored by measuring the *p*-anisidine value (pAV). First, 0.3 g emulsion was mixed with 1.5 mL *n*-hexane : isopropanol mixture (3:1 v/v). The absorbance of the hexane (upper) phase (*Ab*) obtained was first measured at 350 nm using pure hexane as blank. Then, 0.1 mL *p*-anisidine solution (2.5 mol L^{-1} in acetic acid) was added to 0.5 mL hexane phase, mixed, and after 10 min the absorbance at 350 nm (*As*) was measured. The pAV was then determined as shown in Eqn (2):

$$pAV = (1.2As - Ab)/m \quad (2)$$

where *m* is the mass (g) of oil per mL hexane.

Statistical analysis

Statistical analysis was conducted using SPSS software (version 18, PASW Statistics, Chicago, US). A one-way analysis of variance (ANOVA) was conducted using six individual results from two repetitions and the least significant differences were calculated at $P < 0.05$ applying Tukey's b *post hoc* test.

RESULTS AND DISCUSSION

In previous research, the production of liposomes loaded with ferrous sulfate was optimized for encapsulation efficiency.[22] In the present work, the liposomes were added to O/W emulsions stabilized with whey protein or Tween 20. The physical and chemical stability of these emulsions was monitored in time. Lipid oxidation was monitored through the measurement of primary and secondary oxidation products, while emulsion droplet size and zeta potential were checked as indicators for any physical change occurring during incubation.

Liposomes

The physical properties of liposomes are summarized in Table 3. The particle size of empty liposomes was 120 nm, while those containing iron had a slightly larger average size of 137 nm. Similar

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Table 3. Physical characteristics of liposomes

Sample name	Size (nm)	PDI	ζ-potential (mV)	Encapsulation efficiency (%)
Empty liposome	120 ± 3.1[a]	0.15 ± 0.03[a]	−43.61 ± 0.96[a]	–
FS-loaded liposome	137 ± 2.2[b]	0.19 ± 0.06[a]	−52.32 ± 3.79[b]	89 ± 0.01

Mean ± SD, $n = 6$. Values with different letters in column are significantly different according to Tukey's b test ($P < 0.05$). PDI, polydispersity index.

results have been obtained previously with ferrous glycinate[14] and salidroside-loaded liposomes.[23] In these studies, the size difference between loaded and empty liposomes was attributed to the neutralization of the electrostatic charge of iron-loaded liposomes, leading to aggregation. However, the polydispersity index (PDI) of our liposomes was very small, indicating narrow liposome size distributions for both loaded and empty liposomes, which makes aggregation less likely. The slightly larger size of iron-loaded liposomes compared to empty ones may have been due to osmotic pressure compensation, causing the loaded liposomes to swell slightly.

Both empty and $FeSO_4$-loaded liposomes were strongly negatively charged, with zeta potential values of −43.6 and − 52.3 mV, respectively, most probably as a result of the negative surface charge of phospholipids in the liposomal bilayer membrane. This likely contributes to the high physical stability of the liposomes.[11] Although phosphatidylcholine is zwitterionic at pH 6.8, other phospholipids present in lecithin, such as phosphatidylserine and phosphatidylinositol, are negatively charged at neutral pH,[24] and may have caused the negative charge. Nielsen *et al.* observed that the effect of iron addition on the zeta-potential of emulsion droplets was dependent on the type of emulsifier, and on the pH.[27] For instance, at pH 3.0, they observed that iron addition led to a reduction of the positive zeta-potential for protein-stabilized emulsions, and to a less negative zeta-potential for phospholipid-stabilized emulsions. At pH 7.0, however, no clear effect of iron on the zeta-potential could be found.[25] Interestingly, iron-loaded liposomes were even more negatively charged than empty liposomes; although the underlying reason is not clear, this observation contrasts with explanations suggested in literature, i.e., that iron ions would neutralize some negative charges of the phospholipids.[14,23]

The encapsulation efficiency (EE) of ferrous sulfate was 89% in the freshly prepared liposomes (Table 3), which is rather high compared to other publications. For milk fortification, the highest reported encapsulation efficiency was 67%,[14] and in previous work we found 62% using mixed lecithin.[24] The difference in EE of $FeSO_4$-loaded liposome solutions may be related to differences in purity of the phosphatidylcholine source used in these studies. A study by Colletier *et al.*[26] showed that the encapsulation efficiency of proteins in liposomes depends on the type of phospholipid used, with mostly an effect of the type of hydrophilic headgroup, and not so much of the alkyl chains. They suggested that encapsulation could be improved by the establishment of electrostatic interactions between the molecule to encapsulate, and the hydrophilic headgroup of the phospholipids.

To determine the oxidative stability of the liposomes themselves during incubation, both empty and $FeSO_4$-loaded liposomal suspensions were kept in refrigerated conditions for 20 h, and then incubated at 40 °C for up to 96 h. Conjugated diene (CD) values and the change in appearance (color) of the liposome suspensions over time are shown in Fig. 1. After 20 h of storage at 4 °C, CD values of $FeSO_4$-loaded and empty liposomes reached 51 and 38 mmol kg oil^{-1}, respectively. After 96 h of subsequent incubation at 40 °C, the CD values for $FeSO_4$-loaded or empty liposomal suspensions increased to 75 and 45 mmol kg oil^{-1}, respectively. This is in line with the macroscopic appearance of the liposomal suspensions: fresh $FeSO_4$-loaded liposome suspensions had a blue-greenish color, which turned into orange yellowish over the incubation period, showing that ferrous iron (Fe^{+2}) was oxidized to ferric iron (Fe^{+3}). Despite this, the EE% did not change over time (data not shown); from now on we will use the term iron-loaded liposome to do justice to this effect.

The formation of CD hydroperoxides in the liposome suspension can be attributed to oxidizing polyunsaturated fatty acids (PUFAs) present in the phospholipid structure.[11] The zeta potential of liposomes at pH 6.8 was approximately −45 mV, indicating the presence of negatively charged functional groups at the bilayer surface. This could attract (free) ferrous cations, bringing them in close proximity to the PUFAs, which could have promoted oxidative reactions, as illustrated by the higher values found for Fe-loaded liposomes. In addition, lecithin generally contains trace amounts of hydroperoxides,[27] which can promote lipid oxidation via the formation of radicals through hydroperoxide decomposition,[28] which could have negatively influenced the oxidative stability of both samples.

O/W emulsions

Physical characterization

The droplet size distribution and average droplet size in emulsions that are freshly prepared or after 13 days of incubation at 40 °C are presented in Fig. 2 and Table 4. Emulsions stabilized with WPI or Tween 20 had an average volume mean diameter ($d_{4,3}$) of about 0.3 μm at t_0, irrespective of the presence of empty or iron-loaded liposomes. For Tween 20, monomodal distributions were found both initially and after 13 days (Fig. 2(a) and Table 4). The initial bimodal distribution found for whey protein-stabilized emulsions was due to flocculation as is evidenced by the fact that after dilution of emulsion samples in SDS solution, the peak corresponding to the largest droplet population disappeared (Fig. 2(c)). The droplet size of WPI-stabilized emulsions was considerably larger after 13 days of incubation (Table 4 and Fig. 2(d)), and also remain so after SDS treatment which indicates that coalescence occurred over the incubation period (Fig. 2(d)).

For all emulsions, the zeta potential remained stable over the incubation period, and the values were always very negative (Table 4). Whey protein isolate-stabilized emulsions had higher net surface charge (−48 mV) than Tween 20-stabilized emulsions (−34 mV). The negative charge of WPI-stabilized emulsions was expected as the pH of the environment was higher than the isoelectronic point of whey proteins (pI ∼ 5.2) and similar values were found in previous work.[29] The negative surface charge recorded for the Tween 20-stabilized emulsions was somewhat

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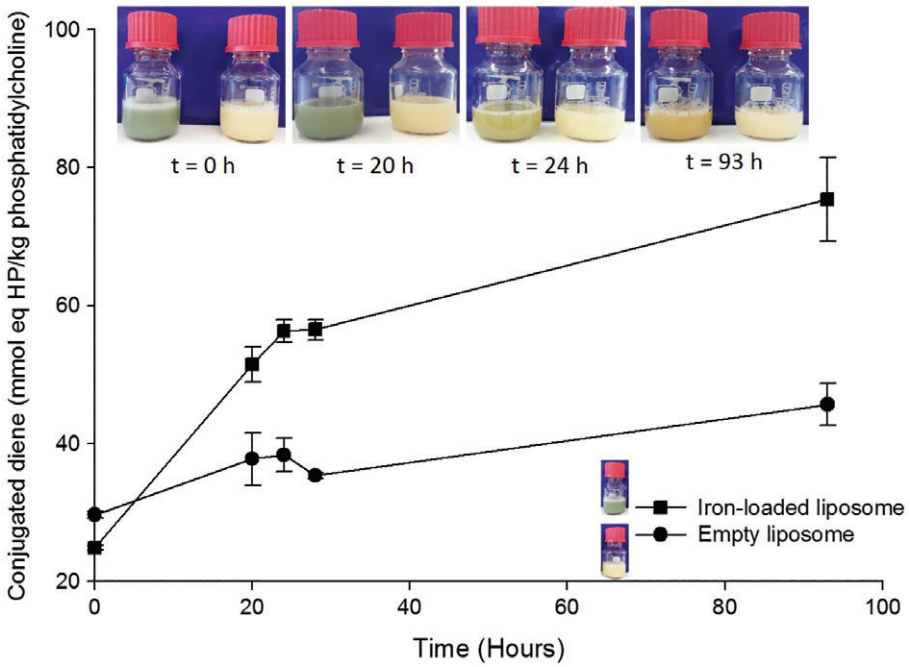

Figure 1. Conjugated diene (CD) hydroperoxide values in liposome suspensions (iron-loaded liposomes (■) or empty liposomes (●)), and macroscopic physical appearance of the suspensions during incubation at 40 °C (first images are always the empty liposomes, and second images the iron-loaded samples; the sample time is indicated underneath). Error bars represent standard deviations ($n = 6$).

surprising, as Tween 20 is a nonionic surfactant. Nonetheless, it was in agreement with results from others,[30,31] who attributed this to preferential adsorption of OH^- species from water onto the emulsion droplet surface at relatively high pH.[32] Some results from our department (unpublished data) also suggest that phosphate ions could bind to the surface of emulsion droplets, thereby inducing such a negative surface charge even with nonionic surfactants.

Oxidative stability of emulsions

Tween 20- and WPI-stabilized emulsions containing liposomal iron, free iron, or empty liposomes were incubated for up to 13 days, and primary (conjugated dienes) and secondary (*p*-anisidine value) lipid oxidation markers were recorded (see Fig. 3, panels a and c for Tween 20, the other two for WPI stabilized emulsions). For the WPI-stabilized emulsions, only the first 5 days of incubation are reported (Fig. 3(b), (d)), as extensive physical destabilization of the emulsions occurred later on, with macroscopic phase separation of the emulsion.

During the first 2 days, in all emulsions, the CD levels remained below 15 mmol kg oil^{-1}, which is relatively low,[33] after which, CD formation increased, reaching around 17–22 mmol kg oil^{-1} in all Tween 20-stabilized emulsions, and around 25–30 mmol kg oil^{-1} in the WPI-stabilized emulsions containing free or liposomal iron. In contrast, in the WPI-stabilized emulsion containing empty liposomes, the CD level remained below 15 mmol kg oil^{-1} over the first 5 days of incubation. The pAV value increased continuously in all emulsions, eventually reaching about 0.5 (AU) in the Tween 20-stabilized emulsions, about 1.5 (AU) in the WPI-stabilized emulsion containing empty liposomes, and about 3.0 in the WPI-stabilized emulsions containing free or liposomal iron.

From these results, a number of points can be made. First, Tween 20-stabilized emulsions were more oxidatively stable than WPI-stabilized emulsions, independently of the presence of iron and / or liposomes. This is particularly clear from the formation of secondary oxidation products, as the pAV was much lower in all Tween-20 stabilized emulsions compared to WPI emulsions. This was in line with the findings of Berton *et al.*,[5] and the difference may be attributed to the characteristics of the interfacial layer, which can act as a barrier between the oil droplet core and the aqueous phase,[4] influencing the interaction with the liposomes and the free iron. Surfactants, and particularly polyoxyethylene sorbitan esters such as Tween 20, form tightly packed layers, 0.5–1 nm thick, at the oil–water interface, and homogeneously cover the oil surface, while proteins usually form thicker (1–15 nm) but more porous layers[34] that only cover 30–40% of the oil droplet surface because of conformational constraints.[35,36] As mentioned before, the charge of the interfacial layer may affect lipid oxidation through counter ion compensation that may bring positively charged metal ions such as Cu^{+2}, Fe^{+2} or Fe^{+3} in close proximity with the PUFA in the oil droplets.[4] In our case, WPI-stabilized emulsions had a higher net surface charge (−48 mV) than Tween 20-stabilized emulsions (−34 mV), which could have affected the concentration of ferrous and ferric ions present near the interface, and through that oxidation reactions.

Second, the addition of iron actively accelerated lipid oxidation in WPI-stabilized emulsions, whereas it only had a minor effect on the formation of CD and secondary lipid oxidation products in Tween 20-stabilized emulsions. This could be explained by the fact that proteins themselves may have been subjected to metal-catalyzed oxidation, which can help propagate oxidation of oil droplets surrounded by a protein-based interfacial layer.[37] The involvement of proteins in the lipid oxidation pathways in emulsions is far from simple. It is also well-known that proteins, such as WPI, can act as antioxidants in such systems, especially when present in the continuous phase. β-Lactoglobulin and α-lactalbumin, which are the major components of WPI, contain cysteyl residues and thus thiol groups, which can scavenge free radicals and thereby inhibit lipid oxidation.[38,39] In the present

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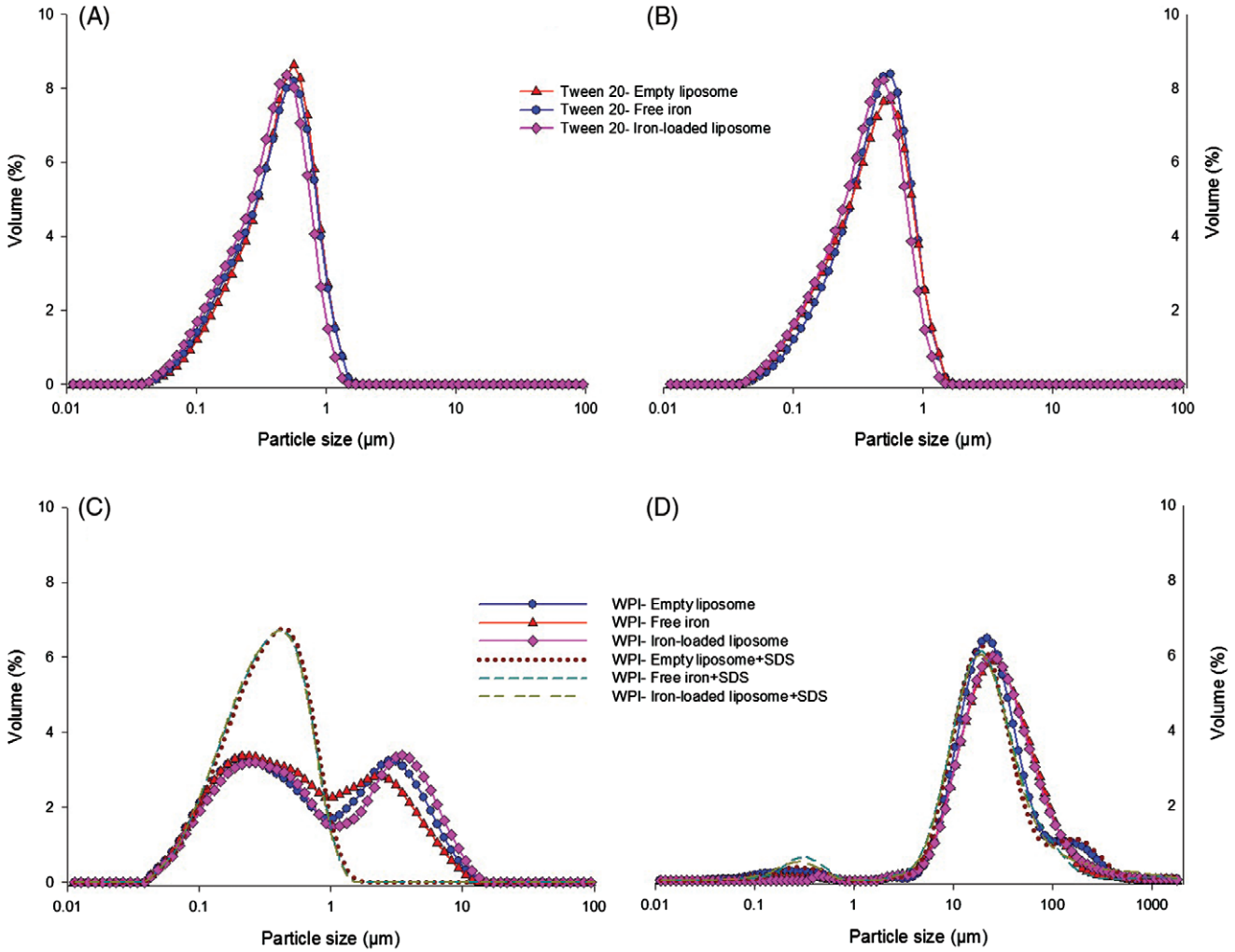

Figure 2. Particle size distribution in emulsions: (a) Tween 20-stabilized, fresh emulsion; (b) Tween 20-stabilized emulsion, after 13 days incubation; (c) WPI-stabilized, fresh emulsion; (d) WPI-stabilized emulsion, after 13 days incubation. Emulsions containing free iron (▲), empty liposomes (●), or iron-loaded liposomes (♦) were incubated at 40 °C under slow rotative agitation. Error bars represent standard deviations ($n = 6$).

Table 4. Physical characteristics of O/W emulsions

		Size (μm) *d* [4, 3]		ζ-potential (mV)	
Surfactant type	Sample name	Fresh	13th day	Fresh	13th day
Tween 20	Empty liposome	$0.30 \pm 0.01^{bc, A}$	$0.30 \pm 0.01^{a, A}$	$-33.23 \pm 1.37^{bc, B}$	$-33.67 \pm 1.66^{c, A}$
	Free FS	$0.28 \pm 0.01^{bc, A}$	$0.27 \pm 0.01^{a, A}$	$-31.23 \pm 0.57^{b, A}$	$-34.35 \pm 1.16^{b, A}$
	FS-loaded liposome	$0.29 \pm 0.01^{b, A}$	$0.29 \pm 0.01^{b, A}$	$-34.23 \pm 1.15^{c, A}$	$-38.28 \pm 3.71^{a, B}$
Whey protein isolate	Empty liposome	$0.29 \pm 0.02^{bc, A}$	$10.05 \pm 8.49^{b, B}$	$-50.48 \pm 0.78^{a, A}$	$-48.42 \pm 1.81^{a, A}$
	Free FS	$0.28 \pm 0.01^{c, A}$	$18.99 \pm 1.37^{b, B}$	$-48.92 \pm 1.72^{a, A}$	$-49.73 \pm 4.09^{a, A}$
	FS-loaded liposome	$0.32 \pm 0.05^{b, A}$	$17.56 \pm 3.68^{c, B}$	$-49.98 \pm 1.49^{a, A}$	$-49.45 \pm 1.35^{a, A}$
Whey protein isolate	Empty liposome + SDS	$0.23 \pm 0.01^{a, A}$	$17.89 \pm 0.47^{c, B}$	ND	ND
	Free FS + SDS	$0.22 \pm 0.01^{a, A}$	$17.84 \pm 0.72^{c, B}$	ND	ND
	FS-loaded liposome + SDS	$0.22 \pm 0.01^{a, A}$	$18.18 \pm 0.82^{b, B}$	ND	ND

Mean ± SD, $n = 6$. Different lower case letters (a–c) in the same column and different upper case letter (A and B) in the same row and category (i.e., for T-Empty liposome Fresh and 13th day) show statistical differences between the samples according to Tukey's b test ($P < 0.05$). +SDS indicates that emulsion samples were diluted in SDS solution prior to particle size measurement to disintegrate droplet flocs that may possibly be present. ND, not determined.

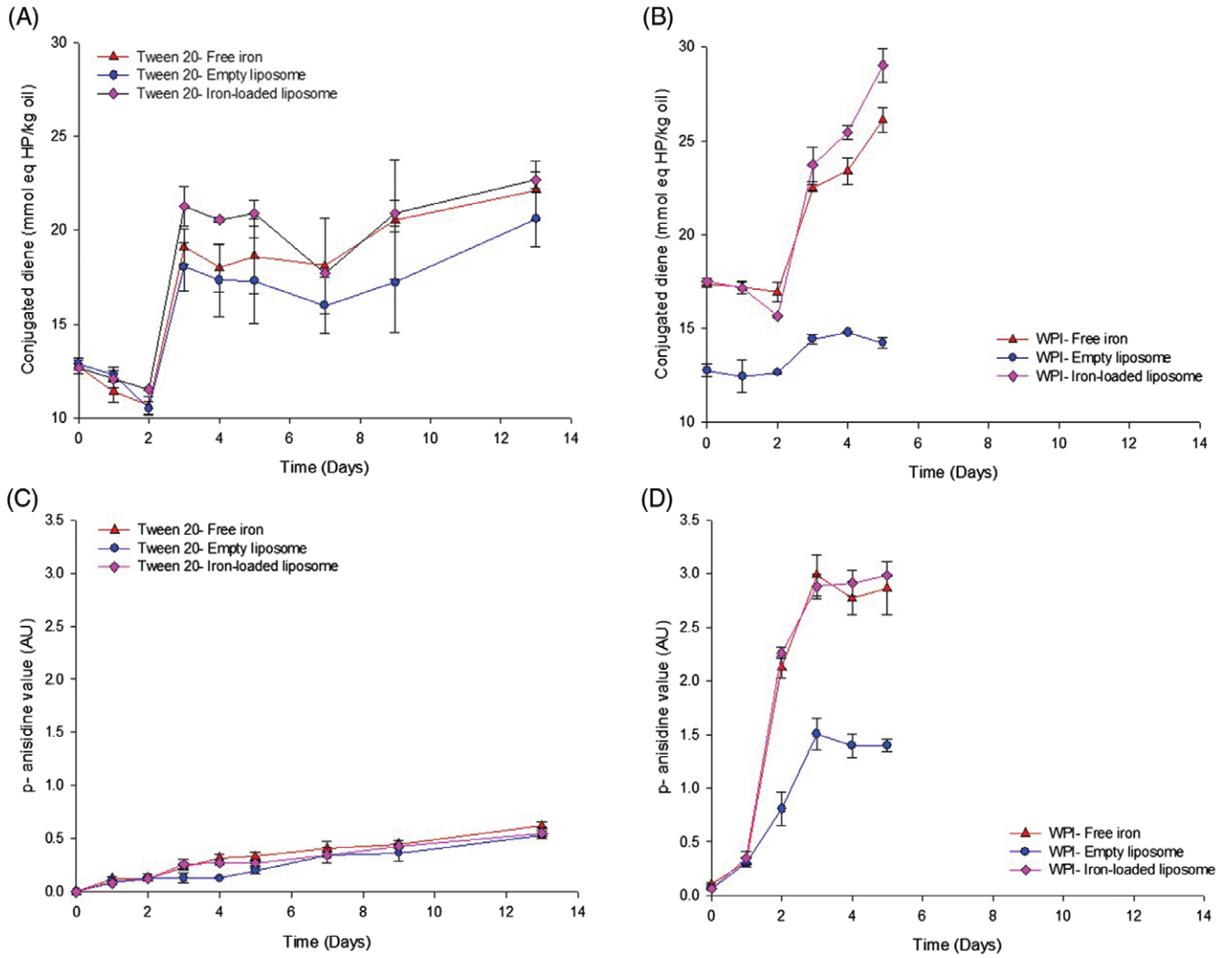

Figure 3. Conjugated diene (CD) hydroperoxide values for: (a) Tween 20-stabilized emulsions and (b) WPI-stabilized emulsions; and *p*-anisidine values for (c) Tween 20-stabilized emulsions and (d) WPI-stabilized emulsions. Emulsions contained free iron (▲), empty liposomes (●), or iron-loaded liposomes (♦) and were incubated at 40 °C under slow rotative agitation. Error bars represent standard deviations ($n = 6$).

work, such an antioxidant effect of WPI may exert in the empty liposome-containing emulsion, which showed lower CD formation from day 3, when the emulsifier was WPI, compared to Tween 20.

Finally, our emulsions were prepared in a phosphate–citrate buffer, of which both components are known to have antioxidant effects due to metal chelation properties.[40] It can thus be expected that even faster oxidation could have occurred with another composition of the continuous phase.

Although the results that were obtained were not as initially hoped for, they have taught us that physically sequestrating iron and preventing it from exerting its pro-oxidant activity through the use of liposomes is challenging when using unsaturated phosphatidylcholine. It is expected that the iron stays inside the liposomes because encapsulation efficiency remained high during storage (89%, Table 3). In WPI-stabilized emulsions, the amount of CD formed after 5 days of incubation was slightly higher with liposomal iron, compared to non-encapsulated iron (Fig. 3(b)), which was somehow unexpected. The reason might be that the liposomes themselves are highly prone to oxidation, especially when they are loaded with iron (Fig. 1). It may be presumed that, in the emulsions fortified with liposomal iron, lipid oxidation may have started in the phospholipid bi-layer, forming hydroperoxides and lipid radical species; then, due to the close contact with iron, decomposition of hydroperoxides formed in the liposomal membrane could proceed rapidly, forming radical species that could in turn attack the PUFA present in the oil droplets. It is interesting to note that the initial oxidative status of the phosphatidylcholine was different from that of the fresh oil: it contained substantial amounts of primary and secondary lipid oxidation products (Table 1), which could have contributed to the rapid oxidation of the liposomes. This illustrates clearly the pivotal role of the components used for encapsulation, which should not result in just a physical buffer zone but also in a chemical one.

CONCLUSION

This study showed that ferrous sulfate could be encapsulated at high efficiency using phosphatidylcholine-based liposomes. The liposomes thus formed were physically stable in time; however the addition of these liposomes to emulsions did not result in higher oxidative stability compared to emulsions to which free ferrous sulfate was added. This was presumably caused by the

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oxidation of the liposomal membrane material itself: the phosphatidylcholine used contained PUFA, and possibly pre-formed hydroperoxides, which were the main oxidation triggers in emulsions fortified with iron-loaded liposomes. It should also be noted that although encapsulation efficiency was high, some non-encapsulated iron was still present. It could thus be interesting to remove any free iron from the liposome suspension before adding it to the emulsions.

The pro-oxidant effect of ferrous ions was reduced when a low molecular weight emulsifier (Tween 20) was used, as compared to proteins (WPI), which supports the hypothesis that such interfacial layers provide a more effective chemical barrier between aqueous phase pro-oxidants and the oil droplet core.

As it is of great interest, from a nutritional viewpoint, to develop foods that not only contain a good fatty acid profile (i.e., high levels of PUFA) but are also fortified in dietary iron, the encapsulation material needs to be carefully considered. For example, using more saturated phosphatidylcholine for liposome preparation, or adding cationic polymers such as chitosan, which could adsorb to the liposomal membrane (in layer-by-layer fashion) and repel metal cations, may be promising approaches to improve oxidative stability to emulsions fortified with ferrous sulfate-loaded liposomes.

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