

Compressibility of nano inclusions in complex fluids by ultrasound velocity measurements.

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Abstract

We present a high precision ultrasonic velocimeter for a small volume sample (one cm³), for a path length of 1 cm achieved. The method used is based on the time-of-flight measurement with an original signal processing technique: the barycenter method. With our system, we have measured the sound velocity with an accuracy of 10⁻⁵. The detection of a difference in velocity between two liquids of about 2 cm/s is achieved. The compressibility of the reference liquid can be then deduced with an accuracy better than 0.2 %. Using this custom-made system, we have studied and characterized complex fluids, systems biomimetic of biological membranes, as well as proteins included in nanometric water droplets. Under these experimental conditions, we have reached the value of protein compressibilities with an accuracy better than 10 %.

I. INTRODUCTION

Ultrasonic velocity and density measurements of a fluid lead to its compressibility. More difficult is the determination of the compressibility of nanometric inclusions (e. g. macromolecules and proteins) solubilized in a fluid at low volume (less than 2 cm³) and at low concentration. The difficulty increases further, when we investigate the compressibility of more complex systems, such as small cavities filled with water, or proteins solubilized in these cavities. For this purpose we have built a dedicated device as well as developed an original methodology.

This device can be utilized for fundamental and complex biological investigations such as protein conformation (folding, aggregation), protein hydration, and vectorization of macromolecules for therapeutic use. To address these problems, we have selected a biomimetic medium such as reverse micelles [1]. They are water nanodroplets, surrounded by a monomolecular film of surfactant and dispersed in an organic solvent. The system can be considered, by certain aspects, as mimetic of biological membranes [2]. The amount of micellar water is experimentally controlled with a good accuracy. Moreover, proteins can be hosted within these micelles. The system constitutes a physical model which reflects the protein behavior as a function of hydration in the vicinity of biological membranes. The compressibility of micelles, or of proteins included within these micelles, is not directly measurable, but it can be deduced from measurements of density and ultrasonic velocity of

the medium. The ultrasonic measurements of velocity are carried out by methods either temporal or spectral [3], leading to an accuracy of about 10^{-5} to 10^{-6} for difference measurements of a volume around 1 ml. The temporal method consists in measuring the time of flight between the emission of a short duration signal and its reception, either by transmission or by reflection. The process to determine the time of flight, uses either zero-crossing of the signal, or the search for a maximum. An alternative method consists in starting the emission when a signal is detected in transmission. In this method, known as the Sing Around Method [4], time is determined by the measurement of the impulse repetition period. Taking into account multiple reflections, this method has to deal with trigger problems, especially in low attenuating media. A third method, referred to as the Pulse Echo Overlap [5-6], consists in visually superimposing the successive echoes on the screen of an oscilloscope. This method is not easily automated, and consequently is of limited interest for our applications. Spectral methods use an acoustic resonator to determine ultrasonic velocity in the medium [7-9]. These methods require very high accuracy mechanics, in particular a quasi-perfect parallelism of the front-face transducers. The principles of velocity measurement are well-known but they can be improved today by technological developments and specific signal processing [10-12].

For our specific applications, we have adapted the time-of-flight method because of its simplicity and of its simplicity of operation. To overcome velocity variations induced by small temperature fluctuations during experimentation, our experimental device is composed of a pair of tandem cells, carefully thermally regulated at 25 ± 0.01 °C, and placed in an temperature-controlled room. By difference measurements, we can detect, very small variations of velocity between two liquids (in the absence or presence of inclusions) and thus deduce the compressibility of these inclusions. To reduce the effects of the the received circuits noise, our procedure is significantly improved by an original signal. The processing is based on the calculation of the barycenter (or energy gravity center) of the emitted signal as well as of that of the received signal. The calculation introduces a filtration effect, which reduces the variance of the speed of sound. Following theoretical developments, we present the experimental system developed in our laboratory, followed by the presentation of the results and of its performances.

II. BACKGROUND

On the assumption of linear acoustics, the adiabatic compressibility β of a medium, is given by the well-known Laplace equation:

$$\beta = \frac{1}{\rho u^2} \quad (1)$$

According to this relation, the compressibility can be determined from two measurements: the measurement of the medium density, ρ , and the measurement of the sound velocity, u , of the same medium. In our experiment, the solution consists of an organic solvent with inclusions. Such inclusions may be reverse micelles, macromolecules or proteins, encapsulated in micelles or in an aqueous solution. While compressibility of the solution may easily be obtained, the compressibility of inclusions must be deduced. For objects of small size (about 10^{-9} m) compared to the acoustic wavelength (about 10^{-4} m) the compressibility of the solution, for small densities variations, we can use the effective medium theory [13]:

$$\beta = \beta_{oil}\phi_{oil} + \beta_{inc}\phi_{inc} \quad (2)$$

where β , β_{oil} and β_{inc} are respectively the solution, the solvent and the inclusion compressibilities. ϕ_{oil} and ϕ_{inc} are respectively the solvent and inclusion volume fractions. Therefore, one has: $\phi_{oil} + \phi_{inc} = 1$. The volume fraction of inclusions ϕ_{inc} is obtained from the mass conservation law:

$$\rho = \rho_{oil}\phi_{oil} + \rho_{inc}\phi_{inc} = \rho_{oil}\phi_{oil} + C_{inc} \quad (3)$$

where ρ , ρ_{oil} and ρ_{inc} are the densities of the solution, the organic solvent and the inclusions in solution, respectively. The mass concentration of inclusions, C_{inc} , is measured by weight at constant volume. The micellar volume fraction is obtained by Eq. 3 :

$$\phi_{inc} = 1 - \phi_{oil} = 1 - \frac{\rho - C_{inc}}{\rho_{oil}} \quad (4)$$

Taking into account the low concentration of inclusions in solution (less than 10 %), one can assume that ρ_{oil} is independent of C_{inc} and equal to the density of pure solvent. Knowing ϕ_{inc} , one can deduce the compressibility of inclusions β_{inc} :

$$\beta_{inc} = \beta_{oil} + \frac{\beta - \beta_{oil}}{\phi_{inc}} = \beta_{oil} \left(1 + \frac{\beta - \beta_{oil}}{\beta_{oil}} \frac{1}{\phi_{inc}} \right) \quad (5)$$

The accuracy of the determination of β_{inc} depends upon the accuracy of β_{oil} , ϕ_{inc} and especially the difference $\beta - \beta_{oil}$. The variation of the solvent compressibility is expressed by the logarithmic derivative of Laplace's relation:

$$\frac{d\beta_{oil}}{\beta_{oil}} = -\frac{d\rho_{oil}}{\rho_{oil}} - 2\frac{du_{oil}}{u_{oil}} \quad (6)$$

where u_{oil} is the velocity in the oil (the organic solvent). For small variations between the densities (i. e. ρ_{oil} and ρ), and the velocities (i. e. u_{oil} and u), Eq. 6 can be written:

$$\frac{\beta - \beta_{oil}}{\beta_{oil}} = -\frac{\rho - \rho_{oil}}{\rho_{oil}} - 2\frac{u - u_{oil}}{u_{oil}} \quad (7)$$

Consequently, to obtain the compressibility of the inclusions dispersed in a solvent, we need to know:

- (i) the compressibility of the solvent, which can be obtained by measurements of the oil density ρ_{oil} and the ultrasonic velocity of the solvent u_{oil} , (see Eq. 1);
- (ii) the compressibility of the solution which can be determined from measurements of the density ρ and the ultrasonic velocity u of the solution; and
- (iii) the volume fraction, ϕ_{inc} , calculated using Eq. 4.

The accuracy on the compressibility of inclusions at low concentration depends primarily on the accuracy of ρ , ρ_{oil} , u , and u_{oil} , in addition to the accuracy of the concentration inclusions of the (Eq. 5 and Eq. 7). In general, the accuracy on the determination of the compressibility is expressed by the partial derivative equation:

$$d\beta = \frac{\partial\beta}{\partial\rho}d\rho + \frac{\partial\beta}{\partial u}du + \frac{\partial\beta}{\partial C_{inc}}dC_{inc} = S_{\beta(\rho)}d\rho + S_{\beta(u)}du + S_{\beta(C_{inc})}dC_{inc} \quad (8)$$

where $S_{\beta(\rho)}$, $S_{\beta(u)}$, and $S_{\beta(C_{inc})}$ represent the coefficients of sensitivity of the factor β as related to variations of ρ , u , and C_{inc} respectively. Thus the accuracy on β is related to a sum of products of the form $S_{\beta(x)}dx$. In the construction of our experimental device, the required accuracy of dx is governed by the value of the coefficient $S_{\beta(x)}$. For example, the accuracy on du must be better than the accuracy on dC_{inc} .

III. EXPERIMENTAL SET UP

The compressibility of inclusions must be evaluated with a accuracy better than 1 %. Such an objective involves a accuracy of $3 \cdot 10^{-4}$ for the weight, and 10^{-5} for the density and velocity measurements.

A. Sample weight measurements

All the samples were prepared by weighing the solutes on a Model 1712 Sartorius balance, in accuracy volumetric flasks (class A ± 0.04 ml), with an accuracy of ± 0.03 mg. The solvents used to make up the volume at 20 °C, were either pure organic solvents or organic solutions of the prepared surfactant. The accuracy of the inclusion concentration is about $3 \cdot 10^{-4}$.

B. Density measurements

For small variations of density (Eq. 7), the required accuracy is obtained by using a digital density meter (Anton Paar DMA 58) with a vibrating tube. Each density measurement is the average of at least 5 repetitions at 25 ± 0.01 °C. The accuracy on the density is better than 10^{-5} .

C. Velocity measurements

The experimental device consists of a pair of tandem measurement cells, built in the same block of stainless steel, and thermally stabilized by circulating water thermally regulated at 25 ± 0.01 °C (Huber HS 40). Furthermore, the unit is placed in a temperature-controlled room. A permanent temperature control is monitored by measurement of the ultrasonic velocity in the reference cell. The first cell is filled with the reference liquid (solvent) and the second cell with the solution. The difference in velocity between the two cells represents the speed-of-sound variation, induced in the solvent by the inclusions.

Each cell comprises two identical lithium niobate piezoelectric transducers. They are glued on stainless steel elements, the parallel faces of which are optically polished (Fig. 1). These elements ensure the impedance matching between the transducer and the medium. They are essential to damp the signal and thus to improve temporal resolution. The thickness of the steel elements is calculated to avoid multiple reflections and interference with the desired experimental signals. These elements introduce a delay which must be taken into account during the calibration procedure. Hence, they are referred to as delay lines. The transmitting transducers emits an acoustic wave into the medium, and the receiving transducers convert the received pressure into a voltage. The excitation signal sent to the two transmitting transducers is a modulated coherent pulse at the resonance frequency of the transducers. The signal duration is equal to exactly n cycles (n is an integer) of the modulating signal, and consequently, does not yield discontinuities at the beginning or end of the impulse. The emission signal is optimized first by digital simulation and then by experiment. Several features of the optimized are:

- (i) the modulating signal frequency corresponds to the center frequency of the transducers ($f = 8$ MHz) in order to optimize energy transfer;
- (ii) the duration of the modulated impulse is equal to 2 cycles of the modulating signal ($n = 2$), and is a compromise between sufficient energy being received and too large of a measurement bias;
- (iii) the delay between two consecutive emissions (1 ms) is selected in order to avoid interactions of multiples echoes; and

- (iv) the amplitude of emitted signal (10 V) is optimized with respect to the signal-to-noise ratio as well as avoid an increase in temperature of the solution.

After propagation in the medium, the two signals provided by the two receiving transducers are sampled simultaneously (at 10 giga-samples per second) by a digital oscilloscope (model Lecroy 9424E) synchronized by a pulse generator. Given the total time of flight (medium plus delay lines), the temporal sampling resolution provides a theoretical accuracy to better than 10^{-5} . The accuracy, which is limited by the sampling rate of our oscilloscope, can be easily improved by the follow procedure. The reception signals are averaged by the oscilloscope over 100 successive acquisitions, in order to improve the signal-to-noise ratio: this amounts to a measurement every 45 seconds. An overall average is also calculated on the result of 10 time-of-flight values. Fig. 2 provides the schematic diagram of the experimental set-up.

A PC computer controls the data acquisition on via an IEEE 488 interface and the signal processing describe below by home-made software (written in C language).

Considering the received signal profile, the precise time of the received wave beginning is difficult to determine. Moreover, the zero-crossing is perturbed by noise and the solution averaging of acquisitions is not a sufficient solution. To overcome this difficulty, the time-of-flight measurement is calculated by an integration of the received signal using the following relation:

$$t_{vol} = t_0 + \frac{\sum_i t_i a_i^2}{\sum_i a_i^2} \quad (9)$$

where t_{vol} is the time of flight, t_0 is the delay between the emission and the beginning of the analysis window, t_i is the time and a_i is the i^{th} sample of the signal at the time t_i . Eq. 9, by which one calculates the energetic gravity center, is known as the barycenter method. It will be noted that the method introduces a bias which is compressed by the velocity difference determination.

D. Calibration

To obtain the time of flight in the medium, one first has to measure the temporal shift introduced by the delay lines. It is also necessary to know the length of the cell. These two parameters lead to velocity u . A calibration is carried out in four steps for each cell. The measurement cell is initially filled of standard liquid of known velocity with a sufficiently high accuracy (1 cm/s). The velocity must be close to that of the medium to explore, in order to minimize the impedance mismatching. Secondly, the time of flight, t_{dl} , in the delay lines is measured in reflection. The total time of flight, t_{tot} , is then measured in transmission, and the time of flight in the medium, t_m , is deduced from the

difference $t_{\text{tot}} - t_{\text{dl}}$. Finally, knowing the velocity of the medium and the time of flight, the length of the cell (d) and, consequently, the variation of compressibility are determined. The accuracy of the distance d is the sum of the accuracy on the velocity of the standard liquid (10^{-6}) and on the time of flight in the medium (about 10^{-5}). However, since d remains constant during a given experiment, an error on its value does not generate errors on the variation of velocity.

IV. EXPERIMENTAL RESULTS

A. Velocimeter performance and application to simple fluids and solutions

The measurement of the velocimeter stability in both the short and medium term was investigated. Fig. 3 represents the evolution of the difference of time-of-flight between the two cells, using the zero-crossing method (a) and the barycenter method (b) for twenty hours. These curves clearly demonstrate the advantages of the barycenter method with respect to the classical method. In the barycenter calculation, the integration obviously reduces the noise. By the barycenter method, the short-term measurements of the time of flight (during a typical experiment of 5 hours), gives a standard deviation of 0.8 ns for one cell and 0.2 ns on the difference between the two cells. These results correspond to a velocity variation of about 1 cm/s (Fig. 4) over a velocity of 1500 m/s. The accuracy thus obtained with our velocimeter is thus about 10^{-6} . The benefit obtained with the barycenter method is 14 dB.

During the validation process, we have measured the ultrasound velocity and density of methanol. The results obtained are in a excellent agreement with literature [14]. In the same way, we have determined the compressibility of a pure oil, isooctane. This value of $\beta_{\text{oil}} = 121.0 \pm 0.2 \cdot 10^{-11} \text{ Pa}^{-1}$ has been used in equation 5. The standard deviation of this measure, is lower than that given in literature [15-16].

In a second step, we have studied a protein (lysozyme of hen egg) solubilized in water. In a typical experiment, the reference cell contains water, while the measurement cell is filled with the protein solution. Fig. 5 illustrates the velocity variation between u_0 (velocity in water) and u (velocity in the solution) versus the protein concentration; in this experiment, the accuracy is better than 2 cm/s. Our results are in good agreement with those of literature: the slope of the curve (du/dC) is 0.259 ± 0.001 , compared to 0.257 [17]. This result corresponds to compressibilities of $4.70 \pm 0.5 \cdot 10^{-11} \text{ Pa}^{-1}$ in our measurement and $4.67 \cdot 10^{-11} \text{ Pa}^{-1}$ in the literature.

B. Application to complex fluids

In this last section, we present the measurement of the speed of sound and the compressibility of more complex fluids composed of 3 or 4 components. The studied fluid is composed of nano-droplets of water surrounded by a monomolecular film of a surfactant (sodium bis [2-ethylhexyl] sulfosuccinate: AOT) and dispersed in a solvent (isooctane); such a system is denominated reverse micelles [1]. The size of these spherical droplets is on the order of several nanometers and depends only on the water concentration, W_0 defined as the water-to-surfactant molar ratio. This parameter is experimentally controlled with accuracy. The droplets are thermodynamically stable [1].

In this experiment the reference cell contains also the pure solvent, while the measuring cell is filled with the micellar solution. The plot of the difference in velocity between the two liquids, versus W_0 is illustrated in Fig. 6. This velocity difference associated to density measurements, enables us to determine the micellar compressibility (see Fig. 7). We observe, an increase of the compressibility versus the number of water molecules per micelle, n_{H_2O} (logarithm scale). The accuracy obtained is around $\pm 1 \cdot 10^{-11} \text{ Pa}^{-1}$. Using the above-described velocimeter, we have been able to give a first estimate of the compressibility of the water in the vicinity of a membrane. We have found a value of $60 \cdot 10^{-11} \text{ Pa}^{-1}$, whereas bulk water yields a compressibility of $45 \cdot 10^{-11} \text{ Pa}^{-1}$. This confirms the unusual physical properties of water in contact with a membrane-mimetic system which mirrors the properties of biological membranes [19].

When adding an additional component to the system (a protein, cytochrome c, inserted into reverse micelles), the determination of the compressibility of the protein is still possible, although more difficult. The sensitivity and the accuracy of such measurements are illustrated in Fig. 8, where we have plotted the protein micellar solution compressibility versus protein volume fraction (ϕ_p). These results show that, even under arduous experimental conditions, the values of compressibility remain measurable and precise for very small variations of the volume fraction of the protein.

V. SUMMARY

We have built a high accuracy apparatus to determine the compressibility of nanometric-size inclusions, dispersed at low concentration in a complex biomimetic system. The measurement of the sound velocity in the medium renders possible estimation of small differences in velocity between a reference liquid and the same liquid containing inclusions of interest. The velocimeter, precisely thermally regulated, also allows, the permanent temperature control of the tandem cells. The difference of velocity measurement reduces the influence of residual variations of the temperature. The method used is based on the time-of-flight measurement. In addition, we make use of an original

signal processing technique: the barycenter method. As a consequence, the precise determination of the speed of sound becomes less sensitive to noise and the variance of the results is strongly decreased when compared to classical temporal methods. In particular, we have measured a reduction in the standard deviation of about 14 dB.

With the described system in hand, we have measured the sound velocity, in low volumes of solvent (around one cm³) with an accuracy of 10⁻⁵. This accuracy permits the detection of a difference of about 2 cm/s in velocity between two liquids. The compressibility of the reference liquid can be then deduced with an accuracy about 0.2 %. In this report, we have been able to measure the compressibility of fluids of increasing complexity, composed of one to four different components, with excellent accuracy. The method has also been applied with success in our laboratory to micellar solutions of biological macromolecules. Work is in progress to determine the compressibility of proteins at very low hydration levels, and thus to reach the protein intrinsic compressibility.

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Figure legends.

Fig 1. Schematic representation of one cell (not actual size): \neg emission, $-$ reception, \textcircled{R} lithium niobate piezoelectric transducers, $-$ stainless steel elements: delay line, $^{\circ}$ cell volume, \pm filling hole, 2 block of steel.

Fig. 2. Block diagram of the experimental set-up.

Fig. 3. The time of flight difference between the two cells as a function of time (over a 20 hour period): (a) by the zero-crossing method and (b) by the barycenter method. (870 points of measurement). Zero-crossing method: the standard deviation is 0.76 ns, barycenter method: the standard deviation is 0.24 ns.

Fig 4. Barycenter method: the time of flight difference (left axis) and the velocity difference (right axis) between the two cells versus time for 5 hours, (110 points of measurement). The minimum is at: -0.219 ns, and the maximum is at: 0.173 ns, and the standard deviation is 0.083 ns.

Fig. 5. Velocity difference between u_0 (velocity in water) and u (velocity in an aqueous solution of lysozyme), versus the protein concentration C . The error bars ($\pm 0.02 \text{ m.s}^{-1}$) is smaller than the size of experimental points.

Fig. 6. Difference between u_0 (the velocity in isooctane) and u (the velocity in a micellar), versus the water to surfactant molar ratio (W_0). The error bars is 0.02 m.s^{-1} .

Fig. 7. Micellar compressibility plotted versus the number of water molecules per micelle, $n_{\text{H}_2\text{O}}$ calculated according to [18] (error bars: $\pm 1 \%$).

Fig. 8. Compressibility of a micellar solution of cytochrome c at $W_0 = 8.2$ versus the protein volume fraction ϕ_p (error bars: $\pm 0.05 \%$).

















