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Molecular acoustic as a new tool for the study of biophysical properties of lipoproteins

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Abstract

The method of measurement of velocity and absorption of ultrasound at a fixed frequency (7.2 MHz) and measurement of density were used to study the physical properties of high- (HDL_3) and low- (LDL) density lipoproteins. We found substantial changes in velocity number [u] and absorption number [$\alpha\lambda$] on temperature, which reflect structural changes in the hydrophobic core of LDL at the thermotropic-phase transition. The absorption number revealed broad changes in temperature for both classes of lipoproteins (LP). The density of LP also depends on temperature but in considerably less degree than the acoustic parameters. The values of acoustic parameters were determined, showing that LDL and HDL_3 greatly differ with respect to adiabatic compressibility. © 1997 Elsevier Science B.V.

Keywords: Ultrasonic velocimetry; Ultrasound absorption; Density; Lipoproteins; Adiabatic compressibility

1. Introduction

Lipoproteins (LP) are supramolecular lipid–protein complexes that are responsible for lipid transport in blood plasma. They consist of a hydrophobic core containing mainly cholesterol esters and triglycerides, surrounded by an amphiphatic phospholipid–cholesterol monolayer accommodating the apoprotein components. Impaired LP metabolism in plasma and in cells, which depends on biophysical and biochemical properties of the particles, is associated with an increased risk for atherosclerosis. So far, considerable information on biophysical LP properties has been obtained from various X-ray, spectroscopic and thermodynamic measurements (see Ref. [1] for review). Among a wide variety of LP, two classes have been most intensively studied so far: low-density lipoproteins (LDL) and high-density lipoproteins (HDL_3). LDL are cholesterol- and cholesteryl ester-rich LP and are supposed to be atherogenic. The LDL are the major sterol transporter in the circulation between blood plasma and the cell membranes. More than 50% of the mass of LDL is cholesterol. HDL_3 contains less cholesterol (about 20% of its mass) and is smaller than LDL. It

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is necessary to note that most of the cholesterol (up to 80%) present in LP is esterified with fatty acids. HDL_3 is supposed to be antiatherogenic (see Ref. [1]). The core of HDL_3 is characterised by a radial arrangement of cholesteryl esters in extended conformation into which the hydrocarbon chains of phospholipids and cholesterol are interdigitated. The core of LDL also contains radial layers of cholesteryl esters and triglycerides, but cholesteryl esters and phospholipids do not interdigitate, or interdigitation is considerably less pronounced than in HDL_3. The free cholesterol resides primarily in the phospholipid monolayer of the surface of the LP. X-ray diffraction studies provide evidence that the core of LDL is less ordered compared to HDL_3 [2]. LDL shows a reversible thermotropic structural transition around 30°C [3,4], which is due to a cooperative order–disorder transformation of cholesteryl esters in the apolar core. The temperature of the phase transition in this temperature range depends on the ratio between cholesteryl esters and triglycerides [5]. In addition, observed reversible thermotropic transitions coincide with structural changes in apolipoprotein B, hence, occurrence is between 40–60°C and transition is between 74 and 82°C. The last transition is irreversible and caused disruption of LDL [6,7]. In contrast to LDL, HDL_3 did not show such a thermotropic behaviour of the cholesteryl esters. However, DSC calorimetry studies showed a thermotropic behaviour of apolipoprotein AI located on the LP surface. A reversible transition of this protein has been shown between 43 and 71°C with the mid-point at 54°C [8].

Detailed information about molecular properties of lipids in LP has been obtained using different spectroscopic techniques and X-ray diffraction method [1]. Here, we report on the application of a new macroscopic method — the measurement of the absorption and velocity of ultrasound, to obtain information on biophysical properties of LP that have not been available so far. The method of ultrasonic velocimetry is based on molecular acoustics and could provide new insight into the supramolecular structure of different LP classes. The usefulness of ultrasonic velocimetry for solving various biophysical problems was shown in a review by Sarvazyan [9]. This method was demonstrated to be very effective for the study of mechanical properties of liposomes [10], phase transitions of lipid bilayers [11,12], protein–lipid interactions [13–16], hydration of liposomes [17], determination of partition coefficients [18], and the study of physical properties of nucleic acids [19] and proteins [20]. The method of ultrasonic velocimetry is based on the relation between the velocity of ultrasound, u, density, ρ, and adiabatic compressibility, β, of the 'solvent' [21].

$$u^2 = \frac{1}{\rho\beta} \tag{1}$$

Both density and adiabatic compressibility can reflect the peculiarities of the structure of LP if they serve as the acoustic medium. Density can be measured by an independent method, e.g., an oscillation densitometer [22], while adiabatic compressibility can be determined by means of ultrasonic velocimetry [9]. Parameter β is directly related to the volume compressibility of a given solution or dispersion, e.g., microemulsion particles such as LP. In this work, we have applied ultrasonic velocimetry to study two important classes of human LP — LDL and HDL_3.

In the present work, we were able to show for the first time that ultrasonic velocity and absorption depend significantly on structural and physical properties of LDL and HDL_3 at their physiological concentrations, as well as on temperature.

2. Materials and methods

2.1. Preparation of LP

LDL was isolated from a single plasma sample by fasting normolipemic volunteers as described earlier [23]. Immediately after blood drawing and centrifugation, the plasma was stabilised with EDTA and sodium azide (1 mg/ml) and subjected to density gradient ultracentrifugation in an SW-40 rotor (Beckmann) for 24 h at 40000 rpm [24]. LDL was harvested from a fraction corresponding to densities 1.025–1.055 g/ml of the density gradient and recentrifugated under identical conditions. All buffers and solutions used for LP preparation contained EDTA and sodium azide (1 mg/ml) and were deoxygenated in vacuo after saturation with nitrogen. All purification steps were performed at 4°C and preparations

were used within one week. The purity of the LDL fraction was assayed by double-decker rocket immunoelectrophoresis and SDS polyacrylamide gel electrophoresis as described in Refs. [25–27]. HDL_3 were also isolated from plasma of fasting normolipemic volunteers. In the first step, serum density was adjusted to 1.070 g/ml by adding NaCl followed by ultracentrifugation at 16°C and 100000 g for 18–20 h. Total HDL_3 was isolated by adding solid NaBr to the lower two-thirds of the tubes up to a density of 1.22 g/ml. Ultracentrifugation proceeded for 25 h at 145000 g and 16°C. HDL_3 was subsequently isolated at a density of 1.125–1.210 g/ml [28]. Protein was measured according to Lowry et al. [29] in the presence of 0.5% (w/w) sodium dodecyl sulphate. Buffers were prepared using double-distilled water. Solutions of LDL or HDL_3 were dialysed overnight against 10 mmol/l phosphate buffer (pH 7.4). Low salt concentrations are important for ultrasonic velocimetry, because both ultrasonic velocity and absorption are sensitive to the concentration of salts [9]. Concentration of LP was determined gravimetrically by dry weight after dialysis.

2.2. Measurement of velocity and absorption of ultrasound

Ultrasonic velocity and absorption were measured using a differential fixed-path velocimeter with acoustic resonators [30,31]. Determination of the resonance parameters was based on measuring the midpoints and widths of the phase-frequency curves of the resonance peaks by means of a home-made electronic circuit based on phase-frequency feedback circuits that were computer-controlled. The resonators contained a sample volume of 0.7 ml and were equipped with magnetic stirrers for continuous stirring of the solution during measurements. One resonator contained the LP solution, while the reference resonator contained the blank (10 mM phosphate buffer). The experiments have been performed at 7.2 MHz. The magnitude of the alternating pressure in the ultrasonic wave was < 0.01 bar. This allowed the avoidance of the influence of ultrasound on structural transition of biocolloids and also the exclusion of any inhomogeneous heating effect from absorption which could distort the standing wave field in the liquid, fill the velocimeter cavity, and shift the resonance peaks. Frequency, f, and half-power width, Δf, of a resonance peak of the acoustic resonator are related to each other, to sound velocity, u, and absorption per wavelength, $\alpha\lambda$, respectively [32].

$$\Delta\frac{u}{u} = \left(\frac{\delta f}{f}\right)(1+\gamma) \tag{2}$$

and

$$\Delta\alpha\lambda = \Omega\Delta\left(\frac{\Delta f}{f}\right) \tag{3}$$

where Δu and δf are changes in sound velocity and resonance frequency, respectively, caused by a change in the physical properties of the measured liquid; γ is a correction term ($\gamma < 0.003$), which can be omitted from Eq. (2) for the type of resonator used in this work [12,33]. Ω is the calibration coefficient for sound absorption. For an ideal resonator $\Omega = \pi$, if the peak width is measured at half of the power level of the peak. For a real system, the value of Ω was obtained by calibration. Calibration has been performed, using the known ultrasonic absorption of 0.1 mol/l $MnSO_4$ [21]. The relative precision of measurements of velocity at single temperature was about 2×10^{-4}% and absorption about 5%. In the case of temperature-dependent measurement, we also determined base line (when both cells were filled with buffer). Reproducibility of base line was approximately $\pm 1 \times 10^{-3}$% for sound velocity and about 5% for absorption. Cells were thermostated with Lauda RK8CS ultrathermostat.

Temperature dependencies of sound velocity and absorption were measured by changing temperature stepwisely. After setting a temperature, the sample was equilibrated in 5 min (the establishment of equilibrium was controlled by measurement of sound velocity). This procedure was repeated for each temperature.

The theory of ultrasonic velocimetry and absorption is described in a number of reviews (see Refs. [9,13,21]). Below are listed the basic equations quoted in this work for the so-called velocity number $[u]$, and absorption number $[\alpha\lambda]$, defined as

$$[u] = \frac{(u - u_0)}{u_0 c} \tag{4}$$

and

$$[\alpha\lambda] = \frac{(\alpha\lambda - \alpha_0\lambda_0)}{c} \quad (5)$$

where c is the concentration of the solution expressed in mg/ml and the index '0' refers to solvent (buffer). Velocity number $[u]$ is related to the adiabatic compressibility of diluted particles. The compressibility of diluted solutions of proteins or liposomes is usually expressed using the value of the apparent compressibility Φ_k [9,20]

$$\Phi_k = \frac{(\beta V - \beta_0 V_0)}{CV} \quad (6)$$

where β, β_0 are the adiabatic compressibilities, V and V_0 are sample volumes and C is the molar concentration. For very dilute solutions

$$\frac{\Phi_k}{\beta_0} \approx -2\frac{(u - u_0)}{u_0 C} - \frac{M}{\rho_0} + 2\Phi_V \quad (7)$$

where M is molecular mass of the dispersed particles, ρ_0 is the density of the solvent and Φ_V is the apparent molar volume of solution, or for specific values

$$\frac{\varphi_k}{\beta_0} = -[u] - \frac{1}{\rho_0} + 2\varphi_V \quad (8)$$

where φ_k/β_0, φ_V are the specific apparent compressibility and volume of solution, respectively. Absorption number $[\alpha\lambda]$ expresses the exponential reduction in the pressure amplitude as the acoustic wave travels through a distance of one wavelength.

2.3. Measurement of density

The hollow oscillator principle for precision density measurement [22] was used to determine the apparent partial specific volumes, φ_V, of LP. The experiments were performed by means of precise densitometer system (DMA 60 and 602, Anton Paar KG, Graz, Austria). The specific volumes were calculated from the densities of the LP solutions ρ_s, of the buffer ρ_0 and the LP concentration, c, according to

$$\varphi_V = \frac{\left[1 - \frac{(\rho_s - \rho_0)}{c}\right]}{\rho_0} \quad (9)$$

Thus, we can see that determination of velocity number $[u]$ and specific volume give possibility to evaluate apparent specific compressibility of LP (see Eq. (8)).

3. Results and discussion

The dependence of velocity and absorption of ultrasound as well as density of LP on temperature was measured in the range of 15–45°C. Results of DSC experiments show that the thermotropic behaviour of LP is reversible in this range [6,7]. Fig. 1 shows a linear decrease of velocity number with temperature for HDL_3 and LDL. We can see that the values for HDL_3 are higher than that for LDL. An important feature of the function $[u](T)$ is the change in the sign of $[u]$ at $T = 44$°C for HDL_3 and $T = 29$°C for LDL. According to Eq. (4), the change in the sign of the parameter $[u]$ and its negative value at higher temperatures means that the ultrasound velocity in LP suspension is lower than that in buffer. This corresponds to increasing of the adiabatic compressibility coefficient of LP suspension (see Eq. (1)). This phenomenon is known from the ultrasonic studies of phase transitions in liposome suspensions and is due to the critical fluctuations in the order parameter of biocolloids [34,35]. Absorption numbers $[\alpha\lambda]$ for both classes of LP monotonously increased with

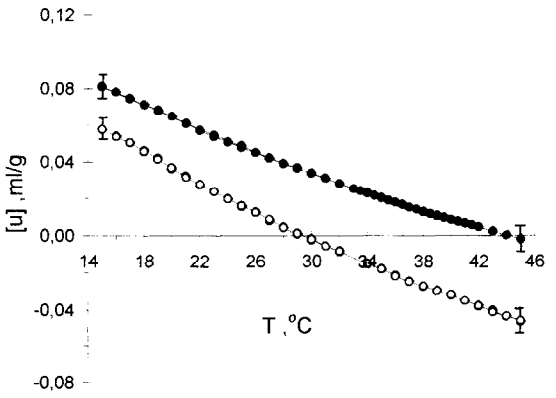

Fig. 1. Dependence of velocity number $[u]$ on temperature: (●) HDL_3, (○) LDL. Error bars represent S.E. calculated from five measurements of the shift of resonance frequencies relative to buffer for the marginal experimental points of temperature interval tested.

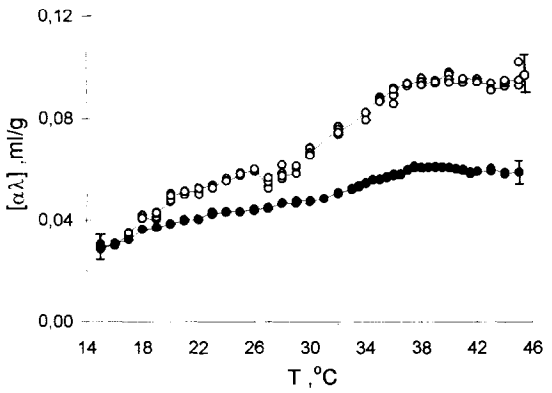

Fig. 2. Dependence of absorption number $[\alpha\lambda]$ on temperature for HDL_3 (●) and LDL (○). Error bars represent S.E. calculated from five measurements of the resonance peak width Δf for the marginal experimental points of temperature interval tested.

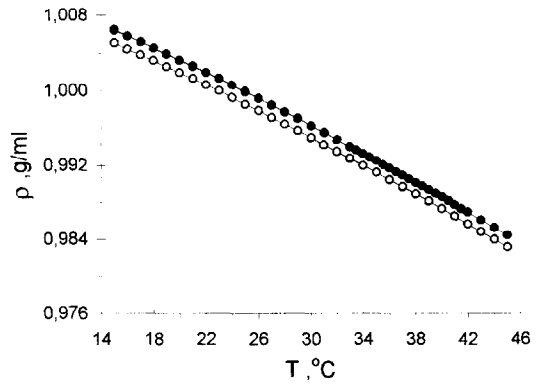

Fig. 3. Dependence of density on temperature: (●) HDL_3, (○) LDL. The S.E. does not surpass 2.5%.

increasing temperature (Fig. 2). For LDL, a slight nonmonotonous behaviour of the absorption number in the region 26–30°C is observed. The differences between the values of $[u]$ for LDL and HDL_3 were statistically significant with $P < 0.001$, according to Student's t-test. The differences between the values of $[\alpha\lambda]$ for LDL and HDL_3 were statistically significant only at higher temperatures, above the phase transition of LDL (the level of statistical significance, according to Student's t-test, was $P < 0.001$ at $T = 45$°C). Densities of both classes of LP monotonously decreased with temperature (Fig. 3). The overall changes in the density with temperature were, however, small and do not exceed 3%. These results are in agreement with data from Ref. [5] in which no significant changes in LDL density have been reported at different temperatures. Using Eq. (9), we calculated and plotted partial specific volumes φ_V of LP as a function of temperature (Fig. 4). We can see that parameter φ_V changes nonmonotonously with temperature for both classes of LP. (The concentrations of LP used in measurements of $H[u, \alpha\lambda]$ and density as a function of temperature was 10 mg/ml) These data, together with the results from measurements of velocity numbers, revealed a general tendency: (1) for both class of LP: the volume compressibility of LP increases with temperature; and (2) volume compressibility is different for LDL and HDL_3 (see Table 1). Using the changes in $[u]$, we can analyse the thermotropic-phase behaviour of LP and compare it with the phase behaviour of liposomes. Interestingly, LP and phospholipids show a typical decrease of $[u]$ below their phase transition temperatures as well as changes in the sign of $[u]$ are observed. It has to be emphasised that most of the free cholesterol in LP is contained in their surface and only minor amount of free cholesterol is accommodated in the LP core, which contain mainly cholesteryl esters. Nevertheless, the LDL shows the same monotonic decrease in $[u]$ compared to cholesterol-rich phospholipid liposomes [34]. Sterol-free phospolipid membranes show a significant change in $[u]$ at the main phase transition temperature. For liposomes, as well as, for homogeneous solutions of n-alkanes, the value of $[u]$ has a minimum at the critical point, which is connected with an anomalous decrease of ultrasonic velocity

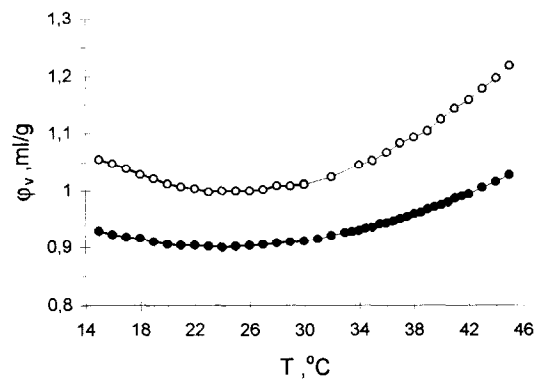

Fig. 4. Dependence of the specific volume φ_V on temperature: (●) HDL_3, (○) LDL. The S.E. does not surpass 2.5%.

Table 1
Acoustical properties of LDL and HDL_3

Class of LP	$T = 25°C$				$T = 37°C$			
	$[u]$ (10^{-2} ml/g)	φ_V (ml/g)	φ_k/β_0 (ml/g)	β (10^{-11} Pa^{-1})	$[u]$ (10^{-2} ml/g)	φ_V (ml/g)	φ_k/β_0 (ml/g)	β (10^{-11} Pa^{-1})
LDL	1.6 ± 0.2	1.0 ± 0.03	0.98 ± 0.09	43.6 ± 4.3	-2.3 ± 0.2	1.08 ± 0.03	1.17 ± 0.09	47.0 ± 3.9
HDL_3	4.4 ± 0.3	0.9 ± 0.03	0.75 ± 0.06	37.1 ± 3.2	1.6 ± 0.1	0.95 ± 0.03	0.87 ± 0.07	39.7 ± 3.3

The values represent mean ± S.E. calculated from five independent measurements of LP suspension.

and with an increase of relaxation time at the phase-transition temperature [35]. Liposomes are characterised by a relatively narrow (~ 2–5°C) region of dramatic changes in $[u]$ [35]. The phase behaviour of LP is also characterised by changes in the sign of $[u]$, however, the behaviour of $[u]$ is substantially different. We did not observe any typical critical phenomena characteristics for homogeneous solution of phospholipids or *n*-alkanes. The changes in absorption number, together with the clearly expressed change in the sign of $[u]$ can be taken as evidence that a thermotropic-phase behaviour also takes place in LP. However, even for LDL, the results of molecular acoustics point to broad changes in lipid ordering, with no clearly expressed phenomena characteristics for the typical phase transition of the hydrocarbon chains in phospholipids. If we take into account possible variations in lipid composition and complex inhomogeneous structure of LP [36], then obtained broad changes are not so surprising.

Thus, we found that velocity numbers $[u]$ and absorption numbers $[\alpha\lambda]$ are different between LDL and HDL_3. It is known that the value $[\alpha\lambda]$ represents the sum of two processes: (1) Classical, and (2) Nonclassical absorption [13,37]. Classical absorption is associated with viscous energy losses and transport of thermal energy along temperature gradient. Nonclassical absorption mechanism is due to thermal, chemical, and/or structural relaxation processes. The characteristic relaxation times for proteins lie below [20] and for lipids [37,38] above the frequency used in our experiments. It is, however, necessary to note that apolipoproteins can induce a shift in the relaxation frequency of the colloid suspension (i.e., at which the absorption per wavelength reaches its maximal value). This was, in particular, demonstrated for structural relaxation of liposomes modified by gramicidin [39]. The exact comparison of $[\alpha\lambda]$ values requires, therefore, the comparison of the maximum absorption values for LDL and HDL_3 determined by means of broad band ultrasonic spectrometry. Recently obtained results using the former method showed that at a frequency range of 1–7 MHz and temperatures of $T = 25$ and 37°C the absorption number of HDL_3 is lower than that for LDL [40], i.e., this is in agreement with the data obtained at a single frequency 7.2 MHz.

Two main physical processes may contribute to $[u]$, namely, compressibility of LP and hydration of their surface. In aqueous dispersions, both the protein surface as well as the polar phospholipid head groups of LP are hydrated. Due to dipole–dipole interactions, the hydrated layer represents a rather ordered structure, which at temperatures around 20°C is characterised by lower compressibility than globular proteins and phospholipid bilayers. At higher temperatures, the ordering of the hydrated LP shell decreases leading to an increase in its compressibility. Specific apparent compressibility (see Eq. (8)) can be defined as follows [20]:

$$\frac{\varphi_k}{\beta_0} = \left(\frac{\varphi_k}{\beta_0}\right)_{LP} + \left(\frac{\varphi_k}{\beta_0}\right)_H \qquad (10)$$

where indexes LP and H represent the contribution of LP themselves and their hydration, respectively. The total specific apparent compressibility can be calculated according to Eq. (8) using measured values of $[u]$ and specific apparent volumes φ_V of LP. The values of $[u]$ and φ_V in Eq. (8) represents the limiting variables (at $c \to 0$). However, due to the practically linear dependence of the difference $u - u_0$ on concentration in the concentration range studied (2–14 mg/ml), the limiting value $[u]$ was simply calculated by dividing $(u - u_0)/u_0$ by concentration c. The values of φ_V also did not practically depend

on concentration c. Having $[u]$ and φ_V, we can evaluate the specific adiabatic compressibility (using Eq. (8)) of LP. The values of adiabatic compressibility of LP can be evaluated using the expression [20]

$$\beta \approx \left(\frac{\varphi_k}{\beta_0}\right)\frac{\beta_0}{\varphi_V} \quad (11)$$

where β_0 is the adiabatic compressibility of the solvent. This value has been determined from Eq. (1) using the measured value of density, ρ_0; velocity of ultrasound has been taken from Ref. [21]. The acoustics parameters we determined for two temperatures $T = 25$°C and 37°C. At these temperatures, the adiabatic compressibility of solvent was 44.5×10^{-11} and 43.4×10^{-11} Pa^{-1}, respectively. The results of the calculation of φ_k/β_0, adiabatic compressibility of LP, β as well as limiting velocity number $[u]$ and φ_V at $T = 25$ and 37°C, are shown in Table 1. The values of φ_k/β_0 and β are higher for LDL in comparison with HDL_3 at both temperatures. This coincides with the higher compressibility of LDL. The compressibility of LP also increases with increasing temperature. This increase is more expressed for LDL. However, the overall changes in compressibility in the range of the temperature interval studied, do not exceed 15%. For comparison, the changes in volume compressibility for unilamellar liposomes during the main phase transition in a similar temperature range approaches 30% [34]. It is also clear from Table 1 that the parameter most sensitive to the structural LP changes is the velocity number. This parameter even changes the sign for LDL at 37°C. This reflects the decrease in the ordering of the hydrophobic core of LDL due to the thermotropic-phase transition. This is in agreement with X-ray and neutron small-angle scattering data [41]. The values of φ_k/β_0 for LP are about one order in magnitude higher than that of globular proteins [20] and have comparable, but slightly lower values, than that of unilamellar liposomes from unsaturated fatty acids [42].

Let us now compare the hydration effect on the values of the specific adiabatic compressibility for LDL and HDL_3. The hydration of spherical particles of basically similar structure and composition should be proportional to the area S of the hydrated surface. Therefore, $(\varphi_k/\beta_0)_H \sim S/M$ and the relation between hydration and the specific apparent compressibility of LDL and HDL_3 is given by:

$$\frac{\left(\frac{\varphi_k}{\beta_0}\right)_H^{LDL}}{\left(\frac{\varphi_k}{\beta_0}\right)_H^{HDL_3}} \approx \left(\frac{d_{LDL}}{d_{HDL_3}}\right)^2 \times \left(\frac{M_{HDL_3}}{M_{LDL}}\right) \approx 0.55 \quad (12)$$

where $d_{LDL} = 21$ nm, $d_{HDL_3} = 8$ nm are diameters of LDL and HDL_3, respectively, $M_{LDL} = 2.5$ MDa, $M_{HDL_3} = 0.2$ MDa are their molecular weights [1]. On the other hand, the relation Eq. (11) for adiabatic compressibility gives $(\beta_{LDL}/\beta_{HDL_3})_{T=25°C} \approx 1.17$ and $(\beta_{LDL}/\beta_{HDL_3})_{T=37°C} \approx 1.18$. We can see that the contribution of hydration is in both cases about 2-fold less, and thus, represents only a minor contribution to the adiabatic compressibility of the LP studied, i.e., the differences in adiabatic compressibility between both types of LP are determined mainly by their structural peculiarities and not the compressibility of hydration shells.

Thus, the measurement of velocity and absorption of ultrasound in parallel with the measurement of density provides new information on the physical properties of LP. Our results show for the first time, the capability of the method of molecular acoustics as a useful tool for the study of physical properties of different classes of LP.

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