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Hen Egg Yolk Low-Density Lipoproteins Film Spreading at the Air−Water and Oil−Water Interfaces

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Hen egg yolk is largely used as an ingredient in food emulsions due to its exceptional emulsifying properties. Low-density lipoproteins (LDL) are the main egg yolk constituents and the most important contributors to yolk emulsifying properties. To better understand the LDL adsorption mechanism and spreading at the interface, we extracted and studied LDL at different interfaces. At the air−water interface, the LDL film isotherm presents three transitions, and two were identified by each lipid class present in LDL. The last transition should be due to apoproteins−lipids complexes. During LDL adsorption, the presence of apoproteins at the LDL surface and the neutral lipid core is necessary. At pH 3 and pH 7, LDL are disrupted and spread quasi-similarly at the air−water interface, contrary to the oil−water interface where LDL spread more at pH 7 than at pH 3.

KEYWORDS: LDL; air−water interface; oil−water interface; spreading; film, pH; adsorption

INTRODUCTION

Low-density lipoproteins (LDL) are considered to be the main contributors to the exceptional emulsifying activity of hen egg yolk, which is a particle dispersion in a protein solution. Yolk can be fractionated, by centrifugation, in plasma (supernatant) and granules (pellet). By comparison with the stability and the granulometry of emulsions containing egg yolk, plasma or granules, similarities were found between emulsions containing egg yolk and those containing plasma (*1*, *2*). Studies were realized with the constituents of plasma: LDL and livetins. Davey et al. (*3*) separated the main constituents of egg yolk (LDL, livetins, granules) and then re-formed, at equal dry matter, mixtures to compare their emulsifying properties to those of egg yolk. They observed that emulsions stabilized by LDL are more stable than emulsions stabilized by granules or livetins and have properties close to emulsions stabilized by egg yolk. Further results confirmed the main role of LDL among the plasma constituents and consequently in egg yolk (*4*−*9*).

LDL are the main constituents of egg yolk: they represent 66% w/w of its dry matter and 24% w/w of its total proteins (*10*, *11*). They have a classical structure of blood very low-density lipoproteins (VLDL) with a core of neutral lipids (NL) surrounded by a layer of apoproteins (Apo) and phospholipids (PL) in contact with the aqueous phase (*12*). By electronic microscopy, LDL appear as spherical particles of about 30 nm diameter with a size distribution from 17 to 60 nm (*13*). Their density is 0.98 (*14*) with 83−89% w/w of lipids and 11−17% w/w of proteins. Lipids are separated in 74% w/w of neutral lipids (triglycerides and cholesterol) and 26% w/w of phospholipids (*15*).

Concerning the apoproteins, the proportion of hydrophobic amino acids (about 40%), like valine, leucine, and methionine, is high (*16*). Thus, LDL apoproteins are considered to be the most hydrophobic known proteins (*17*). Apoproteins are particularly heterogeneous and insoluble, excepted in detergents (*18*). This is the reason why their separation and purification to characterize them remains a problem. They are constituted by six major polypeptides (*19*): the most important with a molecular mass of about 130 kg/mol represents more than 70% w/w of apoproteins, another with a molecular mass of 15 kg/mol represents about 20% w/w, and the four others, in minority, have molecular masses of 60−95 kg/mol. Apoproteins are glycoproteins (*19*) out of which the proportion of sugars was estimated at 2.4% w/w with 1.3% w/w of hexose, 0.7% w/w of hexosamine, and 0.38% w/w of sialic acid. The isoelectrical point of apoproteins has not been determined, but that of LDL was estimated in the range of pH 6.5−7.3 (*19*). The insolubility of apoproteins in the absence of detergent and the difficulty in purifying each apoprotein are problems in characterizing their secondary structures. The proportions of α-helices, β-sheets, and random coil, reported by different authors, depend on studies. These variations are due to measurements on apoprotein mixtures or to a variation of solvents used to solubilize apoproteins. Therefore, the secondary structures of apoproteins in LDL are not known.

Concerning the interfacial properties of LDL, Shenton (*9*) suggested that LDL break at the oil−water interface, the lipid core should coalesce with the oil phase, and apoproteins and phospholipids should spread at the interface. Apoproteins (*20*, *21*) or phospholipids (*22*) could be most responsible for LDL emulsifying properties. The destructuration of LDL could be explained by a decrease of proteins−proteins interactions (*9*).

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However, other authors think that the interfacial adsorption of LDL should be due to proteins−lipids complexes existing in LDL structure and their interactions with the oil−water interface (*23*, *24*).

A recent study (*25*) was realized at the air−water interface, using Langmuir balance, to compare films of LDL with films of neutral lipids, phospholipids, and total lipids extracted from LDL. During the compression of LDL film, three transitions were observed out of which two were determined by comparison with the compression of pure lipid films. The transition observed at 19 mN/m corresponds to the collapse of neutral lipids, and the transition at 54 mN/m corresponds to phospholipid collapse. LDL being composed of three constituents, the transition observed at 41 mN/m was attributed, by deduction, to the apoproteins. These apoproteins being insoluble, it was not possible to study them separately. The three constituents spread to form an interfacial film, and the compression induces their collapses sequentially. So, this study has confirmed the disruption of LDL at the interface.

At the oil−water interface, the interfacial properties of LDL should be attributed to apoproteins and phospholipids adsorbed at the interface, because neutral lipids should coalesce with oil droplets (*9*). However, in the air−water interface case, these properties should be due to the three constituents that coexist at the interface (*25*). Consequently, to better understand these differences, we studied the adsorption mechanism of LDL at the air−water interface and we compared the LDL film spread at the air−water interface with that spread at the oil−water interface.

MATERIALS AND METHODS

Low-Density Lipoproteins. LDL were extracted from hen egg yolk and purified according to the method of Anton et al. (*26*). Fresh hen eggs were manually broken, and albumen was eliminated. Yolks were carefully rolled on a filter paper (Whatman, Springfield Mill, England) to remove albumen and chalazes adhering to the vitellin membrane. This membrane was then perforated to collect unspoiled egg yolk in a beaker cooled in iced water. Yolk was fractionated into plasma and granules according to the method of McBee and Cotterill (*27*). Yolk was diluted with an equal volume of a 0.17 M NaCl solution and stirred with a magnetic stirrer for 1 h at 4 °C. This solution was then centrifuged at 10 000*g* for 45 min at 4 °C, and the supernatant (plasma) was separated from the sediment (granules). Plasma was again centrifuged under the same conditions for a complete removal of granules. Ammonium sulfate (40% w/w of saturation) was added to plasma, which was then stirred 1 h at 4 °C and centrifuged at 10 000*g* for 30 min at 4 °C. The precipitate was discarded and the supernatant was dialyzed against deionized water for at least 36 h, and then centrifuged at 10 000*g* for 30 min at 4 °C. The resulting floating material containing LDL was pooled. The floating material was dissolved in a 0.05 M Tris buffer (pH 7) and applied on a glass column (4.5 cm × 100 cm) of Ultrogel AcA 34 (Sepracor/IBF, Villeneuve-La-Garenne, France) to separate LDL and livetins. LDL eluted in the exclusion volume, contrary to livetins that delay in the gel. Optical density at 214 nm was recorded to monitor the effluent from column. The protein concentration in LDL solution was determined using the Markwell method (*28*).

Extraction of Lipids from LDL Egg Yolk. Total lipids were extracted from LDL solution. One volume of LDL solution was added to one volume of a 0.12 M NaCl solution and two volumes of a mixture of hexane/2-propanol (3:2 v/v) and then centrifuged at 1000*g* at 10 °C for 10 min. The upper organic phase was stored, and two additional extractions were carried out on the lower aqueous phase. The organic phases resulting from the three extractions were pooled, and the solvent was evaporated.

The extracted total lipids were separated in neutral lipids and phospholipids following Juaneda and Rocquelin's method (*29*). Total lipids were dissolved in dichloromethane and then applied on a silica Sep-Pack column (Waters, Millipore, France). Neutral lipids were first eluted with chloroform, and phospholipids were eluted second with methanol. Solvents were finally evaporated, and the samples were dried under a nitrogen stream.

Extraction of Apoproteins from LDL Egg Yolk. The LDL solutions were extracted with diethyl ether−ethanol (1:3 v/v) for 12 h at −20 °C and centrifuged at 1500*g* for 10 min at −20 °C. The solvent was removed, and the precipitate was extracted again with several volumes of diethyl ether−ethanol for 10 min, and then centrifuged in the same conditions. Three extractions were carried out. The protein mixture was dissolved in a 0.05 M sodium dibasic phosphate buffer, pH 12, by ultrasonication during 30 min. To check the protein mixture composition, we compared its SDS-polyacrylamide gel electrophoresis (10% acrylamide) with LDL electrophoresis and obtained the same bands.

Liposomes and Emulsion Containing Extracted Total Lipids. We prepared liposomes of phospholipids and emulsion of extracted total lipids by ultrasonication. Lipids, in dichloromethane solvent, were deposited in 1 mL tubes. The solvent was then dried under a nitrogen stream. We added 0.05 M Tris buffer, pH 7, to these lipid films and waited overnight at room temperature for hydration. After a vortex agitation of 2 min, we ultrasonicated 15 min at 15 W in continuous mode for phospholipids and 30 min for total lipids in ice-cold water. The suspensions were then centrifuged 15 min at 2500*g* at 10 °C to avoid possible lipid aggregates or titan particles coming from the sonication probe. We obtained liposomes at 1 g/L of phospholipids and emulsion at 1 g/L of total lipids.

Particle Sizing Measurements. We measured the hydrodynamic diameters of liposomes and oil droplets of emulsion containing total lipids by dynamic light scattering (DLS) using a Malvern Nanosizer Instrument (Malvern, UK). Samples were deposited into a 1 cm spectroscopic plastic cell and then transferred to the Nanosizer thermostated at 20 °C. At each condition, the hydrodynamic diameter was measured in triplicate. Each diameter measurement corresponds to five autocorrelation functions recorded during 20 s.

Isotherm Measurements at the Air−Water Interface. Surface tension isotherms of spread interfacial layers of LDL, extracted apoproteins (Apo), and mixes (Apo−PL and Apo−NL) were studied at pH 3 or 7. Surface pressure versus surface area isotherms were performed at the air−water interface on a Langmuir trough apparatus (Nima Technology Ltd., Coventry, England) with a compression ratio of 11.7. The trough was filled with a 0.05 M Tris buffer solution, pH 7, or 0.05 M glycine buffer solution, pH 3. The samples were spread with a microsyringe (Hamilton) on the surface of these solutions. After being spread, samples were allowed to stand 1 h for equilibrium before compression. Isotherms were recorded at 20 °C at a compression speed of 76 cm^2 min^{-1}. We deposited on the maximum available area of 700 cm^2, 70 μL of LDL solution at 0.25 g protein/L in 0.05 M Tris buffer solution, pH 7, or 0.05 M glycine buffer solution, pH 3. For the Apo samples, we deposited 12 μL of apoproteins at 5.6 g/L in 0.05 M sodium dibasic phosphate, pH 12, in 0.05 M Tris buffer solution, pH 7. For the Apo−NL and Apo−PL mixtures, first 12 μL of apoproteins at 5.6 g/L in 0.05 M sodium dibasic phosphate, pH 12, was deposited, and second phospholipids or neutral lipids in dichloromethane solution were deposited, respecting their mass proportions in LDL (Apo = 15% w/w, NL = 63% w/w, and PL = 22% w/w). These samples were deposited on pH 7 buffer solution, and LDL solution and Apo−PL mixture were deposited on pH 3 and 7 solution. We deposited 90 μL of total lipids at 1 g/L in emulsion or in dichloromethane solution, and 25 μL of phospholipids at 1 g/L in liposome form or in dichloromethane solution on pH 7 buffer solution to study LDL film spreading. Measurements were carried out in triplicate, and the mean standard error was ±0.5 mN/m.

Interfacial Tension at the Oil−Water Interface. The interfacial tensions were measured using an IT Concept drop tensiometer (Longessaigne, France). The two samples were in a quartz cuvette (4 × 2 × 1 cm) thermostated at 20 °C. We used purified sunflower oil. The oil drop volume was 6 μL. The LDL solution was fixed at 5 μg protein/mL in 0.05 M Tris buffer solution, pH 7. Once equilibrium interfacial tension was reached, then the pH was adjusted at 3 and the

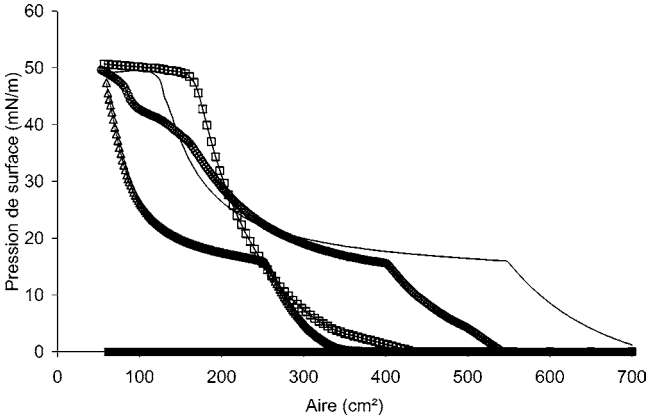

Figure 1. Compression isotherms at pH 7, at the air–water interface, films obtained by deposit, on the surface of the same quantity of total lipids in emulsion (△) and in dichloromethane solution (thin line); and of the same quantity of phospholipids in liposomes (thick line) and in dichloromethane solution (□); and LDL solution (○).

new equilibrium interfacial tension was obtained. At the end, the pH was readjusted to 7 and the final equilibrium interfacial tension was measured.

Another LDL solution was fixed at 5 μg protein/mL in 0.05 M glycine buffer solution, pH 3. The same scheme of pH modification was followed (pH 3, then pH 7, and then pH 3). For each interfacial film, the three measurements were realized on the same film having undergone pH modifications. Measurements were carried out in triplicate, and the mean standard error was ±0.8 mN/m.

RESULTS

LDL Spreading Mechanism at the Air−Water Interface. We compared the spreading of total lipids in emulsified form to that in dichloromethane solution, and the spreading of phospholipids in liposome form to that in dichloromethane solution, by measuring their compression isotherms obtained by Langmuir balance (**Figure 1**). The total lipid isotherms in emulsified form or in dichloromethane solution were similar, but the spreading of total lipids deposited in emulsion was delayed as compared to that of total lipids deposited in dichloromethane solution.

Concerning the phospholipids, in the form of liposomes they have not modified the surface pressure, whereas phospholipids solubilized in dichloromethane exhibited a compression isotherm significant of a correct spreading (**Figure 1**). This indicates that they were not disrupted and that they did not spread at the air−water interface. It is likely that liposomes have plunged in the water during the deposit. We checked that, after a long time of equilibrium (3 h), the surface pressure climbed out, suggesting a diffusion and a subsequent adsorption at the air−water interface (data not shown). Consequently, the presence of phospholipids alone on the surface of liposomes is not sufficient for the disruption and spreading of the structure. Apoproteins should have an important role in the LDL adsorption, like a first step of anchorage at the interface precipitating the disruption of the structure.

Determination of the Second Transition. In this section, we wanted to determine more exactly the second transition (at 41 mN/m), which had been attributed by deduction to the apoproteins. The air−water interface isotherms of apoprotein alone or in a mixture with phospholipids or neutral lipids, respecting the natural proportions occurring in LDL, Apo = 15% w/w/PL = 22% w/w or Apo = 15% w/w/NL = 63% w/w, were compared to the LDL isotherm. To facilitate the detection of transitions in the different isotherms, we calculated the elastic

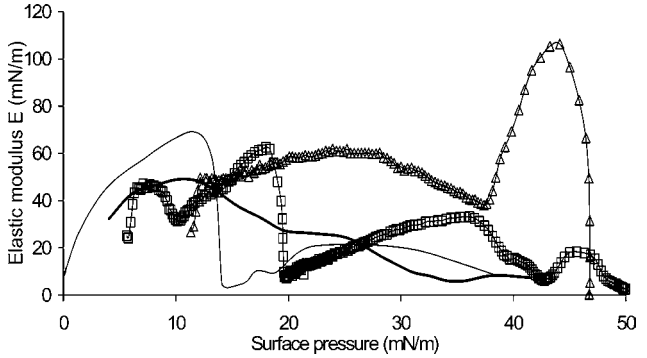

Figure 2. Evolution of elastic moduli against surface pressure of films of LDL (□), apoproteins (thick line), Apo–NL mixture (thin line), and Apo–PL mixture (△). These moduli were calculated from derivation of compression isotherms: $E = dA/d \ln P$, where dA is an average of area difference and d ln P is the neperien logarithm difference of surface pressure. The Apo–NL and Apo–PL mixtures were deposited in the same LDL proportions (Apo = 15% w/w, NL = 63% w/w, and PL = 22% w/w).

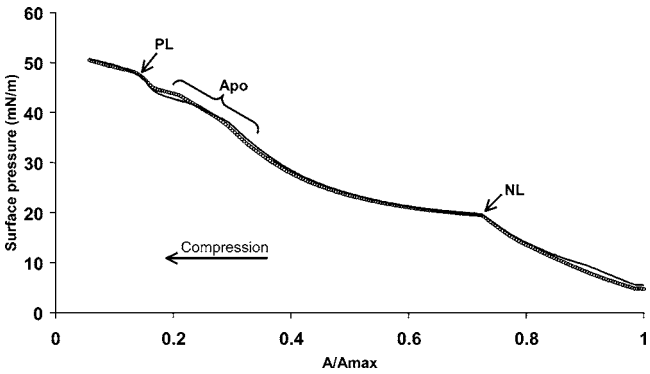

Figure 3. Compression isotherms of LDL spread films measured using Langmuir film balance. The surface pressure of these films was recorded at pH 7 (line) and pH 3 (○) as a function of area/area max. The LDL film isotherms exhibit three transitions indicated by arrows: NL (neutral lipids), Apo (apoproteins), and PL (phospholipids). Arrow indicates compression way.

moduli ($E = d\pi/dA$) because the transitions of apoproteins were not accentuated. We compared the top peaks observed on the curves $E = f(\pi)$ (**Figure 2**). The apoproteins present a main transition at 11 mN/m when they were alone, at 25 mN/m in mixture with neutral lipids, and at 25 mN/m in mixture with phospholipids, whereas LDL present three transitions at 18, 36, and 41 mN/m in LDL. The apoproteins alone have collapsed at surface pressures lower than when they were in a mixture with phospholipids, neutral lipids, or in LDL. Consequently, this transition should not be due to apoproteins alone, but rather to apoproteins−lipids complexes.

LDL Spreading at the Air−Water Interface, at pH 3 and 7. To characterize the LDL spreading, we deposited the same quantity of LDL at the surface of the Langmuir trough filled with buffer solutions at pH 7 or pH 3. The films formed at the two pH's were then compressed. The two isotherms (**Figure 3**) showed the three transitions generally observed (*25*), which confirms LDL spread at the interface and all of the LDL constituents present at the interface. We found that isotherms were quasi-similar, whatever the pH used. Therefore, in our case, the pH seems to not modify spreading properties of LDL forming the air−water interfacial film.

LDL Films at the Oil−Water Interface at pH 3 and 7. We also studied LDL at the oil−water interface using a drop

Table 1. Variations of Equilibrium Interfacial Tensions (γ_{eq}) against pH on LDL Interfacial Films at the Oil–Water Interface[a]

		pH of LDL film formation	modification of this pH	return to initial pH
film formed at pH 7	pH	pH 7	pH 3	pH 7
	γ_{eq} (mN/m)	3.5	6.5	3.5
film formed at pH 3	pH	pH 3	pH 7	pH 3
	γ_{eq} (mN/m)	9.5	3.5	3.5

[a] Two films were formed by LDL diffusion in the aqueous phase at pH 7 and pH 3. Modifications of pH were realized on each film. In these two cases, the interfacial tension was measured at pH of film formation, after modification of this pH, and after return to initial pH.

tensiometer. LDL interfacial films were formed by LDL diffusion and adsorption from two aqueous phases at pH 3 and pH 7. The evolutions of their equilibrium interfacial tension, γ_{eq}, as a function of pH modifications in the aqueous phase, were followed (**Table 1**). In the case of LDL film formed at pH 7, the equilibrium tension is of about 3.5 mN/m (±0.5 mN/m). It increased significantly during the pH modification from pH 7 to 3 and went back to its initial equilibrium tension when the pH returned to 7. Consequently, this reversibility is significant of the absence of loss of surfactant agents from the interfacial film at the end of the pH modification.

For LDL film formed at pH 3, the equilibrium interfacial tension is of about 9.5 mN/m. During the pH modification from pH 3 to 7, this tension decreased significantly to 3.5 mN/m and remained steady when the pH returned to 3. This tension being below the initial tension, we deduced that there was no surface agent lost during this experience; otherwise the tension should have been superior.

We compared the equilibrium tensions at pH 7 and pH 3 on each film separately and concluded that LDL have decreased this tension more at pH 7 than pH 3 for an equivalent quantity of LDL adsorbed. So, at the oil−water interface, LDL spread more at pH 7 than at pH 3.

DISCUSSION

Apoproteins Are Necessary for LDL Spreading at the Air−Water Interface. The different LDL constituents and their organization could occur in the LDL adsorption mechanism. In this work, we specifically studied the role of each constituent. We first deposited total lipids in dichloromethane solution or in emulsion form, and phospholipids in dichloromethane solution or in liposome form. The emulsion and the liposomes have a spherical structure surrounded by phospholipids. Furthermore, the hydrodynamic diameters obtained (50 nm for liposomes and 100 nm for oil droplets of total lipids) were close to that of LDL (30 nm). The main differences between emulsion droplets or LDL, on one side, and liposomes, on the other, are the presence of surface apoproteins and of a neutral lipid core. So, we can suggest that the apoproteins present on the LDL surface initiate the LDL disruption mechanism by their initial anchorage and denaturation leading to the destabilization of the external layer of the LDL. This phenomenon could then be followed by a deformation of the particle due to the formation of a neutral lipid lens conducive to the spreading of the LDL constituents.

Consequently, we can suggest, in the same way as Martinet et al. (*25*), that the kinetics of LDL film formation at the air−water interface is governed by a diffusion process of spherical LDL into the bulk phase, and then an irreversible transformation process at the interface, changing intact LDL into surface tensioactive structures of aggregates and/or individual protein and lipid molecules. We can add that the LDL structure allows the transport through the aqueous phase, thanks to the low density provided by the neutral lipid core, and to the interface, of these amphiphilic constituents normally insoluble in their molecular form in water. The initial anchorage by apoproteins then initiates the spreading of LDL.

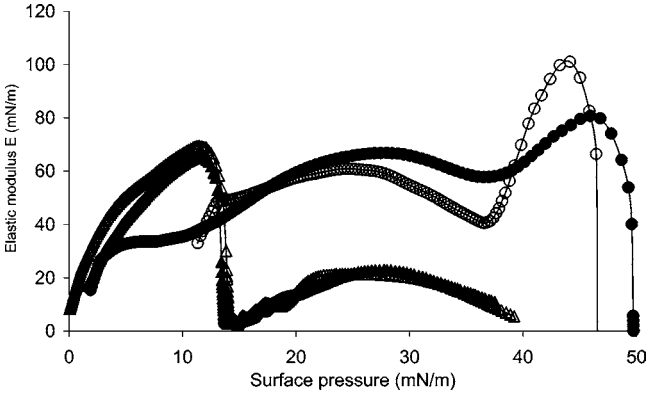

Figure 4. Evolution of elastic moduli against surface pressure of films of Apo–PL mixtures at pH 7 (○) and pH 3 (●) and films of Apo–NL mixture at pH 7 (△) and pH 3 (▲). These moduli were calculated from derivation of compression isotherms: $E = dA/d \ln P$, where dA is an average of area difference and $d \ln P$ is the neperien logarithm difference of surface pressure. The Apo–PL mixtures were deposited in the same LDL proportions (Apo = 15% w/w and PL = 22% w/w).

Comparison of LDL Films at the Air−Water and Oil−Water Interfaces. We observed in this study that the pH (3 or 7) does not modify the spread of LDL at the air−water interface, whereas, at the oil−water interface, the LDL spread more efficiently at pH 7 than at pH 3. Concerning this last experiment, we have to mention that the films were formed from LDL diffusion. Consequently, we did not know the exact quantity adsorbed at the interface, and the comparison between these two films with compositions and/or quantities of materials that may be different could be subject to discussion. So, we preferred to compare different conditions for each film.

Our hypothesis is that, during the pH modification from pH 3 to pH 7, the LDL film should collapse, preventing its modification during the initial pH return. We deduced that, at pH 3, LDL spread less than pH 7. The film being formed at pH 3, when the pH increases to 7, LDL should spread more due to charge modifications, but the LDL quantity on the interfacial surface being not modified, the film should collapse and this phenomenon should be irreversible. In the case of the Apo−PL mixture deposited at the air−water interface, we observed an important hysteresis after the phospholipid collapse, during film relaxation (data not shown). The complexes formed should not disrupt instantaneously to come back to their initial state. So, it seems that these phenomena should be similar between these two interfaces.

The LDL film spreading at the oil−water interface was close to that of the Apo−PL film at the air−water interface. The calculations of elastic moduli of Apo−PL and Apo−NL isotherms (**Figure 4**) have shown a shift of apoproteins and phospholipids transitions between pH 7 and pH 3 in the Apo−PL mixture, but similar apoproteins and neutral lipids transitions, whatever the pH, in Apo−NL mixture. These results were similar to those obtained with the difference of LDL spreading at the oil−water interface depending on pH. Moreover, our results were obtained in the same way as those of Shenton (*9*),

suggesting that apoproteins and phospholipids are at the oil−water interface, but not neutral lipids.

So, at the oil−water interface, the interfacial properties of LDL should be due to apoproteins and phospholipids present at the interface, and neutral lipids should coalesce with oil droplets (*9*). However, at the air−water interface, these properties should be due to the three individual LDL constituents that stay at the air−water interface alone or in association (*25*).

Conclusion. In the isotherms of LDL at the air−water interface, the second transition should not be due to apoproteins alone, but to apoproteins−lipids complexes.

At pH 7 and pH 3, LDL spread quasi-similarly at the air−water interface, opposite from the oil−water interface where LDL spread more at pH 7 than at pH 3, similarly to the Apo−PL mixture at the air−water interface.

At the air−water interface, LDL form interfacial films constituted of neutral lipids, apoproteins, and phospholipids, contrary to the oil−water interface where LDL form interfacial films constituted of apoproteins and phospholipids.

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