A correlation between secondary structure and rheological properties of low-density lipoproteins at air/water interfaces

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Abstract The secondary structure of apolipoprotein B-100 is studied within the bulk phase and at the air/water interface. In these “in viro” experiments, infrared reflection absorption spectroscopy (IRRAS) study was performed at the air/water interface while circular dichroism (CD) was conducted in the bulk phase. In the bulk phase, the conformational structure containing a significant amount of β -structure, whereas varying amount of α -helix, unordered structures, and β -sheet were observed at the air/water interface depending on the low-density lipoprotein (LDL) film interfacial pressure. The present IRRAS results demonstrate the importance of interfacial pressure-induced structural conformations on the apoB-100. A correlation between the secondary structure of the apoB-100 protein and the monomolecular film elasticity at the air/water interface was also established. The orientation of apoB-100 with respect to the LDL film-normal was found to depend on the interfacial pressure exhibited by the monomolecular film. These results may shed light on LDL’s pivotal role in the progression of atherosclerotic coronary artery disease as demonstrated previously by clinical trials.

Keywords Atherosclerotic disease · Apolipoprotein B-100 · IRRAS · Langmuir monolayer

1 Introduction

The low-density lipoproteins (LDL) are spherical particles organized into two major compartments, a hydrophobic core, comprised primarily of cholesteryl esters, and triglycerides surrounded by a surface monolayer of phospholipids with free cholesterol and a unique copy of apolipoprotein B-100 (apoB-100) as a major protein constituent [1–3]. ApoB-100 is one of

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the largest proteins known, containing about 4563 amino acid residues with molecular mass of about 515 kDa with a mean diameter of 17–60 nm [4]. It is also known that a single copy of the apoB-100 is wrapped around the LDL particle surface [4]. ApoB-100 is a single-chain protein with sites of hydrophobicity immersed in the lipid phase to facilitate molecular transport and targeting in a hydrophilic environment [5]. Since it is not soluble in aqueous media, this led to hinder the understanding of structural basis of apoB-100 function, although its primary structure is already known [6–8].

Chemical and physical changes of the LDL particles, due to oxidation, are associated with the formation of macrophage cells that initiate cell foams in blood vessels [8]. Thus, the accumulation of these potent materials induces secondary structure modifications of apoB-100 and an increase in the endothelial cells' stiffness [9]. Then, as a result of these subsequent biological processes within the artery inner membrane, an abrupt increase in blood pressure appears [8, 9]. The latter process progresses further to atherosclerotic plaques in the vascular membrane arterial wall [8, 9]. This in turn affects the apoB-100 secondary structure by modifying the inner artery layer elastic properties [10].

Effects of temperature, ionic strength, or lipid composition have been long known to change the apoB-100 secondary structure (reviewed in [11]). These particular effects were studied extensively using bulk and interfacial infrared (IR) technique [11]. For example, Scanu et al. have firstly applied IR spectroscopy to study thermal effects on secondary structures of the apoB-100 [12]. On the other hand, another thermal effects study by Coronado-Gray and Antwerpen using CD spectra showed that 25% α -helix spans the secondary structure of apoB-100 [4]. Later, many studies have been carried out to study the effects of different environmental parameters like ionic strength or lipid compositions on apoB-100 conformations using different techniques [13–15]. However, thermal dependence-structural studies of LDL have gained more attention on bulk properties than the interfacial conformations of the apoB-100 film at the air/water interface [4, 12]. Few experimental works have been devoted to studying pressure effects on the secondary structure of apoB-100 proteins at the air/water interface [12–14]. Yet such studies may infer valuable information about the pathological effects because increasing the LDL film interfacial pressure may simulate the artery blood pressure increase [16, 17]. In the present work, we investigate the effect of interfacial pressure on the secondary structure and elasticity of apoB-100 at the air/water interface mimicking the interior pressure sustained within blood vessel's arterial walls [9, 16]. We employ the well-known IRRAS and CD techniques to pursue these experiments. This simple biological interface, which models the properties of LDL-particles at the endothelial cell interface, may shed a light on biological and clinical trials to understand at least some aspects of atherosclerotic disease risk [16]. Also, a correlation between the apparent apoB-100 secondary structure and LDL film elasticity at the air/water interface will be discussed in detail.

2 Materials and methods

2.1 Materials

Low-density lipoprotein (LDL) in solution was purchased from Sigma-Aldrich (Germany). Solution of LDL in 150 mM NaCl and 0.01% EDTA, pH 7.4 was shipped in a wet ice container and stored between 4 and 8 °C until used. The LDL solution was used without further purification. Tris-HCl was also purchased from Sigma Chemical. All reagents and

solvents used in this study were of analytical grade, which were also obtained from Sigma-Aldrich. Water was purified with a Millipore desktop system.

2.2 Monolayer preparation for interfacial pressure and IRRAS measurements

LDL solution was dissolved in 0.15 M NaCl with 0.01 EDTA and adding an appropriate amount of 0.05 M Tris-HCl buffer solution (maintaining pH 7.4) at a concentration of 0.3 mg/ml for both pressure–area (π -A) isotherms and IRRAS measurements. In all experiments carried out in this study, the subphase was an aqueous buffer solution of the same composition as mentioned above.

2.3 π -a isotherms

The Langmuir troughs, monolayer deposition, and compression method was used to measure the surface pressure (π) utilizing the well-known method of Wilhelmy balance [R&K, Germany]. The setup consisted of a computer-interfaced film balance yielding digital data of the interfacial pressure in units of mN/m. Reproducibility of the results was checked by repeating each measured curve three times. The measured curves were almost superimposed with a standard error of ± 0.6 mN/m. Before each measurement, the trough was cleaned thoroughly with chloroform and pure water. Also, before spreading the LDL solutions, cleanness of the bare air/water interfacial pressure was always checked to be at zero pressure. For all measurements, a fixed amount of LDL solution (30–50 μ l) was gently spread at the air/water interface with a micro-syringe (Hamilton, USA) Hamilton Robotics, Reno, NV 89502 U.S.A. over the maximum surface area (10×30 cm²) provided by the trough. The LDL film was allowed to relax for at least 1 h prior to monolayer compression at an rate of 0.25 cm².s⁻¹. The subphase temperature was held at 25.0 ± 1 °C by a water circulation bath (Model RK20).

2.4 Secondary structure of the LDL: IRRAS and CD studies of monolayer and bulk phases

2.4.1 Polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS) measurements of the LDL monolayer

A home-built Teflon trough was used to monitor simultaneously the interfacial pressure and the IRRAS spectra of the spread LDL films. The same experimental conditions were also applied here as mentioned above Section 2.2. Then, the monolayer was compressed until the desired surface pressure was reached. Then another 30 min was permitted for protein film relaxation before IRRAS spectra were collected. PM-IRRAS spectra were recorded using a Bruker IFS66 spectrometer (Bruker, Karlsruhe, Germany) equipped with an external MCT (Hg-Ca telluride) detector. A p-polarized beam at an angle of 62° with respect to the surface normal was used. Spectra were recorded with a spectral resolution of 4 cm⁻¹ and collected using 200–400 scans during 5–10 min. It is worth noting that measurements at two light polarizations (p & s) and at different incidence angles may distinguish between distinct protein conformations. Therefore, measurements using p-polarized light at an angle of incidence equal to 62° is performed in order to maximize the amount of reflected light from the monomolecular film at the air/water interface when compared with the s-polarized light, (see [18] and the references therein). The spectra from buffer solution were subtracted from the sample spectra

in order to eliminate the water vapor signal. To reduce relative humidity fluctuations, the setup was placed in a glass-sealed container. Details of the experimental setup and the data acquisition can be found in [19].

2.4.2 Circular dichroism spectroscopy

Circular dichroism spectroscopy is known as a powerful tool in structural biology to determine the possible structures of different proteins, peptides, or polypeptides [20]. This method has emerged as an essential and effective method for examining a protein's secondary structure in aqueous media or when interacting with other molecules in solutions. Thus, changing the protein's secondary structure may lead to a change or a shift in both the position and intensity of CD bands [21].

CD spectra were collected using a Jasco Model J715 Circular Dichroism Spectrometer using a 0.5-mm path-length cell. The LDL samples were prepared in the buffer solution approximately at a concentration of 0.3 mg/ml. All measurements were performed at room temperature. The CD spectra were measured in the wavelength range of 265–190 nm. Similar CD spectra were obtained for the buffer solution alone and used to correct the baseline of apoB-100 spectra. The molar ellipticity ϵ was determined according to the following equation:

$$\epsilon = \frac{MRW \times \phi_{\lambda}}{10 \times L \times C}$$

where MRW is the mean amino acid residue weight of the protein, ϕ_{λ} is the measured ellipticity at wavelength λ , L is the path length of the cuvette, and C is the protein concentration [20, 21].

2.4.3 CD and IRRAS data analysis

After background subtraction, spectra were baseline-corrected and smoothed with two points Savitsky–Golay routine using Origin software. IR position and band area were determined by calculating the area under each curve centered at a certain IR-peak between two minima.

3 Results and discussion

3.1 Bulk phase CD spectroscopy

Typical CD spectra of LDL in H₂O buffered solution are shown in Fig. 1. Data were collected at room temperature, well below the phase-transition temperature of the LDL particles in the range of 260–190 nm. In this buffered solution, the CD spectrum possessed a shallow minimum at 217 nm and maximum at 196 nm, suggesting that the major conformations of the protein are random or β -structures [20]. Fitting the CD spectra using Brahm's basis set [21, 22] reveals approximately $31\% \pm 5\%$ random coil, $21\% \pm 5\%$ β -sheets, and $32\% \pm 5\%$ β -turn with up to 16% α -helix structure. These results are consistent with the previously collected data on both native and oxidized LDL apoB-100 [3, 4, 12]. In the following, these results will be discussed in light of the available literature data on the effects of temperature, oxidation, and ionic strength on the secondary structure changes of apoB-100 [3, 4, 12].

Coronado-Gray and Antwerpen [4] have shown that gradual heat of LDL particles gradually altered the apoB-100 secondary structure, while further heating above the phase-transition

Fig. 1 CD spectra of LDL in a buffered solution (150 mM NaCl, and 0.01% EDTA, pH 7.4) at a concentration of 0.3 mg/ml. Measurements were performed at $T = 25\text{ }^{\circ}\text{C}$

temperature leaves the apoB-100 secondary structure unchanged up to $T = 45\text{ }^{\circ}\text{C}$. This group has investigated the bulk secondary structure of apoB-100 in two types of LDL particles, namely normal LDL (N-LDL) and triglyceride-rich LDL (T-LDL). Their result shows that above the phase-transition of apoB-100 core, the apoBs of N-LDL and T-LDL had a similar secondary structure. Li et al. used the multiple-interaction model to study the conformation of apoB-100 when exposed to surfaces containing saccharides and alkanesulfonate ligands [21]. Their CD analysis revealed that the α -helical structure of apoB-100 in glucose solution has decreased to 34.2% compared to 39.3% for LDL dissolved in buffer solution only, with a constant proportion of β -strands in both solutions. Also, the percentage of turns and unordered structures was increased in glucose solution compared to buffer solution. Their study has shown that a decrease in α -helix structure and an increase in β -turns and unordered structures when added to a saccharide solution indicated a destruction of the highly ordered α -structures as a result of the interactions between LDL and saccharides. On the other hand, Sneek et al. [14] have examined conformational changes of apoB-100 of large aggregates of SMase-modified LDL at acidic pH. This study mimicked the extracellular pH of atherosclerotic lesion decrease during atherogenesis. The effect of SMase treatment on the conformational changes of apoB-100 at pH 5.5 and 7.50 was studied by CD, revealing that the conformational changes of apoB-100 are required for particle aggregation and implying that changes in LDL lipid composition are responsible for these structural changes. These observations can be linked to the increase of surface hydrophobicity of the modified LDL particles. Another study of Parasassi et al. [23] has revealed the possible loss of apoB-100 secondary structures and conformations as a result of oxidation in a hydroperoxide-rich environment. Their analysis of the total CD spectra indicated a large amount of α -helix ($\sim 40\%$) and smaller content of β structures ($\sim 13\%$) and about 27% random coils were obtained. These data showed LDL particles containing different structures of a denatured apoB-100 are hidden in the lipophilic environment. Also, these structural changes were due to the penetration of hydroperoxide into the outer LDL surface, thereby strongly altering the lipid packing and its physical state. Another attempt to predict the possible LDL oxidation by oxidative stress like Cu^{2+} is presented by Brunelli et al. [24]. They have proposed a unique mechanism through stabilizing apoB-100 conformation by the antioxidant 17- β -estradiol (E2). This has emerged in the context of possible protection exerted by estrogens against cardiovascular disease. They used a 1:6 molar ratio of apoB-100 to E2 as an optimum experimental condition for a maximum resistance oxidative agent. At this ratio, their results provided evidence that is originated from the conformational changes of apoB-100 upon interacting with the lipidic surface of LDL

particle. Moreover, the outcome of this study is summarized as follows: the CD measurements revealed that apoB-100 undergoes a conformational modification and progressive loss of secondary structure during lipid peroxidation, while the increase of E2 modifies and increases apoB-100 secondary structure and the presence of E2 makes this protein more resistant to structural modifications [24].

Analogous to the above results, human apolipoproteins AII, CII, E3, and AI, were also studied by means of CD [25]. These results indicated a well-preserved relationship between the secondary structure of the amphipathic α -helices and their ability to recover their native secondary structure after thermal denaturation. The pressure–area isotherms obtained in this study with direct observation of the monolayer with a Brewster angle microscope of these apoproteins monolayers at the air/ionic water subphase revealed that a phase-transition occurs in all of them, even at high lateral interfacial pressures.

As in many previous reports, apoB-100 has always been proposed to wrap around the LDL particle; then its conformations and binding properties can change in relation to particle shape and size. These changes are associated with the altered apoB-100 conformations that must accommodate coverage of increasing interfacial area effects along with decreasing LDL particle size. The above results indicate clearly that the structural conformations of apoB-100 on the LDL surface depend more strongly on the physio-chemical state of the lipoprotein core [1–6]. Next, we examine the physical effects of the interfacial pressure on the elasticity of the LDL monolayers at the air/water interface.

3.2 Pressure–area isotherms of LDL/elasticity of LDL particles

It is known that apoB-100 is not soluble in water, remaining at the interface when spread on a buffered subphase, which in turn forms a stable Langmuir monolayer at the air/water interface [13, 15, 26]. Interfacial π -A isotherms for the LDL monolayer on a buffered subphase are depicted in Fig. 2. Several features are clearly recognized from this isotherm. The region of 15 mN/m is a typical behavior of the low compressibility condensed phase [25, 26]. On further compression of the LDL film and upon a pressure increase, we find a wide plateau between 35 and 39 mN/m; however, the LDL-monolayer does not show a collapse, where a typical second phase transition appears. This observed Phase transition occurs at to $2.50 \times 10^3 \text{ nm}^2/\text{LDL}$ particle, and an inflection point is reached at about 39 mN/m. We noticed that when increasing the number of LDL particles in the monolayer above ca. $8 \times 10^3 \text{ nm}^2/\text{particles}$, the gas-condensed phase co-existence region appears. This behavior could be explained analogous to Gibbs monolayers at the air/water interface by protein solubilization into the subphase buffer solution. However, this seems not to be the case, because an LDL film of $3 \times 10^3 \text{ nm}^2/\text{particles}$ left for more than 1 h at interfacial pressure of 33 mN/m exhibited a net loss at of only 5 mN/m. This means that the loss of the interfacial pressure is negligible for the current compression times. Also, keeping the protein at the same concentration in the monolayer, we have performed cycles of compression up to $\pi = 30 \text{ mN/m}$, then expansion down to $\pi = 0 \text{ mN/m}$, and further recompression back to 30 mN/m (data not shown). We found that the new isotherm preserved the common feature of the one obtained before the compression-expansion procedure but did not retrace exactly the existing isotherm. This hysteresis phenomenon is very common in the Langmuir monolayers technique at the air/water interface where some LDL particles may sink in the subphase as a result of the compression cycle. Results of LDL is similar to other human apoproteins (AII, CIII, E3 and AI) isotherms as presented by Bolanos-Garcia et al [25]. For smaller areas available for the proteins, the pressure rapidly decreases and

a film collapse at an extreme value of 42 mN/m is observed. This suggests that a high percentage of LDL particles remain at the air/buffer interface. Dauphas et al. [13] have studied the structure of LDL-hen egg yolk film at the air/water interface. Their isotherms suggested three transitions at $\pi = 19, 41$, and 51 mN/m, which corresponds to neutral lipid, apoprotein-lipid, and phospholipid, respectively. Also, their AFM images of the film structures on mica substrate for different pressure revealed that for low interfacial pressure (i.e., $\pi = 22$ mN/m), the film was homogenous, whereas at intermediate pressure (i.e., $\pi = 30$ mN/m), the film becomes heterogeneous with circular domains surrounded by co-concentric domains with irregular edges. Finally, at a very high interfacial pressure (i.e., $\pi > 44$ mN/m) these well-structured images changed into a more heterogeneous morphology and structures composed of smooth and well-circular domains. The same group then studied the structures and rheological properties of LDL extracted from hen egg yolk spread at the air/water interface but with different pH values and pointed out that the ionic strength plays less of a role in the interfacial isotherms of LDL, but a dominant role in their structures as observed by AFM. On the other hand, the condensed phase observed in the current study remains stable even at very high lateral pressures (approx. 42 mN/m). Only after this point, a collapse appears in the monolayer at a limiting area of 1.24×10^3 nm²/LDL particle. The higher collapse pressure, which appears at a very condensed monolayer, indicates that intermolecular interactions between the LDL particles provide a great degree of stabilization of LDL monolayer at air/water interface [25, 26]. From this limiting area, one can deduce the LDL particle size. This is done by assuming that all of the LDL are spherical-like particles tightly arranged on the air/water interface with an estimated mean diameter per particle of 20 nm. This result is in good agreement with many previous results as pointed out in the Introduction [10, 27, 28]. The result indicates that the size of LDL particles is not only affected by the oxidation [10] but also due to its adsorption and compression at the air/water interface; however, the compression elasticity of the LDL film is also likely to be affected by these external stimuli [13, 25, 26]. Johs et al. [27] investigated a 3D model of apoB-100 structure using spectroscopic and small-angle neutron scattering in combination with advanced shape reconstruction modeling to generate a three-dimensional model of lipid-free apoB-100 protein. The detailed analysis of the secondary structure by this unique computational approach has predicted that apoB-100 is composed of five consecutive domains with alternating α -helix and β -strand regions. In their attempts to reconstruct a spherical-like model for LDL particles, they also found that the LDL diameter of the ring-shaped structure roughly corresponds to the diameter of an intact LDL particle on the order of 25 nm. Other topological AFM-based studies on native- and ox-LDL particles on densely immobilized Au surfaces showed that measuring the short and long axis of the observed ellipsoid-like LDL particles lies between 20 and 30 nm [28].

In order to compare the effects of the interfacial pressure on the LDL elasticity, the dynamic elastic modulus C_s^{-1} was calculated from the π -A isotherms using:

$$C_s^{-1} = -A \frac{d\pi}{dA}$$

where $d\pi/dA$ is the slope for each step of π -A isotherm and A is the area per LDL particle at particular pressure. Then, the π -A isotherm of LDL was used to determine the interfacial dilatational elasticity [26]. Elasticity is plotted against selected interfacial pressure values in Fig. 2b.

The elastic modulus–pressure curves at the selected pressure values show an increase in the elasticity up to $C_s^{-1} = 57$ mN/m at $\pi = 20$ mN/m, and then decrease down to $C_s^{-1} = 37$ mN/m

Fig. 2 **a** Interfacial pressure–area isotherm of LDL at the air/water interface. The subphase is the buffer solution mentioned in the caption of Fig. 1. The subphase temperature was kept constant at $T = 25^\circ\text{C}$. A collapse pressure of LDL monolayer appears at $\pi \approx 43$ mN/m. **b** The inverse compressibility as calculated from the pressure–area isotherm. The *solid line* is a guide for the eyes and does not represent any empirical model

at $\pi = 25$ mN/m, pointing to the first transition. After this transition, which was followed by a further decrease in the protein matrix elasticity, the curve reaches the interfacial elasticity minimum of $C_s^{-1} = 15$ mN/m at $\pi = 40$ mN/m, indicative of second phase transition. These elasticity values should be correlated with the apoB-100 secondary structures as discussed below. Along these line of evidence, the elasticity values for the hen yolk LDL speared at the air/water interface are discussed by Martinet et al. [26] in terms of total and individual component of LDL particles as follows. Their results for LDL film elasticity were found to have a value of $C_s^{-1} = 8$ mN/m at $\pi = 20$ mN/m and increase up to $C_s^{-1} = 22$ mN/m at $\pi = 25$ mN/m, then at $\pi = 30$ mN/m a value of $C_s^{-1} = 35$ mN/m. Finally, at a very high of pressure $\pi = 54$ mN/m, a minimum value was found to be 0.1 mN/m [26]. Also, they have investigated the effects of other LDL components on the elastic modulus of the monomolecular film with a final conclusion that the dynamical behavior of the LDL monolayer elasticity originated from the LDL-phospholipid component composing the spherical particles [25, 26]. Next, we proceed to correlate the observed LDL film elasticity with the apoB-100 structural conformations at the interface (see below).

3.3 Influence of interfacial pressure on apoB-100 secondary structure: Peak assignment and band area

To further characterize potential changes in apoB-100 conformational changes during compression, LDL films at the air/buffer interface were investigated by IRRAS at different interfacial pressures as shown in Fig. 3. In general, the amide I and amide II bands are the most informative part of the IRRAS spectra to gain insight into the possible conformations of the secondary structure of proteins and their orientations with respect to the monolayer normal at the air/water interface. In order to explain the different interfacial behaviors of LDL films, we looked for possible differences in the conformations and orientation of apoB-100 at the air/buffer interface at selected interfacial pressures [18, 29]. Since the whole LDL protein was able to form a stable monomolecular layer at the water interface, then the use of PM-IRRAS spectroscopy as described in the Materials and Methods section was a suitable technique to examine the possible changes of the protein secondary structure. From the whole pressure

range, we have selected three pressure values in this particular experimental work to investigate their role on the IR-spectrum. The LDL monolayer showed a clear IRRAS spectrum assigned to an amide I band located between 1700 and 1600 cm^{-1} (mostly a signaling spectrum from $\text{C}=\text{O}$ stretching mode of the carbonyl group of the apoB-100 backbone) centered at 1650 cm^{-1} with a small shoulder at 1631 cm^{-1} and an amide II band located between 1600 and 1500 cm^{-1} (mostly a signaling spectrum from $\text{C}-\text{N}$ stretching and $\text{C}-\text{N}-\text{H}$ bending vibration modes of the apoB-100 backbone) centered at 1540 cm^{-1} (see Fig. 3a). In fact, the peak intensity of a particular frequency of these bands can be correlated with a defined protein conformation [29].

The IRRAS spectra of pure LDL monolayers on the saline buffer at three distinct interfacial pressures are shown in Fig. 3a and a zoom around amide I band is presented in Fig. 3b. On the three spectra shown in the figure, the amide I peak is located at 1650 cm^{-1} and the amide II peak at 1540 cm^{-1} . The band at 1650 cm^{-1} is characteristic for α -helices while and shoulder at 1631 cm^{-1} is associated mostly with the presence of β -sheet structures evolving at the air/buffer interface [18, 29]. Moreover, the occurrence of different peak intensities at amide I and amide II confirms that apoB-100 proteins are present at the air/buffer interface. We have also plotted the three spectra horizontally on the same scale in order to compare their peak intensities and the their bandwidths (see Fig. S1 in supplementary information provided online).

Fig. 3 a The IRRAS spectra of pure LDL monolayer on the saline buffer subphase at three selected interfacial pressures: $\pi = 20, 25$, and 30 mN/m . The subphase temperature was kept constant at $T = 25^\circ\text{C}$. The curves are shifted vertically for clarity (b). A zoom of the IRRAS spectra of the amide I band peak around 1655 cm^{-1} . c Curve deconvolution of amide I band at $\pi = 30\text{ mN/m}$. Similar analysis is applied for other pressures. d The second derivative of IRRAS spectrum pinpoints the position of the different assigned apoB-100 structures

In the whole range of frequencies of amide I band for the three specific pressures $\pi = 20$, 25, and 30 mN/m (see Fig. 3a), different peak intensities were observed, suggesting that the increase of the interfacial pressure induced conformational and orientation changes in the protein backbone at the air/buffer interface [30]. Domon et al. have investigated the case of annexin A6 protein-lipid monolayer, show that increasing the interfacial pressure, in turn, increased the intensities of amide I and amide II with no significant change in their band shape [31]. Next, we show how the spectral analysis of these two important bands may lead to more detailed information about the lipid organization and conformational changes of LDL particles at the air/buffer interface.

IRRAS spectra at $\pi = 30$ mN/m exhibits seven bands assignable to the apoB-100 substructures, which are located at 1696, 1683, 1670, 1650, 1643, 1631, and 1620 cm^{-1} (see Fig. 3c and d). In the case of pressure $\pi = 20$ or 25 mN/m, the IRRAS spectra showed only five bands located at 1696, 1670, 1650, 1643, and 1620 cm^{-1} . A deconvolution analysis of the amide I band of the human plasma LDL apoB-100 for these three pressure values yields the corresponding parameters, i.e., band position and secondary structure percentage for each spectral component. The deconvolution analysis is exemplified for the amide I at the pressure $\pi = 30$ mN/m as shown in Fig. 3c. The second derivative determination of different band assignments is presented in Fig. 3d. A summary of these parameters is given in Table 1.

Our discussion of various band assignments observed in this study is based on the review article presented by Barth and the references therein [29]. The low-frequency bands at 1620 and 1631 cm^{-1} arise from the hydrogen bonds within the β -structure; therefore, it is usually assigned for a particular β -strand and β -sheet respectively. The same type of β -structures are also observed in oxidized LDL by copper ions in buffered solutions [32]. The band at 1650 cm^{-1} is undoubtedly assigned to an α -helix. On the other hand, the bands at 1670 cm^{-1} can be assigned to β -turn structures on the basis of previous data [32]. The higher-frequency bands 1683 and 1696 cm^{-1} are assigned to either a β -sheet or turn as explained in the work of Gallego-Nicasio et al. [32]. As pointed out in this reference, there is some disagreement among different research groups regarding the band components between 1670 and 1696 cm^{-1} in the IR-spectra of proteins or peptides. Theoretical calculations in this particular IR-spectra region indicted a strong presence of antiparallel β -sheets [33], whereas other experimental works assigned it to β -turns as a more realistic contribution for a possible protein conformation [34]. Thus, the mysterious discrepancy in the identification of these bands results in a problematic discussion about these β -structures between various

Table 1 Secondary structure percentage corresponding to the amide I band decomposition of LDL infrared spectrum at $T = 25$ °C in 150 mM NaCl and 0.01% EDTA and 0.05 M Tris-HCl buffer subphase, pH 7.4

Band Position (cm^{-1})	Secondary Structure	$\pi = 20$ mN/m	$\pi = 25$ mN/m	$\pi = 30$ mN/m
1696 ± 3	β -turns	16	10	6
1683 ± 4	β -sheet	-	-	4
1670 ± 5	β -turns	15	12	5
1650 ± 3	α -helix	26	35	38
1643 ± 4	Unordered	21	17	12
1631 ± 4	β -sheet	-	-	8
1620 ± 3	β -strands	22	26	27

scientists in the field. However, the band at 1696 cm^{-1} cannot be assigned to an anti-parallel β -sheet because the high-frequency component does not undergo the isotopic shift typically produced in that kind of structure by changing the pressure from $\pi = 20\text{ mN/m}$ to $\pi = 30\text{ mN/m}$. Then, as discussed in Ref. [32], a more generalized assignment would be β -turns, i.e., extended structures not forming β -sheets. The band observed at 1643 cm^{-1} is usually absent in other proteins or polypeptide assemblies, which is usually assigned to unordered structures. In fact, in non-denatured LDL proteins in buffer solution, this particular band has only been found in apolipoproteins assigned to “low-frequency β -sheets” or to β -structures less accessible to external solution [32].

From a comparison between the results at $\pi = 20$ and 30 mN/m , it is evident that the secondary structure of apoB-100 is affected by the monolayer elasticity. It should be noted that this is the first result that addresses the monolayer pressure effect on the conformation of LDL particles, while the only studies that have been conducted so far are the temperature or oxidation influence on the bulk conformations of LDL particles by different IR techniques [22, 23]. One of our most interesting results is that the surface pressure induced an increase in the α -helix structures while abolishing different β -structures at the air/water interface. As the monolayer is compressed from $\pi = 20$ to 30 mN/m , the amide I component at 1650 cm^{-1} becomes more intense. Simultaneously, the peak at 1631 cm^{-1} gains more intensity at the expense of the other peaks found at $\pi = 20$ and 25 mN/m . It is also worth noting that the interfacial IRRAS at $\pi = 30\text{ mN/m}$ results for the apoB-100 secondary structure conformations show that as the interfacial pressure is increased, some of the LDL particles may accumulate into the subphase by changing their secondary structure conformations in order to adapt to the new environment. These conformational changes adapted by the apoB-100 may hinder or minimize the unfavorable direct contact between the protein's residue and the aqueous solution as a result of the amphiphilic nature of this protein [25].

These results, about the possible apoB-100 conformational changes utilizing the PM-IRRAS technique at the air/water interface, may shed some light on the LDL secondary structures' role in progression of atherosclerotic disease. Here, we shall discuss the existing data from other reports, which are mainly emerging from the FTIR and related techniques. In this regard, Gallego-Nicasio et al. [32] have presented compositional and structural changes of lipids and apolipoproteins during *in vitro* oxidations of LDL by IR spectroscopy. The conformational changes of apoB-100 during Cu^{+2} -induced oxidation was shown in two steps: initially, oxidized LDL generates intermolecular β -structures and later the ordered secondary structures decrease during the oxidation course leading to less-ordered loops and turns as a result of protein fragmentation. A report by Herzyk et al. [35] pointed to qualitative information about the apoB-100 structural changes in native LDL acquired from the second and fourth derivatives of LDL by transmission infrared spectra. These authors concluded that the percentage of the β -sheet structure in apoB-100 is less than the α -helical structure as a result of the oxidation. Later, Goormaghtigh et al. [36] re-investigated the secondary structure of apoB-100 present in LDL by transmission and attenuated total reflection infrared spectroscopy (ATR). Their findings were focused on the amount of β -sheets present in the sample, which turned out to be 41% of the total structures presented in apoB-100 backbone. In fact, in this report, there is no other information about the other possible structures within the protein backbone. A computer analysis of the apoB-100 primary sequence revealed the existence of internal repeats of both amphipathic α -helices and putative β -sheet structures [37]. A decade later, with the identification of LDL oxidation as a key risk factor for cardiovascular disease, studies of structural modifications upon oxidation have become increasingly prevalent. On the

other hand, the early stages of conformational changes due to oxidation have been confirmed using the same IR-technique by the group of Goormaghtigh [38]. Their measurements showed that predominantly β -sheet region exists along the apoB-100 backbone in this early stage of oxidation, later as the oxidation progress it undergoing conformational changes to a β -strand structure. Also, in this type of study the α -helical regions were not discussed or focused on. Very recently, Blume et al. have explored the relationship between apoB-100 secondary structure and its rheological properties [39]. Thermally induced secondary structure changes of apoB-100 were observed by FTIR, focusing on the peak intensities of intermolecular β -sheets in relation to gel formation of egg yolk proteins. These FTIR-rheological measurements demonstrated a clear connection between the temperature onset of the protein secondary structure changes.

To discuss the relationship between the LDL monolayer elasticity and the apparent LDL backbone secondary structure, we proceed in the following way. Several studies including theoretical, experimental, and computational methods have proposed that the amino acid sequence of apoB-100 is composed of an alternating five subdomains of amphipathic α -helical and β -sheet structure (NH_2 - β α_1 - β_1 - α_2 - β_2 - α_3 -COOH) [11–13], with up to 40% β -sheet structures and 25% α -helical domains [11]. These studies have also suggested that the β -sheets of apoB-100 are oriented parallel to the phospholipid monolayer of LDL while the amphipathic α -helices, which contribute to the high affinity of apoB-100 for LDL interface, are not parallel to the air/water interface [12, 13]. Besides these known featured secondary structures usually present in these types of proteins, apoB-100 contains also a unique and well-preserved extended structure characterized by bands centered at 1620 and 1696 cm^{-1} as confirmed by the IR spectra. To elucidate the participation of the compressed LDL particle of different structures towards the monolayer elastic behavior, we look for the influence of the backbone structures along the sequence of apoB-100 upon compression. It is likely that the apolipoproteins' hydrophobicity and affinities are the essential properties that have a significant effect on the observed LDL-monolayer elasticity at the air/water interface because apoB-100 is flexible and contains several β -sheets and α -helical segments along its backbone, which may behave as independent sub-units. Several results from different research groups have pinpointed that hydrophobic forces are the main driving force for the formation of apolipoprotein monolayers at the bare air/water interface [15]. This led us to fact that apolipoproteins contain fewer amphipathic α -helical segments is a contributing factor to the LDL-monolayer elasticity as evidenced from our current data. Also, the data showed that the ability of these proteins to form more α -helical structures, while the percentage of β -structures are almost constant upon compression, which in turn reduces the overall monolayer elasticity. Although it is probable that the α -helical percentage per sequence causes a difference in their affinities toward the monolayer elasticity, the role of their α -helix subdomain orientation along the protein backbone cannot be ruled out in this situation, since the average hydrophobicity and orientation may be affected by compression, which alters the percentage and location of α and β structures along the apoB-100 backbone. Thus, the mixed β -strands or sheets that appeared in the LDL structure, which make an excellent anchor for apoB at the air/water interface, may also contribute to the monolayer's elasticity. Thus, this interplay between the hydrophobicity and structural content of the apolipoproteins may play a critical role in LDL-monolayer elasticity. It is also possible that other helical or sheet amphiphilicity properties, such as length and number of α -helices or β -sheets per protein residue, are important factors when adsorbed at the bare air/water interface. However, these intrinsic features are not the only factors at play, because adsorption at the air/water interface is further modulated by electrostatic interactions

Fig. 4 Representation of the external interfacial pressure effect on the α -helix backbone orientation with respect to monolayer normal. The graph indicates a parallel helical orientation with respect to the LDL film with increasing interfacial pressure

and polar effects as well as entropic penalty when flexible proteins or peptides binds to interfaces.

3.3.1 Future study: Orientation of a single α -helical structure with respect to the monolayer normal

In case of a single α -helix protein, the tilt angle (ϕ) between the mean helix backbone and the interface normal (see Fig. 4) can be estimated from the ratio of the intensity of amide I peak over the intensity of amide II peak [30]. In this case of apoB-100 at the air/buffer interface, for the selected pressure values presented in this study, the AMIDI/AMIDII ratio values are always greater than 3.5. These amide peak ratio values correspond to approximately an α -helix protein oriented parallel to the air/water interface (see Fig. S1 in supplementary information provided online) while they become straightened via the establishment of protein–lipid or protein–protein interactions due to their presence at the interface [30]. A precise value of these tilt angles and pressure values need more experimental work including different effects of phospholipids properties like charge, tail lengths, and chain saturations. Moreover, the effect of cholesterol may also be included to shed more insight into these organization and structural changes at interfaces in connection to atherosclerotic disease.

4 Conclusions

In summary, the structures formed by LDL particles both at the air/water interface and within the spreading buffer have been investigated. The bulk secondary structures in buffered solution were found to be consistent with the available literature data. At the air/water interface, the LDL film seems to form a well-defined and reproducible film. A clear correlation was found between the apoB-100 protein secondary structure and the monolayer elasticity at the air/water interface. We found that the maximum elasticity was obtained at a pressure of 20 mN/m where the apoB-100 exhibited mixtures of α -helices and various β -structures. The transition

observed in the LDL film relative to a molecular rearrangement of the LDL particles in association with the apoB-100 structural conformations could arise for different reasons: it could be a transition to a more complicated secondary structure including a mixture of β - and α -helix structures, or it might be a change in the orientation of the helical backbone with respect to the monolayer normal, as seen from the ratio of amide I and amide II bands of PM-IRRAS spectra. These results may reflect possible evidence of the interfacial pressure in association with the apoB-100 secondary structure changes in cardiovascular risk.

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